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The Involvement of Endothelial Progenitor Cell Dysfunction in the Angiographically Defined Coronary Atherosclerotic Patients

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Objectives: To evaluate the levels of endothelial progenitor cell-colony forming units in the angiographically defined coronary atherosclerotic patients. **Methods:** The 10 ml blood was drawn from the peripheral vein of 12-man patients that 4-stable angina, 4 acute myocardial infarction (AMI), and 4 healthy people. Peripheral blood mononuclear cells were isolated by Ficoll density-gradient centrifugation and EPC-CFUs was assayed after two plating and a 6-day culture on fibronectin-coated, 72 well plates, as described. eNOS enzyme titers were determined by ELISA according to the protocol in the cell culture. **Results:** The people were 52 ± 2.12 years. The number of EPC-CFUs increases with the accordance of patients with stable angina, AMI, healthy people with the statistical significance ($H = 15.8$, $p < 0.001$): stable angina (2.6 ± 0.47 colony/well), AMI (6.7 ± 0.81 colony/well), healthy people (10.5 ± 1.34 colony/well). Furthermore, the Kruskal–Wallis test of eNOS enzyme levels in patients with stable angina (5.2 ± 0.61 pg/ml), AMI (8.7 ± 1.49 pg/ml), and healthy people (13.7 ± 2.48 pg/ml). The significant difference ($H = 5.7$, $p < 0.010$) was observed among the three groups. The number of EPC-CFUs had a direct significant correlation ($r = 0.621$, $p < 0.001$) with the eNOS enzyme levels of this culture. **Conclusions:** The number of EPC-CFUs and eNOS enzyme levels decrease at patients with stable angina, indicate more than endothelial dysfunction.

Keywords: Endothelial Progenitor Cell, Colony Forming Units, Endothelial Nitric Oxide Synthetase, Coronary Atherosclerosis, Coronary Artery Disease.

Introduction

Coronary artery disease (CAD) is the leading cause of death in Worldwide. CAD has many risk factors, such as hypercholesterolemia, smoking, diabetes mellitus, arterial hypertension and aging, endothelial dysfunction by inducing impairment of endothelial progenitor cell (EPC) in peripheral blood [1, 2]. In 1997, Asahara et al. isolated an EPC from the human peripheral blood of adults which differentiate in vitro into endothelial and contribute to neoangiogenesis after tissue ischemia in vivo [3]. EPCs are capable of proliferating and differentiating into endothelial cells. Impaired function of EPC to circulation contributes to coronary artery disease, and patients with reduced numbers of EPC-colony forming units (EPC-CFUs) are at increased risk for the development of coronary atherosclerosis. However, other markers typical for the EPC lineage can be found on circulating EPC, although with varying intensity. These cells form EPC-CFU over time that stains positive for several endothelial markers such as endothelial nitric oxide synthetase (eNOS), plasma vascular endothelial growth factor (VEGFR) and others, but also express markers of hematopoietic lineage such as CD45 [4].

In the absence of a phenotypic definition, functional endothelial colony forming assays have been developed, whereby cultured peripheral mononuclear cells form colonies exhibiting mature endothelial cell characteristics. Formation of such colonies from the peripheral blood has been taken as inferential evidence that circulating EPCs exist within the adult circulation. Based on the premise that colonies arise via clonal expansion of a single precursor cell, the enumeration of endothelial colonies has been used to estimate the frequency of circulating EPCs [5]. Such colonies and have been used in a large number of both pre-clinical and clinical studies in order to determine the relationship between putative EPCs and cardiovascular disorders. The endothelial progenitor cell-colony forming units (EPC-CFUs) assay described by Hill et al. [6] has been used most extensively. In support of their vasculoprotective role, EPC-CFUs are reduced in patients with overt atherosclerosis, and cardiovascular risk factors. An increased capacity to generate EC-CFUs is associated with better vascular function [6], and furthermore EPC-CFUs are increased in response to cardiovascular stress, such as coronary artery bypass surgery [7], myocardial ischemia [8] and infarction [9] and angioplasty [10]. But, Inadequate coronary collateral development in patients with CAD is likewise associated with reduced numbers of circulating EPC [11].

Therefore, the patient with CAD that reduced numbers of circulating EPC, leads to complications of plaque rupture, suddenly thrombosis, etc. After that, the patient occurred unstable angina and AMI. A common feature of the characterization of various EPC subtypes is eNOS expression. Indeed, most groups have identified eNOS mRNA or protein expression as well as eNOS activity in certain types of EPC. The EPC-CFUs had a higher expression level of eNOS enzyme and the expressions of EPC markers were elevated during culture. The eNOS enzyme levels might indicate that the EPC-CFU was differentiating towards mature endothelial cells [4].

According to this hypothesis, we sought to evaluate the levels of endothelial progenitor cell-colony forming units in the angiographically defined coronary atherosclerotic patients.

Materials and Methods

Patients and control subjects

All subjects (n = 12) underwent a quantitative coronary angiography. The 8 patients subjects contained stable angina pectoris (n = 4) with stable stenosis and acute myocardial infarction (n = 4) with acute occlusion in the coronary arteries. Control subjects consisted of 4 persons without significant stenosis in their coronary arteries and healthy volunteers free from clinical symptoms such as angina and cardiovascular risk factors.

Quantitative coronary angiography

Cardiac catheterization was performed according to the guidelines for the percutaneous coronary intervention of the American College of Cardiology Foundation and the American Heart Association [12].

Preparation of blood samples

10ml blood was drawn from the peripheral veins after cardiac catheterization. Mononuclear cells were isolated with the use of a Ficoll density gradient (SepMate 50, Stem Cell Technologies Inc, USA) according to standard protocols. Additional blood samples were obtained for routine analyses.

Culture of EPC-CFUs

In an endothelial basal medium (Stem Cell Technologies) with supplements, 5-106 mononuclear cells were seeded on human fibronectin-coated plates (Sigma Aldrich). After 48 hours, 1-106 nonadherent cells were transferred into new fibronectin-coated wells to avoid contamination with mature endothelial cells and no progenitor cells. After six days in vitro, EPC-CFUs in at least six wells were counted by two independent investigators after Giemsa staining. EPC-CFUs are expressed as absolute numbers of colonies per well. The eNOS enzyme titers determined by enzyme-linked immunosorbent assay according to the manufacturer's recommended protocol (R and D Systems, Inc. USA).

Statistical analysis

Data were expressed as the mean (M) ± standard deviation (SD). Data were analyzed using the Kruskal–Wallis test (H test) in the nonparametric statistics. Statistical analysis was performed using IBM SPSS statistic program (IBM software, Inc. USA). Statistical significance was defined as $p < 0.050$.

Ethical statement

All procedures performed in studies involving human participants were by the ethical standards of the institutional (MNUMS, 6/3/201506, approved on Jan 01, 2015) and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Results

Patient characteristics

The patients and control subject details are shown in Table 1. The 12 people individuals were enrolled consisting of 8 patients with CAD, AMI, and 4 controls. Patients had various cardiovascular risk factors such as hypertension, and dyslipidemia and had been administered medications corresponding to each disease (Table 1).

Hypercholesterolemia and hypertension were among patients with stable angina and AMI. The total white blood cell, neutrophil, and lymphocyte counts were similar between the 3 groups (all $p > 0.10$).

Culture of EPC-CFUs

On day 6 of the assay, count the number of colonies per well for each sample. The EPC-CFUs is defined as a central core of round cells with radiating elongated spindle-like cells at the periphery (Figure 1).

Table 1. Placental morphometric parameters

Variables	Stable angina (n = 4)	AMI (n = 4)	Healthy people (n = 4)	p-value*
	(Mean ± SD)	(Mean ± SD)	(Mean ± SD)	
Age	56 ± 4.3	53 ± 2.7	52 ± 3.2	0.563
WBC (cells/μl)	6.3 ± 0.7	6.6 ± 1.3	5.9 ± 1.1	0.463
Lymphocyte (cells/μl)	2.05 ± 0.73	2.12 ± 1.32	1.95 ± 0.89	0.140
Lymphocyte (cells/μl)	2.05 ± 0.73	2.12 ± 1.32	1.95 ± 0.89	0.140
Neutrophil (%)	62%	64%	61%	0.520
Hypercholesterolemia	2 (17%)	2 (17%)	-	0.251
Hypertension	1 (8%)	1 (8%)	-	0.363
Aspirin	4 (33%)	3 (8%)	-	0.178
Statin	2 (17%)	1 (8%)	-	0.466
Family history of CAD (%)	25%	50%	25%	0.160

* Kruskal–Wallis test

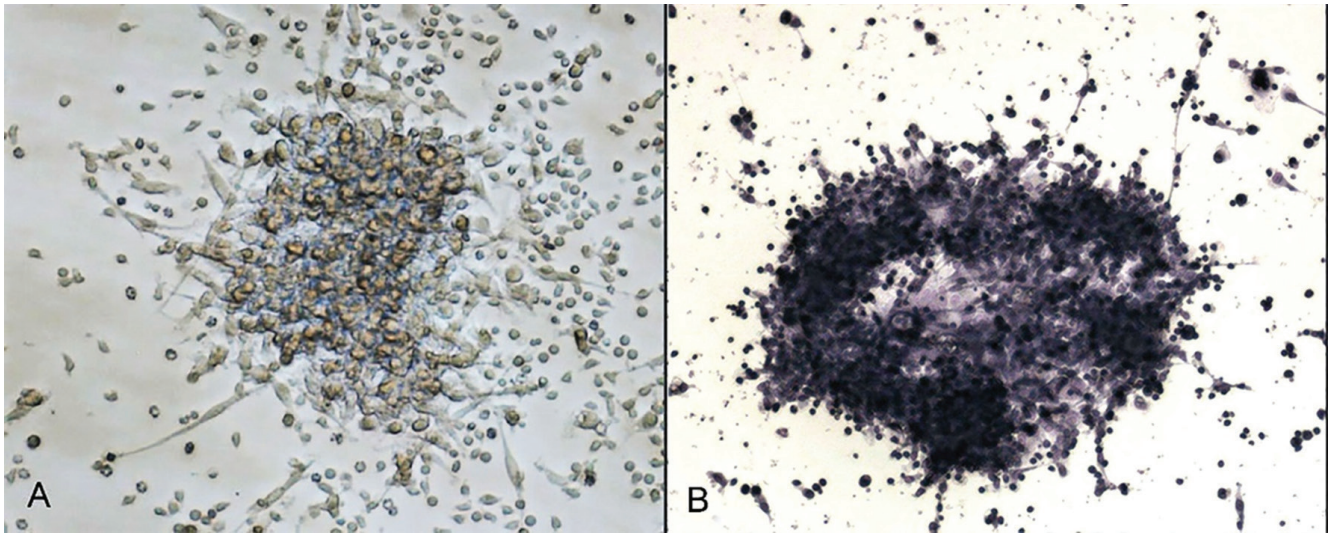

Figure 1. EPC-CFUs (A. 6 days, B. Giemsa staining, CFU-Hill medium, x100).

The number of EPC-CFUs increases with the accordance of patients with stable angina, AMI, healthy people with the statistical significance ($H = 15.8$, $p < 0.001$): stable angina (2.6 ± 0.47 colony/well), AMI (6.7 ± 0.81 colony/well), healthy people (10.5 ± 1.34 colony/well) (Figure 2).

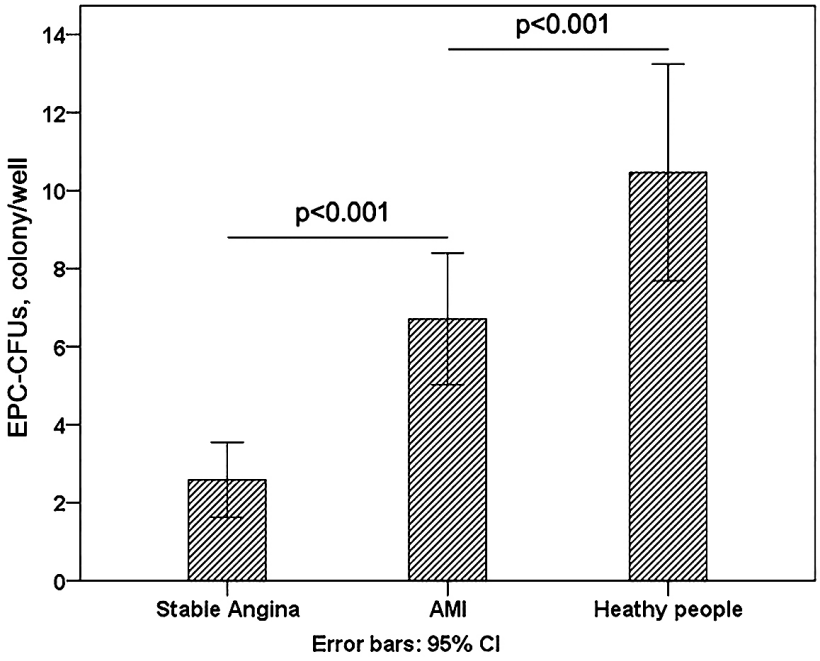

Figure 2. EPC-CFUs per well (each group of stable angina, AMI and healthy people).

The regeneration of endothelial cells with senescent and damaged decreases stable angina more than AMI and healthy groups. Therefore, it may be a possible plaque rupture of coronary atherosclerosis.

Functional evaluation of EPC-CFUs

The Kruskal–Wallis test of eNOS enzyme levels in patients with stable angina pectoris (5.2 ± 0.61 pg/ml), acute myocardial infarction (8.7 ± 1.49 pg/ml) and healthy group (13.7 ± 2.48 pg/ml). The significant difference ($H = 5.7$, $p < 0.010$) was observed among the three groups (Figure 3). Furthermore, the number of

EPC-CFUs had a direct significant correlation
($r = 0.621$, $p < 0.001$) with the eNOS enzyme levels of this culture.

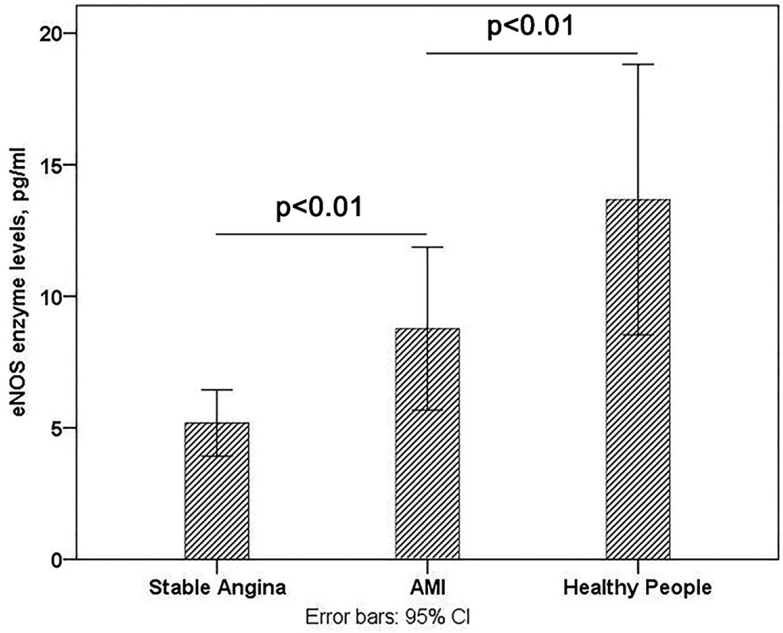

Figure 3. The levels of eNOS enzyme (each group stable angina, AMI and healthy people).

Discussion

The identification and characterization of EPC have been the most challenging and controversial. In general, 2 approaches have been used to isolate EPCs: (1) culture and colony assays and (2) selection of subpopulations based on surface markers.

Although the markers for identification of EPC populations vary between studies, it has been agreed that there are lineage and functional heterogeneities within the EPC population. There are at least two different types of EPCs: the early and late EPCs. Early EPCs are usually referred to as the angiogenic EPC population obtained from short-term cultures of 4-7 days in vitro. These early EPC-CFUs and possess many endothelial characteristics, such as harboring markers of CD31, and eNOS [13]. We used the method of culture of EPC-CFUs. Because The EPC-CFUs possibly determine morphology feature using a light microscope without need surface marker due to this method have the selective medium of EPC-CFUs [14]. EPC-CFUs is defined as a central core of round cells with radiating elongated spindle-like cells at the periphery in light microscopy [15]. However, a consensus has not yet been reached for a true definition of the EPCs, and the controversy regarding the identification of these cells still exists. Generally, EPCs are defined by the ability to express the antigens shared by hematopoietic and EPCs, such as CD133, CD34, CD14, CD45, vascular endothelial growth factor-2 receptor (KDR), von Willebrand Factor (vWF) [16].

In our study, the identification of EPC-CFU not uses based on flow cytometry (FACS) was more precise than culture assays. But we sought to use that the identification of EPC-CFU assay to presented different features of colony morphology and eNOS enzyme.

Despite the above results, the most interesting finding was that EPC-CFUs expressed the eNOS and could produce NO. EPC-CFUs had almost the same expression level of eNOS mRNA. From the western-blot results, we also found that the eNOS was expressed in EPC-CFUs, though at a lower level compared with the type of EPC. Furthermore, both types of EPCs could produce NO steadily. From the function level, EPCs should have the properties of differentiation into endothelial cells and contribution to neovascularization. And as one of the important functions of endothelial cells, the capability to produce NO could become the most reliable indicator of EPCs. Thus, we presumed that the expression of eNOS and the production of NO, as reliable biological features, could be used to identify EPCs [17]. Therefore, we evaluated the number of EPC colonies per well using the method of EPC-CFUs. After that, EPC identified to

determine the eNOS enzyme in the culture of CFU, their assessed function of EPC.

Our study, EPC-CFUs have 10.5 ± 1.34 colony/well in the healthy people, was like a result of Tagawa et al. [18] (12±1.4 colony/well). The EPC-CFUs significantly was ($F = 17.3$, $p < 0.001$) stable angina group less than AMI and healthy group. This result similar was George [19] and Aragona et al. [20] As a result, endothelial dysfunction activates coronary atherosclerosis, decreases circulating EPC with created EPC-CFUs. Recently in the study, the number of circulating EPC has increased the first week after AMI. Therefore, the levels of EPC-CFU increase in the AMI group were similar to the results of Shintani et al [21].

Circulating EPCs are mobilized endogenously in response to tissue ischemia or exogenously by cytokine therapy with several other inflammatory molecules such as hypoxia-induced factor and matrix metalloproteinase, which thereby augment the neovascularization of ischemic tissues. Moreover, AMI is the most established acute pathological stimulus for EPC mobilization. Indeed, AMI leads to an increase in EPC number, which correlates positively with VEGF levels. A positive association of increased CD133+ cells and IL-8, an inflammatory chemokine with angiogenic properties, is also reported in patients with AMI. After an AMI, progenitor/stem cells are mobilized from bone marrow, released into peripheral blood, and subsequently homed in the myocardium [22].

Cells were further characterized in cultures as EPC-CFUs. The number of released cells reached a maximum of 7 days after the onset of AMI parallel to a significant increase in plasma levels of VEGF. Further that, Massa et al. [23] number of EPC after 7 days of AMI decreased than patients with stable angina, healthy groups. A decreased number of EPC after AMI has indicated poor complications. Because, the decreased number of circulating EPC, loss regeneration during senescent, damage of EPC and neoangiogenesis, collateral circulation. Therefore, the risk of mortality has a higher rate [24]. As an important product of EPCs, eNOS in the enzyme of EPCs and EPC-CFUs was measured as total amounts of eNOS enzyme [17, 25].

The term EPC describes a group of cells existing in diverse stages of differentiation ranging from hemangioblasts to differentiated endothelial cells [26]. However, conflicting results have been reported in the field, and the identification and characterization of EPCs in vascular biology is still a subject of much discussion. Yoder [27] has concluded that EPCs could be defined by three general approaches in the human system: method of culture and the morphological criteria; monoclonal antibodies and fluorescence-activated cell sorting (FACS) analysis to enumerate specific cell populations; colony-forming assay in vitro, though all of these approaches have defects. In most reports, the combination of different surface markers was embraced for the identification of EPCs. But there has been a great deal of controversy associated with which of these surface markers or combination of them can delineate the definitive profile for EPCs.

Despite the above results, the most interesting finding was that both types of cells expressed the eNOS and could produce NO. The EPC-CFUs had almost the same expression level of eNOS mRNA [28]. From the western-blot results, we also found that the eNOS was expressed in both colony and types of EPCs, though at a lower level compared with human aortic endothelial cells. Furthermore, both colony and types of EPCs could produce NO steadily. From the function level, EPCs should have the properties of differentiation into endothelial cell and contribution to neovascularization. And as one of the important functions of endothelial cells, the capability to produce NO could become the most reliable indicator of EPCs. Thus, we presumed that the expression of eNOS and the production of NO, as reliable biological features, could be used to identify EPC-CFUs [29]. The eNOS enzyme levels determined healthy group more than stable angina and AMI groups. This result was similar like Güven et al [30]. The weak differentiation of EPCs indicated less colony of EPC in group of stable anginas.

Impaired EPCs levels during the acute phase were associated with the absence of major adverse clinical outcomes. The authors suggested that patients with low EPC count have a reduced capacity for angiogenesis, repair of endothelial damage, and formation of collateral vessels [31]. Similarly, other researchers reported a better prognosis related to the higher EPC count during the ischemic event [32].

There are some limitations in this study

We cultured EPC-CFUs from human peripheral blood. This study not used common phenotypes such as expressing some endothelial antigen. However, they also presented different features of colony morphology, eNOS enzyme. Therefore, we have evaluated the number of EPC-CFUs that defined as a central core of round cells with radiating elongated spindle-like

cells at the periphery. In addition, the eNOS enzyme identifies the determined culture of EPC-CFUs. Stimulating chemokine uses to improve circulating EPC in the stable angina pectoris. The EPC increases endothelial function, as a result, improves secondary prevention and prevention to acute myocardial infarction, etc. Therefore, we try to study about stimulating chemokine of circulating EPC and EPC captured bio-stent.

Conclusions

Patients with stable angina showed a decrease of circulating EPCs. Especially, the leading poor prognosis reason by low eNOS enzyme and EPC-CFUs count during patient with stable angina.

Conflict of Interest

The authors declare no conflict of interests.

Acknowledgments

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