No Age-Related Change in Circulating Endothelial Progenitor Cells in Healthy Subjects

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SUMMARY

Endothelial progenitor cells were isolated from peripheral blood obtained from 32 healthy volunteers without cardiovascular risk factors who ranged in age from 20 to 61 years (mean [$\pm$ SD] age, 34.1 ± 9.6 years). The fractions of CD34⁺ endothelial progenitor cells expressing kinase insert domain receptor-1, CD62E, or CD31 were analyzed with flow cytometry. Correlation analysis demonstrated that there was no significant correlation between subject age and the fraction of circulating CD34⁺ mononuclear cells expressing kinase insert domain receptor-1 ($P = 0.324$; $r = -0.180$). Similarly, there was no significant correlation between subject age and the fraction of circulating CD34⁺ mononuclear cells expressing CD62E ($P = 0.496$; $r = -0.125$) or the fraction of circulating CD34⁺ mononuclear cells expressing CD31 ($P = 0.245$; $r = -0.212$). In conclusion, the experimental results showed that there was no age-related change in the basal level of circulating endothelial progenitor cells in healthy subjects without cardiovascular risk factors. (Int Heart J 2006; 47: 95-105)

Key words: Stem cell, Endothelial progenitor cell, Age, Cytokine

STIMULATION of neovascularization is a therapeutic goal to rescue tissue from critical ischemia.¹⁾ A growing body of evidence has demonstrated that neovascularization does not exclusively rely on proliferation of localized endothelial cells but also requires bone marrow-derived circulating stem cells.²⁻⁶⁾ Endothelial progenitor cells, a subset of these stem cells, improve angiogenesis, reendothelialization after vascular injury, and increase ventricular function after myocardial infarction.⁷⁻¹⁰⁾ These cells can be cultured from the circulating mononuclear cell fraction and exhibit typical endothelial surface markers and properties.³⁾ Experimental studies demonstrate that impaired neoangiogenesis in

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ischemic tissues and impaired reendothelialization of vascular lesions are functions of advanced age.¹¹⁻¹⁴⁾ Extensive studies have been performed on patients with coronary artery disease or ischemic peripheral vascular disease.^{8-10,15,16)} Scheubel, *et al*¹⁴⁾ demonstrated that there was an age-dependent depression in the number of circulating endothelial progenitor cells in patients undergoing coronary artery bypass grafting. However, Vasa, *et al*¹⁷⁾ demonstrated that the number and migratory activity of circulating endothelial progenitor cells were inversely correlated with risk factors for coronary artery disease. Moreover, Vasa, *et al*¹⁸⁾ demonstrated that the number and migratory activity of circulating endothelial progenitor cells were reduced in patients with coronary artery disease. Thus, age-related changes in the number of circulating endothelial progenitor cells may be secondary indicators of underlying coronary artery disease or vascular dysfunction.^{14,19)} Exogenous vascular endothelial growth factor has been shown to mobilize bone marrow-derived endothelial progenitor cells and increase the number of circulating endothelial progenitor cells.²⁰⁻²⁴⁾ Rivard, *et al*¹¹⁾ showed that in the murine ischemic hindlimb model, the expression of vascular endothelial growth factor protein in ischemic tissue was significantly reduced in old mice compared with young mice. However, there were no significant differences in the basal expression of vascular endothelial growth factor in nonischemic limbs for unoperated old mice compared with young mice.¹¹⁾ Whether there is an age-related change in the basal level of circulating endothelial progenitor cells in healthy subjects has not been investigated. It remains to be determined if there is an age-dependent depression in the number of circulating endothelial progenitor cells in healthy subjects without cardiovascular risk factors.¹²⁾ This study hypothesized that there is no age-related change in the basal level of circulating endothelial progenitor cells in healthy subjects without cardiovascular risk factors. Accordingly, the present study investigated the basal level of circulating endothelial progenitor cells in healthy subjects of different ages without cardiovascular risk factors.

METHODS

Study population: Thirty-two healthy volunteers were studied. The subjects consisted of 14 men and 18 women, ranging in age from 20 to 61 years (mean $\pm$ SD] age, 34.1 ± 9.6 years), with no evidence of systemic disease in patient history, physical examination, or laboratory examination. No subject smoked, abused alcohol, or had liver disease, renal disease, inflammatory disease, malignancy, any risk factors for atherosclerosis or coronary artery disease, and no subject was taking any medications including oral contraceptives, or was pregnant. Informed consent was obtained from all study subjects. The Institutional Review Commit-

tee on Human Research at our institution approved the study protocol.

Isolation, characterization of endothelial progenitor cells, and flow cytometry analysis: Blood samples were obtained from all study subjects during a fasting state upon entering the study. Peripheral blood (10 mL) was drawn into blood collection tubes containing sodium heparin (Vacutainer™; Becton Dickinson; Franklin Lakes, NJ). The mononuclear cells fractions were isolated by density-gradient centrifugation with Ficoll 400 (density 0.057 g/mL) (Ficoll-Plaque™ Plus, Amersham Biosciences, Sweden). The mononuclear cells were washed twice with a phosphate buffer solution and centrifuged, followed by incubation with 1 mL of blocking buffer (2% bovine serum albumin in phosphate buffer solution) for 30 minutes at 4°C. The viability of the mononuclear cells was identified by staining 20 μ L of mononuclear cells with 180 μ L of 0.25% trypan blue. The concentration of mononuclear cells in peripheral blood was $0.84 \pm 0.24 \times 10^6$ cells/mL and the viability of mononuclear cells was $96.1 \pm 5.8\%$.

To detect CD34 expression in the isolated nucleated mononuclear cells, the mononuclear cells were incubated for 30 minutes at 4°C in a dark room with phycoerythrin-conjugated monoclonal antibody against human CD45RA (Becton Dickinson, PharMingen) and fluorescein isothiocyanate-labeled monoclonal antibody against human CD34 (Becton Dickinson) (20 μ L of antibody against 1×10^6 cells). Mean levels of expression of CD34⁺ cells in the isolated nucleated mononuclear cells were measured using the specific mononuclear cell gates in forward-sideward light scatter plots. The mean level of expression of CD34⁺ in the isolated nucleated mononuclear cells was $0.67 \pm 0.38\%$.

To identify endothelial marker protein expression, mononuclear cells were incubated for 30 minutes at 4°C in a dark room with mouse monoclonal antibody against human kinase insert domain receptor-1 (Sigma) and subsequently, the phycoerythrin-conjugated goat anti-mouse polyclonal antibody (Becton Dickinson, PharMingen) and the fluorescein isothiocyanate-labeled monoclonal antibody against human CD34 (Becton Dickinson). Furthermore, the phycoerythrin-conjugated mononuclear antibody against human CD31 (Becton Dickinson) and the phycoerythrin-conjugated monoclonal antibody against human CD62E (Becton Dickinson, PharMingen) were also used for each blood sample. Dual-stained cells expressing CD34 and kinase insert domain receptor-1, CD34 and CD62E, or CD34 and CD31 were judged to be endothelial progenitor cells. To assess the extent of nonspecific association of protein with mononuclear cells, a control tube containing isotype-identical antibodies (IgG1-fluorescein isothiocyanate and IgG1-phycoerythrin, Simultest™ Control r1/r1, Becton Dickinson) was used for each blood sample and served as control. After treatment and washing twice with phosphate buffer solution, the cells were fixed in 1% paraformaldehyde. Two-color flow cytometric analyses were performed using a fluorescence-acti-

vated cell sorter (FACSCalibur™ system; Becton Dickinson). Each analysis included 30,000 gated events. Events representing mononuclear cells were differentiated from other contaminating cells by virtue of their “clustering” in a discrete region on the basis of size and granules (based on the forward light scatter and 90° side light scatter). Quadrants were set on the basis of fluorescein isothiocyanate and phycoerythrin isotype controls and cells without antibody incubation. The percentage of CD34⁺ cells expressing endothelial marker proteins was defined as the fraction of cells exhibiting specific binding (ie, kinase insert domain receptor-, CD62E-, or CD31-positive) minus that exhibiting nonspecific binding (ie, the percentage defined with the IgG1-fluorescein isothiocyanate and IgG1-phycoerythrin conjugate).

Blood sampling and measurement of plasma cytokine levels: Blood samples were also drawn into an evacuated tube containing K₃ ethylenediamine tetra acetic acid (Vacutainer, Becton Dickinson; Franklin Lakes, NJ). Mixtures of blood and K₃ ethylenediamine tetra acetic acid were immediately centrifuged (model 5400; Kubota Corp; Tokyo, Japan) at 3,000 revolutions per minute for ten minutes at room temperature. The plasma was immediately separated and frozen at -80°C until the assay was performed.

The human plasma interleukin-18 concentrations were quantified using a commercially available enzyme-linked immunosorbent assay (MBL, Nagoya, Japan). The human plasma vascular endothelial growth factor concentrations were quantified with a commercially available ELISA (Pierce Endogen, Pierce Chemical Co., Rockford, IL, USA). The human plasma tumor necrosis factor- α , granulocyte-macrophage colony-stimulating factor, and erythropoietin concentrations were measured with a highly sensitive ELISA (R and D Systems, Minneapolis, MN, USA). The samples were processed according to the manufacturer's instructions. All standards and samples were tested in duplicate.

Total plasma homocysteine levels were measured using an AxSYM homocysteine assay (Abbott Laboratories, Abbott Park, IL, USA). Furthermore, plasma concentrations of high-sensitivity C-reactive protein were measured by immunonephelometry using the BN™ system (Dade Behring Inc., Newark, DE, USA); the low detection limit in this test is 0.175 mg/L. Blood samples were also taken to obtain whole blood counts, and biochemical and electrolyte measurements using standard laboratory methods.

Statistical analysis: Continuous variables are presented as the mean $\pm$ SD. Continuous variables were compared using Wilcoxon's paired rank sum test. The relationships between the expressions of endothelial marker proteins on CD34⁺ cells and subject age were analyzed using Spearman's correlation. Statistical analyses were performed with SAS for Windows version 8.02 (SAS Institute, Cary, NC). A probability value < 0.05 was considered statistically significant.

RESULTS

Correlation between subject age and different endothelial marker proteins on CD34⁺ cells: The baseline characteristics of the study subjects are summarized in Table I. The endothelial progenitor cells were identified in peripheral blood and confirmed by identifying the expressions of the endothelial marker proteins (kinase insert domain receptor-1, CD31, and CD62E) with flow cytometry (Fig-

Table I. Baseline Clinical Characteristics of Study Subjects

Age, years	34.1 ± 9.6
Men, n (%)	14 (43.8%)
Body mass index (kg/m ²)	20.7 ± 1.9
Total cholesterol, mg/dL	166.3 ± 17.4
Total triglycerides, mg/dL	83.7 ± 29.9
Leukocytes, 10 ³ cells/ μ L	6093.8 ± 1137.3
Red blood cells, 10 ⁶ cells/ μ L	4.58 ± 0.43
Platelets, 10 ⁴ cells/ μ L	24.1 ± 4.8
Homocysteine, μ mol/L	9.0 ± 2.3
High-sensitivity C-reactive protein, mg/L	0.54 ± 0.51
Vascular endothelial growth factor, pg/mL	51.7 ± 12.4
Granulocyte-macrophage colony-stimulating factor, pg/mL	1.11 ± 0.11
Tumor necrosis factor- α , pg/mL	1.04 ± 0.44
Erythropoietin, mIU/mL	7.16 ± 2.68
Interleukin-18, pg/mL	79.9 ± 48.6

Figure. Endothelial progenitor cell characteristics of a normal volunteer. The expression of CD31, CD62E, and kinase insert domain receptor-1 in endothelial progenitor cells was analyzed using a fluorescence-activated cell sorter and compared with isotype controls. Quadrants were set on the basis of fluorescein isothiocyanate and phycoerythrin isotype controls and cells without antibody incubation (two left figures in the lower panel).

ure). Correlation analysis demonstrated that there was no significant correlation between subject age and the fraction of circulating CD34⁺ mononuclear cells expressing kinase insert domain receptor-1 ($P = 0.324$; $r = -0.180$) (Table II). Similarly, there was no significant correlation between subject age and the fraction of circulating CD34⁺ mononuclear cells expressing CD62E ($P = 0.496$; $r = -0.125$) or the fraction of circulating CD34⁺ mononuclear cells expressing CD31 ($P = 0.245$; $r = -0.212$). Correlation analysis also demonstrated that there was no significant correlation between subject age and the leukocyte or mononuclear cell counts.

Correlation among different endothelial marker proteins on CD34⁺ cells: Correlation analysis demonstrated that there was a significant and direct relationship between the fraction of circulating CD34⁺ mononuclear cells expressing kinase insert domain receptor-1 and the fraction of circulating CD34⁺ mononuclear cells expressing CD62E ($P < 0.0002$; $r = 0.619$). Furthermore, there was a significant and direct relationship between the fraction of circulating CD34⁺ mononuclear cells expressing kinase insert domain receptor-1 and the fraction of circulating CD34⁺ mononuclear cells expressing CD31 ($P < 0.0001$; $r = 0.671$). There was also a significant and direct relationship between the fraction of circulating CD34⁺ mononuclear cells expressing CD31 and the fraction of circulating CD34⁺ mononuclear cells expressing CD62E ($P < 0.0001$; $r = 0.875$).

Correlation between subject age and different cytokines: Correlation analysis demonstrated that there was no significant correlation between subject age and

Table II. Spearman's Correlation Coefficients Between Age and Endothelial Progenitor Cells or Cytokines

	Age	Vascular endothelial growth factor	Granulocyte macrophage colony stimu- lating factor	Tumor necro- sis factor- α	Erythropoietin	Interleukin-18
CD34 ⁺ /kinase insert domain receptor-1 ⁺ mono- nuclear cells	-0.180	0.032	0.103	0.004	-0.275	-0.055
CD34 ⁺ /CD62E ⁺ mononu- clear cells	-0.125	0.088	0.377*	0.006	-0.146	0.137
CD34 ⁺ /CD31 ⁺ mononu- clear cells	-0.212	0.019	0.174	-0.140	-0.312	0.063
Vascular endothelial growth factor	0.252					
Granulocyte macrophage colony stimulating factor	-0.019					
Tumor necrosis factor- α	0.098					
Erythropoietin	0.271					
Interleukin-18	0.171					

* $P = 0.033$

plasma levels of vascular endothelial growth factor ($P = 0.164$; $r = 0.252$) (Table II). Similarly, there was no significant correlation between subject age and plasma levels of granulocyte-macrophage colony-stimulating factor ($P = 0.916$; $r = -0.019$), tumor necrosis factor- α ($P = 0.595$; $r = 0.098$), or erythropoietin ($P = 0.134$; $r = 0.271$)

Correlation among different endothelial marker proteins on CD34⁺ cells and different cytokines: Correlation analyses demonstrated that there was no significant correlation between the fraction of circulating CD34⁺ mononuclear cells expressing kinase insert domain receptor-1 and the plasma levels of vascular endothelial growth factor ($P = 0.864$; $r = 0.032$), granulocyte macrophage colony stimulating factor ($P = 0.576$; $r = 0.103$), erythropoietin ($P = 0.127$; $r = -0.275$), tumor necrosis factor- α ($P = 0.981$; $r = 0.004$), or interleukin-18 ($P = 0.764$; $r = -0.055$) (Table II). Notably, there was also no significant correlation between the fraction of circulating CD34⁺ mononuclear cells expressing CD31 and the plasma levels of vascular endothelial growth factor ($P = 0.918$; $r = 0.019$), granulocyte macrophage colony stimulating factor ($P = 0.342$; $r = 0.174$), erythropoietin ($P = 0.082$; $r = -0.312$), tumor necrosis factor- α ($P = 0.446$; $r = -0.140$), or interleukin-18 ($P = 0.730$; $r = 0.063$). Additionally, there was no significant correlation between the fraction of circulating CD34⁺ mononuclear cells expressing CD62E and the plasma levels of vascular endothelial growth factor ($P = 0.633$; $r = 0.088$), erythropoietin ($P = 0.426$; $r = -0.146$), tumor necrosis factor- α ($P = 0.975$; $r = 0.006$), or interleukin-18 ($P = 0.453$; $r = 0.137$). However, there was a significant and direct relationship between the fraction of circulating CD34⁺ mononuclear cells expressing CD62E and plasma levels of granulocyte-macrophage colony-stimulating factor ($P = 0.033$; $r = 0.377$).

Comparison of circulating endothelial progenitor cells between men and women:

There was no difference between men and women in terms of age (33.1 ± 7.8 versus 34.8 ± 11.0 years, $P = 0.993$), serum cholesterol levels (171.8 ± 15.7 versus 162.1 ± 17.9 mg/dL, $P = 0.089$), triglyceride levels (84.6 ± 20.1 versus 82.9 ± 36.3 mg/dL, $P = 0.593$) and levels of high-sensitivity C-reactive protein (0.599 ± 0.414 versus 0.489 ± 0.589 mg/L, $P = 0.136$), leukocyte counts (5892.9 ± 930.2 versus 6250 ± 1279.8 cells/uL, $P = 0.413$), and the fraction of circulating CD34⁺ mononuclear cells expressing endothelial marker proteins (kinase insert domain receptor-1, $0.721 \pm 0.664\%$ versus $0.788 \pm 0.896\%$, $P = 0.687$; CD62E, $2.097 \pm 1.652\%$ versus $1.872 \pm 1.580\%$, $P = 0.555$; CD31, $15.2 \pm 17.3\%$ versus $10.8 \pm 10.1\%$, $P = 0.398$).

DISCUSSION

Age and circulating endothelial progenitor cells: Rivard, *et al*⁽¹¹⁾ showed that there were no significant differences in the basal expression of vascular endothelial

growth factor in nonischemic limbs for unoperated old mice compared with young mice. However, vascular endothelial growth factor protein expression in ischemic tissues was upregulated as early as day 3 after surgical induction of hindlimb ischemia, reached a maximum by day 7, and decreased thereafter. Additionally, the level of vascular endothelial growth factor protein was significantly reduced in old mice compared with young mice for all time points studied after surgical induction of hindlimb ischemia. Rivard, *et al*¹¹⁾ reported that impaired angiogenesis in old animals resulted from impaired endothelial function and a low expression of vascular endothelial growth factor in ischemic tissues. Vascular endothelial growth factor (VEGF) is the fundamental regulator of differentiation in the hemangioblast, the common precursor for endothelial and hemopoietic cells.²⁰⁾ In the presence of VEGF, a population of mesodermal cells from chicken embryos in the gastrulation stage, with the expression of the VEGF receptor Flk-1/kinase insert domain receptor, differentiated in endothelial lineage. Conversely, hemopoietic differentiation occurred with the absence of VEGF. Therefore, VEGF is the instructive signal that results in the differentiation of hemangioblasts into endothelial lineage. Furthermore, VEGF, which is upregulated in the ischemic myocardium of patients with myocardial infarction, has been shown to mobilize endothelial progenitor cells in both mice and humans.²¹⁻²⁴⁾ Scheubel, *et al*¹⁴⁾ showed that there was an age-dependent depression in the number of circulating endothelial progenitor cells in patients while undergoing coronary artery bypass grafting. However, Vasa, *et al*¹⁸⁾ demonstrated that the number and migratory activity of circulating endothelial progenitor cells were reduced in patients with coronary artery disease. Moreover, Vasa, *et al*¹⁷⁾ demonstrated that the number and migratory activity of circulating endothelial progenitor cells were inversely correlated with risk factors for coronary artery disease. This study showed that there was no age-related change in the basal level of circulating endothelial progenitor cells in healthy subjects without cardiovascular risk factors. Thus, the age-related change in the level of circulating endothelial progenitor cells in patients with coronary artery disease may be the result of impaired endothelial function and may be secondary indicators of underlying coronary artery disease or vascular dysfunction.¹⁹⁾

Circulating endothelial progenitor cells and cytokines: Erythropoietin has been shown to be a potent physiologic stimulus for endothelial progenitor cell mobilization.²⁵⁾ Tumor necrosis factor- α has also been shown to be a potent inhibitor of endothelial cell growth *in vitro* and to be angiogenic *in vivo*.²⁶⁾ Takahashi, *et al*²⁷⁾ demonstrated that granulocyte-macrophage colony-stimulating factor therapy in mice increases the number of circulating endothelial progenitor cells. This study showed that there was no significant correlation between age and plasma levels of VEGF, granulocyte-macrophage colony-stimulating factor, tumor necrosis

factor- α , or erythropoietin. These findings might explain in part why there was no age-related change in the basal level of circulating endothelial progenitor cells in healthy subjects without cardiovascular risk factors.

Although exogenous VEGF, erythropoietin, and granulocyte-macrophage colony-stimulating factor administration have been shown to mobilize bone marrow-derived endothelial progenitor cells and increase circulating endothelial progenitor cells,²⁵⁻²⁷⁾ the relationship between endogenous plasma levels of VEGF, erythropoietin, and granulocyte-macrophage colony-stimulating factor and the number of circulating endothelial progenitor cells has remained unclear. Vasa, *et al*¹⁸⁾ showed that statin treatment increased the number of circulating endothelial progenitor cells in patients with stable coronary artery disease without affecting endogenous serum levels of VEGF, granulocyte-macrophage colony-stimulating factor, or tumor necrosis factor- α . This study showed that only the fraction of circulating CD34⁺ mononuclear cells expressing CD62E was significantly correlated with plasma levels of granulocyte-macrophage colony-stimulating factor. However, there was no significant correlation between the fraction of circulating CD34⁺ mononuclear cells expressing kinase insert domain receptor-1 or CD31, and the plasma levels of VEGF, granulocyte-macrophage colony-stimulating factor, erythropoietin, or tumor necrosis factor- α . These findings were consistent with those of a previous study.¹⁸⁾

Clinical implications: The results of the present study indicate that in the presence of normal plasma levels of VEGF, granulocyte-macrophage colony-stimulating factor, erythropoietin, and tumor necrosis factor- α , there was no age-dependent depression in the number of circulating endothelial progenitor cells in healthy subjects without cardiovascular risk factors. However, a previous study showed that there was an age-dependent depression in the number of circulating endothelial progenitor cells in patients while undergoing coronary artery bypass grafting.¹⁴⁾ Therefore, clinical studies involving patients with various cardiovascular diseases and normal subjects in terms of endothelial progenitor cells should consider age as an important confounding factor for analysis.

Study limitations: Three limitations of this study deserve consideration. First, endothelial progenitor cell kinetics was not investigated. However, Vasa, *et al*¹⁸⁾ showed that there were no spontaneously occurring changes in endothelial progenitor cell numbers in healthy volunteers. Second, data were not provided regarding whether there was an age-related change in the number of circulating endothelial progenitor cells following exogenous VEGF, granulocyte-macrophage colony-stimulating factor, or erythropoietin administration in healthy volunteers. Finally, as aging promotes atherosclerosis and vascular dysfunction, we did not include subjects in their 70s or 80s who were most likely to have undetected atherosclerosis and vascular dysfunction.

Conclusions: The results of this study demonstrate that there was no age-related change in the basal level of circulating endothelial progenitor cells in healthy subjects without cardiovascular risk factors.

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