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Role of circulating endothelial progenitor cells in the reparative mechanisms of stable ischemic myocardium

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ABSTRACT

Background: Mobilization of endothelial progenitor cells (EPCs) into circulation from bone marrow in patients with acute myocardial infarction has strong scientific evidence; less is known about EPC mobilization in patients with stable coronary artery disease (CAD). The aim of this study was to investigate the association of stable ischemic heart disease with EPC levels in tissue and blood.
Methods: Fifty-five consecutive patients admitted to a single treatment center for valve or coronary artery bypass grafting (CABG) surgeries were included in the study. Blood samples were collected in the morning before surgery and analyzed by flow-cytometry to determine peripheral EPC levels (EPC/ml). Tissue EPC (CD34 + VEGFR2+) levels were assessed on a right atrial appendage segment.
Results: Mean age was 76 ± 5 years, 48% were men, and 53% had CAD The number of CD34+ VEGFR2+ cells in the tissue of patients with CAD was significantly higher ($p < 0.005$) and circulating EPC showed a tendency to be reduced by approximately 20% in peripheral blood of patients with CAD when compared to those without CAD.
Conclusion: Patients with stable CAD had higher EPC density values (EPC/mm2) and were more likely to have lower EPC blood levels when compare with normal controls.

1. Introduction

Since the identification of endothelial progenitor cells (EPCs) by Asahara et al. in 1997 [1], significant progress has been made in determining the cellular and molecular attributes of EPCs and their potential for treatment of human disease. Mobilization of EPCs during angiogenesis and vascular repair have been described in several experimental clinical settings [2,3], however translation from bench to bedside is never straightforward. Recent investigation of the pathophysiological and therapeutic implications of EPCs suggests their involvement after ischemia and in atherosclerotic patients. EPCs are not innocent biomarkers but active players in cardiovascular system. The number of circulating EPCs are elevated in patients with an acute coronary syndrome, such as acute myocardial infarction (MI) [4–6] or unstable angina (UA) [7], due to increased mobilization from the bone marrow into the bloodstream. In myocardial infarction, the number of circulating EPCs is markedly increased, returning to basal level only after approximately 60 days [8]. The functional role of the bone marrow cells in MI is attributable to the angiogenic properties and release of growth factors and cytokines, but also to their ability to restore the population of cardiac progenitor cells by selective homing to specific areas of myocardial injury and conversion to the phenotype of cardiac side- population cells [9–10]. In fact, bone marrow-derived hematopoietic cells may generate cardiomyocytes within the infarcted myocardium in some animal models [11], however, if the involvement of EPC in acute coronary syndrome has been largely discussed. Less is known about EPC mobilization in patients with stable coronary artery disease (CAD) [12]. The aim of this study was to investigate whether if there is an association of stable CAD with EPC levels in tissue and blood.

2. Methods

Consecutive patients admitted between January 2013 and January 2015 at the University Hospital of Pisa for surgical procedures on the aortic valve, mitral valve, ascending aorta and for coronary artery bypass grafting (CABG) were included in the study. Exclusion criteria were: patients requiring emergency or repeat operations, patients with severe COPD or cancer or those who refused to be enrolled. Preoperative diagnosis of cardiac disease was assessed clinically, by means of 2-D transthoracic echocardiography and coronary angiography; echo- Doppler studies were also used to evaluate brachiocephalic vessels and limb arteries. Patients with 1 vessel or 2 vessels disease at angiography (defined as presence of a significant stenosis >75% in one of the major coronary arteries at coronary angiography) or with previous revascularizations were classified as having

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coronary artery disease. Indication for surgery was based on the most recent guidelines according to the specific pathology diagnosed at the end of each patient work up. The research was approved from the responsible review committee and informed consensus was provided for each patient. EPC (CD34 + VEGFR2 +) levels in blood and cardiac tissue samples were evaluated for each patient. EDTA-peripheral blood samples were collected in the morning before surgery, and analyzed by flow-cytometry. Tissue samples were obtained from the right atrial appendage, and stored in formalin.

2.1. Surgical techniques

In all patients the heart was approached through a median sternotomy and all operations were performed by means of moderately hypothermic standard cardiopulmonary bypass. Arterial and vein grafts were prepared in a standard fashion when CABG was required. After systemic heparinization with heparin 300 IU/kg of heparin (reaching a target activated clotting time over 480 s), the ascending aorta was cannulated followed by cannulation of either the right atrium or both venae cavae. Prior to insertion of a cannula in the right auricle, a small sample of the top tip of the right atrial appendage was resected for subsequent analysis. The original study protocol designated resection of the tissue samples from the left ventricle, but this location was subsequently considered either unpractical or unsafe and therefore not performed. Taking small samples of the right atrial tissue did not either alter the planned procedure nor had any influence on the results of the operation.

2.2. Immunohistochemical evaluation of tissue EPC

Tissue samples from the right atrial appendage were cut into 1 mm thick slices and fixed in 10% formalin for 24–48 h and embedded in paraffin; from each slice a three micrometer section was obtained, dewaxed in xylene and rehydrated in ethanol and distilled water. Non-specific peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide for 15 min. Antigen retrieval was performed by microwaving the sections for 15 min in citrate /EDTA buffer (pH 7.8). Detection of CD34 and VEGFR2 double positive cells was accomplished by primary incubation of samples with an anti-CD34 mouse monoclonal antibody (mAb, clone QBEnd/10, Cell Marque, prediluted) and anti-VEGFR2 (KDR/Flk-1) rabbit mAb (clone 55B11, Cell Signaling, dilution 1:200) followed by secondary antibody incubation (HRP Polymer anti-Mouse IgG; AP Polymer anti-Rabbit IgG) and immunohistochemical detection (AEC [red color] used to detect HRP; BCIP/NBT [purple/blue color], used to detect AP. EPCs were quantified by light microscopy by determining the number of CD34+/VEGFR2+ double positive medium sized spindle cells/mm2 present in the myocardial tissue at epicardial level. Positivity for CD45 was not one of exclusion criteria since some circulating (hem)angioblasts (including EPCs) are CD45 positive and CD133+/VEGFR2+ cells represent progenitors of endothelial cells at an earlier stage when compared to CD34+/VEGFR2+ [13]. Co-expression of these two markers, together with morphology, allows to exclude mature endothelial cells and monocytes/macrophages. Mature endothelial cells lying on endocardium and small vessels do not co-express VEGFR2. The latter together with CD34+ (VEGFR+, CD34+ double positive) lying on endocardium and small vessels may represent EPCs generated at sites of vascular injury from circulating CD34+ cells [14]. In addition mature endothelial cell of small lymphatic vessels inside the myocardial tissue do not express CD34, moreover, monocytes/macrophages inside the myocardial tissue are round medium to large sized cells that do not co-express CD34 and VEGFR2. Finally stained sections were independently evaluated by two pathologists (C.S. and A.G.N), both blinded to the clinical data. Discrepancies in interpretation were resolved by consensus conference at a double-headed microscope.

2.3. Evaluation of circulating endothelial progenitor cells

EDTA-peripheral blood samples were processed within 4 h after collection. Briefly, 100 μl of samples of peripheral venous blood of patients were incubated with PerCP-conjugated anti-human CD34/45 mAb and AlexaFluor 647-conjugated anti-VEGFR2 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) in the dark at 4 °C, according to manufacturers' instruction. Red cells were then lysed with 1× BD FACS Lysis solution (BD Pharmigen, Oxford, UK) for 5 min at room temperature. Alexa Fluor and PerPC isotype-matched antibodies were used as negative control. Validation of the assay was performed adopting the gating strategy defined by the International Society of Haematotherapy and Graft Engineering (ISHAGE) guidelines and CD45dimCD34+ cells were quantified for VEGFR2 [15]. A minimum of 100 CD34+ events were collected. Quantitative fluorescence analyses were performed with a FACS-Calibur instrument and data were processed using Cell Quest Software (BD Pharmigen). Each analysis included at least 500,000 events. The number of EPCs was expressed as absolute number of cells per ml of blood.

2.4. Statistical methods

Continuous data are expressed as mean ± SD, categorical data are expressed as frequency or percentage. *t*-test was used in paired data. The normality of distributions of EPC blood numbers was assessed using a one-sample Kolmogorov–Smirnov test. The interaction between the number of CD34+ VEGFR2+ cells and CAD was examined by binary logistic regression model. Differences of $p < 0.05$ were considered statistically significant.

3. Results

Fifty-five patients were included in the study. The mean age of study participants was 76 ± 5, 46% were male, and 53% had CAD. Baseline characteristics for patients with and without coronary artery disease were similar. 21% of patients had positive family history, 80% had hypertension, 22% of patients were smoker and 23% of patients were obese. (Table 1) The number of CD34+ VEGFR2+ cells in the tissue of patients with CAD was significantly higher when compared with control subjects (30cells mm^2 vs 20 cells/mm^2 $p < 0.005$) and circulating EPC showed a tendency to be reduced by approximately 20% in peripheral blood of patients with CAD when compared with no CAD patients (Figs. 1–2) Binary Logistic Regression showed that concentration of EPC was higher in patients with coronary disease when corrected for risk factors ($p = 0.035$). (Table 2).

4. Discussion

There is a large amount of data in the literature demonstrating that the extent of the EPC pool is an indicator of cardiovascular health and that EPCs negatively correlate with severity of both peripheral and coronary atherosclerosis especially in acute settings [16]. In 2007 Nonaka-Sarukawa M et al. showed that the number of CD34+ MNCs was decreased in severe CHF and increased in mild CHF; suggesting that impaired EPC recruitment might be involved in the pathophysiology of severe CHF. In the same year Michowitz Y et al. showed that EPC levels are independent predictors of all-cause mortality among patients with CHF [17,18]. Moreover several studies have shown that the number of circulating EPCs is strictly related to the presence of risk factors and other systemic diseases [19]. Recently Felice et al., showed that the presence of depressive disorders is associated with a significantly

Table 1
Patient's characteristics.

	CAD 0 ($n = 24$)	CAD 3 ($n = 30$)	*p*-Value
Age (years, mean ± SD)	75 ± 7	76 ± 5	0.7*
Gender (male/female)	7/17	19/11	**0.016**†
Cardiovascular risk factors			
CAD family history, n (%)	4 (16%)	14 (47%)	**0.024**†
Diabetes, n (%)	3 (12%)	6 (20%)	0.07†
Hypertension, n (%)	21 (87%)	27 (90%)	>0.99†
Dyslipidemia, n (%)	8 (33%)	17 (57%)	0.10†
Active smokers, n (%)	2 (8%)	10 (33%)	0.05†
Biochemical parameters			
Fasting glucose (mg/dl)	92 ± 10.5	101 ± 20	0.32*
Total cholesterol (mg/dl)	202 ± 56	160.8 ± 53	0.14*
LDL cholesterol (mg/dl)	135 ± 51	78 ± 6.5	0.05*
HDL cholesterol (mg/dl)	53.6 ± 15	44.5 ± 17	0.34*
Triglycerides (mg/dl)	106.8 ± 38	131 ± 51	0.32*
C-reactive protein (mg/l)	0.37 ± 0.38	0.29 ± 0.25	0.63*
Creatinine (mg/dl)	1.12 ± 0.37	0.9 ± 0.19	0.18*
HbA1C	36.7 ± 14	54.5 ± 23.7	0.08*
Peak-hsTn (ng/ml)	24.5 ± 6.6	82.8 ± 7.8	0.26*
Therapy at the day of EPC analysis			
Statins, n (%)	9 (37%)	22 (73%)	0.17†
ACE inhibitors, n (%)	7 (29%)	23 (77%)	0.07†
Beta-blockers, n (%)	4 (16%)	18 (60%)	0.07†
Oral antidiabetics, n (%)	3 (12%)	4 (13%)	>0.99†
Nitrates, n (%)	1 (4%)	3 (10%)	0.42†
Insulin, n (%)	1 (4%)	4 (13%)	0.42†
Diuretics, n (%)	15 (62%)	18 (60%)	>0.99†
Antiplatelet agents, n (%)	9 (37%)	23 (76%)	0.17†
Anticoagulants, n (%)	5 (21%)	0 (100%)	0.48†

CAD, coronary artery disease; LDL, low density lipoprotein; HDL, high density lipoprotein; hsTn, high sensitive troponine. Data are expressed as number (n), percentage (%) and as mean and SD.

* Independent-sample *t*-test.

† Fischer's exact test.

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