Journal of Human Hypertension (2007) 21, 558–563
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www.nature.com/jhh

ORIGINAL ARTICLE

Circulating endothelial cells in preeclampsia

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Endothelial dysfunction plays an important role in the pathogenesis of preeclampsia. Increased number of circulating endothelial cells (CECs) have previously been reported after various diseases associated endothelial injury. The aim of this study was to evaluate the CECs in patients with preeclampsia and to demonstrate any association between CECs and homocysteine, which is another marker of vascular injury. The study included 20 preeclamptic, 15 hypertensive women, 15 healthy pregnant and 15 healthy non-pregnant women. All subjects had normal renal function. Systolic and diastolic blood pressures, serum homocysteine levels were measured. To isolate CECs, peripheral blood was first incubated with anti-CD-146 antibody and subsequently conjugated to immunomagnetic beads. Cells were stained with acridine and counted. Preeclamptic patients had elevated numbers of CECs (13.2 ± 5.2 cells/ml) compared with hypertensive patients (6.9 ± 0.8 cells/ml), healthy pregnants (5.2 ± 1.4 cells/ml) and non-pregnant controls (4.0 ± 1.8 cells/ml), ($P < 0.0001$). Serum homocysteine level in preeclamptic patients (9.5 ± 2.8 μmol/l) was significantly higher compared with healthy pregnants (6.0 ± 0.6 μmol/l), was not different from hypertensive patients (11.5 ± 2.3 μmol/l, $P > 0.05$), but it was lesser compared with non-pregnant controls (12.2 ± 3.3 μmol/l, $P < 0.0001$). Also, significant correlation between CECs and systolic blood pressure ($P < 0.0001$, $r = 0.63$), diastolic blood pressure ($P < 0.0001$, $r = 0.64$) and serum homocysteine ($P < 0.01$, $r = 0.55$) levels were found in preeclamptic patients. CECs as a marker of endothelial injury were significantly higher in patients with preeclampsia than in hypertensive patients, healthy pregnants and normal controls. Further studies are needed for the prognostic and potential importance of CECs in preeclampsia.
Journal of Human Hypertension (2007) **21,** 558–563;
doi:10.1038/sj.jhh.1002199; published online 12 April 2007

Keywords: endothelial cells; homocysteine; preeclampsia

Introduction

Preeclampsia is a pregnancy-specific disorder that is clinically associated with hypertension, proteinuria, oedema and it adversely affects 7–10% of all pregnancies in the United States.[1] It is characterized by abnormal vascular response to placentation that is associated with increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation system and endothelial cell dysfunction.[2] An initiating event in preeclampsia has been postulated to be reduced placental perfusion that leads to widespread dysfunction of the maternal vascular endothelium. Levels of cellular fibronectin, vascular cell adhesion molecule (VCAM)-1, intracellular cell adhesion molecule (ICAM)-1, E-selectin, CD31 and von Willebrand factor have been documented to be elevated in preeclamptic patients even before its clinical manifestation.[3] Additionally, several studies have suggested that women who develop preeclampsia can be at increased risk of cardiovascular complications later in life.[4]

An increased number of circulating endothelial cells (CECs) has previously been reported after the onset of various diseases that are associated with endothelial injury, including myocardial infarction, septic shock, sickle cell crisis, antineutrophil cytoplasmic autoantibody (ANCA)-associated small vessel vasculitis, thrombotic thrombocytopenic purpura (TTP), systemic lupus erythematosus and Behçet's disease.[5–8] An increase in the number of CECs has been used for assessment of vascular damage and it seems to be a marker of disease activity in recent years.[6,8] Hyperhomocysteinemia is now widely recognized as an independent risk factor for cardiovascular disease – homocysteine level was found to be increased and was associated with cellular fibronectin in preeclampsia.[9] In previous studies, it was reported that homocysteine is toxic for endothelial cells and it induces apoptosis of endothelial cells.[10,11]

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Received 21 December 2006; revised 5 March 2007; accepted 6 March 2007; published online 12 April 2007

In this study, we hypothesized that there might be an increase in CECs in preeclamptic patients and it can be associated with plasma homocysteine level as a marker of vascular injury.

Subjects and methods

Patients and controls

Twenty patients with preeclampsia, 15 patients with uncomplicated essential hypertension, 15 healthy pregnants and 15 age-matched non-pregnant controls with no history of preeclampsia were recruited for the study. Preeclampsia was defined as development after 20 weeks' gestation of systolic blood pressure $\geqslant$140 mm Hg and/or diastolic blood pressure $\geqslant$90 mm Hg and proteinuria of $>$300 mg/24 h in previously normotensive women. All the cases were subjected to routine investigations, for example, haematocrit, urinalysis and blood chemistry (blood urea nitrogen, creatinine, Na, K, fasting blood glucose, uric acid, albumin) and serum homocysteine level. Exclusion criteria were signs and symptoms of any clinical infection or any invasive procedure during the month before blood collection, diabetes mellitus, chronic renal disease and neoplastic or autoimmune diseases. The patients with chronic or acute renal failure were not included. Blood pressure was measured in a sitting position, after at least a 5-min resting period, using a manual sphygmomanometer in the morning of the visits. Five of 20 preeclamptic patients were taking antihypertensive therapy (α-methyl dopa) during blood collection. In pregnant subjects with or without preeclampsia, there was no patient on aspirin or folic acid. The study was approved by the local Ethics Committee of Ankara University School of Medicine and all participants gave informed consent.

Collection of blood specimen

Blood collection was performed by atraumatic puncture of the antecubital vein with a 21G butterfly needle. The first 5 ml of blood was discarded to avoid contamination of endothelial cells traumatically released from the punctured vessel wall. Four milliliter samples of blood from preeclamptic patients, healthy pregnants and controls were drawn into ethylenediaminetetraacetic acid (EDTA)-containing tubes.

Isolation of CECs

To isolate CECs, we used indirect cell separation technique. Four milliliter whole blood was taken and immediately processed after collection. The blood was diluted by 4 ml PBS/BSA (phosphate-buffered saline/bovine serum albumin). Peripheral blood was incubated with 10 μl primary mouse anti–CD-146 antibodies (S-Endo1) (Alexis Biochemicals, Lausanne, Switzerland) for 30 min at 4°C with tilting and rotation. Heterogeneous suspension was centrifuged at 2000 *g* for 5 min and serum was separated. Three milliliter of PBS/BSA with citrate was added to suspension and centrifuged again. This step was repeated twice and serum was separated again to remove all unbound antibody. Thus, the cells were covered with primary mouse anti-CD-146 antibody.

Immunomagnetic beads (Dynabeads M 450 sheep anti-mouse IgG, Dynal Biotech Inc., Oslo, Norway) were washed according to Dynabeads washing procedure before use. Fifty microlitres of immunomagnetic beads were added to antibody-coated cell suspension. At the same time, 20 μl Fc receptor blocking reagent (Miltenyi Biotech, Bergisch Gladbach, Germany) was added to the suspension to prevent nonspecific binding of leucocytes to magnetic beads and to increase binding specificity. Heterogeneous suspension was completed to original volume with PBS/BSA with citrate and was incubated for 45 min at 4°C with tilting and rotation. The washing tube was then placed in a magnetic particle concentrator (MPC-L, Dynal Biotech Inc., Oslo, Norway) for 1 min and supernatant was pipetted off. The step of washing with citrated PBS/BSA and of removing supernatant was repeated 4–5 times. Final dilution was determined as 50 μl.

Immunomagnetic beads were also added to heterogeneous cell suspension, not anti-CD-146 antibody-coated, as negative controls. These measurements were completely negative, suggesting that nonspecific binding to immunomagnetic beads did not occur.

Identification and enumeration of CECs

Final suspension was mixed with 50 μl acridine orange (FluoroQuench, One Lambda Inc., CA, USA) and endothelial cells were counted in a Nageotte chamber using an immunofluorescent microscope (Zeiss Axiostar Plus, Oberkochen, Germany in Immunology Laboratory). Endothelial cells were clarified based on morphology and binding to beads. They are 20–50 μm in diameter, larger than other blood cells including lymphocytes, had a well-delineated round and oval cell shape and more than five beads attached (Figure 1). Assay variability was less than 5%. We did not perform a subsequent step with Ulex-Europaeus-Lectin-1 (UEA-1) staining, because it was demonstrated that further staining seems not necessary and identifying cells on the basis of morphology and adherence to beads alone is enough.[8] In addition, it had been previously shown that there was a very strong correlation between acridine counting alone and immunomagnetic isolation with subsequent UEA-1 stain.[12]

Serum homocysteine level was measured by HPLC (high-performance liquid chromatography) using Thermo Finnigan Spectra System (San Jose, CA, USA). Intra-assay variability was 3.9% and interassay variability was 5.8% for serum homocysteine concentrations.

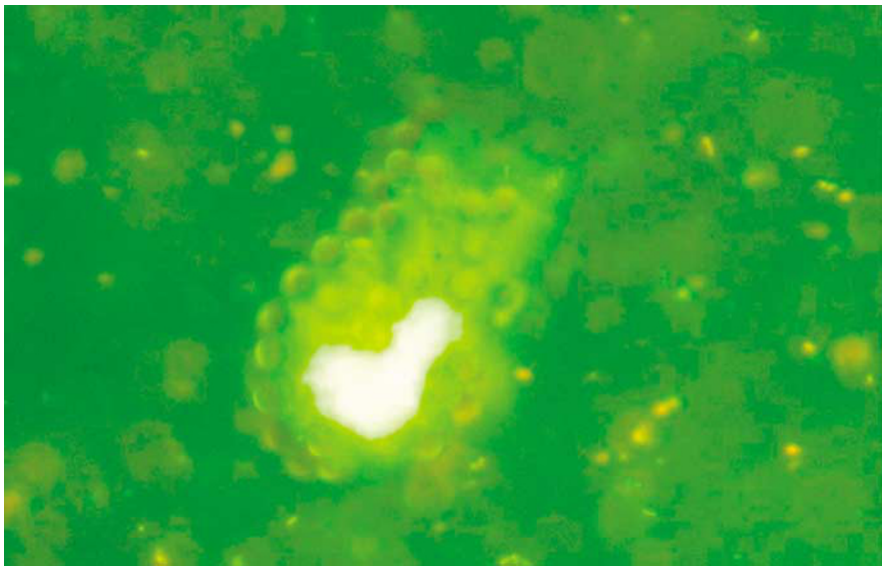

Figure 1 Endothelial cell were 20–50 μm in diameter, were well-delineated round or oval in cell shape, and had more than five beads attached.

All laboratory work was performed in a blinded fashion with respect to the identity of the samples.

Statistical analysis

All values are expressed as mean $\pm$ s.d., unless otherwise specified. All data passed the normality test ($P>0.05$) as determined by the Kolmogorov–Smirnov test. Preeclamptic and hypertensive patients, healthy pregnants and control groups were assessed by one-way analysis of variance (ANOVA) test. Scheffe *posthoc* test was used to adjust for multiple comparisons where appropriate. Logarithmic transformation was applied to reduce variance among the groups for CECs. Correlations between systolic and diastolic blood pressure, the number of CECs and serum homocysteine levels in each group were studied by Pearson correlation analysis, and P value <0.05 was regarded as statistically significant. For power calculation in a one-way ANOVA study, sample sizes of 20, 15, 15 and 15 are obtained from the four groups whose means are to be compared. The total sample of 65 subjects achieves 83% power to detect differences among the means versus the alternative of equal means using an F-test with a 0.05 significance level. The size of the variation in the means is represented by their standard deviation, which is 1.33. The common standard deviation within a group is assumed to be 3.[13] The sample size required for the study was calculated based on the primary outcome variable, that is the CECs. Although 15 patients seem to be sufficient for the groups, 20 patients in preeclampsia group were arbitrarily recruited. Statistical analysis was performed using SPSS, version 11.5 for Windows (Chicago, IL, USA).

Results

Demographic data and study parameters in the groups are presented in Table 1. No significant difference was observed except for systolic and diastolic blood pressure between the groups.

The number of CECs in preeclamptic patients was significantly higher than those in healthy pregnants, non-pregnant controls and hypertensive patients ($P<0.0001$). Also, there was a significant difference between hypertensive patients and non-pregnant controls ($P=0.001$) (Table 1).

Systolic and diastolic blood pressures in patients with preeclampsia were significantly higher than in healthy pregnants and controls ($P<0.0001$), but were not significantly different from those in hypertensive patients ($P>0.05$). The measured serum homocysteine levels in preeclamptic patients were significantly higher than in healthy pregnants ($P=0.02$), significantly lesser than controls ($P=0.038$), and it was not significant for hypertensive patients ($P>0.05$) (Table 1).

In pregnant subjects with or without preeclampsia, a statistically significant positive correlation was found between the number of CECs and systolic ($P<0.0001$, $r=0.63$) and diastolic ($P<0.0001$, $r=0.64$) blood pressures. Also, significant positive correlation was found between the number of CECs and serum homocysteine level ($P=0.01$, $r=0.56$) in preeclamptic patients. Correlation between homocysteine level and the number of CECs in preeclamptic patients is presented in Figure 2.

Serum uric acid levels in preeclamptic patients (6.6 ± 1.7 mg/dl) was significantly higher than hypertensive patients (4.5 ± 0.6 mg/dl, $P<0.0001$) and healthy pregnants (3.7 ± 0.8 mg/dl, $P<0.0001$). Correlation between serum uric acid levels and the

Table 1 Demographic features and study parameters of the groups

	Preeclampsia, N = *20*	*Hypertension,* N = *15*	*Healthy pregnant,* N = *15*	*Healthy control,* N = *15*	P-*value*[*]
Age (years)	29.1 ± 5.9	29.1 ± 4.1	27.7 ± 4.6	27.0 ± 5.0	NS
Parity	2 (1–8)	2 (1–6)	2 (1–6)	2 (1–5)	NS
The number of previous deliveries	1 (0–6)	1 (0–3)	1 (0–4)	1 (0–3)	NS
Gestational age (weeks)	34.5 ± 0.4	—	33.4 ± 0.5	—	$P > 0.05$
Systolic blood pressure (mm Hg)	143.5 ± 9.8[a,b]	139.5 ± 6.5[c,d]	113.0 ± 9.2[e]	123.7 ± 8.0	$P < 0.0001$
Diastolic blood pressure (mm Hg)	97.0 ± 5.7[f,g]	96.6 ± 5.7[h,i]	68.3 ± 6.7	75.0 ± 6.3	$P < 0.0001$
Homocysteine (μmol/l)	9.5 ± 2.8[j,k]	11.5 ± 2.3[l]	6.0 ± 0.6[m]	12.2 ± 3.3	$P < 0.0001$
CECs (cell/ml)	13.2 ± 5.2[n,o,p]	6.9 ± 0.8[r]	5.2 ± 1.4	4.0 ± 1.8	$P < 0.0001$*
Serum creatinine (mg/dl) (μmol/l)	0.8 ± 0.2 (70 ± 17)	0.8 ± 0.3 (70 ± 26)	0.7 ± 0.2 (61 ± 17)	0.8 ± 0.2 (70 ± 17)	NS

Abbreviations: CECs, circulating endothelial cells; NS, not significant.
[a]$P < 0.0001$ vs preeclampsia, [b]$P < 0.0001$ vs healthy control, [c]$P < 0.0001$ vs healthy pregnant, [d]$P = 0.001$ vs healthy control, [e]$P = 0.026$ vs healthy control, [f]$P < 0.0001$ vs healthy pregnant, [g]$P < 0.0001$ vs healthy control, [h]$P < 0.0001$ vs healthy pregnant, [i]$P < 0.0001$ vs healthy control, [j]$P = 0.02$ vs healthy pregnant, [k]$P = 0.038$ vs healthy control, [l]$P < 0.0001$ vs healthy pregnant, [m]$P < 0.0001$ vs healthy control, [n]$P < 0.0001$ vs hypertension, [o]$P < 0.0001$ vs healthy pregnant, [p]$P < 0.0001$ vs healthy control, [r]$P = 0.001$ vs healthy control *calculated from log-transformed CECs values.

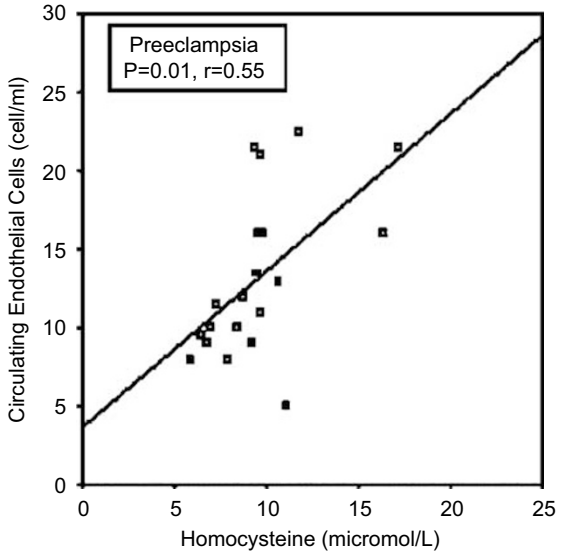

Figure 2 Correlation between homocysteine level and the number of CECs in preeclamptic patients.

number of CECs was not significant for all groups. In pregnant patients with preeclampsia, serum albumin levels (2.6 ± 0.4 g/dl) was significantly lesser than healthy pregnants (3.2 ± 0.2 g/dl, $P < 0.0001$) and patients with hypertension (4.1 ± 0.3 g/dl, $P < 0.0001$). No correlation was found between CECs and albumin levels in all groups.

Discussion

In the present study, we demonstrated that the number of CECs was significantly increased in preeclamptic patients in comparison with healthy pregnants, hypertensive patients and non-pregnant controls. In addition, plasma homocysteine level was significantly associated with CECs in preeclamptic patients.

In recent years, the number of CECs has been reported to be increased in various immune-mediated diseases including TTP, Behçet's disease, systemic lupus erythematosus and vasculitis.[5–8] In their study, Koc *et al.*[14] reported that an increase in CECs in haemodialysis patients seems to predict the development of cardiovascular and vascular events, and to be independent of other known markers of inflammation or endothelial dysfunction. Preeclampsia is a disease in which impaired maternal endothelial function seems to be central in the pathogenesis and has been observed to persist for several years following the preeclamptic pregnancy. In their study, Ronnback *et al.*[15] demonstrated that pulse wave reflection and thus, systemic arterial stiffness were increased in pregnant women with preeclampsia. Moreover, sVCAM-1, sICAM-1, E-selectin, von Willebrand factor (vWf), fibronectin, factor VII antigen and thrombomoduline were found to be significantly increased in severe preeclamptic patients when compared with normal pregnant subjects.[3] The results of these studies support the concept of generalized vascular dysfunction in preeclampsia. In this regard, we thus hypothesized that CECs, as a new marker for endothelial injury, can be increased in preeclampsia and that this new marker can be further used for determining the degree of the disease activity. This study is the first report evaluating CECs in preeclampsia.

Several mechanisms that may explain the relationship between hyperhomocysteinemia and cardiovascular disease have been suggested; however, endothelial dysfunction may be one of the most important. A meta-analysis including 25 investigations evaluated the relationship between plasma homocysteine and preeclampsia. Serum homocysteine level was found to be higher in preeclamptic patients than in uncomplicated pregnancies; however, the results were heterogeneous. In eight studies, serum homocysteine levels were measured

consecutively during the pregnancy (before preeclampsia) and in 17 of them plasma homocysteine levels were evaluated after manifestation of preeclampsia. In these studies, plasma homocysteine concentrations were mildly elevated in normotensive pregnancies before the development of preeclampsia and significantly elevated once preeclampsia was established.[16] In our study, the serum homocysteine level was significantly increased in preeclamptic patients compared with normal pregnants. However, healthy pregnant subjects had a lower level of homocysteine than nonpregnant subjects, as expected. In preeclamptic patients, plasma homocysteine level was found to be associated with the number of CECs, and this can be a finding to support that in hyperhomocysteinemia even a mild elevation during the preeclampsia seems to be related to some extent to the endothelial injury.

In this study, blood pressures were significantly higher in preeclampsia, and both systolic and diastolic blood pressures were found to be associated with CECs in pregnant subjects. In previous studies, it was demonstrated that blood pressure is one of the most important prognostic factors for preeclampsia and it predicts the severity of the disease and complications. It is also a good indicator of the endothelial dysfunction in preeclampsia. Thus, demonstrating such a relation between blood pressure and CECs in our study appears to be meaningful.

In preeclamptic patients, the degree of hyperuricemia and hypoalbuminemia might have a prognostic significance;[17,18] however, we did not show any association between CECs and uric acid or albumin. Longitudinal study might be more suitable for comparison of these factors.

Our study evaluated preeclamptic and healthy pregnants in late gestation. The next step is to investigate women from early gestation with serial blood sampling to determine whether or not CECs are increased before preeclampsia develops clinically. Such an investigation would also allow us to determine if an increase in CECs would identify women at risk of preeclampsia at a later gestational age. The relationship between CEC number and severity of preeclampsia is also not known.

There are several limitations of this study. As the number of cases is small and cross-sectional, the accuracy of the results must be verified in future longitudinal studies containing a large number of cases. In addition, we do not know the optimal cut-off value for the number of CECs indicating endothelial injury, as this has not been determined previously. In this study, our patients had a mild form of preeclampsia and they were presented with proteinuria and hypertension. Therefore, more severe cases of preeclampsia with different clinical forms and even eclampsia cases should be further investigated in future studies of CECs.

What is known about this topic

- Several studies have supported the concept of generalized vascular dysfunction in preeclampsia.[1,2]
- Serum homocysteine level was found to be higher in preeclamptic patients than in uncomplicated pregnancies.[12]
- An increase in the number of CECs has been used for assessment of vascular damage and it seems to be a marker of disease activity in recent years.[5,7]

What this study adds

- This study shows that CECs as a marker of endothelial injury were significantly higher in patients with preeclampsia than in hypertensive patients, healthy pregnants and normal controls.
- At the same time, plasma homocysteine level was increased in preeclamptic patients in concordance with the CECs.

Conflict of interest

None declared.

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