European Review for Medical and Pharmacological Sciences 2018; 22: 2767-2777

The relationship between number and function of EPCs and concentration of VEGF165 and SDF-1 in coronary artery spasm

Z.-Q. SHENG[1,2], Y.-F. LI[2], K.-L. ZHENG[2], H.-H. LU[2], J. XIE[1], H. WU[1], B. XU[1]

[1]Department of Cardiology, Drum Tower Clinical Medical Hospital, Nanjing Medical University, Nanjing, China
[2]Department of Cardiology, the Second Affiliated Hospital of Nantong University, Nantong, China

Abstract. – OBJECTIVE: We aimed at investigating the functions of endothelial progenitor cells (EPCs) in the pathophysiology of coronary artery spasm (CAS) and coronary artery disease (CAD), as well as to evaluate the correlation of these diseases with the number and function of EPCs, the plasma concentration of vascular endothelial growth factor 165 (VEGF165) and stromal cell-derived factor 1 (SDF-1).

PATIENTS AND METHODS: Participants were recruited into three groups, CAS, CAD and the control. The number and functions of early EPCs and outgrowth endothelial cells (OECs) were determined in peripheral blood samples, and the endothelial function was evaluated by measuring endothelium-dependent flow-mediated vasodilation (FMD).

RESULTS: No differences of baseline characteristics were found among CAS, CAD, and the control groups. The OECs isolated from CAS and CAD exhibited significant decrease in the percentage of CD34+/CD45- population, OEC colony formation, OEC proliferation and OEC tubulogenesis, nitric oxide (NO) production, endothelial nitric oxide synthase (eNOS) activity, and the phosphorylation level at Ser1177 of eNOS, compared with OECs isolated from control participants. Meanwhile, FMD was significantly reduced in CAS and CAD (CAS, 4.1% ± 1.9%; CAD, 4.3% ± 1.8%; control, 11.2% ± 3.5%). FMD was positively correlated to OEC functions including NO production, eNOS phosphorylation, colony formation, and proliferation. No differences of plasma VEGF165 and SDF-1 concentrations were recorded among these three groups. Similarly, there was no correlation between plasma VEGF165 (and SDF-1) concentration and EPC number and function.

CONCLUSIONS: EPCs show the potential of repairing damaged endothelium in CAS and CAD.

Key Words
Coronary artery spasm (CAS), Coronary artery disease (CAD), Endothelial progenitor cells (EPCs), Flow-mediated vasodilation (FMD), Correlation.

Introduction

Coronary artery spasm (CAS) has been an important and often overlooked etiology of chest pain. CAS plays an essential role in the pathogenesis of vasospastic angina, myocardial infarction, sudden death, and other ischemic heart diseases[1]. However, the precise mechanism underlying CAS remains unknown. Various pathophysiological abnormalities have been observed in patients with CAS, including endothelial dysfunction[2], autonomic system dysfunction, oxidative stress[3], genetic susceptibility, inflammation, and smooth muscle hypercontraction[4]. It has been demonstrated that endothelial dysfunction represents a main pathophysiological cause of coronary spasm[5]. The endothelium is a single-cell lining on the internal surface of blood vessels, cardiac valves, and numerous body cavities. Previous studies have shown that the vascular endothelium is a multifunctional organ, which exhibits complex metabolic capabilities, including modulation of cellular adhesion, vascular tone, thrombosis resistance, vessel wall inflammation, and smooth muscle cell proliferation[6]. Thus, it is important to maintain the integrity of the vascular endothelium and a well-balanced release of numerous vasoactive substances for a normal vascular function[7]. Therefore, the endothelial failure and imbalance of vasoactive factors contribute to the pathogenesis of vascular disease[7].

Increasing evidence suggests that the injured endothelial monolayer may be regenerated by endothelial progenitor cells (EPCs), which accelerates re-endothelialization[8]. Reduced levels and impaired adhesive ability of circulating EPCs significantly contribute to endothelial dysfunction[9]. Till now, no uniform definition of EPC population is established[10]. However, it has been demonstrated that EPCs may express surface protein mark-

Corresponding Author: Biao Xu, MD; e-mail: xubiao@medmail.com.cn

ers CD34, KDR and CD133, which are also detected in hematopoietic progenitor cells[11]. Based on the morphology as well as the cultured duration, EPCs have been classified into early EPCs, which appear within 4 to 7 days following plating, and late outgrowth endothelial cells (OECs), which appear as late as 14 to 21 days in culture[12]. Early EPCs and OECs originate from diverse bone marrow-born mononuclear cell populations. Briefly, early EPCs develop from a heterogeneous population of CD45 positive ($CD45^+$) hematopoietic cells, including $CD34^+/CD45^+$ hematopoietic progenitors as well as $CD45^+/CD14^+$ monocytes[13]. Contrarily, OECs are a homogenous population of rare circulating cells that derive from CD45 negative non-hematopoietic non-monocytes ($CD45^-/CD14^-$)[14,15]. Furthermore, these cultured EPCs always show different characteristics. For example, OECs (but not early EPCs) are able to directly form vascular networks *in vitro* [16] and construct perfused vessels *in vivo*[17]. Nevertheless, no investigation has reported the functions of EPCs in the development of CAS.

Since different EPCs exhibit various biological functions, we hypothesized that distinct EPCs populations would play different roles in CAS. Therefore, we investigated the roles of different EPC populations in human CAS by analyzing the clinical samples in this research.

Patients and Methods

Patients

We recruited participants from June 2016 to December 2016. The patients admitted to the Second Affiliated Hospital of Nantong University fell into the CAS group (30 patients) and the CAD group (22 patients), following the Japanese Circulation Society Guidelines for Diagnosis and Treatment of Patients with Vasospastic Angina (Coronary Spastic Angina) (JCS 2013). Patients in CAS group were those with < 50% stenosis in major coronary artery on coronary angiography, while those in CAD group had at least one lesion with >70% stenosis in major coronary artery on coronary angiography. We also recruited 20 subject as the control group, who used to experience chest pain, without the observation of >50% stenosis in major coronary artery on coronary angiography and proved negative on acetylcholine provocation test. Exclusion criteria were as follows: (1) > 65 years old; (2) peripheral arterial diseases, proliferative retinopathy and neoplastic disease; (3) acute coronary syndrome; (4) coagulopathies and/or hematopoietic system diseases; (5) history of surgical within 8 weeks of the study; (6) left ventricular ejection fractions (LVEF) <45% (ventricular dysfunction); (7) cardiomyopathy, valvular heart disease; (8) cardiac syndrome X; (9) coronary slow flow.

Ethics Statement

This study was approved by the Ethics Review Board for Clinical Studies of Nanjing Drum Tower Hospital. All patients provided written informed consent and the research was carried out conforming to the principles expressed in the Declaration of Helsinki.

EPC Isolation, Cultivation and Quantification

EPCs were determined and cultured as described previously[18]. After placement of an arterial sheath, 50 mL of whole blood were obtained for EPC isolation. Mononuclear cells (MNCs) were isolated by density gradient separation using Histopaque-1077 (Hao Yang Biological, Tianjin, China). To obtain early EPCs, 3×10^7 isolated mononuclear cells per well were planted into six-well tissue culture plates precoated with human fibronectin (Gene Operation, Lansing, MI, USA), and cultured in endothelial cell growth medium-2 (EGM-2MV, Lonza Walkersville, MD, USA) containing hydrocortisone, hEGF, VEGF, hFGF-B, IGF-1, ascorbic acid, and 5% fetal bovine serum (FBS, Gibco, Rockville, MD, USA). After incubation for 24 hours, the unattached cells were removed. The remaining cells were evaluated for their ability to ingest 1,1-dioctadecyl-3,3,39,39-tetramethylin-docarbocyanide-labeled acetylated low-density lipoprotein (acLDL) (Sigma-Aldrich, St. Louis, MO, USA) and to bind isothiocyanate ulex europaeus agglutinin lectin (UEA-1) (Sigma-Aldrich, St. Louis, MO, USA). After 7 days of culture, early EPC colonies were calculated by scanning culture plates. Three microscopic fields of view were assessed by random field under 200× magnification imaging with an inverted microscope from Carl Zeiss (Axio Observer Al, Jena, Germany). The calculation of EPC was performed by an observer who was blinded to the clinical profile of the patients. To obtain OECs, 3×10^7 mononuclear cells per well were seeded into human fibronectin pre-coated plates. On the 21st day of *in vitro* culture, the number of OECs colonies was quantified as above.

Flow Cytometry

The EPCs were quantified by performing flow cytometry as described previously[16], and the cells were subject to assessment of $CD34^+/KDR^+$ population, early EPCs population ($CD34^+/CD45^+$) and OECs population ($CD34^+/CD45^-$)[11]. In brief, cells were isolated from whole blood using density gradient separation; then, these cells were gated by forward and side scatter to select mononuclear cells by removing erythrocytes, granulocytes, and cell debris. The obtained cells were incubated with the indicated antibodies against human antigens: phycoerythrin-Cy5-conjugated CD45 (BD Pharmingen, San Diego, CA, USA), fluorescein isothiocyanate-conjugated CD133 (BD Pharmingen, San Diego, CA, USA), phycoerythrin-conjugated KDR (BD Pharmingen, San Diego, CA, USA), and fluorescein isothiocyanate-conjugated CD34 (BD Pharmingen, San Diego, CA, USA). Fluorescent isotype-matched IgG1 antibodies were used as the negative controls. Each analysis was conducted in duplicates and included 100 000 events.

EPC Proliferation Assay

EPC proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as reported previously[19]. EPCs were incubated with MTT (0.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 4 h. The produced blue formazan was solubilized with dimethyl sulfoxide (DMSO) and the absorbance at 550/650 nm was measured. Each experiment was performed in triplicate. The absorbance values of each well were measured with a microplate spectrophotometer (Molecular Devices; Sunnyvale, CA, USA).

EPCs Migration Assay

The migratory ability of early EPCs and OECs was measured in a modified Boyden chamber (8 μm pore size, Corning, Corning, NY, USA) transwell assay[19]. Briefly, 4×10^4 EPCs were seeded in the top chamber of 24-well trans-well plates with a polycarbonate membrane (8 μm pores) covered by serum-free medium, and another special medium (50 ng/mL VEGF, Sigma-Aldrich, St. Louis, MO, USA) was added in the lower chamber. After incubation for 24 hours, the membrane was washed with phosphate-buffered saline (PBS) for three times and fixed with 4% paraformaldehyde. Additionally, the upper side of membrane was wiped gently. After that, the membrane was stained using hematoxylin for 10 min. The migration of OECs was assessed by measuring the area containing migrated cells as a percentage of the total area in five random fields (100x). The data were expressed as mean ± standard deviation (SD).

OEC Tube Formation

OEC tube formation assay was conducted as described previously[20]. ECM matrix gel solution was thawed overnight at 4°C, mixed with ECM matrix diluent buffer (Sigma-Aldrich, St. Louis, MO, USA), placed in a 96-well plate (Corning, Corning, NY, USA) and incubate at 37°C for about 1 hour. After that, the OECs (10^4) were placed on matrix solution with EGM-2 MV medium, and incubated at 37°C for about 20 hours. Tube formation was observed under an inverted light microscope (100×). Four typical fields were taken, and the results were expressed as mean ± SD.

Measurement of NO Production

Early EPCs of 7 days and OECs of 21 days were starved by serum deprivation for 24 hours. The production of NO was estimated by the concentration of NO in the culture supernatant. The NO concentration was analyzed by the nitrate reductase assay. The protocol conformed to the manufacturer's instructions (Nanjing Jiancheng Technology, Nanjing, China).

Growth Factor Detection

The cytokines VEGF165 (vascular endothelial growth factor 165) and SDF-1 (stromal cell-derived factor-1) were analyzed by ELISA (enzyme-linked immunosorbent assay) in stored plasma specimens according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA).

Western Blot

Protein extracts were prepared and Western blots were performed as previously described[21]. Proteins from EPCs were obtained with RIPA lysis buffer (Beyotime, Shanghai, China) containing a protease inhibitor cocktail. These protein samples were subjected to electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked by 5% slim milk for 30 min and incubated with indicated primary antibodies: anti-eNOS (Cell Signaling Technology, Danvers, MA, USA), anti-p-eNOS (Ser1177, Cell Signaling Technology, Danvers, MA, USA), at 4°C overnight. The membranes were washed and

Table I. The clinical baseline characteristics of patients in this study.

Group	Age (years)	Gender (M/F)	Smoking (%)	Diabetes (%)	LVEF (%)	BMI (kg/m^2)	Systolic (mm Hg)	Diastolic (mm Hg)	Glucose (mg/L)
CAS	54±8	19/11	23.33	13.33	62.87± 6.11	23.7± 3.78	131.37± 18.42	84.37± 13.29	10.5± 2.1
CAD	56±7	13/7	25.00	15.00	63.25±6.62	23.45± 4.04	131.55± 23.09	84.85± 12.64	10.7±1.8
Control	56±6	13/7	25.00	15.00	63.84±6.16	23.45± 4.62	132.64± 22.31	85.26± 14.07	10.6± 1.7

Group	Creatinine (mg/dL)	TG (mg/dL)	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	Statin (%)	ACEI (%)	Asprine (%)
CAS	3.88± 0.91	143.2± 46.0	173.7±29.7	89.2± 26.6	48.3± 14.3	46.67	66.67	100
CAD	3.92± 1.05	147.3±48.6	180.3± 31.3	94.6± 27.4	48.6± 16.2	50.00	70.00	100
Control	3.97± 0.95	145.0±68.7	174.1± 27.8	91.9±24.7	47.1± 13.9	45.00	65.00	100

The data were represented mean± standard deviation (SD). CAS, coronary artery spasm; CAD, coronary artery disease; Control, the control participants; M/F, male or female; LVEF: left ventricular ejection fractions; BMI: body mass index; Systolic: systolic blood pressure; Diastolic: diastolic blood pressure; TG: triglycerides; TC: total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; Medication history included ACEI, statins and aspirine had been continuously used in the last month before the test.

subsequently incubated with secondary antibody (1:1000) (Thermo Fisher Scientific, Waltham, MA, USA) for approximately 1 hour. Protein bands were detected by employing an enhanced chemiluminescence system (Cell Signaling Technology, Danvers, MA, USA). β-actin was used as an internal control. The semi-quantitative analysis of protein bands was conducted using the Image J software (Rawak Software, Dresden, Germany).

Endothelium-Dependent Flow-Mediated Vasodilation

Endothelium-dependent flow-mediated vasodilation (FMD) was evaluated using a 7.5-MHz linear array transducer (Hewlett-Packard Sonos 5500, Andover, MA, USA) to scan the brachial artery in longitudinal section. The measurement of FMD was performed strictly as described previously[22]. To minimize the mental stress, it was necessary to make the patients as comfortable as possible, and the procedure was performed in a quiet air-conditioned room (25°C). FMD was calculated as the maximal post-occlusion diameter relative to the averaged pre-occlusion diameters.

Statistical Analysis

All data were presented as mean ± standard deviation (SD) for numeric variables and expressed as number or percentage for categorical variables. $p<0.05$ was considered as statistically significant. Differences in baseline characteristics of underlying diseases and treatments were compared using x^2-test. The correlation between independent variables was analyzed by performing simple linear regression and multiple regression analyses. The GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) and Statistical Product and Service Solutions (SPSS) 19 software packages (IBM, Armonk, NY, USA) were used for statistical analysis.

Results

Baseline Characteristics of the Patients

In this study, 72 patients were recruited, with a mean age of 52 ± 9 (range from 39 to 75) years and 46 (63.9%) of patients were male. Their baseline characteristics were summarized in Table I. No significant differences were found among the CAS, CAD, and control group regarding age, sex, body mass index, smoking, systolic blood pressure, diastolic blood pressure, diabetes, serum levels of total cholesterol, triglycerides, high-density lipoproteins, low-density lipoproteins and creatinine ($p>0.05$ for all, Table I). Furthermore, no significant differences were found in administrated drugs, including aspirin, angiotensin-converting enzyme inhibitors (ACEI), angiotensin II receptor blockers and statins ($p>0.05$ for all, Table I).

Characterization of EPCs

The initially planted cells were round (Figure 1A). Up to the 7th day, the attached cells began to be clustered and these cells were called early EPCs (Figure 1B). Then, we found that OECs appeared as clusters on the 21st day after plating, and showed cobblestone appearance (Figure 1C). Both types of EPCs took up DiI-acLDL and showed lectin binding affinity (Figure 1D, 1E).

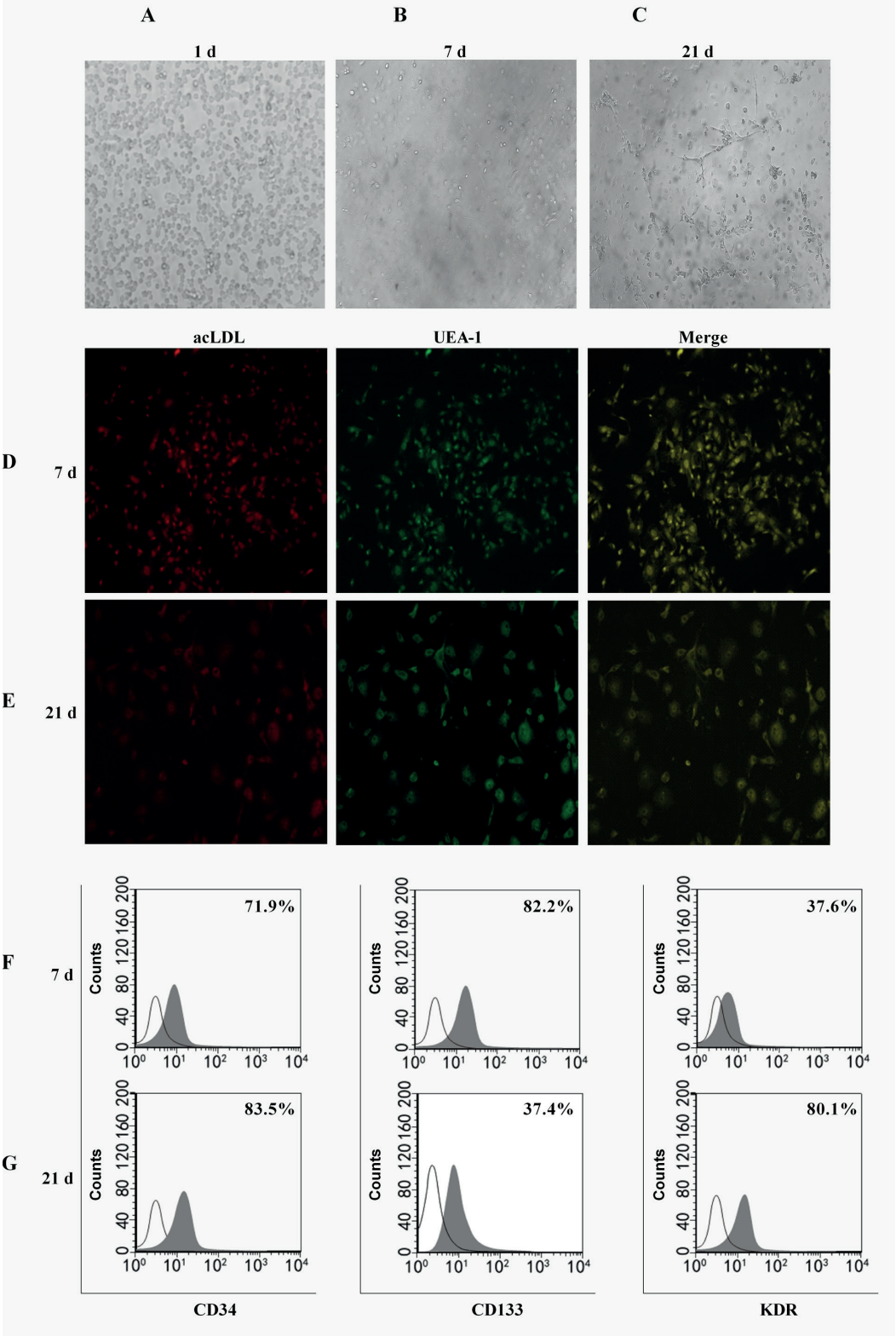

Figure 1. The phenotype of endothelial progenitor cells (EPCs). (**A**, **B**, **C**,) Representative photos of cultured cells on the 1st, 7th (early EPCs), and 21st day (OECs, outgrowth endothelial cells). (**D**, **E**,) The cells on the 7th and 21st day were incubated with UEA-1 and acLDL and imaged with a microscope (x100). (**F**, **G**,) Representative early EPCs and OECs from blood samples on the 7th and 21st day were analyzed by flow cytometry; these cells were determined by detecting the surface markers CD34, CD133, and KDR in cell populations of low cytoplasmic granularity.

Table II. The circulating EPC levels and function in the three groups.

	CAS (n = 30)		CAD (n = 22)		Control (n = 20)	
EPC levels						
CD34+/KDR+(%)	0.59 ± 0.24		0.33 ± 0.20###		0.62 ± 0.19	
CD34+/CD45+(%)	0.21 ± 0.018		0.14 ± 0.009###		0.22 ± 0.015	
CD34+/CD45-(%)	0.05 ± 0.003***		0.04 ± 0.002###		0.07 ± 0.005	
Early EPCs colonies	5.11 ± 1.27		3.0 ± 1.11###		5.08 ± 1.45	
OECs colonies	2.65 ± 0.88***		2.13 ± 0.95###		4.02 ± 1.16	
EPC function	**Early EPCs**	**OECs**	**Early EPCs**	**OECs**	**Early EPCs**	**OECs**
Proliferation	0.51 ± 0.06	0.25 ± 0.03***	0.32 ± 0.05	0.21 ± 0.04###	0.48 ± 0.05	0.36 ± 0.05
Migration	18.93 ± 5.12	12.17 ± 2.6	10.38 ±2.92	8.15 ± 1.84###	17.77 ±2.16	12.54 ± 1.90
Tubulogenesis		18.97 ± 2.48***		13.78 ± 2.37###		23.22 ± 2.95

The data were expressed mean±SD; ***p<0.001, CAS versus the control group; ###p<0.001, CAD vs. the control group. CAS, coronary artery spasm; CAD, coronary artery disease; Control, the health people; EPC, endothelial progenitor cell; EPCs, endothelial progenitor cells; OECs, outgrouth endothelial cells.

Finally, we determined the expression of CD133 and KDR in EPCs by flow cytometry. Compared with the EPCs on the 7th day, we observed that CD133 was significantly downregulated by approximately 50% and KDR was upregulated by more than 40% in OPCs on the 21st day (Figure 1F, 1G).

The Number and Function of EPCs in CAS, CAD and the Control Group

Compared with control participants, patients with CAS were found to exhibit significantly decreased $CD34^+/CD45^-$ population, OEC colonies, OEC proliferation, and OEC tubulogenesis (p<0.001 for these variables, Table II). However, no differences were recorded in early EPC colonies, $CD34^+/KDR^+$, $CD34^+/CD45^+$, early EPC migration, and OEC migration (p>0.05 for these variables). On the other hand, we found that compared with the controls, patients with CAD were found to have a significantly decrease in $CD34^+/KDR^+$ population, $CD34^+/CD45^+$ population, $CD34^+/CD45^-$ population, as well as the early EPCs colonies, OECs colonies, early EPCs proliferation, OECs proliferation, early EPC migration, OEC migration, as well as OEC tubulogenesis (p<0.001 for these variables). Taken together, the number of EPC was larger in CAS than in CAD, and the functions of EPC was better in CAS than in CAD.

NO and eNOS in the CAS, CAD and Control Group

OECs from patients with CAS and CAD (Figure 2 and Table III) exhibited a significant downregulation in the level of NO production, eNOS activity as well as the protein phosphorylation (Ser1177) of eNOS, compared with OECs obtained from control participants (p<0.001 for these variables, Table III). Similarly, early EPCs from patients with CAD also exhibited a significant decrease in the

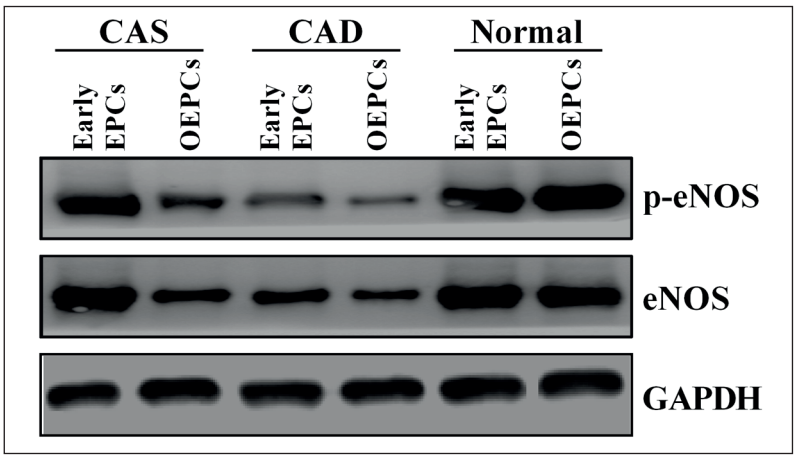

Figure 2. The expression of eNOS and p-eNOS in EPC cells from typical CAS and CAD samples. The expression of eNOS and p-eNOS in representative early EPCs and OECs from blood samples on the 7th and 21st day were determined by Western blot. The GAPDH was used as an internal control.

Table III. NO concentration and phosphorylation of eNOS in the OECs.

	CAS (n=28)		CAD (n=24)		Control (n=21)	
	Early EPCs	OEPCs	Early EPCs	OEPCs	Early EPCs	OEPCs
NO (μM)	11.51±3.67	7.64±1.70***	6.56±3.26###	6.97±2.34###	10.39±4.66	13.52±0.19
Total eNOS protein (Fold of control)	1.04±0.05	0.63 ±0.05***	0.68±0.07###	0.44±0.08###	1.00±0.08	1.00±0.03
P-eNOS protein (Fold of control)	0.98±0.07	0.61±0.08***	0.67±0.02###	0.45±0.08###	1.00±0.01	1.00±0.02
eNOS mRNA (Fold of control)	1.03 ±0.04	0.52±0.07***	0.64±0.08###	0.45±0.06###	1.00±0.03	1.00±0.04

The data were expressed mean±SD; ***p<0.001, OECs of CAS versus OECs of the control group; ###p<0.001, early EPCs or OECs of CAD vs. early EPCs or OECs of the control group. NO, nitric oxide; eNOS, endothelial nitric oxide synthase; P-eNOS protein, phosphorylation of endothelial nitric oxide synthase.

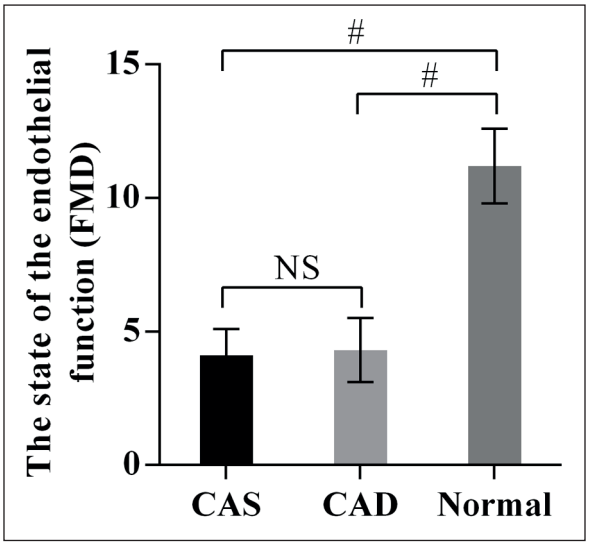

Figure 3. The state of the endothelial function was analyzed by FMD in CAS, CAD and control groups. Data was presented as mean ± SD; NS, no significance; @p< 0.001, compared with the control group.

level of NO production, eNOS activity, and the protein phosphorylation atSer1177 of eNOS compared with early EPCs isolated from the control group (p<0.001 for these variables). However, there was no difference of these variables between early EPCs isolated from CAS and controls (p>0.05 for these variables).

The Relationship Between FMD and EPCs Levels and Function

The state of the endothelial function was assessed by FMD. The results showed that patients with CAS and CAD had significantly decreased FMD compared with the control group, and there was no difference between CAS and CAD (CAS, 4.1% ± 1.9%; CAD, 4.3% ± 1.8%; controls, 11.2% ± 3.5%) (Figure 3). Further analysis demonstrated that FMD was significantly correlated with OEC functions including NO production, eNOS phosphorylation (Ser1177), colony formation, and proliferation (p<0.001 for these variables, Table IV).

Table IV. Relationship between FMD and EPC levels and functions.

	CAS (n=28)		CAD (n=24)		Control (n=21)	
	Spearman r	*p*-value	Spearman r	*p*-value	Spearman r	*p*-value
NO	0.6170	<0.001	0.4918	< 0.05	0.9297	<0.001
Total eNOS	-0.1522	>0.05	-0.2191	>0.05	-0.0174	>0.05
P-eNOS	0.8384	< 0.001	0.7537	< 0.001	0.8259	<0.001
eNOS mRNA	0.0679	>0.05	-0.3293	>0.05	0.1416	>0.05
OECs colonies	0.6041	<0.001	0.8808	< 0.001	0.9348	<0.001
EPC function						
Proliferation	0.6802	<0.001	0.8247	<0.001	0.8848	<0.001
Migration	0.2554	>0.05	0.3928	>0.05	0.4493	>0.05
Tubulogenesis	-0.0268	>0.05	0.0593	>0.05	0.0544	>0.05

We determined the relationship between FMD and OEC number, function. CAS, coronary artery spasm; CAD, coronary artery disease; Control, the health people; EPC, endothelial progenitor cell; OEC, outgrouth endothelial cell; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; P-eNOS protein, phosphorylation of endothelial nitric oxide synthase.

The Relationship Between Growth Factors and EPC Number and Function

Finally, we determined the correlation between the concentration of growth factors and EPC number and function. No significant differences were recorded in plasma VEGF165 concentration (CAS, 52.83 ± 8.17; CAD, 53.26 ± 8.34; 51.69 ± 8.78) in these three groups (p> 0.05 for all, Table V). Moreover, no significant correlation was recorded between plasma VEGF165 concentration and EPC number and function (p> 0.05 for all, Table VI). In addition, similar results demonstrated that there was no significant correlation between plasma SDF-1 concentration and EPC number and function (p> 0.05 for all, Table VII).

Table V. The levels of growth factors in the three groups.

	CAS (n=30)	CAD (n=22)	Control (n=20)
VEGF165 (pg/mL)	52.43±8.17NS	53.26±8.34 NS	51.69±8.78
SDF-1 (pg/mL)	6.12±1.04 NS	6.25± 1.21 NS	6.09±0.99

CAS, coronary artery spasm; CAD, coronary artery disease; Control, the health people; VEGF165, vascular endothelial growth factor; SDF-1, stromal cell-derived factor-1; NS, no significance. The VEGF165-related data were expressed as mean ± SD and the SDF-1 related data were showed as percentage; NS (p>0.05), CAS or CAD *vs*. Control.

Table VI. Relationship between VEGF165 concentration and EPC levels and functions.

	CAS (n=28)		CAD (n=24)		Control (n=21)	
	Spearman r	*p*-value	Spearman r	*p*-value	Spearman r	*p*-value
EPC levels						
CD34+/KDR+	0.2459	>0.05	0.1716	>0.05	0.4521	>0.05
CD34+/CD45+	0.5161	>0.05	0.3589	>0.05	0.6835	>0.05
CD34+/CD45-	0.4123	>0.05	0.5922	>0.05	0.4455	>0.05
Early EPC colonies	0.6619	>0.05	0.4582	>0.05	0.3782	>0.05
OEC colonies	0.4753	>0.05	0.5276	>0.05	0.0336	>0.05
EPC function						
Early EPC proliferation	0.7307	>0.05	0.4442	>0.05	0.3111	>0.05
OEC proliferation	0.5438	>0.05	0.6123	>0.05	0.5571	>0.05
Early EPC migration	0.7291	>0.05	0.6703	>0.05	0.4005	>0.05
OEC migration	0.2877	>0.05	0.3364	>0.05	0.6325	>0.05
OEC tubulogenesis	-0.2015	>0.05	-0.2149	>0.05	-0.2887	>0.05

Table VII. Relationship between SDF-1 concentration and EPC levels and function.

	CAS (n=28)		CAD (n=24)		Control (n=21)	
	Spearman r	*p*-value	Spearman r	*p*-value	Spearman r	*p*-value
EPC levels						
CD34+/KDR+	0.3114	>0.05	0.1716	>0.05	0.4866	>0.05
CD34+/CD45+	0.4325	>0.05	0.7093	>0.05	0.6764	>0.05
CD34+/CD45-	0.4060	>0.05	0.4522	>0.05	0.3238	>0.05
Early EPC colonies	0.5937	>0.05	0.5631	>0.05	0.4312	>0.05
OEC colonies	0.4482	>0.05	0.3477	>0.05	0.4614	>0.05
EPC function						
Early EPC proliferation	0.7019	>0.05	0.3124	>0.05	0.6937	>0.05
OEC proliferation	0.5987	>0.05	0.5456	>0.05	0.3315	>0.05
Early EPC migration	0.3546	>0.05	0.2999	>0.05	0.3154	>0.05
OEC migration	0.7701	>0.05	0.4154	>0.05	0.5463	>0.05
OEC tubulogenesis	-0.2893	>0.05	-0.2607	>0.05	-0.6755	>0.05

Discussion

It has been demonstrated that various mechanisms and precipitating factors may play a role in the pathogenesis of vasospastic angina, including endothelial failure, smooth muscle hypersensitivity, increased oxidative stress, upregulated autonomic tone, gentle inflammation, decreased magnesium, and genetic susceptibility[21-33]. The integrity of the vascular endothelium and a proper release of numerous vasoactive factors both contribute to normal vascular physiology, while its dysfunction plays a role in the pathogenesis of vascular disease[34-36]. In our work, patients with CAS and CAD exhibit significantly reduced FMD compared with the control group, suggesting that endothelial function is abnormal in patients with CAS and CAD.

EPCs are bone marrow-derived cells, which are able to develop into functional mature endothelial cells, regenerating the diseased endothelium[37]. Levels of circulating EPCs have been revealed to be correlated with endothelial functions and cardiovascular risk factors. Therefore, the levels of circulating EPCs may be used to identify patients with high cardiovascular risk. Moreover, decreased levels of circulating EPCs can be considered as an independent indicator to predict progression of the atherosclerotic disease as well as the development of cardiovascular events[38-44]. Thus, these studies support that EPCs mediated endogenous vascular repair plays an important role in regulating the clinical progression of CAD. However, no research has reported the role of circulating EPCs in patients with CAS. In this study, EPCs insolated from patients with CAS, compared with cells from the control participants, showed impaired migration and tubulogenesis and lower levels of OECs. On the contrary, our results indicated that no such relationship is determined between CAS patients and control participants on early EPCs number and functions. It has been reported that early EPCs are originated from the hematopoietic system and they fail to form perfused vessels[45]. Previous researches[10,12] have demonstrated that OECs have high proliferative potential, and OECs can improve perfused vessels in immune deficient mice[45]. In this regard, it is likely that the early EPCs and OECs play distinct roles in the complex process of neovascularization. Particularly, the principal activity of early EPCs would be the secretion of growth factors and pro-angiogenic cytokines and OECs would be the main participator in the proliferation of numerous endothelial elements involved in the new vessels[16,46]. Collectively, the improvement of OEC functions may provide novel therapeutic strategies for CAS treatment.

In this study, OECs from patients with CAS were found to have significantly decreased NO production, eNOS expression, and eNOS phosphorylation at Ser1177 compared with OECs insolated from the control participants; no differences were found in early EPCs between CAS and control groups. Furthermore, the data also demonstrated that in OECs, NO production and phosphorylation of eNOS at Ser1177 were significantly associated with FMD while no such relationship was found in early EPC. It has been reported[41] that NO often plays a key role in the functions of EPCs as a signaling molecule. The activation of eNOS catalyzes NO production, which plays a central role in maintaining cardiovascular homeostasis. The NO released in the coronary endothelium exerts paracrine effects on cardiomyocytes, predominantly prolonging relaxation[47]. Additionally, NO often acts as a positive regulator of EPC activity, and tube formation of cultured OECs is partly dependent on NOS function[48]. When eNOS expression is reduced by oxidized low-density lipoprotein both in mature endothelium and EPCs, both the survival and the adhesive properties of these cells would be impaired[49]. Similarly, eNOS deficient mice display suppressed ischemia triggered angiogenesis as well as reduced EPC mobilization[50]. Consequently, these results suggest that OECs influence the development of CAS and CAD probably by modulating the phosphorylation of eNOS, which is closely associated with NO production. It might be a promising treatment to recover CAS and CAD by enhancing the eNOS activity in OECs.

If the EPC is defined as a progenitor cell for the endothelial lineage, we may assume that the following fundamental properties would be identified in this cell population: (1) a circulating cell population which may proliferate and differentiate into the endothelial lineage; (2) the ability to form capillary-like tubes *in vitro*; (3) the ability to regenerate stable human blood vessels (cells must secrete a basement membrane) that would be integrated as part of the host circulatory system to form the intima of arterial, venous, as well as capillary structures when they are implanted into tissues. However, it has been demonstrated that circulating endothelial colony-forming cells (ECFCs) and other bone marrow-derived cells fail to exhibit these features mentioned above[10]. Taken together, identifying a unique cell surface marker would facilitate a convenient isolation and enrichment method of EPCs, which present all the indicated characteristics. In a word, the identification of EPCs remains a research focus in the field of coronary artery diseases.

Conclusions

We demonstrated that endothelial regeneration might play a central role in the pathophysiological mechanisms of CAS and CAD. Levels and function of EPCs, particularly the OECs, should be taken as a promising target in treating the patients with CAS and CAD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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