Original Article

Circulating Endothelial Progenitor Cells, C-Reactive Protein and Severity of Coronary Stenosis in Chinese Patients with Coronary Artery Disease

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We sought to investigate whether numbers and activity of circulating endothelial progenitor cells (EPCs) correlate with severity of coronary stenosis as well as cardiovascular risk factors in patients with stable coronary artery disease (CAD). Number of circulating EPCs was analyzed in 104 consecutive patients with proven or clinically suspected CAD. Adhesive and migratory activity was also determined. The number of EPCs was lower in patients with a single diseased coronary artery (Group II, $n=35$, $p<0.05$ *vs.* Group I) or multiple diseased arteries (Group III, $n=25$, $p<0.01$ *vs.* Group I, $p<0.05$ *vs.* Group II) compared to those with normal coronary arteries (Group I, $n=44$). The number of EPCs was also related with angiographic Gensini score ($r=-0.355$, $p=0.006$). In addition, concentrations of C-reactive protein (CRP) were elevated in patients with CAD, and positively correlated with Gensini score ($r=0.476$, $p=0.001$). As for the risk factors, the number of EPCs was also inversely correlated with age ($p=0.001$), high sensitivity-CRP ($p=0.012$), hypertension ($p=0.042$) and family history of CAD ($p=0.043$). Most importantly, the migratory capacity of EPCs was compromised in patients with CAD, and inversely correlated with the angiographic Gensini score ($r=-0.315$, $p=0.021$). EPCs isolated from patients with CAD also showed an impaired adhesive activity ($p<0.05$). In conclusion, in patients with stable CAD, reduction in the number and impairment in the function of circulating EPCs were correlated with the severity of coronary stenosis. CRP may play an important role in reducing the number of EPCs and accelerating atherosclerosis. Given the important role of EPCs in neovascularization of ischemic tissue, a decrease in the number and activity of EPCs may contribute to the impaired vascularization in patients with CAD. (*Hypertens Res* 2007; 30: 133–141)

***Key Words*: endothelial progenitor cell, C-reactive protein, coronary artery disease, cardiovascular risk factors**

Introduction

Following the first description of the isolation of putative progenitor endothelial cells for angiogenesis by Asahara in 1997 (*1*), increasing evidence has indicated that bone marrow–derived circulating endothelial progenitor cells (EPCs) are involved in the process of vasculogenesis. These EPCs were considered to be originated from hematopoietic stem cells, which are positive for CD133 and the vascular endothelial

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Table 1. Characteristics of Patients with Stable Coronary Artery Disease Classified According to the Number of Diseased Vessel

	Total	CAG(−)	Single vessel disease	Multiple vessel disease	*p* value
n	104	44	35	25	
Age (years)	63.9±10.6	61.5±9.98	63.03±10.01	64.60±10.48	0.23
BMI (kg/m^2)	24.69±3.67	23.80±3.24	25.07±3.96	25.80±3.80	0.11
Gender (male, *n* (%))	77 (74.0)	25 (57)	29 (82)	23 (92)	<0.01
Medical history (*n* (%))					
Hypertension	55 (52.9)	18 (45)	20 (57.1)	17 (68)	0.21
Diabetes mellitus	12 (11.5)	1 (2.3)	6 (17.1)	5 (20.0)	0.049
Current smokers	37 (35.6)	16 (36.3)	13 (37.1)	8 (32)	0.87
Alcohol drinkers	14 (13.4)	2 (4.5)	6 (17.1)	6 (24)	0.02
Family history of CAD	27 (26.0)	9 (20.5)	13 (37.1)	5 (20)	0.37
LVMI (g/cm^2)	119.33±28.78	114.3±25.46	120.43±20.81	126.64±26.62	0.51
Blood tests					
Plasma fasting glucose (mmol/l)	5.75±1.11	5.49±0.68	5.84±1.27	6.07±1.40	0.15
Serum cholesterol (mmol/l)	4.81±1.05	4.81±0.90	4.80±1.22	4.82±0.85	0.89
Serum triglyceride (mmol/l)	1.71±0.90	1.66±0.90	1.73±1.11	1.76±0.53	0.88
Serum HDL-cholesterol (mmol/l)	1.26±0.41	1.36±0.49	1.18±0.31	1.18±0.34	0.39
Serum LDL-cholestrol (mmol/l)	2.79±0.78	2.77±0.70	2.81±0.94	2.82±0.70	0.92
Uric acid (umol/l)	346.27±85.65	334.35±75.14	348.11±84.01	364.69±103.61	0.65
Ccr (ml/min/1.73 m^2)	76.55±21.32	83.53±22.27	75.74±17.72	65.40±19.96	0.03

CAD, coronary artery disease; CAG(−), normal coronary angiography; BMI, body mass index; LVMI, left ventricular mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Ccr, creatinine clearance.

growth factor receptor 2 (KDR) in the early stage.

The integrity and functional activity of the endothelial monolayer play a critical role in atherosclerosis. Risk factors such as hypertension, smoking, or diabetes function through a final common pathway potentially involving oxidative injury to endothelial cells. These processes can lead to the formation of atherosclerotic lesions, plaque rupture, and finally myocardial infarction (*2*). Recent studies suggest that the injured endothelial monolayer is regenerated by circulating bone marrow–derived EPCs (*3*). However, risk factors reduce the number and functional activity of these circulating EPCs (*4*). The impairment of EPCs by risk factors may contribute to atherosclerotic disease progression.

The results of our preliminary experiment indicated that the number of circulating EPCs was lower in patients with coronary artery disease (CAD) than their age-matched controls. This is in agreement with the results of Vasa *et al*. (*4*). However, whether the number and activity of circulating EPCs is correlated with the severity of coronary stenosis has not been determined. The present study measured the number and activity of circulating EPCs in patients with stable CAD to examine if there is a relationship between EPCs and angiographically determined severity of coronary artery stenosis, as well as cardiovascular risk factors.

Methods

All experiments in this study were approved by the ethical committee of the Shanghai Institute of Hypertension and conducted in accordance with the Declaration of Helsinki. All subjects provided their informed written consent prior to participation.

Study Subjects

One hundred and four consecutive patients with chest pain and ST-T changes regarded as definite or probable stable angina pectoris were admitted to the Cardiology Department of Ruijin Hospital Shanghai, P.R. China. All patients underwent coronary angiography after admission. Patients with malignant disease, peripheral vascular disease, proliferative retinopathy or recent (<2 months) acute coronary syndrome, myocardial infarction, inflammation, bleeding or blood transfusion were not included in the study, since these conditions and procedures could have influenced the number of EPCs. Pre-menstrual women were excluded from this study. None of the patients had been treated with statins or erythropoietin in the preceding 2 months.

Participants underwent physical examination and blood tests to assess cardiovascular risk factors including age, gender, body mass index (BMI), smoking and alcohol drinking

Table 2. Correlations between CRP Levels, Risk Factors and Number of Circulating Endothelial Progenitor Cells in Patients with Stable Coronary Artery Disease

Risk factors	Standard regression coefficient (SEM)	*p* value
Age	−0.390 (0.001)	0.001
ln hs-CRP	−0.229 (0.003)	0.012
Hypertension	−0.166 (0.005)	0.042
Family history of CAD	−0.170 (0.005)	0.043

hs-CRP, high sensitivity C-reactive protein; ln, natural logarithm; CAD, coronary artery disease.

Table 3. Multivariate Predictors of the Presence of Coronary Artery Disease

Risk factors	OR	95% confidence interval	*p* value
Gender (male)	4.624	1.093–19.562	0.037
ln hs-CRP	3.151	1.466–6.772	0.001
Diabetes	11.922	0.961–147.950	0.044
EPCs number	0.64	0.487–0.842	0.001

OR, odds ratio; hs-CRP, high sensitivity C-reactive protein; ln, natural logarithm; EPC, endothelial progenitor cells.

status, blood pressure, plasma fasting glucose, serum total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, triglyceride, creatinine and uric acid.

Definition of Risk Factors for CAD

The patients' characteristics were obtained from medical records with regard to history of hypertension, hypercholesterolemia, diabetes, alcohol consumption and smoking. Patients with a systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg (JNC 7 definition) or current use of antihypertensive medication or use of antihypertensive medication for more than 1 year were categorized as hypertensive. Hyperlipidemia was defined as a total cholesterol concentration exceeding 5.7 mmol/l, or the current use of lipid-lowering drugs, and diabetes mellitus was defined using the American Diabetes Association criteria. Height and weight were measured, and BMI (kg/m^2) was calculated.

Determination of Left Ventricular Mass from Echocardiography

Calculations of left ventricular mass (LVM) were done echocardiographically using a Hewlett Packard ultrasound instrument with a 2.5-MHz phased array transducer. The following parameters were detected: left ventricular end-diastolic dimension (LVDd), interventricular septal thickness (IVST), and posterior wall thickness (PWT). The LVM was calculated on-line from the formula designated as the standard of the American Society of Echocardiography and derived from Devereux *et al*. (*5*), *i.e.*,

$$\text{LVM (g)} = 1.04\ \{(\text{LVDd} + \text{IVST} + \text{PWT})^3 - \text{LVDd}^3\} - 13.6.$$

LVMI (left ventricular mass index) was calculated by dividing LVM by body surface area.

Coronary Angiography

Coronary angiography was performed using the Judkins approach. Quantitative coronary angiographic (QCA) analysis was performed by an independent laboratory, and significant coronary stenosis was defined as a decrease in luminal diameter of greater than or equal to 50% in one or more of the major epicardial coronary arteries or their primary branches (left anterior descending [LAD], left circumflex [LCX] and right coronary artery [RCA]). Patients with stenosis of 50% or more of the left main coronary artery were considered to have double-vessel disease.

Determination of the Severity of Coronary Atherosclerosis

The severity of coronary stenosis in the patients was estimated by the number of affected vessels (> 50% of luminal diameter) and the coronary score of Gensini (*6*). The Gensini system scores the narrowing of the coronary artery lumen as follows: a score of 1, 1–25% narrowing; 2, 26–50% narrowing; 4, 51–75% narrowing; 8, 76–90% narrowing; 16, 91–99% narrowing; and 32, total occlusion. The score is then multiplied by a factor that incorporates the importance of the lesion's position in the coronary arterial tree: for example, 5 for the left main coronary artery, 2.5 for the proximal LAD or LCX, 1.5 for the mid LAD, and 1 for the distal LAD or mid-distal LCX.

Determination of the Serum Concentrations of High Sensitivity C-Reactive Protein, Intercellular Adhesion Molecule-1 and Vascular Cell Adhesion Molecule Type-1

Serum concentrations of high sensitivity C-reactive protein (hs-CRP) were determined by particle-enhanced immunonephelometry using the N High Sensitivity CRP Reagent (Dade Behring Inc., Marburg, Germany). This assay has a sensitivity limit of 0.175 mg/l.

Serum concentrations of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule type-1 (VCAM-1) were determined by the ELISA technique using sICAM-1 and sVCAM-1 ELISA kits (DIACLONE, Besançon, France) according to the manufacturer's instruction. The sensitivity limit was 8 ng/ml for ICAM-1 and 0.6

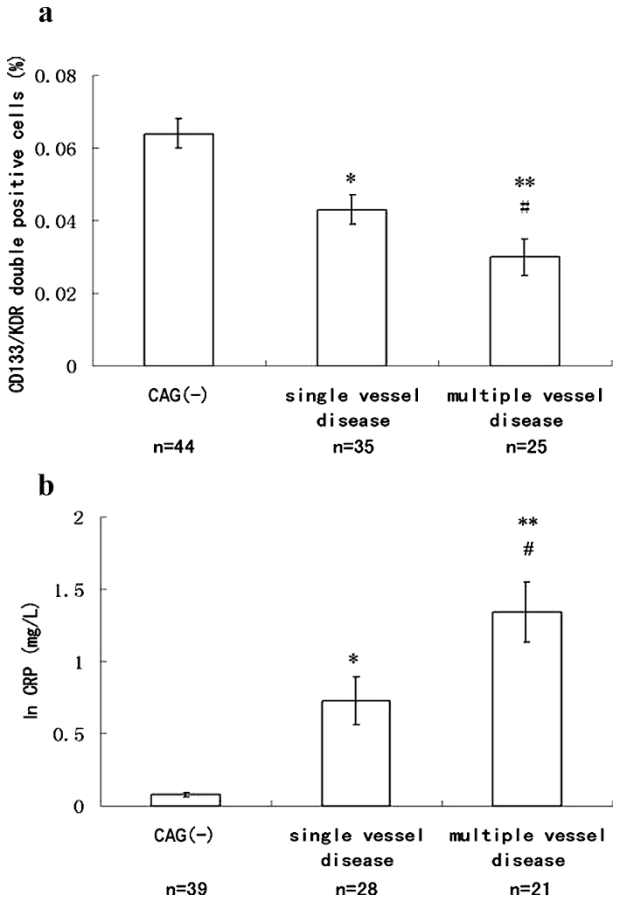

Fig. 1. *EPC number and CRP level in patients with different numbers of stenosed coronary vessels. Comparative analysis of the number of CD133/KDR double-positive precursor cells (a) and serum concentrations of hs-CRP (b) in patients with angiographically normal coronary arteries (CAG(−) group) and single or multiple diseased vessels.* $^{*}p < 0.05$, $^{**}p < 0.01$, *compared to the CAG(−) group,* $^{\#}p < 0.05$, *compared to the single-vessel disease group.*

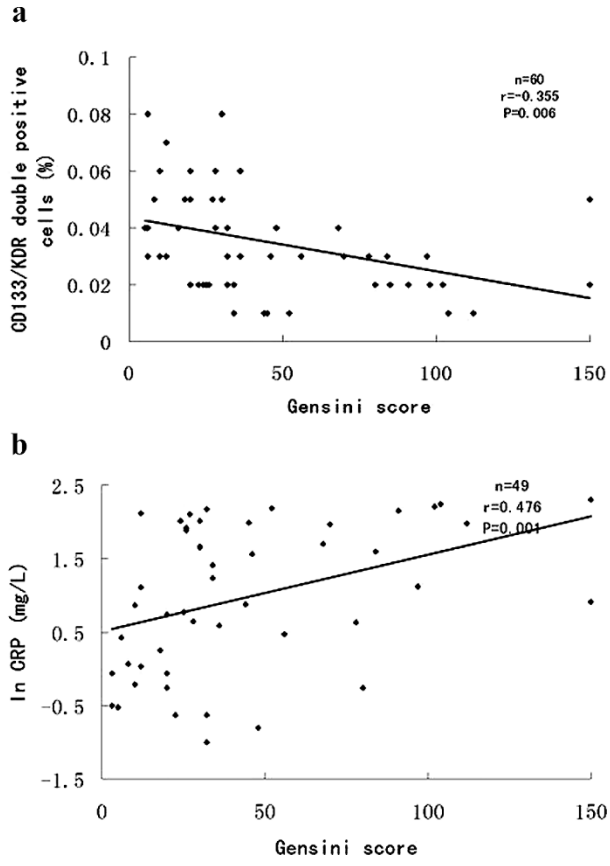

Fig. 2. *Correlations among CRP level, EPC number and coronary angiographic Gensini score in patients with stable coronary artery disease. a: Linear regression analysis for the number of CD133/KDR double-positive precursor cells and Gensini score (*$n = 60$, $r = -0.355$, $p = 0.006$*). b: Linear regression analysis for the hs-CRP levels and Gensini score (*$n = 49$, $r = 0.476$, $p = 0.001$*). Only those with single or multiple vessel disease were analyzed.*

ng/ml for VCAM-1.

Flow Cytometry Studies

Before coronary angiography, a Judkins catheter was introduced *via* the femoral artery to obtain blood samples. One hundred and fifty microliters of whole blood treated with heparin as an anticoagulant was incubated for 30 min in the dark with APC-labeled monoclonal antibody against human KDR (R & D, Minneapolis, USA) and the PE-labeled monoclonal antibody against human CD133 (MACS, Bergisch Gladbach, Germany). Isotype-identical antibodies IgG1-PE and IgG1-APC (Becton Dickinson, Franklin Lakes, USA) served as controls. The analysis was done using a FACSCalibur flow cytometer with CellQuest software (BD Pharmingen, San Diego, USA). The viable lymphocyte population was analyzed and each analysis included 60,000 events. The cell surface expression of KDR was determined by flow kilometric analysis using 620–650 nm wavelength laser excitations and monitoring emitted fluorescence with a detector optimized to collect peak emissions at 660–670 nm, and the cell surface expression of CD133 was determined using 488/575 nm excitation and emission wavelengths.

EPCs Culture Assay

Mononuclear cells (MNCs) were isolated by density-gradient centrifugation with ficoll from 10 ml of peripheral blood in 93 patients. Immediately after isolation, 1×10^7 mononuclear cells were plated on 6-well culture dishes coated with human fibronectin (BD Biosciences, San Jose, USA) and maintained in endothelial basal medium supplemented with EGM SingleQuotes and 10% FCS (Cambrex, Rome, Italy). After 4 days in

Table 4. Multivariate Analysis on Gensini Score in Patients with Stable Coronary Artery Disease

Risk factors	Standard regression coefficient	*p* value
Age	0.35	0.001
Gender	0.241	0.002
CD133/KDR double-positive cells	−0.282	0.002
Ccr (ml/min)	0.225	0.039
ln hs-CRP	0.327	0.001

hs-CRP, high sensitivity C-reactive protein; ln, natural logarithm; Ccr, creatinine clearance rate.

culture, nonadherent cells were removed by a thorough washing with PBS. The culture was maintained through day 7, and adherent cells were subjected to cytochemical analysis.

Characterization of EPCs

Fluorescent chemical detection of EPCs was performed on attached MNCs after 7 days in culture. Direct fluorescent staining was used to detect dual binding of FITC-labeled Ulex europaeus agglutinin (UEA)-1 (Sigma, St. Louis, USA) and 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (DiI)–labeled acetylated low-density lipoprotein (ac-LDL; Molecular Probe Co., Eugene, USA). Cells were first incubated with ac-LDL at 37°C and later fixed with 2% paraformaldehyde for 10 min. After being washed, the cells were reacted with UEA-1 (10 mg/l) for 1 h. After the staining, samples were demonstrated by laser scanning confocal microscope (LSCM; Leica, Mannheim, Germany). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs.

Migration Assay

Isolated EPCs were detached using 0.25% tyrosine, harvested by centrifugation, resuspended in endothelial growth medium-2 (EGM-2), and counted. Transwell migration assays were performed in in 24-well culture dishes with Costar Transwells (6.5-mm diameter, 8-μm pore size; Corning Costar, Cambridge, USA). EPCs (2×10^4) were added to the upper chamber and 0.6 ml EGM with human recombinant VEGF (50 ng/ml) (Peprotech EC, London, UK) was added to the bottom chamber. After 24 h incubation at 37°C, the lower side of the filter was washed with PBS and fixed in 70% ethanol at 4°C. For quantification of migrated cells, cells were stained with hematoxylin and eosin. Migrated cells in the lower chamber were counted manually in 5 random microscopic fields.

Adhesion to Fibronectin

Fibronectin (100 μg/ml) was coated onto 24-well plates and left for 2 h at 37°C, and 1×10^5 EPCs were added to each well and left to attach for 30 min. Nuclei of adherent cells were stained with hematoxylin. Attached cells were counted manually in 5 random microscopic fields.

Statistical Analysis

Data are expressed as the means±SD. We used a natural logarithm transformation of hs-CRP (ln hs-CRP) to meet the demands of normal frequency distribution. One-way ANOVA was used to compare the mean values of variables among the three groups of patients with different numbers of stenosed coronary vessels, whereas χ^2 was used to compare proportions. Multivariate stepwise regression analysis was used for data analysis of the relation between various risk factors and the number of CD133/KDR double-positive cells. The interaction between the number of CD133/KDR double-positive cells and either inflammatory factors or severity of coronary stenosis was examined by partial correlation or analysis of covariance adjusted for age, gender and BMI. The relation between functional activity of EPCs and severity of coronary stenosis was estimated by partial correlation or analysis of covariance adjusted for age and gender. Values of $p<0.05$ were considered significant.

Results

Clinical Characteristics of Patients

A total of 104 patients with proven or clinically suspected CAD (mean age, 63.9±10.6 years; 74% men) were enrolled. Table 1 summarizes the characteristics of patients classified according to the number of diseased coronary arteries. Group I comprises 44 patients (61.5±9.98 years) with angiographically normal coronary arteries, group II included 35 patients with single vessel disease (63.03±10.01 years), and group III consisted of the remaining 25 patients (64.60±10.48 years) with multiple vessel disease. There were no differences in age, BMI, hypertension, current smoking status, family history of CAD, LVMI, plasma fasting glucose or serum lipid levels among the three groups, but significant differences in gender, diabetes, alcohol drinking status and creatinine clearance (Ccr) were found.

Risk Factors and hs-CRP Levels as Predictors of EPCs Number

A multivariate regression model was constructed to examine the relationships between the number of CD133/KDR double-positive cells and the risk factors. Age ($p=0.001$), hs-CRP ($p=0.012$), hypertension ($p=0.042$) and family history of coronary artery disease ($p=0.043$) were finally selected as independent predictors of the EPCs number (Table 2). Risk factors such as gender ($p=0.476$), diabetes ($p=0.773$), smoking ($p=0.512$), alcohol consumption ($p=0.903$), plasma fasting glucose ($p=0.581$), serum total cholesterol ($p=0.669$),

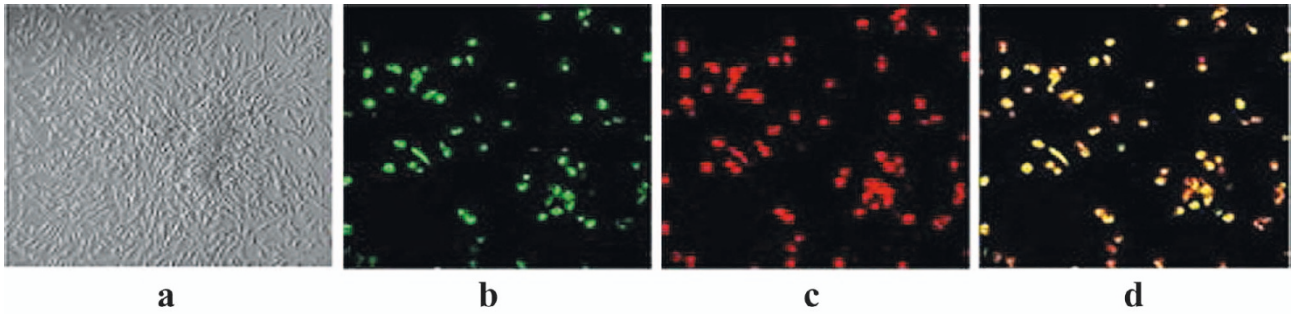

Fig. 3. *Characterization of endothelial progenitor cells. a: An endothelial progenitor cell colony with central clusters of cells surrounded by spindle-shaped cells. Magnification: ×100. b, c, d: Double-staining of mononuclear cells with lectin binding (green, exciting wavelength of 477 nm) and DiLDL uptake (red, exciting wavelength of 543 nm) under a laser scanning confocal microscope. Double-positive cells appearing yellow in the overlay were identified as differentiating EPCs. Magnification: ×200.*

triglyceride ($p=0.448$), LDL-cholesterol ($p=0.859$), HDL-cholesterol ($p=0.270$), uric acid ($p=0.157$), LVMI ($p=0.106$), VCAM-1 ($p=0.842$), ICAM-1 ($p=0.450$) and BMI ($p=0.110$) were not significant predictors of the number of circulating EPCs. hs-CRP was transformed to the natural log scale in order to improve the symmetry and comparability of effect estimates.

EPCs, hs-CRP and Risk Factors as Predictors of CAD

A multiple logistic regression analysis was performed with the presence/absence of CAD as the dependent variable and EPCs and risk factors as the predictive variables. Analysis showed that gender (male) (odds ratio [OR] 4.624, 95% confidence interval [CI] 1.093–19.562; $p=0.037$), ln hs-CRP (OR 3.151, 95% CI 1.466–6.772; $p=0.001$), diabetes mellitus (OR 11.922, 95% CI 0.961–147.950, $p=0.044$) and the number of CD133/KDR double-positive cells (OR 0.640, 95% CI 0.487–0.842; $p=0.001$) were significant predictors of CAD (Table 3). Variables excluded by the model were age ($p=0.936$), hypertension ($p=0.953$), smoking ($p=0.242$), alcohol consumption ($p=0.315$), family history of CAD ($p=0.499$), serum fasting glucose ($p=0.269$), serum cholesterol ($p=0.605$), LDL-cholesterol ($p=0.737$), LVMI ($p=0.248$), uric acid ($p=0.248$), Ccr ($p=0.946$), ICAM-1 ($p=0.398$), von Willebrand factor [vWF] ($p=0.831$) and BMI ($p=0.127$).

Relationship between EPCs Number, hs-CRP and Severity of CAD

When patients were classified according to the number of angiographically diseased coronary arteries, the EPCs number was significantly lower in patients with a single diseased artery (Group II, $n=35$, CD133/KDR double-positive cell number$= 0.043\pm0.004\%$, $p<0.05$ *vs*. Group I) and with multiple diseased arteries (Group III, $n=25$, CD133/KDR double-positive cell number$=0.030\pm0.005\%$, $p<0.01$ *vs*. Group I; $p<0.05$ *vs*. Group II) compared to patients with angiographically normal coronary arteries (Group I, $n=44$, CD133/KDR double-positive cell number$=0.064\pm0.004\%$) (Fig. 1a), while ln normalized hs-CRP levels were higher in Group II ($n=28$, ln hs-CRP$=0.725\pm0.125$ mg/l, $p<0.05$ *vs*. Group I) and Group III ($n=21$, ln hs-CRP$=1.343\pm0.206$ mg/l, $p<0.01$ *vs*. Group I; $p<0.05$ *vs*. Group II) than that in Group I ($n=39$, ln hs-CRP$=0.077\pm0.015$ mg/l) after adjustment for age, gender and BMI (Fig. 1b).

The Gensini scoring system assigned a higher score to proximal lesions than to distal lesions, so the severity of coronary stenosis was further defined by the Gensini score. The partial correlation coefficients adjusted for age, gender and BMI were obtained to evaluate the correlations between Gensini score and the number of CD133/KDR double-positive cells as well as serum hs-CRP and adhesion molecules. Significant partial correlation coefficients were noted between angiographic Gensini score and CD133/KDR double-positive cells ($n=60$, $r=-0.355$, $p=0.006$) (Fig. 2a), and ln normalized hs-CRP ($n=49$, $r=0.476$, $p=0.001$) (Fig. 2b).

By multiple stepwise (backward) regression analyses, a reduced number of CD133/KDR double-positive cells and ln normalized hs-CRP remained independent predictors of a high Gensini score (Table 4).

There were no significant differences in the serum levels of ICAM-1 and VCAM-1 among the three groups, and there were no correlations between Gensini score and serum levels of ICAM-1 and VCAM-1 (data not shown).

EPCs Characterization

MNCs isolated and cultured for 7 days formed distinct colonies and exhibited endothelial characteristics including positivity for DiLDL-uptake and lectin binding by using LSCM (Fig. 3).

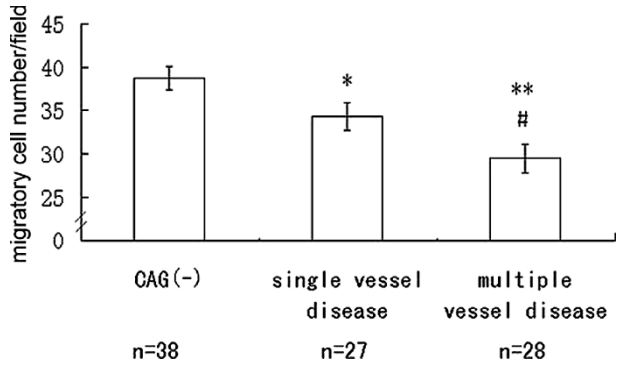

Fig. 4. *Comparative analysis of the migratory function of EPCs in patients with angiographically normal coronary artery and single or multiple diseased vessels.* *p < *0.05,* **p < *0.01, compared to the CAG(−) group,* #p < *0.05, compared to the single-vessel disease group.*

Migratory Capacity of EPCs and Severity of Coronary Stenosis

The migratory function of EPCs in response to VEGF, which is believed to be important during neovascularization, was evaluated using a transwell insert. As illustrated in Fig. 4, the migratory capacity of EPCs was compromised in patients with a single diseased artery (Group II, $n=27$, $p<0.05$ *vs*. Group I) and in those with multiple diseased arteries (Group III, $n=28$, $p<0.01$ *vs*. Group I) as compared with patients with angiographically normal coronary arteries (Group I, $n=38$). Moreover, the migratory capacity of EPCs was inversely correlated with the angiographic Gensini score ($n=55$, $r=-0.315$, $p=0.021$).

Adhesive Capacity and Severity of Coronary Stenosis

EPCs isolated from patients with CAD ($n=55$) revealed an impaired adhesive activity compared to those from patients with normal coronary arteries ($n=38$, $p<0.05$), while no significant correlation was found between adhesive function and angiographic Gensini score ($n=55$, $r=-0.196$, $p=0.159$).

Discussion

In the present study we measured the number and angiogenic activity of circulating EPCs in order to assess the relation between EPCs and the severity of coronary stenosis in patients with stable CAD.

The level of circulating EPCs in blood samples can be directly quantified by low cytometry measurement of the percentage of CD133/KDR double-positive or CD34/KDR double-positive cells. Unlike the progenitor marker CD34, CD133 is not expressed on mature endothelial cells (*7*); therefore, CD34/KDR double-positive cells also represent shedded cells of the vessel wall, whereas CD133/KDR double-positive cells more likely reflect immature progenitor cells.

The results of the present study demonstrated that the number of circulating EPCs, as measured by the number of CD133/KDR double-positive cells, was lower in patients with double or triple vessel disease than in those with single vessel disease. In addition, the number of EPCs was inversely correlated with the angiographic Gensini score. In our study, the severity of coronary stenosis was assessed by the number of diseased coronary arteries or by angiographic Gensini score, and both of the results demonstrated correlations between the number of EPCs and coronary stenosis. A previous study by Vasa *et al*. (*4*) suggested that the number of peripheral blood EPCs was significantly reduced in patients with CAD compared with control subjects. These results are in agreement with those of the present study, although the earlier study employed only a small group of patients and did not investigate the correlation between the number of circulating EPCs and the severity of CAD. Normal endothelium is indispensable for the regulation of vascular tone and maintenance of vascular homeostasis. The presence of endothelial dysfunction reflects a specific atherogenic vascular milieu, which is associated with perfusion abnormalities and cardiovascular events (*8*). Given that several experimental studies have indicated that bone marrow–derived EPCs contribute to the neovascularization of ischemic tissue in adults (*9*, *10*), the reduction in the number of circulating EPCs might contribute to reduced endothelial repair. On the other hand, chronic endothelial damage may result in the continuous exhaustion of circulating EPCs. In this study we did not perform experiments to differentiate the mechanisms by which the number of EPCs was reduced in CAD. Potential impairments in the production, mobilization or half-life of EPCs or continuous exhaustion of circulating EPCs in patients with coronary artery disease should be investigated in future studies.

Another important finding of our study is that the number of CD133/KDR double-positive precursor cells was inversely correlated with the serum level of CRP. A positive correlation between the CRP concentration and number of EPCs was previously reported in a clinical study by George *et al*. (*11*). Their study also employed patients with unstable angina, and their results suggested that, in acute coronary syndrome, chemokines such as VEGF may be released and EPCs may be transiently mobilized from bone marrow. Verma *et al*. demonstrated in an *in vitro* experiment that CRP significantly reduced the number of EPC cells, inhibited the expression of endothelial cell–specific markers, significantly increased EPC apoptosis, and impaired EPC–induced angiogenesis (*12*). Moreover, Suh *et al*. reported that CRP directly inhibits the angiogenic activity of EPCs and decreases the secretion of various arteriogenesis-related chemo-cytokines (*13*). The present study is the first to demonstrate in a clinical setting that serum CRP concentrations were independently correlated with the number of EPCs in patients with stable angina pectoris. The results of our study further confirm the effects of CRP

on the EPC number. Taken together, these findings suggest that CRP may reduce the number of functional EPCs in the bloodstream, thereby leading to increased risk of cardiovascular diseases.

The present study also confirms previous findings that the number of circulating EPCs correlates with conventional cardiovascular risk factors such as age, hypertension and family history of CAD. There are controversial findings regarding the correlation between individual risk factors and EPCs. In the study by Vasa *et al*. (*4*), smoking and LDL-cholesterol were found to be correlated with the number of EPCs, a finding in contradiction to the present research. In another study by Schmidt-Lucke *et al*. (*14*), age and a positive family history of CAD were found to be the only independent predictors of a reduced number of EPCs. Possible reasons for these discrepancies may include differences in the study subjects and surface markers of EPCs. In the present study, patients with acute coronary syndrome were excluded so that acute tissue ischemia (hypoxia) and neovascularization might be avoided. Secondly, some of the difference in results may be attributable to differences between ethnic groups, since our study included only Chinese subjects. Finally, when EPCs are quantified as CD34/KDR double-positive cells as in the previous study, they are likely to include shedded cells of the vessel wall, whereas when they are quantified as CD133/KDR double-positive cells as in the present study, they are more likely to reflect immature progenitor cells. The mechanisms by which risk factors reduce the number of circulating EPCs remain to be determined. It has been reported that cardiovascular risk factors may also lead to endothelial dysfunction. Therefore, it may be speculated that conventional risk factors not only participate in the endothelial injury but also play a role in the impairment of endothelial progenitor cells, thereby advancing the process of atherosclerosis.

Taking these results together, we speculate that either traditional risk factors or CRP may reduce the number of functional EPCs in the blood stream, thereby leading to endothelial dysfunction and the acceleration of atherosclerotic lesioning.

Given the low numbers of circulating EPCs, chemoattraction may be of utmost importance to the recruitment of reasonable numbers of progenitor cells to the ischemic or injured tissue. Among all the growth factors, VEGF is the most critical for vasculogenesis and angiogenesis, and recently collected data indicate that VEGF is an important factor for the migration of circulating EPCs (*15*). Our data suggest that the migratory capacity of EPCs was compromised in patients with a single diseased artery and those with multiple diseased arteries as compared with patients with angiographically normal coronary arteries. Moreover, the migratory capacity of EPCs was inversely correlated with the angiographic Gensini score in patients with stable CAD. The adhesion assays provide insight into the mechanism by which EPCs function *in vivo*, which has been shown to involve extension into the interstitium (cell-matrix adhesion) (*16*). In this study, the ability of EPCs to adhere to fibronectin was significantly lower in patients with CAD. However, there was no significant correlation between adhesive function and angiographic Gensini score.

In summary, a reduction in the number and functional impairment of EPCs might contribute to the reduction in endothelial repair in patients with coronary artery disease. Because of the strong relationship between the severity of CAD and EPCs in the blood stream, we speculate that one cause of CAD might be an increasing inability of these EPCs to keep up with the endothelial damage.

One limitation of the present study was the lack of follow-up on the participants, which prevented us from examining the association between EPCs and the development of atherosclerosis. Moreover, the number of patients included was not sufficient to evaluate the relationship between EPCs and risk factors; a future study with a larger cohort will be needed. Finally, the functional activity of EPCs was tested *in vitro*, and the results of this analysis may not necessarily correlate well with the *in vivo* response. The direct mechanism of the correlation requires further investigation.

References

1. Asahara T, Murohara T, Sullivan A, *et al*: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275**: 964–967.
2. Libby P, Ridker PM, Maseri A: Inflammation and atherosclerosis. *Circulation* 2002; **105**: 1135–1143.
3. Shi Q, Rafii S, Wu MH, *et al*: Evidence for circulating bone marrow–derived endothelial cell. *Blood* 1998; **92**: 362–367.
4. Vasa M, Fichtlscherer S, Aicher A, *et al*: Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001; **89**: E1–E7.
5. Devereux RB, Reichek N: Echocardiographic determination of left ventricular mass in man. Anatomic validation of the method. *Circulation* 1977; **55**: 613.
6. Gensini GG: A more meaningful scoring system for determining the severity of coronary heart disease. *Am J Cardiol* 1983; **51**: 606.
7. Fan CL, Li Y, Gao PJ, Liu JJ, *et al*: Differentiation of endothelial progenitor cells from human umbilical cord blood CD 34+ cells *in vitro*. *Acta Pharmacol Sin* 2003; **24**: 212–218.
8. Poredos P: Endothelial dysfunction in the pathogenesis of atherosclerosis. *Clin Appl Thromb Hemost* 2001; **7**: 276–280.
9. Kalka C, Masuda H, Takahashi T, *et al*: Transplantation of *ex vivo* expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci U S A* 2000; **97**: 3422–3427.
10. Murohara T, Ikeda H, Duan J, *et al*: Transplanted cord blood–derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest* 2000; **105**: 1527–1536.
11. George J, Goldstein E, Abashidze S, *et al*: Circulating endothelial progenitor cells in patients with unstable angina:

association with systemic inflammation. *Eur Heart J* 2004; **25**: 1003–1008.
12. Verma S, Kuliszewski MA, Li SH, *et al*: C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. *Circulation* 2004; **109**: 2058–2067.
13. Suh W, Kim KL, Choi JH, *et al*: C-reactive protein impairs angiogenic functions and decreases the secretion of arteriogenic chemo-cytokines in human endothelial progenitor cells. *Biochem Biophys Res Commun* 2004; **321**: 65–71.
14. Schmidt-Lucke C, Rossig L, Fichtlscherer S, *et al*: Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation* 2005; **111**: 2981–2987.
15. Shintani S, Murohara T, Ikeda H, *et al*: Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 2001; **103**: 2776–2779.
16. Vajkoczy P, Blum S, Lamparter M, *et al*: Multistep nature of microvascular recruitment of *ex vivo*–expanded embryonic endothelial progenitor cells during tumor angiogenesis. *J Exp Med* 2003; **197**: 1755–1765.