Alterations of Circulating Endothelial Cell and Endothelial Progenitor Cell Counts around the Ovulation

Sunao Tanaka, Takayuki Ueno, Fumiaki Sato, Yoshitsugu Chigusa, Nobuko Kawaguchi-Sakita, Masahiro Kawashima, Noriyoshi Fujisawa, Kenichi Yoshimura, Satoshi Teramukai, Hiroshi Fujiwara, Masatoshi Fujita, and Masakazu Toi

Departments of Surgery (Breast Surgery) (S.T., T.U., N.K.-S., M.K., N.F., M.T.), Target Therapy Oncology (F.S.), Gynecology and Obstetrics (Y.C., H.F.), and Clinical Trial Design and Management, Translational Research Center (K.Y., S.T.), and Human Health Sciences (M.F.), Graduate School of Medicine, Kyoto University, 54 Kawaharacho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Context: Circulating endothelial cells (CECs) and progenitor cells (CEPs) have been intensively studied as a promising tool for treating ischemic diseases and monitoring cancer treatments, but how the menstrual cycle affects the variation in their counts remains unclear.

Objective: The aims of the study were to determine the influence of the menstrual cycle on the number of CECs and CEPs and to investigate the association of their counts with circulating hormones and angiogenesis-associated factors.

Design: CEP and CEC counts by flow cytometry and the CellSearch system and circulating factor levels were measured eight times during the menstrual cycle in 18 volunteers. The menstrual cycle was divided into six phases based on hormone concentrations.

Results: CEP counts peaked in the periovulatory and middle luteal phases with a drop in the early luteal phase. CEC counts showed no significant variation. There were significant correlations between the CEP counts and the serum concentrations of estradiol (E2), LH, and granulocyte colony-stimulating factor (G-CSF) ($P < 0.0001$, $P < 0.0001$, and $P = 0.01$, respectively). The difference in CEP counts between two adjacent phases was significantly correlated with that in E2, LH, G-CSF, and serum vascular endothelial growth factor ($P < 0.0001$, $P < 0.0001$, $P = 0.02$, and $P = 0.006$, respectively).

Conclusion: CEP counts peaked in the periovulatory and middle luteal phases, with a drop in the early luteal phase, and were correlated with serum E2, LH, and G-CSF concentrations. Consideration of the variation in CEP counts would be important for the clinical application of CEPs. **(*J Clin Endocrinol Metab* 97: 4182–4192, 2012)**

The vessel wall controls vascular homeostasis via lining cells and circulating bone marrow-derived progenitor cells. Over the last decade, various experimental studies have demonstrated that circulating bone marrow-derived progenitor cells are capable of differentiating into mature, functional endothelial cells and inducing neovascularization at the level of the ischemic tissue, the growing tumor, and the site of wound healing (1, 2). In particular, a subset of circulating premature cells, designated as circulating endothelial progenitor cells (CEPs), is considered to contribute to endothelial cell regeneration and neovascularization.

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2012-1736 Received March 21, 2012. Accepted August 14, 2012.
First Published Online September 4, 2012

Abbreviations: BBT, Basal body temperature; CEC, circulating endothelial cell; CEP, circulating endothelial progenitor cell; E2, estradiol; eL, early luteal phase; Epo, erythropoietin; F, follicular phase; G-CSF, granulocyte colony-stimulating factor; lL, late luteal phase; M, menstrual phase; mL, middle luteal phase; O, periovulatory phase; PlGF, placental growth factor; VEGF, vascular endothelial growth factor; sVEGFR1, soluble VEGF receptor 1.

CEPs are defined as nonendothelial cells that display clonal expression and stemness characteristics and differentiate into endothelial cells (3). These cells play multifaceted regulatory roles in the adult vascular system and participate in a number of physiological functions including homeostasis, ischemic tissue vasculogenesis, and tumor angiogenesis (4). CEPs were discovered in the 1970s in a study designed to detect cells that possess the ability to repair injured denuded vasculature (5). In 1997, CEPs isolated from peripheral blood were demonstrated to incorporate into foci of neovascularization in the adult, promoting processes such as regenerative neoangiogenesis (1). Recently, CEPs have been studied as a promising tool for treating ischemic disease and cancer. It has been reported that the introduction of vascular endothelial growth factor (VEGF)-transduced CEPs into the circulation significantly improved blood flow recovery and capillary density in an ischemic mouse model (6). Transplantation of adult progenitor cells has a beneficial effect on postinfarction remodeling processes in patients with acute myocardial infarction (7). In animal tumor models, interferon-β-transduced CEPs attenuated tumor growth (8). To use CEPs in clinical situations, efficient collection of CEPs from donors would be required. CEPs are mobilized from the bone marrow to the circulation in response to cytokines, growth factors, and hormones. It has been reported that some factors such as VEGF, granulocyte colony-stimulating factor (G-CSF), and erythropoietin (Epo) can induce the differentiation of mobilized stem cells into CEPs and subsequently enhance CEP proliferation, migration, and homing to peripheral tissues (9–11). Furthermore, it is known that changes in CEP counts are affected by hormonal status, body weight, and factors associated with lifestyle such as smoking, exercise, weight, caffeine, and alcohol consumption (12). The ovarian sex steroid hormones estrogen and progesterone are primarily uterotrophic and control the cyclical patterns of uterine cell proliferation and vascular growth that occur throughout the menstrual cycle. Given the synchronized nature of cyclic vascularization, it is assumed that angiogenesis-associated growth factor expression is induced by steroid hormones to regulate blood vessel formation including that of CEPs in reproductive organogenesis (13, 14). However, whether the cyclical systemic variations of these factors during the menstrual cycle is linked to the variation in CEP counts is unknown.

Circulating endothelial cells (CECs) are considered as mature endothelium and generated by vascular turnover. We reported that CEC counts altered during chemotherapy in breast cancer patients and that their counts were associated with the therapeutic response (15). CECs and CEPs have also been studied as monitoring marker for treatment response (16). Thus, it is important to clarify the physiological variation of CECs and CEPs for the clinical application.

The aims of this study were to determine the influence of the menstrual cycle on the CEP and CEC counts and to investigate the association of their counts with circulating sex steroid or angiogenesis-associated factor levels during the normal menstrual cycle.

Subjects and Methods

Study population

This study was approved by the institutional ethical committee, and oral and written informed consent was obtained from all volunteers. Eighteen menstruating women (ages, 23–51 yr; average, 36.3 yr) were recruited for this study. All volunteers were nonsmokers.

Study design

Peripheral blood samples were collected eight times during the menstrual cycle to perform CEP and CEC counts and to measure the concentrations of hormones and angiogenesis-associated factors from each volunteer. The menstrual cycle was divided into the following six phases based primarily on hormone concentrations [estradiol (E2), progesterone, LH], basal body temperature (BBT), and the period of menstruation: 1) menstrual (M) phase—the period of menstruation as declared by the volunteers; 2) follicular (F) phase—the phase between the menstrual phase and the periovulatory phase determined by the criterion described below; the first E2 peak is observed during the follicular phase; 3) periovulatory (O) phase— estimated ovulation based on hormone levels (immediately after LH surge, after the first E2 peak, before elevation of progesterone levels) and BBT (during elevation of BBT). The luteal phase was determined by the phase between the periovulatory phase and the menstrual phase in addition to the progesterone peak, the second E2 peak, high BBT, and the approximate duration of the luteal phase (approximately 14 d $\pm$ 2 d).

The luteal phase was divided into the following three phases: 4) early luteal (eL) phase—the first third of the luteal phase; 5) middle luteal (mL) phase—the middle third of the luteal phase; and 6) late luteal (lL) phase—the last third of the luteal phase.

Blood was drawn into 10-ml CellSave tubes (Veridex, LLC, North Raritan, NJ) for CEP and CEC counts, 5-ml vacuum tubes (Terumo Corporation, Tokyo, Japan) for serum measurements, and 3-ml EDTA tubes (Terumo Corporation) for plasma measurements.

Sample preparation

Serum and plasma were obtained from 18 and eight volunteers, respectively. Specimens were centrifuged at 3000 rpm for 15 min at 4 C. All samples were aliquoted after collection and frozen at −80 C until use.

Growth factor, cytokine, and chemokine detection

Plasma concentrations of VEGF and SDF-1α and serum concentrations of VEGF, soluble VEGF receptor 1 (sVEGFR1), sVEGFR2, G-CSF, Epo, placental growth factor (PlGF), endoglin,

and Tie-2 were determined using ELISA kits according to the manufacturers' instructions. The Epo ELISA kit was purchased from Uscn Life Science Inc. (Wuhan, China), and the other ELISA kits were purchased from R&D Systems (Minneapolis, MN). The coefficient of variation was 4.6–6.5% for VEGF, 3.4–3.9% for SDF-1α, 2.6–3.8% for sVEGFR1, 2.9–4.2% for sVEGFR2, 1.1–2.8% for G-CSF, 3.6–7.0% for PlGF, 2.8–3.2% for endoglin, and 4.4–5.0% for Tie-2. The cross-reactivity with human proteins was reported not to be significant for VEGF, SDF-1α, sVEGFR1, sVEGFR2, G-CSF, Epo, PlGF, endoglin, and Tie-2. The VEGF ELISA was reported to be interfered with by recombinant VEGFR1 and -2 at the concentration of 500 and 4000 pg/ml, respectively. Absorbance was read at 450 nm using a microplate reader (SpectraMax; Molecular Devices, Sunnyvale, CA). Samples were measured in duplicate.

Measurement of hormone concentrations

Serum concentrations of E2, progesterone, FSH, and LH were measured by an electrochemiluminescence immunoassay method in a Modular E170 automatic analyzer (Roche Diagnostics, Mannheim, Germany). The coefficient of variation for each hormone was less than 10%.

Flow cytometry

Mononuclear cells were isolated by density centrifugation using Ficoll–Paque Plus gradients (GE Healthcare Bio-Science, Uppsala, Sweden). The mononuclear cells were rinsed twice with PBS and then incubated with allophycocyanin-labeled monoclonal antihuman CD31 antibody (clone AC 128), fluorescein isothiocyanate-conjugated monoclonal antihuman CD34 antibody (clone AC136), phycoerythrin-conjugated monoclonal antihuman CD133 antibody (clone AC133) (all from Miltenyi Biotec, Bergisch Gladbach, Germany), and peridinin chlorophyll protein-conjugated monoclonal antihuman CD45 antibody (clone 2D1; BD Biosciences, San Jose, CA). Samples were finally resuspended in 400 μl of PBS and measured with a FACSCalibur flow cytometer (BD Biosciences). The following populations were considered: CEPs, defined as $CD31^+CD34^+CD133^+CD45^{dim}$ cells, and CECs, defined as $CD31^+CD45^-$ cells. Data were expressed as cells per million mononuclear cells (17, 18).

CellSearch system

The standardized CellSearch technique (Veridex, LLC) has been reported previously (15). Briefly, CECs expressing CD146 were immunomagnetically enriched and stained with the nuclear dye 4′,6-diamidino-2-phenylindole, CD105, and CD45. A fluorescein isothiocyanate-conjugated anti-CD34 antibody (clone AC136; Miltenyi Biotec) was used in the additional channel of the system. Analysis was performed using an image cytometer, for which CECs were defined as $CD146^+CD105^+CD45^-$ cells.

Basal body temperature and the period of menstruation

Each volunteer measured her BBT every morning throughout the menstrual cycle including the days of blood collection. The period of menstruation was recorded in the body temperature records.

Statistical analysis

Continuous variables are described as mean ± SD. The highest value in each phase was used for the analysis if two or more measurements were performed in one phase. Data were adjusted by dividing each value by the mean value of each factor in each woman excluding hormone concentrations because variation in each factor between individuals was large and this adjustment increased statistical stability, resulting in statistical reliability. Statistical analysis was performed using Wilcoxon's test for comparisons between phases of the menstrual cycle and Pearson's correlation coefficient for the correlations between factors. All analyses were performed using the JMP version 9.0.0 software package (SAS Institute, Inc., Cary, NC). All P values were two-sided, and $P < 0.05$ was considered statistically significant. For multiple comparisons, the Bonferroni correction was applied, and in the figures, results with statistical significance by the Bonferroni correction are shown in *italics* and results with $P < 0.05$ are shown in a *nonitalic font*. The permutation under the null correlation was performed to further assess the association of cyclic variation in each factor. A 100,000-permutation test was performed except for the serum concentration of Epo and the plasma concentrations of VEGF and SDF-1α due to the small sample number.

Results

Serum hormone levels during the menstrual cycle

Serum hormone levels were measured in 18 women volunteers, of whom two women exhibited a perimenopausal hormonal status, one exhibited an anovulatory hormonal pattern, three did not exhibit a normal hormonal pattern, and one took oral contraceptives. Thus, a total of 11 women were included in the subsequent analyses (ages, 23–45 yr; average, 36.3 yr). BBT in each individual is shown in Supplemental Fig. 1 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Cyclic alterations of circulating pituitary and ovarian hormones were confirmed by the periovulatory peaks for serum E2 and LH concentrations and by the middle luteal increases for serum E2 and progesterone concentrations (Fig. 1).

Alterations of CEP and CEC counts during the menstrual cycle determined by flow cytometry

CEP counts were higher in the O and mL phases than in the other phases, indicating that CEP counts dropped in the eL phase after ovulation (O *vs.* eL, $P = 0.003$; and eL *vs.* mL, $P = 0.01$; Fig. 2A). Differences in CEP counts between two adjacent phases (ΔCEP) were identified by two peaks in "O–F" and in "mL–eL" (Fig. 2B).

The CEC counts and the differences of CEC counts between two adjacent phases (ΔCEC) did not exhibit sig-

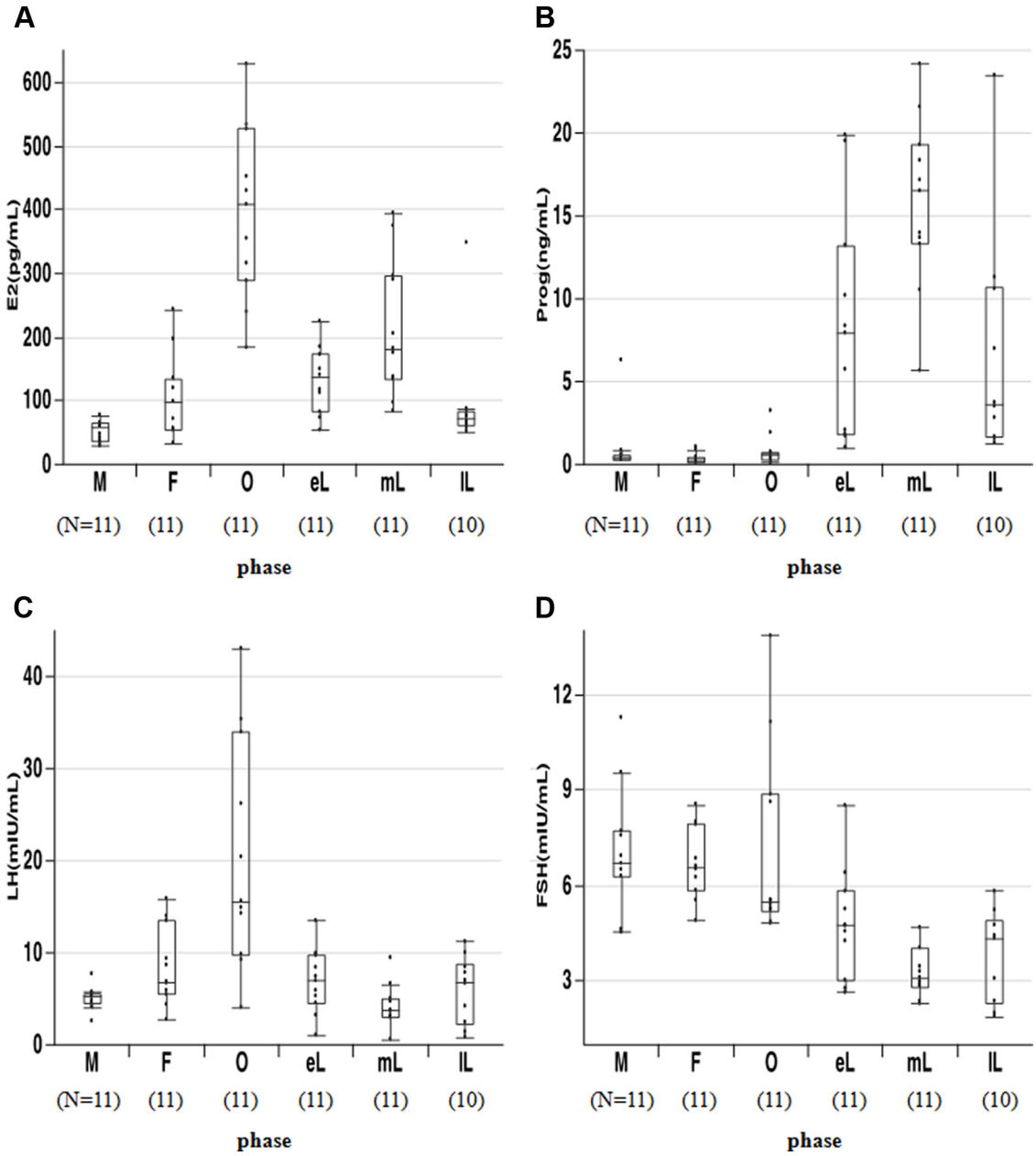

FIG. 1. Serum hormone levels during the menstrual cycle (A, E2; B, progesterone; C, LH; D, FSH). Normal menstrual alterations are indicated by the O phase peaks in serum E2 and LH concentrations, and the mL phase increases in serum E2 and progesterone (Prog) concentrations. The individual values are presented as *dots*.

nificant alterations during the menstrual cycle (Fig. 2, C and D).

Alterations of CEC counts during the menstrual cycle determined by the CellSearch system

Alterations of CEC counts by the CellSearch system were examined. CEC counts were increased in the mL phase (eL *vs.* mL, $P = 0.02$; mL *vs.* lL, $P = 0.03$; and mL *vs.* M, $P = 0.04$; Fig. 2E). ΔCEC in "mL–eL" was larger than those between other phases (Fig. 2F). The alterations of CD34-positive CEC counts as determined by the CellSearch system were similar to those of the CEC counts by the CellSearch system (data not shown).

Alterations of angiogenesis-associated factor levels during the menstrual cycle

The concentrations of angiogenesis-associated factors during the menstrual cycle are shown in Table 1. The concentration of VEGF in serum was significantly lower in the lL phase (O *vs.* lL, $P = 0.003$; M *vs.* lL, $P = 0.01$; and eL *vs.* lL, $P = 0.01$; Fig. 3A). The concentration of VEGF in plasma was lower in the F phase than in the other phases (M *vs.* F, $P = 0.02$; F *vs.* O, $P = 0.03$; and F *vs.* lL, $P = 0.045$; Fig. 3C). The concentrations of serum G-CSF varied throughout the menstrual cycle. The concentration of G-CSF was higher in the O phase than in the other phases (O *vs.* M, $P = 0.042$; Fig. 3E).

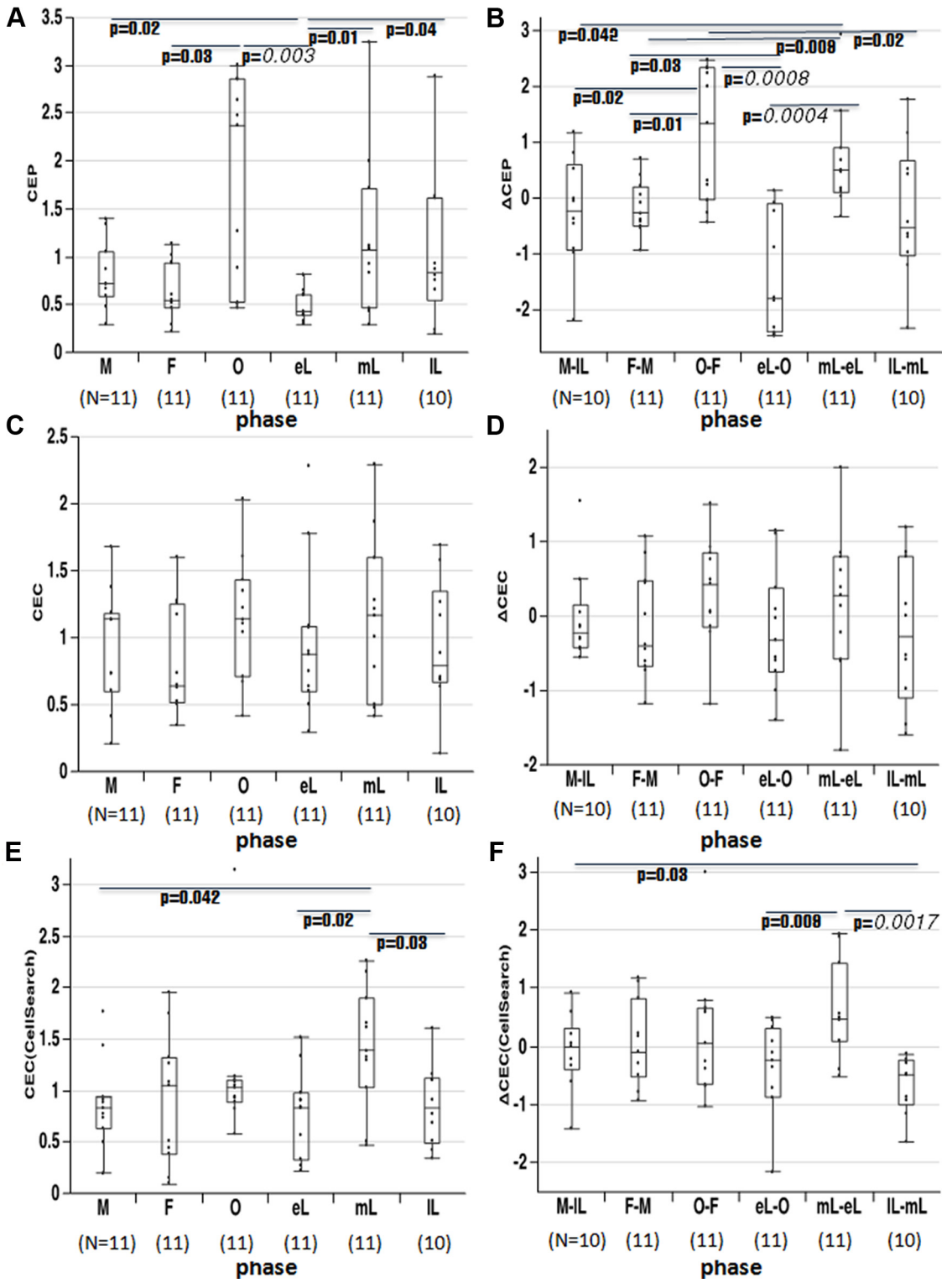

FIG. 2. The alterations of CEP and CEC counts during the menstrual cycle. The counts of CEPs, defined as $CD31^{+}CD34^{+}CD133^{+}CD45^{dim}$ cells, increased in the O and mL phases (A), and the CEC counts determined by the CellSearch system increased in the mL phase (E). The counts of CEC, defined as $CD31^{+}CD45^{-}$ cells, did not exhibit such a pattern (C). The difference in counts between two adjacent phases was indicated by Δ (ΔCEP and ΔCEC) (B, D, and F). *P* values with statistical significance by the Bonferroni correction are shown in *italics*.

TABLE 1. Angiogenesis-associated factor concentrations during phases of the menstrual cycle

	M	F	O	eL	mL	lL
n	11	11	11	11	11	10
Serum						
G-CSF (pg/ml)	21.95 ± 10.17	24.79 ± 23.99	30.95 ± 14.42	27.85 ± 14.02	28.88 ± 12.60	20.41 ± 10.89
VEGF (pg/ml)	303.22 ± 211.50	266.69 ± 164.65	304.37 ± 164.65	297.03 ± 187.33	269.42 ± 163.80	257.93 ± 177.37
Endoglin (ng/ml)	2.80 ± 1.00	2.68 ± 1.05	2.57 ± 1.18	2.44 ± 0.94	2.58 ± 1.02	2.59 ± 1.26
sVEGFR1 (pg/ml)	83.54 ± 34.88	76.97 ± 27.81	78.11 ± 32.35	73.10 ± 33.78	83.34 ± 27.34	78.81 ± 30.57
sVEGFR2 (pg/ml)	7,993.05 ± 5,016.75	5,969.84 ± 1,952.22	6,344.88 ± 1,445.76	6,128.06 ± 2,056.42	5,946.17 ± 1,684.39	5,976.91 ± 1,663.18
Tie-2 (ng/ml)	13.98 ± 1.91	11.73 ± 1.50	12.31 ± 1.58	12.05 ± 1.50	12.95 ± 1.78	12.64 ± 1.85
PlGF (pg/ml)	11.25 ± 1.23	11.24 ± 1.29	11.00 ± 0.96	10.03 ± 1.33	10.54 ± 0.87	10.28 ± 1.40
Epo (pg/ml)[a]	1,318.0 ± 1,833.7	8,245.4 ± 22,503.3	8,015.4 ± 2,138.8	17,451.1 ± 46,226.6	3,157.4 ± 7,188.2	3,964.6 ± 9,500.7
Plasma						
SDF-1α (pg/ml)[b]	2,351.45 ± 304.73	2,265.12 ± 423.59	2,265.82 ± 421.437	2,235.64 ± 358.534	2,292.49 ± 470.017	2,255.68 ± 471.45
VEGF (pg/ml)[b]	33.74 ± 4.49	26.02 ± 3.68	34.36 ± 1.93	29.93 ± 4.84	29.95 ± 3.53	36.15 ± 6.55

Values are reported as mean ± SD.

[a] Epo levels were not measured in one volunteer.

[b] The concentrations of SDF-1α and VEGF in plasma were measured in six samples.

The concentrations of other factors including PlGF, SDF-1α, sVEGFR1, sVEGFR2, Epo, endoglin, and Tie-2 did not exhibit any significant patterns during the menstrual cycle (Table 1).

Correlations between CEP counts and the concentrations of hormones and other angiogenesis-associated factors during the menstrual cycle

There were correlations between CEP counts and E2, LH, and G-CSF concentrations ($r = 0.47$, $P < 0.0001$, in Fig. 4A; $r = 0.46$, $P < 0.0001$, in Fig. 4C; $r = 0.32$, $P = 0.01$, in Fig. 4E, respectively). Correlations between ΔCEP and ΔE2, ΔLH, ΔG-CSF, and ΔVEGF were also observed ($r = 0.58$, $P < 0.0001$, in Fig. 4B; $r = 0.49$, $P < 0.0001$, in Fig. 4D; $r = 0.30$, $P = 0.02$, in Fig. 4F; $r = 0.34$, $P = 0.006$, in Fig. 4H, respectively). There was also a correlation between the concentration of G-CSF and that of E2 ($r = 0.31$; $P = 0.01$; data not shown). The permutation test further supported the correlation of CEP counts with E2, LH, and G-CSF concentrations ($P < 0.001$, $P = 0.025$, and $P = 0.014$, respectively) (Table 2). In addition, CEP counts exhibited correlations with serum VEGF and PlGF levels ($P = 0.016$ and $P = 0.008$, respectively) (Table 2).

Discussion

Components of the female reproductive system undergo a number of programmed angiogenic processes that are coupled with the cyclic evolution and decline of the ovaries and endometrium. In the normal ovary, angiogenesis likely plays an important role in folliculogenesis, ovulation, and corpus luteum function. At the time of ovulation, the basement membrane separates the highly vascular theca cell layer from the avascular granulosa cell layer lining the inside of the follicular degenerates, and dynamic capillary growth occurs directed inward from the theca. Consequently, the most intensive angiogenesis can be detected during the periovulatory period. The dynamic growth is completed by the end of the third day after ovulation, and this may explain the O phase peak and the subsequent drop of CEP counts in the eL phase, which may be a reflection of CEP consumption during the ovulatory phase. A previous study determined that vascular space within the human corpus luteum increased from the eL to mL phase, which may explain the mL phase increase of CEPs (19). Similar to the ovary, the endometrial vascular architecture changes throughout the menstrual cycle in parallel to the changes in the uterine epithelium and stroma. During the late F phase and throughout the luteal phase, a complex subepithelial capillary plexus develops, which may also be associated with the mL phase peak of CEP counts. Because CEPs are derived from the bone marrow but CECs are from existing vasculature, CECs do not seem to show a changing pattern similar to that of CEPs.

Recent studies have provided evidence that the physiological cycle of estrogen regulates CEP kinetics; *i.e.* differentiation, proliferation, migration, apoptosis, mobilization, and ultimately incorporation into foci of neovascularization in the developing endometrium (20, 21). It is known that estrogen increases the growth of cultured CEPs isolated from human peripheral blood. In humans, CEP counts are increased by hormone replacement therapy consisting of an E2-based medication and the progesterone-derived drug norethisterone (22). Consistently, premenopausal women have higher CEP counts than postmenopausal women (22). E2 also affects the production of key proangiogenic factors such as VEGF (23) and G-CSF (24), which have been suggested to increase CEP counts. These findings support our result of a significant correlation between CEP counts and serum E2 concentrations.

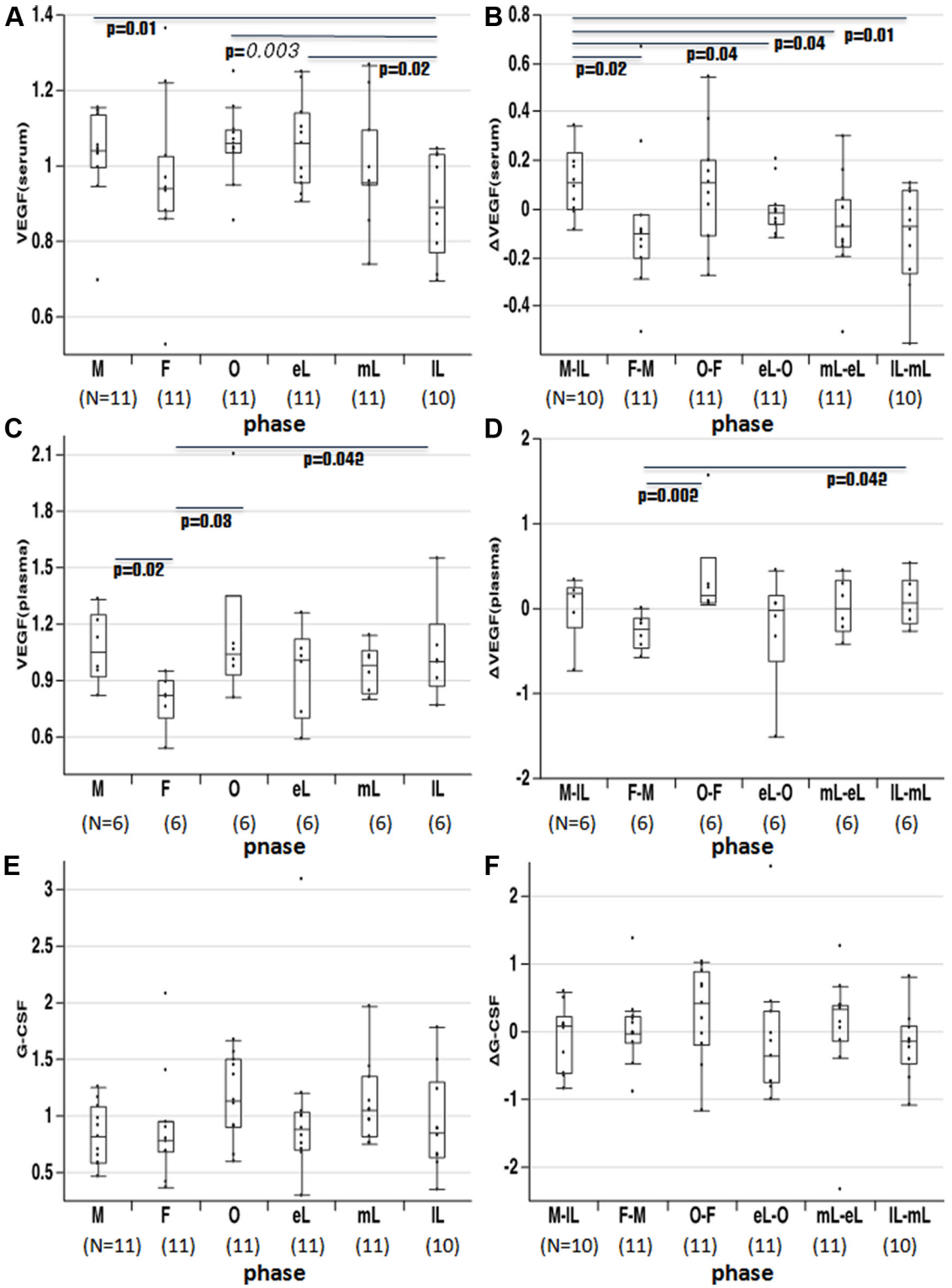

FIG. 3. The alteration of VEGF in plasma and serum and G-CSF levels during the menstrual cycle. The level of VEGF in plasma was lower in the F phase (C). The level of VEGF in serum was higher in the O phase and lower in the lL phase (A). The levels of G-CSF increased in the O phase (E). The difference in concentrations between two adjacent phases was indicated by Δ (ΔVEGF and ΔG-CSF) (B, D, and F). *P* values with statistical significance by the Bonferroni correction are shown in *italics*.

VEGF is a protein with growth-promoting activity in vascular endothelial cells. Several studies suggest that VEGF plays important roles in angiogenesis in the female reproductive tract (25, 26). It is known that estrogen increases VEGF expression in uterine tissues, endothelial cells, and vascular smooth muscle cells (27, 28). In this study, the levels of VEGF in plasma decreased in the F phase and increased in the O phase; this is similar in pat-

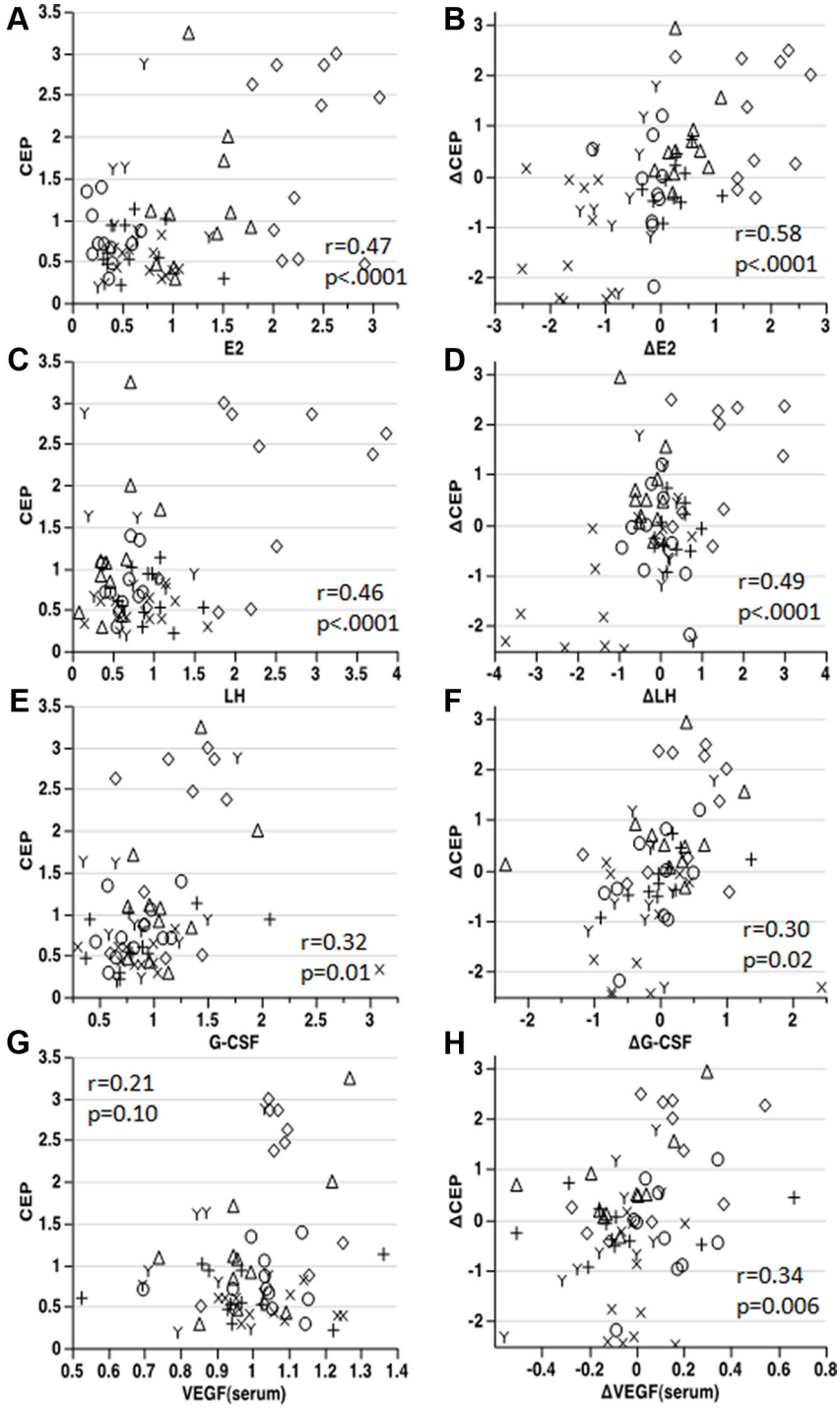

FIG. 4. Correlation between CEP counts and the levels of hormones and angiogenesis-associated factors. There were correlations between CEP counts and serum E2 (A), LH (C), and G-CSF (E) concentrations ($P < 0.0001$, $P < 0.0001$, and $P = 0.01$, respectively). No correlation was observed between CEP counts and serum VEGF concentrations (G) ($P = 0.10$). There were correlations between differences in CEP between two adjacent phases (ΔCEP) and differences in serum E2 (ΔE2) (B), LH (ΔLH) (D), G-CSF (ΔG-CSF) (F), and VEGF (ΔVEGF) (H) concentrations between two phases ($P < 0.0001$, $P < 0.0001$, $P = 0.02$, and $P = 0.006$, respectively). A, C, E, and G, ○ = M, + = F, ◇ = O, × = eL, △ = mL, Y = lL. B, D, F, and H, ○ = M–lL, + = F–M, ◇ =O–F, × = eL–O, △ = mL–eL, Y = lL–mL.

tern to that of the variation of E2 levels. Because there are some conflicting results regarding VEGF variations throughout the menstrual cycle (29), further studies are required to investigate the association of the variation of VEGF levels with the menstrual cycle.

In agreement with our finding, some studies reported that serum G-CSF concentrations were higher in the ovulatory phase than in the other phases (30, 31), suggesting that G-CSF is associated with ovulation. We also found that G-CSF levels were correlated with E2 levels. A previous study demonstrated that mice injected ip with E2 exhibited increased levels of G-CSF in the bronchoalveolar lavage, suggesting that E2 affects G-CSF levels *in vivo* (24). In this study, the alteration of G-CSF levels was similar to that of CEP counts (Fig. 4). Mice that received recombinant human G-CSF exhibited elevated CEP counts (11, 32). Patients with chronic ischemic heart disease who receive G-CSF have increased CEP counts (33). G-CSF was reported to activate the VEGF/VEGFR1 pathway and to promote CEP mobilization *in vivo* (34). These findings raise the possibility that G-CSF increases CEP counts either directly or indirectly.

Recently, the clinical applications of CEPs were investigated. It was reported that autologous progenitor cell transplantation can be used for the treatment of patients with acute myocardial infarction (7). CEPs also have potential uses in anticancer treatment as a vehicle for the delivery of toxic genes, suicide genes, anticancer drugs, and angiogenesis inhibitors (35). The efficient collection of CEPs would be critical for such clinical applications. Our results suggest that consideration of the menstrual cycle (*e.g.* collection in the mL phase) would increase the efficiency of CEP collection. In addition, CEPs and CECs have been studied as biomarkers for tumor progression and for monitoring therapeutic effects (15, 16). The consideration of the physiological variation of CEPs is required for such

TABLE 2. Permutation test

	CEP												
CEC	0.019	CEC											
CellSearch CEC	0.006	0.097	CellSearch CEC										
E2	<0.001	0.067	<0.001	E2									
P	0.321	0.263	0.181	0.345	P								
FSH	0.391	0.125	0.075	0.182	<0.001	FSH							
LH	0.025	0.272	0.459	0.001	0.011	<0.001	LH						
G-CSF	0.014	0.141	0.267	0.001	0.138	0.262	0.377	G-CSF					
serum VEGF	0.016	0.460	0.316	0.133	0.453	0.027	0.132	0.145	serum VEGF				
PlGF	0.008	0.014	0.453	0.297	0.066	0.005	0.064	0.039	0.112	PlGF			
Tie2	0.457	0.471	0.060	0.380	0.133	0.072	0.444	0.185	0.122	0.420	Tie2		
endoglin	0.130	0.418	0.025	0.228	0.188	0.195	0.121	0.335	0.220	0.247	0.271	endoglin	
sVEGFR1	0.186	0.414	0.401	0.446	0.404	0.063	0.276	0.014	0.428	0.004	0.375	0.072	sVEGFR1
sVEGFR2	0.085	0.405	0.465	0.239	0.275	0.121	0.477	0.306	0.029	0.186	0.002	0.034	0.036

P, Progesterone.

applications. However, because it is unknown whether the alterations of CEP counts in patients with ischemic disease or cancer are similar to those in healthy people, such studies are warranted for clinical considerations.

There are inconsistent results regarding the variation of CEP levels during the menstrual cycle (36–40). These differences could be due to differences in determining the phases in the menstrual cycle, the frequency of blood collection, identifying markers for CEPs, standardization of the data, and selection of healthy women with a normal menstrual cycle. Our study employed the most frequent sampling among studies reported, enabling more reliable data in terms of determining the phases of the menstrual cycle. The frequent collection also enabled disclosure of more precise alterations of CEP counts and angiogenesis-associated factors during the menstrual cycle. There is a controversy regarding the surface markers that identify CEPs. The identification of a unique combination of markers specific and selective for primary endothelial progenitor cells would promote studies on the clinical and biological features of CEPs.

One of the biggest limitations of this study is its small sample size. This study recruited 18 healthy volunteers, which is the largest number of volunteers among reported studies, and seven volunteers who did not show a normal hormonal pattern were excluded from the analysis because we believe that strict determination of the menstrual cycle is crucial for the reliable analysis. The large variation in each factor between individuals is another limitation. To increase the statistical stability and the reliability of data, we have adjusted the data by dividing each value by the mean value in each individual. In addition, to avoid false significance, we have used the Bonferroni correction and performed different statistical analyses to corroborate the analytical results. Additional research with a larger sample size is necessary to validate the present study.

In conclusion, we demonstrated that CEP counts peaked in the O and mL phases with a drop in the eL phase, and were closely correlated with serum E2, LH, and G-CSF concentrations. Additional studies are required to further clarify the clinical and biological significance of the fluctuations of CEP counts during the menstrual cycle.

Acknowledgments

We thank all the volunteers who participated in the study. We are grateful to Ms. Masako Ishida for her help in sampling.

Address all correspondence and requests for reprints to: Takayuki Ueno, Department of Surgery (Breast Surgery), Kyoto University, 54 Kawaharacho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: takayuki@kuhp.kyoto-u.ac.jp.

This work was supported by the Innovative Techno-Hub for Integrated Medical Bio-imaging Project of the Special Coordination Fund for Promoting Science and Technology, from the

Ministry of Education, Culture, Sports, Science and Technology, Japan.

Disclosure Summary: The authors have nothing to declare.

References

1. **Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM** 1997 Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967
2. **Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM** 1999 Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85: 221–228
3. **Urbich C, Dimmeler S** 2004 Endothelial progenitor cells functional characterization. Trends Cardiovasc Med 14:318–322
4. **Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T** 1999 Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 5:434–438
5. **Ausprunk DH, Knighton DR, Folkman J** 1975 Vascularization of normal and neoplastic tissues grafted to the chick chorioallantois. Role of host and preexisting graft blood vessels. Am J Pathol 79: 597–618
6. **Iwaguro H, Yamaguchi J, Kalka C, Murasawa S, Masuda H, Hayashi S, Silver M, Li T, Isner JM, Asahara T** 2002 Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation 105:732–738
7. **Leistner DM, Fischer-Rasokat U, Honold J, Seeger FH, Schächinger V, Lehmann R, Martin H, Burck I, Urbich C, Dimmeler S, Zeiher AM, Assmus B** 2011 Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5-year results suggest long-term safety and efficacy. Clin Res Cardiol 100:925–934
8. **Xiao HB, Zhou WY, Chen XF, Mei J, Lv ZW, Ding FB, Li GQ, Zhong H, Bao CR** 10 November 2011 Interferon-β efficiently inhibited endothelial progenitor cell-induced tumor angiogenesis. Gene Ther doi: 10.1038/gt.2011.171
9. **Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM** 1999 VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 18:3964–3972
10. **Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S** 2003 Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 102:1340–1346
11. **Ozüyaman B, Ebner P, Niesler U, Ziemann J, Kleinbongard P, Jax T, Gödecke A, Kelm M, Kalka C** 2005 Nitric oxide differentially regulates proliferation and mobilization of endothelial progenitor cells but not of hematopoietic stem cells. Thromb Haemost 94:770–772
12. **Everaert BR, Van Craenenbroeck EM, Hoymans VY, Haine SE, Van Nassauw L, Conraads VM, Timmermans JP, Vrints CJ** 2010 Current perspective of pathophysiological and interventional effects on endothelial progenitor cell biology: focus on PI3K/AKT/eNOS pathway. Int J Cardiol 144:350–366
13. **Maybin JA, Battersby S, Hirani N, Nikitenko LL, Critchley HO, Jabbour HN** 2011 The expression and regulation of adrenomedullin in the human endometrium: a candidate for endometrial repair. Endocrinology 152:2845–2856
14. **Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E** 1993 Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin Invest 91:2235–2243
15. **Ali AM, Ueno T, Tanaka S, Takada M, Ishiguro H, Abdellah AZ, Toi M** 2011 Determining circulating endothelial cells using CellSearch system during preoperative systemic chemotherapy in breast cancer patients. Eur J Cancer 47:2265–2272
16. **Bidard FC, Mathiot C, Degeorges A, Etienne-Grimaldi MC, Delva R, Pivot X, Veyret C, Bergougnoux L, de Cremoux P, Milano G, Pierga JY** 2010 Clinical value of circulating endothelial cells and circulating tumor cells in metastatic breast cancer patients treated first line with bevacizumab and chemotherapy. Ann Oncol 21: 1765–1771
17. **Bertolini F, Shaked Y, Mancuso P, Kerbel RS** 2006 The multifaceted circulating endothelial cell in cancer: towards marker and target identification. Nat Rev Cancer 6:835–845
18. **Duda DG, Cohen KS, Scadden DT, Jain RK** 2007 A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. Nat Protoc 2:805–810
19. **Lei ZM, Chegini N, Rao CV** 1991 Quantitative cell composition of human and bovine corpora lutea from various reproductive states. Biol Reprod 44:1148–1156
20. **Fontaine V, Filipe C, Werner N, Gourdy P, Billon A, Garmy-Susini B, Brouchet L, Bayard F, Prats H, Doetschman T, Nickenig G, Arnal JF** 2006 Essential role of bone marrow fibroblast growth factor-2 in the effect of estradiol on reendothelialization and endothelial progenitor cell mobilization. Am J Pathol 169:1855–1862
21. **Hamada H, Kim MK, Iwakura A, Ii M, Thorne T, Qin G, Asai J, Tsutsumi Y, Sekiguchi H, Silver M, Wecker A, Bord E, Zhu Y, Kishore R, Losordo DW** 2006 Estrogen receptors α and β mediate contribution of bone marrow-derived endothelial progenitor cells to functional recovery after myocardial infarction. Circulation 114: 2261–2270
22. **Bulut D, Albrecht N, Imöhl M, Günesdogan B, Bulut-Streich N, Börgel J, Hanefeld C, Krieg M, Mügge A** 2007 Hormonal status modulates circulating endothelial progenitor cells. Clin Res Cardiol 96:258–263
23. **Mueller MD, Vigne JL, Minchenko A, Lebovic DI, Leitman DC, Taylor RN** 2000 Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors α and β. Proc Natl Acad Sci USA 97:10972–10977
24. **Wang Y, Cela E, Gagnon S, Sweezey NB** 2010 Estrogen aggravates inflammation in *Pseudomonas aeruginosa* pneumonia in cystic fibrosis mice. Respir Res 11:166
25. **Krüssel JS, Casañ EM, Raga F, Hirchenhain J, Wen Y, Huang HY, Bielfeld P, Polan ML** 1999 Expression of mRNA for vascular endothelial growth factor transmembraneous receptors Flt1 and KDR, and the soluble receptor sflt in cycling human endometrium. Mol Hum Reprod 5:452–458
26. **Torry DS, Holt VJ, Keenan JA, Harris G, Caudle MR, Torry RJ** 1996 Vascular endothelial growth factor expression in cycling human endometrium. Fertil Steril 66:72–80
27. **Bartoli M, Platt D, Lemtalsi T, Gu X, Brooks SE, Marrero MB, Caldwell RB** 2003 VEGF differentially activates STAT3 in microvascular endothelial cells. FASEB J 17:1562–1564
28. **Oh MJ, Lee JK, Lee NW, Shin JH, Yeo MK, Kim A, Kim IS, Kim HJ** 2006 Vascular endothelial growth factor expression is unaltered in placentae and myometrial resistance arteries from pre-eclamptic patients. Acta Obstet Gynecol Scand 85:545–550
29. **Malamitsi-Puchner A, Sarandakou A, Tziotis J, Stavreus-Evers A, Tzonou A, Landgren BM** 2004 Circulating angiogenic factors during periovulation and the luteal phase of normal menstrual cycles. Fertil Steril 81:1322–1327
30. **Makinoda S, Mikuni M, Furuta I, Okuyama K, Sagawa T, Fujimoto S** 1995 Serum concentration of endogenous G-CSF in women during the menstrual cycle and pregnancy. Eur J Clin Invest 25:877–879
31. **Makinoda S, Mikuni M, Sogame M, Kobamatsu Y, Furuta I, Yamada H, Yamamoto R, Fujimoto S** 1996 Erythropoietin, granulocyte-colony stimulating factor, interleukin-1 β and interleukin-6 during the normal menstrual cycle. Int J Gynaecol Obstet 55:265–271
32. **Yoshioka T, Takahashi M, Shiba Y, Suzuki C, Morimoto H, Izawa**

A, Ise H, Ikeda U 2006 Granulocyte colony-stimulating factor (G-CSF) accelerates reendothelialization and reduces neointimal formation after vascular injury in mice. Cardiovasc Res 70:61–69
33. **Honold J, Lehmann R, Heeschen C, Walter DH, Assmus B, Sasaki K, Martin H, Haendeler J, Zeiher AM, Dimmeler S** 2006 Effects of granulocyte colony simulating factor on functional activities of endothelial progenitor cells in patients with chronic ischemic heart disease. Arterioscler Thromb Vasc Biol 26:2238–2243
34. **Ohki Y, Heissig B, Sato Y, Akiyama H, Zhu Z, Hicklin DJ, Shimada K, Ogawa H, Daida H, Hattori K, Ohsaka A** 2005 Granulocyte colony-stimulating factor promotes neovascularization by releasing vascular endothelial growth factor from neutrophils. FASEB J 19: 2005–2007
35. **Wei J, Blum S, Unger M, Jarmy G, Lamparter M, Geishauser A, Vlastos GA, Chan G, Fischer KD, Rattat D, Debatin KM, Hatzopoulos AK, Beltinger C** 2004 Embryonic endothelial progenitor cells armed with a suicide gene target hypoxic lung metastases after intravenous delivery. Cancer Cell 5:477–488
36. **Fadini GP, de Kreutzenberg S, Albiero M, Coracina A, Pagnin E, Baesso I, Cignarella A, Bolego C, Plebani M, Nardelli GB, Sartore S, Agostini C, Avogaro A** 2008 Gender differences in endothelial progenitor cells and cardiovascular risk profile: the role of female estrogens. Arterioscler Thromb Vasc Biol 28:997–1004
37. **Lemieux C, Cloutier I, Tanguay JF** 2009 Menstrual cycle influences endothelial progenitor cell regulation: a link to gender differences in vascular protection? Int J Cardiol 136:200–210
38. **Masuda H, Kalka C, Takahashi T, Yoshida M, Wada M, Kobori M, Itoh R, Iwaguro H, Eguchi M, Iwami Y, Tanaka R, Nakagawa Y, Sugimoto A, Ninomiya S, Hayashi S, Kato S, Asahara T** 2007 Estrogen-mediated endothelial progenitor cell biology and kinetics for physiological postnatal vasculogenesis. Circ Res 101:598–606
39. **Matsubara K, Abe E, Matsubara Y, Kameda K, Ito M** 2006 Circulating endothelial progenitor cells during normal pregnancy and pre-eclampsia. Am J Reprod Immunol 56:79–85
40. **Robb AO, Mills NL, Smith IB, Short A, Tura-Ceide O, Barclay GR, Blomberg A, Critchley HO, Newby DE, Denison FC** 2009 Influence of menstrual cycle on circulating endothelial progenitor cells. Hum Reprod 24:619–625