Mobilization of endothelial progenitor cells in acute cardiovascular events in the PROCELL study: Time-course after acute myocardial infarction and stroke

Ander Regueiro a,⁎, Elisa Cuadrado-Godia b, Carlos Bueno-Betí c, Maribel Díaz-Ricart d, Anna Oliveras e, Susana Novella c, Gemma González Gené f, Carole Jung g, Isaac Subirana h, Jose Tomás Ortiz-Pérez a, Mercé Roqué a, Xavier Freixa a, Julio Núñez f, Gines Escolar d, Jaume Marrugat c, Carlos Hermenegildo c, Miguel Angel Valverde g, Jaume Roquer b, Juan Sanchis f, Magda Heras a

a Cardiology Department, Thorax Institute, Hospital Clinic, IDIBAPS, Universitat de Barcelona, Barcelona, Spain
b Neurology Department, Hospital del Mar, IMIM (Hospital del Mar Medical Research Institute), DCEXS Universitat Pompeu Fabra, Barcelona, Spain
c INCLIVA Biomedical Research Institute, Hospital Clínic de Valencia, Department of Physiology, Universitat de València, Valencia, Spain
d Hemotherapy-Hemostasis Department, Biomedical Diagnostics Center CDB, Hospital Clinic, IDIBAPS, Universitat de Barcelona, Barcelona, Spain
e Nephrology Department, Hospital del Mar, Universitat Autònoma de Barcelona, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain
f Laboratory of Molecular Physiology and Channelopathies, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain
g Epidemiology and Cardiovascular Genetic Group, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain

Original article

ABSTRACT

The mobilization pattern and functionality of endothelial progenitor cells after an acute ischemic event remain largely unknown. The aim of our study was to characterize and compare the short- and long-term mobilization of endothelial progenitor cells and circulating endothelial cells after acute myocardial infarction or atherothrombotic stroke, and to determine the relationship between these cell counts and plasma concentrations of vascular cell adhesion molecule (VCAM-1) and Von Willebrand factor (VWF) as surrogate markers of endothelial damage and inflammation. In addition, we assessed whether endothelial progenitor cells behave like functional endothelial cells. We included 150 patients with acute myocardial infarction or atherothrombotic stroke and 145 controls. Endothelial progenitor cells [CD45−, CD34+, KDR+, CD133+] and circulating endothelial cells [CD45−, CD31+, CD146+, CD133+] were measured in controls (baseline only) and in patients within 24 h (baseline) and at 7, 30, and 180 days after the event. Myocardial infarction patients had higher counts of endothelial progenitor cells and circulating endothelial cells than the controls (201.0/mL vs. 57.0/mL; p < 0.01 and 181.0/mL vs. 62.0/mL; p < 0.01). Endothelial progenitor cells peaked at 30 days post-infarction (201.0/mL vs. 369.5/mL; p < 0.01), as did VCAM-1 (573.7 ng/mL vs. 701.8 ng/mL; p < 0.01). At 180 days post-infarction, circulating endothelial cells and VWF decreased, compared to baseline. In stroke patients, the number of endothelial progenitor cells — but not circulating endothelial cells — was higher than in controls (90.0/mL vs. 37.0/mL; p = 0.01; 105.0/mL vs. 71.0/mL; p = 0.11). At 30 days after stroke, however, VCAM-1 peaked (628.1/mL vs. 869.1/mL; p < 0.01) but there was no significant change in circulating endothelial progenitor cells (90.0/mL vs. 78.0/mL; p < 0.34). At 180 days after stroke, circulating endothelial cells and VWF decreased, compared to baseline. Cultured endothelial progenitor cells from controls and myocardial infarction patients had endothelial phenotype characteristics and exhibited functional differences in adhesion and Ca2+ influx, but not in proliferation and vasculogenesis. In myocardial infarction patients, VCAM-1 levels and mobilization of endothelial progenitor cells peaked at 30 days after the ischemic event. Although a similar VCAM-1 kinetic was observed in stroke patients, endothelial progenitor cells did not increase. Endothelial progenitor cells had mature endothelial capabilities in vitro.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Acute myocardial infarction (AMI) and atherothrombotic stroke are hallmark examples of endothelial damage complicated by superimposed thrombosis [1,2]. After an endothelial injury, circulating endothelial cells (CECs) are detached from the intimal monolayer [3] and

---

**Keywords:**
- Endothelial progenitor cell
- Cell-adhesion molecule
- Myocardial infarction
- Stroke

**Abbreviations:**
- AMI, acute myocardial infarction; CECs, circulating endothelial cells; EPCs, endothelial progenitor cells; NIHSS, National Institutes of Health Stroke Scale; NSTEMI, non-ST-segment myocardial infarction; STEMI, ST-segment elevation myocardial infarction; TIA, transient ischemic attack; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; VWF, Von Willebrand factor.

* Corresponding author at: Cardiology Department, Hospital Clinic, IDIBAPS, University of Barcelona, Villarroel 170, 08036 Barcelona, Spain. Tel.: +34 93 227 93 05.

E-mail address: aregueiro@clinic.ub.es (A. Regueiro).
endothelial progenitor cells (EPCs) are mobilized from the bone marrow into the peripheral circulation. EPCs can then differentiate into mature endothelial cells, initiating an ideal self-reparative process [4–6]. Increased CEC counts have been described in cardiovascular disease. A higher number of CECs predict adverse events after an acute coronary syndrome [7]. The CEC count has been shown to correlate with other markers of vascular disease such as Von Willebrand factor (VWF) in the acute phase after a myocardial infarction [8]. On the other hand, a reduction in EPCs has been associated with the presence of cardiovascular risk factors [9,10] and with a worse prognosis after ischemic events [11–14]. Systemic inflammation could play a role in the peripheral mobilization of EPCs [15]. Vascular cell adhesion molecule (VCAM)-1 is expressed and upregulated on endothelial cells of atherosclerotic lesions and is a marker of inflammation in atherosclerosis [16]. Information about long-term EPC mobilization and CEC counts after acute ischemic events is scarce because previous studies have been limited to the acute and subacute phase. To our knowledge, no studies have analyzed both cell subtypes after AMI or stroke. In addition, it is unknown if the EPC and CEC counts are correlated with known markers of endothelial injury and activation at different time points. Describing the long-term kinetics of EPC mobilization after two different ischemic events is important to better understand the pathophysiology of endothelial injury and repair.

This study had three objectives: (1) to characterize and compare the short- and long-term mobilization pattern of EPCs and CECs following AMI or stroke; (2) to determine the relationship between cell counts and plasma concentrations of VCAM-1 and VWF at different time points; and (3) to assess whether EPCs behave like functional endothelial cells in terms of cell adhesion, growth curve, vasculogenesis, and intracellular calcium signals triggered by endothelial activators.

2. Methods

The PROCELL study was a multicenter, prospective, population-based, case–control study paired by sex and age. The study protocol was approved by the Institutional Ethics Committees of the three participating hospitals in Spain. All patients gave a written informed consent to participate.

2.1. Study population

Between February 2009 and July 2012, we included 150 patients with AMI or stroke and 145 controls. Controls were recruited from a cross-sectional study nested in the REGICOR cohort study [17]. All included patients participated in follow-up at 7, 30, and 180 days.

2.1.1. AMI patients

We enrolled 100 consecutive patients with AMI. Inclusion criteria were age ≤75 years, with a first AMI and more than one traditional cardiovascular risk factor. Exclusion criteria were previously documented coronary artery disease and established statin therapy, because statins can modify EPC and CEC kinetics. All AMI patients received treatment according to current guidelines, including atorvastatin (40 mg per day at admission, modified during follow-up to achieve <70 mg/dL LDL).

2.1.2. Stroke patients

The study enrolled 50 consecutive patients with acute ischemic stroke or transient ischemic attack (TIA) of atherothrombotic origin. Inclusion criteria were age ≤75 years and initial severity <20 on the National Institutes of Health Stroke Scale (NIHSS). Exclusion criteria were previously documented stroke, disability (modified Rankin scale >2), or established statin therapy, which can modify EPC and CEC kinetics. All patients with stroke/TIA were admitted to a stroke unit and a vascular neurologist used the NIHSS to categorize three groups of stroke severity (mild < 7, moderate = 7–14, and high < 14). All strokes were assessed by an initial computed tomography scan performed at admission. Control neuroimaging was performed using computed tomography or magnetic resonance imaging. Under current guidelines, all stroke patients received the same treatment as patients with AMI.

2.1.3. Control group

Participants in the REGICOR cohort study [18] who were free of selected cardiovascular risk factors (hypertension, dyslipidemia, diabetes) were invited to participate in this study. Controls were matched by age and sex.

2.2. Endothelial progenitor cell and circulating endothelial cell counts

Blood samples were collected into low-molecular-weight heparin tubes and processed twice within 4 h of extraction. Circulating EPC and CEC counts were determined by flow cytometry (FC5000 cytomter, Beckman–Coulter, Madrid, Spain). EPCs were defined as negative for CD45 and positive for CD34, KDR, and CD133 [CD45 − CD34 + KDR + CD133 + ]; CECs were defined as negative for CD45 and positive for CD146 and CD31 [CD45 − CD146 + CD31 + ], as previously described [19]. Counts were calculated by multiplying the ratio of EPCs and CECs obtained in the flow cytometry analysis by the number of leukocytes/mL in the blood sample to obtain the absolute number of EPCs and CECs per 1 mL of whole blood.

2.3. Endothelial progenitor cell culture and characterization

Peripheral blood samples were collected from controls upon inclusion and from AMI patients within 24 h of the event. Blood was recovered in heparinized tubes and processed within 2 h. Unfortunately, the hospital from which stroke patients were recruited was too far from the lab to process the samples within this time limit; in this group, therefore, EPCs were not isolated and cultured in vitro. Mononuclear cells were isolated by density gradient centrifugation. After isolation, mononuclear cells were seeded on fibronectin-treated culture dishes. After 24 h, nonadhered cells were removed and attached cells were further cultured up to 30 days, as previously described [20].

2.4. Functional characterization of cultured endothelial progenitor cells

2.4.1. Cell adhesion

EPC adhesion was defined as the cells’ ability to adhere to a fibronectin matrix. Two independent observers counted adhered cells in 6 random squares. Data were expressed as percentage of adhered cells related to total number of seeded cells.

2.4.2. Cell proliferation

Cell-cycle phases were determined by propidium iodide staining to determine cell DNA content and flow cytometry. To determine the proliferative response to the growth factors contained in EGM-2 culture media, confluent EPC cultures were starved for 48 h, stimulated for 18 h, and then proliferative cells were counted. The EPCs were recovered, fixed, and stained with propidium iodide–RNase solution (Immunostep, Salamanca, Spain) for 15 min. Stained cells were analyzed with FC500 cytometer and Infinicyt software (Cytognos, Salamanca, Spain).

2.4.3. Growth curve

To determine the expansion capacity over time, EPCs were seeded on 24-well plates (1.5 × 10² cells/well), and media was changed daily. For 6 days, cells were harvested daily, pelleted, and re-suspended in Trypan blue solution (Sigma Aldrich). Viable EPCs were determined by counting cells in a modified Neubauer chamber.

2.4.4. Vasculogenesis

The ability of EPCs to form capillary-like structures was determined by seeding 1.5 × 10³ EPCs on Matrigel-pretreated plates. Total length of capillary-like structures is expressed in micrometers.
2.4.5. Measurement of intracellular [Ca\(^{2+}\)]

Cytosolic Ca\(^{2+}\) signals were determined at room temperature (−24 °C) in cells loaded with 4.5 μM fura-2 · AM for 30 min, as previously described [21]. Videomicroscopic measurements of [Ca\(^{2+}\)] were obtained using an Olympus IX70 inverted microscope (Hamburg, Germany) with a 40× oil-immersion objective (Olympus). A Polychrome IV monochromator (Till Photonics, Martinsried, Germany) supplied the excitation light (340 and 380 nm), which was directed toward the cells in the field of view by a 505DR dichromatic mirror (Omega Optical, Brattleboro, VT). Images were passed through a 535DF emission filter (Omega Optical) and collected by a digital charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan), using the AquaCosmos software program (Hamamatsu Photonics). The 340/380 nm ratio images were computed every 5 s. Cytosolic [Ca\(^{2+}\)] increases were presented as the ratio of emitted fluorescence (510 nm) after excitation at 340 nm and 380 nm, relative to the ratio measured before cell stimulation (340/380 ratio). To evaluate time-dependent differences in EPC response to vascular endothelial growth factor (VEGF), we measured Ca\(^{2+}\) signals at 0, 7, and 180 days post-AMI. Relative changes in intracellular Ca\(^{2+}\) were measured in EPCs loaded with the fluorescent Ca\(^{2+}\) indicator fura-2. To distinguish endoplasmic reticulum Ca\(^{2+}\) release from Ca\(^{2+}\) influx, EPCs were exposed to VEGF (100 ng/mL) in the absence of extracellular Ca\(^{2+}\) and after addition of Ca\(^{2+}\) to the external medium.

2.5. Vascular cell adhesion molecule and Von Willebrand factor

Peripheral blood samples were withdrawn from controls (baseline only) and in patients within 24 h after the ischemic event, and at 7, 30, and 180 days. VWF antigen and plasma levels of soluble VCAM-1 were measured by enzyme-linked immunoabsorbent assay (DG-EIA VWF®, Grifols; Barcelona, Spain and Millipore Corporation, Billerica, MA, USA, respectively).

2.6. Outcome measures and endpoints

2.6.1. Primary objectives

- To obtain EPC and CEC counts in controls (baseline) and <24 h after an AMI or a stroke and at 7, 30, and 180 days of follow-up.
- To compare EPC and CEC counts between controls and patients.

2.6.2. Secondary objectives

- To test correlation of EPC count with VCAM-1 levels, and CEC count with VWF levels.
- To test correlation of EPC and CEC counts with severity of the ischemic event as assessed by magnetic resonance imaging and degree of disability.
- To characterize cultured EPC function: cell adhesion, proliferation, growth curve, vasculogenesis, and intracellular Ca\(^{2+}\) signals in response to VEGF, in controls and AMI patients at 0, 7, and 180 days after the event.

2.7. Statistical analysis

Mean, standard deviation, and Student t test were computed for continuous normally distributed variables, and frequencies and chi-square test were performed for categorical variables. CEC and EPC counts are presented as median and interquartile range. Comparison between groups was performed using unpaired Mann–Whitney U test. Linear mixed models, which take into account the repeated measures within individuals, were performed to assess changes over time in AMI and stroke cases. All the models were adjusted by age, taking “CEC” and “EPC” as the response variable and study group (AMI/Stroke), time (baseline, 7 days, 30 days, and 180 days) and group-time interaction as factors. The variable of main interest was the group:time interaction, which assessed whether changes in response time differed between AMI and stroke patients. In each model, the response was logarithmically transformed using normality assumptions. Patients with zero values in response were assigned randomly to values near zero, and multiple imputation techniques were used to summarize the estimated results. Data analyses were performed using version 3.0.1 of the R statistical program (R Development Core Team).

3. Results

3.1. Study population

The study flowchart is depicted in Fig. 1. The demographics and clinical characteristics of the study population are shown in Table 1. Patients with AMI were younger (53.7 vs. 64.5 years; p < 0.01) and had a lower prevalence of hypertension (27% vs. 68%; p < 0.01), diabetes mellitus (20% vs. 68%; p < 0.01), hypercholesterolemia (11% vs. 28%; p < 0.01), and a higher prevalence of cigarette smoking (74% vs. 56%; p < 0.01) than patients with stroke. Following discharge, AMI patients were more frequently treated with aspirin (99% vs. 78%; p < 0.01), clopidogrel (95% vs. 24%; p < 0.01), beta-blockers (85% vs. 6%; p < 0.01), and angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (85% vs. 4%); than were patients with stroke. The prescription of statins at discharge was similar between AMI and stroke patients (100% vs. 98%; p = 0.94).

3.1.1. AMI patients

One hundred consecutive AMI patients were recruited. Eighty-seven presented with ST-segment elevation myocardial infarction (STEMI), and 13 with non-STEMI. Coronary angiography was performed in all patients. In the STEMI group, primary percutaneous coronary intervention was the revascularization method in 66 (76%) patients and fibrinolysis in 15 (17%). Seven (54%) patients initially treated with fibrinolysis were subsequently treated with percutaneous coronary intervention. Killip > 1 was observed in 13 (13%) patients. In-hospital left-ventricular ejection fraction measured by cardiac magnetic resonance imaging was 50.6 ± 11.8%. Infarct size, expressed as percentage of left ventricular mass, was 17.2 ± 13.8%, with a 45% prevalence of microvascular obstruction (≥ 1 segment).

3.1.2. Stroke patients

Fifty patients were recruited, 35 (70%) with a diagnosis of stroke and 15 (30%) with TIA. The prevalence of classical vascular risk factors is summarized in Table 1. Neurological symptoms were classified as total anterior circulation stroke in 4 (8%) patients, partial anterior circulation stroke in 21 (42%), posterior circulation stroke in 10 (20%), lacunar stroke in 14 (28%), and transient monocular blindness in one patient (2%). Initial severity was mild in 44 (88%), moderate in 2 (4%), and high in 4 (8%) patients. The stenosis degree in the symptomatic intracranial arteries was 30% to 50% in 14 (34%), 51% to 70% in 10 (20%), > 70% in 21 (42%). Two patients had complete occlusion of the symptomatic artery. Two patients (4%) were treated with intravenous thrombolysis and 12 patients (24%) with delayed surgical or endovascular revascularization therapies. Disability was present in 9 (18%) patients at discharge, but persisted in only one patient (2%) at 180 days.

3.2. EPC and CEC counts and kinetics

3.2.1. AMI patients

At baseline, EPC and CEC counts were higher than in controls. During follow-up, both counts were also higher at 7, 30, and 180 days, compared to controls (Table 2). Over time, intra-subject EPC peaked at day
30, and decreased thereafter up to 180 days post-event; CEC counts showed a progressive decrease in patients with AMI (Fig. 2).

3.2.2. Stroke patients  
At baseline, EPC count — but not CEC count — was higher in stroke patients than in controls (Table 3). At 180 days of follow-up, there was no significant change in intra-subject EPC and CEC count over time for patients with stroke (Fig. 2).

3.2.3. Comparison between AMI and stroke patients  
Patients with AMI had higher EPC and CEC counts than stroke patients at all time-points measured. The differences were statistically significant, except for CECs at 30 days (supplementary appendix, Table 4).

3.3. Correlation of EPC and CEC count according to severity of disease  
There was no relationship between the number of EPCs or CECs and infarct size, microvascular obstruction, or troponin peak (data not shown). Similarly, baseline EPC or CEC count was not associated with stroke severity, OXFORD classification, or degree of stenosis. However, there was a trend to higher EPC counts at 7 days in stroke patients with a high degree of stenosis (>70%), compared to mild stenosis (138.0/mL vs. 57.0; p = 0.07).

3.4. Correlations of EPCs and CECs with VCAM-1 and VWF  
VCAM-1 levels and VWF plasmatic concentrations and intrasubject kinetics are shown in Tables 2, 3, and 4 (supplementary appendix) and Fig. 3. Correlation coefficients between VCAM-1 and EPC counts, and between VWF and CEC counts were not statistically significant at any time point (Table 5, supplementary appendix).

3.4.1. AMI patients  
At baseline, VCAM-1 levels were similar in AMI patients and controls, peaking in the patient group at 30 days of follow-up. Intra-subject VCAM-1 and EPC changes were similar in these patients, both reaching their highest level at 30 days of follow-up (Figs. 2A and 3A). Plasma concentration of VWF was higher in AMI patients than in controls at baseline, and had decreased continuously at 180 days of patient follow-up. Intra-subject VWF concentration kinetics and that of CECs were similar, with their highest level at baseline, decreasing thereafter (Figs. 2C and 3C).

3.4.2. Stroke patients  
VCAM-1 levels at baseline were similar between stroke patients and controls. VCAM-1 peaked after 30 days of patient follow-up. There were no similarities between intra-subject EPCs and VCAM-1 kinetics during follow-up (Figs. 2B and 3B). VWF was significantly higher in stroke patients at baseline than in controls. Plasma VWF concentration decreased from baseline until 180 days of follow-up in these patients and in intra-subject analysis (Figs. 2D and 3D).

3.5. Characterization of cultured EPCs  
The first EPC colonies appeared at 13 ± 2 days after mononuclear seeding in two groups, controls and AMI patients. In order to ensure the successful isolation of EPC from blood samples in both groups,

Table 1  
Demographics and clinical characteristics of all study groups.

<table>
<thead>
<tr>
<th></th>
<th>AMI n = 100</th>
<th>AMI controls n = 98</th>
<th>p value</th>
<th>Stroke n = 50</th>
<th>Stroke controls n = 47</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53.7 (10.2)</td>
<td>54.7 (9.6)</td>
<td>0.48</td>
<td>64.5 (9.4)</td>
<td>64.4 (9.3)</td>
<td>0.94</td>
</tr>
<tr>
<td>Males</td>
<td>85 (85.0%)</td>
<td>83 (84.7%)</td>
<td>0.95</td>
<td>42 (84.0%)</td>
<td>42 (84.0%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Hypertension</td>
<td>27 (27.0%)</td>
<td>0</td>
<td>–</td>
<td>34 (68.0%)</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>20 (20.0%)</td>
<td>0</td>
<td>–</td>
<td>21 (42.9%)</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>11 (11.0%)</td>
<td>0</td>
<td>–</td>
<td>14 (28.0%)</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Current cigarette smoker</td>
<td>74 (74.0%)</td>
<td>0</td>
<td>–</td>
<td>28 (56.0%)</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.10 (1.03)</td>
<td>5.11 (0.63)</td>
<td>0.96</td>
<td>5.15 (1.32)</td>
<td>5.07 (0.75)</td>
<td>0.71</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.33 (0.85)</td>
<td>3.28 (0.53)</td>
<td>0.69</td>
<td>3.33 (1.01)</td>
<td>3.3 (0.67)</td>
<td>0.61</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.89 (1.52)</td>
<td>0.95 (0.39)</td>
<td>0.01</td>
<td>1.82 (0.99)</td>
<td>0.89 (0.38)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

AMI: acute myocardial infarction; LDL: low density lipoprotein.
leukocyte antigen CD45, endothelial antigen KDR, and progenitor antigen CD34 expression were tested by flow cytometry (Fig. 4A) and VWF expression by immunofluorescence (Fig. 4B). All cultures were positive for the expression of VWF and KDR, and negative for CD45 antigen expression. CD34 was expressed in 45% of cultured cells. No differences in antigenic expression on EPC between controls and AMI patients were found (data not shown).

Further functional characterization was carried out in EPCs obtained from controls and AMI patients. The regeneration of damaged endothelial monolayer relies on the ability of EPCs to adhere, proliferate, and form tubular structures in vitro (vasculogenesis). Cultured EPCs were able to adhere to a fibronectin matrix in both groups, and the percentage of attached EPCs was higher in AMI patients at baseline than in the healthy controls (32.35% vs. 23.12%, respectively; p < 0.01; Fig. 5A). No significant differences were observed in proliferative, growth, and vasculogenic capacities between EPCs isolated from controls and from AMI patients (Fig. 5B–D).

We also tested whether EPCs from healthy controls (Fig. 6A) and AMI patients (Fig. 6B–D) elicited intracellular Ca2+ increases in response to VEGF. In the absence of extracellular Ca2+, VEGF elicited Ca2+ release from the endoplasmic reticulum (first peak), followed by Ca2+ influx (second peak) upon readdition of Ca2+ to the external medium. VEGF-mediated Ca2+ entry was increased in the EPCs obtained from AMI patients, peaking at day 0 (Fig. 6B). The SOCE response at

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>AMI patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>7 days</td>
</tr>
<tr>
<td>EPC/mL</td>
<td>57.0 [17.0:100.8]</td>
<td>201.0 [66.3:420.0]</td>
</tr>
<tr>
<td>CEC/mL</td>
<td>62.0 [19.0:118.0]</td>
<td>181.0 [109.0:360.9]</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>532.1 [360.8:747.7]</td>
<td>573.2 [394.0:881.5]</td>
</tr>
<tr>
<td>VWF</td>
<td>85.6 [70.9:108.8]</td>
<td>145.4 [102.5:180.4]</td>
</tr>
</tbody>
</table>

AMI: acute myocardial infarction; EPC: endothelial progenitor cells; CEC: circulating endothelial cells; VCAM: vascular cell adhesion molecule; VWF: Von Willebrand factor.

* p value < 0.01 between controls and AMI patients.
† p value < 0.05 between controls and AMI patients.

**Fig. 2.** Linear mixed model showing intra-subject changes in EPCs and CECs over time. EPC count in AMI patients (Panel-A), and stroke patients (Panel-B). CECs count in AMI patients (Panel-C), and stroke patients (Panel-D).
7 days (Fig. 6C) and 180 days (Fig. 6D) did not differ between the healthy controls and AMI patients.

4. Discussion

Major findings of our study can be summarized as follows: (1) EPC and CEC counts in AMI and stroke patients were higher at baseline and after 7, 30, and 180 days, compared to healthy controls (baseline). (2) In patients with AMI, there was a peak in EPC and VCAM-1 levels at 30 days after the ischemic event. CEC counts decreased progressively from baseline to six-month follow-up. (3) In stroke patients, a peak in VCAM-1 at 30 days after the ischemic event was not accompanied by any change in EPC count from baseline to 30-day follow-up.

4.1. EPC and CEC mobilization after AMI or stroke

EPC mobilization after AMI was first described by Shintani [22], but follow-up was limited to only 7 days. To our knowledge, the present study is the first to describe the kinetics of EPCs and CECs for up to 6 months after AMI or stroke. We observed an increased number of EPCs and CECs immediately after the ischemic event, compared to the healthy controls, and 6 months after the event the number of EPCs and CECs remained higher in the patient population. Although the number of EPCs is inversely correlated with cardiovascular risk factors

---

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Stroke patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>7-day follow-up</td>
</tr>
<tr>
<td>EPC/mL</td>
<td>37.0 [9.0:84.0]</td>
<td>90 [38.0:171.0]*</td>
</tr>
<tr>
<td>CEC/mL</td>
<td>71.0 [32.0:146.0]</td>
<td>105.0 [39.5:193.5]</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>721.3 [510.1:978.8]</td>
<td>719.7 [565.0:1045.4]</td>
</tr>
<tr>
<td>VWF</td>
<td>92.1 [69.1:110.3]</td>
<td>147.9 [114.0:165.6]*</td>
</tr>
</tbody>
</table>

EPC: endothelial progenitor cells; CEC: circulating endothelial cells; VCAM: vascular cell adhesion molecule; VWF: Von Willebrand factor.

* $p$ value $< 0.05$ between controls and stroke patients.
† $p$ value $< 0.01$ between controls and stroke patients.

---

Fig. 3. Linear mixed model showing intra-subject changes in VCAM-1 and VWF over time. VCAM-1 in AMI patients (Panel-A), and VCAM-1 in stroke patients (Panel-B). VWF in AMI patients (Panel-C), and VWF in stroke patients (Panel-D).
Fig. 4. Isolated EPC characterization from controls and AMI patients. EPC phenotype was assessed by flow cytometry and immunofluorescence. A) CD45, CD34, and KDR expression by flow cytometry. In gray, isotype controls; in black, stained cells. B) i. VWF expression, ii. immunofluorescent nuclei staining (DAPI), and iii. merged images. Total magnification 200×; scale bar represents 100 μm. Data are representative of 5 different cultures from each group.

Fig. 5. Functional characterization of cultured endothelial progenitor cells. A) Adhesion of EPCs to a fibronectin matrix after 30 min of incubation was expressed as a percentage of total seeded cells. B) EPCs were starved for 48 h and then stimulated with complete EGM-2 media for 18 h. We measured the number of proliferative EPCs induced by growth factors present in the culture media. Results represent the ratio of proliferative cells to stimulated and starved EPC cultures. C) EPC growth capacity was measured for 6 days until cultures reached confluence. Growth kinetics of EPCs was expressed as total number of cells. D) Vasculogenesis was expressed as EPC ability to form capillary-like structures on Matrigel matrix. Results represent total capillary-like structures’ length per power field. **p < 0.01 by Student t test.
and predicts the occurrence of cardiovascular events and death in stable patients [12], an increase in EPCs after an acute ischemic event that persists for 6 months of follow-up may be explained by persistent inflammatory stimuli [23]. In contrast with our findings, Massa et al. [24] reported that 60 days after a myocardial infarction there were no significant differences in EPC count between patients and healthy controls. This discrepancy may be explained by the smaller sample size in their earlier study.

EPC mobilization differed between AMI and stroke patients. In patients with AMI, the number of EPCs increased after 7 days and peaked at 30 days after an ischemic event in our study, and not immediately afterwards or at 7 days, as reported previously [25]. However, comparison between studies is not possible because of differences in methodology, including patient selection criteria and EPC definition. Unlike the increase in the number of EPCs observed in AMI patients, in patients with stroke there was no increase at 7 or 30 days of follow-up. After ischemic stroke, EPC count is heterogeneous across the literature, again due to differences in methodologies [13,25]. All the previous studies have included all stroke subtypes, with a higher proportion of cardioembolic and cryptogenic strokes that might contribute to the heterogeneity of the results. Our study included only atherothrombotic strokes, because this stroke subtype is the most closely related to ischemic heart disease and endothelial dysfunction. Differences in the number of EPCs between AMI and stroke patients at baseline and after 7 days and 30 days of follow-up could be attributed to bone marrow senescence related to higher atherosclerotic burden and comorbidities in the patients with stroke enrolled in our study. At discharge, 99% of AMI patients and 98% of stroke patients received statins. High-dose statin treatment has been reported to mobilize EPCs into the circulation [26]. Statin treatment could contribute to the mobilization of EPCs observed in our study; however, the difference in the kinetics of EPC counts between groups does not seem related to statin therapy, as both groups received treatment in similar proportions.

Patients with AMI were treated more frequently with beta-blockers than were patients with stroke. Catecholamine signaling is beneficial in mobilizing EPCs after the acute ischemic event; however, without pharmacological intervention, there is an adrenergic desensitization. Beta-blockers resensitize the heart and enhance the release of EPCs [27]. In contrast with the difference in EPC mobilization between stroke and AMI patients, CECs decreased from baseline until 6 months of follow-up in both groups of patients. Beyond 6 months, the number of CECs in patients remained higher than baseline CECs in controls, due to chronic endothelial dysfunction and damage.

It is well known that the endothelium has site-specific characteristics, with unique structural and functional properties in each vascular bed [28]. As a result of the phenotypic heterogeneity of the endothelium, direct comparison of EPC and CEC counts between AMI or stroke patients may be difficult to interpret.

4.2. Relationship of EPCs and CECs with VCAM-1 and VWF

Interestingly, the progressive increase in EPCs with a peak at 30 days resembled VCAM-1 kinetics in AMI but not in stroke. VCAM-1 expression is induced by several stimuli in arterial endothelial cells and is a critical feature of vascular inflammation [29,30]. VCAM-1 values can predict an increased risk of new vascular events in acute cardiovascular...
disease, and at the same time reflect endothelial cell activation with a specific role in atherosclerotic plaque development due to monocyte and T-lymphocyte recruitment. In human coronary arteries, increased expression of VCAM-1 is observed in areas of plaque neovascularization [31]. Furthermore, VCAM-1 is upregulated at the earliest stages after an ischemic event with little constitutive expression. The parallelism between VCAM-1 behavior and EPC count in patients with AMI could reflect endothelial recruitment. However, in patients with stroke, the absence of an increase in the number of EPCs despite VCAM-1 changes might be related to an impaired mobilization of EPCs secondary to bone marrow senescence in patients with high atherosclerotic burden, older age, and increased comorbidities. The difference between AMI and stroke patients in the VCAM-1 and EPC relationship might be secondary to the severity of the ischemic injury. The observed decrease in CECs followed a pattern similar to that of VWF, a biomarker of endothelial damage or dysfunction caused by inflammatory and vascular diseases that has been correlated with cardiovascular outcomes [32]. VWF plasmatic concentration was higher in AMI and stroke patients at baseline and after follow-up, compared with controls (baseline), as a consequence of not only the ischemic event but also the chronic endothelial dysfunction.

4.3. EPC differentiation from mature endothelial cells

In controls and AMI patients, EPCs exhibited not only morphological and immunological endothelial characteristics but also endothelial behavior, as they were able to adhere to a fibronectin matrix, proliferate upon stimulation, grow and form capillary-like structures in vitro, and respond to VEGF with typical intracellular Ca2+ signals. The second peak observed in Intracellular Ca2+ as a response to VEGF upon re-addition of Ca to the external medium reflects the store-operated Ca2+ entry (SOCE) that is triggered following the depletion of intracellular Ca2+ stores (mainly the ER), and has been related to the expansion of EPCs in vitro [33] and to tube formation [34]. The increased adhesion in samples from AMI patients is the first step for EPCs to induce reendothelialization after an ischemic event [35]. Interestingly, store-operated Ca2+ entry generated by VEGF was greater in EPCs obtained from AMI patients at day 0 than at 7 and 180 days. Most likely, this difference reflects the increased expression of the VEGF receptor under post-ischemic conditions [36].

4.4. Limitations

In order to properly culture EPCs, blood samples from patients should be processed within 2 h. Stroke patients were recruited in a hospital more than 450 km from the lab processing the samples to be cultured in vitro. Therefore, only blood samples from AMI patients and healthy controls could be used for cultures and functional characterization. Soluble VCAM-1 was chosen from among several markers of inflammation and endothelial activation; future studies could be useful to evaluate the correlation between cell numbers and various markers of endothelial damage. In addition, future experiments that correlate serial in vitro analysis with cell function could add useful information to better understand the pathophysiology of EPCs after an acute cardiovascular event.

5. Conclusions

Our study contributes to understanding the pathophysiology of endothelial repair in different vascular beds. In AMI patients, EPC mobilization and VCAM-1 levels peaked at 30 days after the ischemic event; an equivalent increase in EPCs was not observed in stroke patients, despite similar VCAM-1 kinetics. The different patterns of EPC release could be due to a decreased mobilization from the bone marrow secondary to a higher cardiovascular burden in stroke patients. Cultured EPCs had a mature endothelial capability and may contribute to atherosclerotic lesion repair. CEC and EPC quantification is a complex procedure. Further research is needed to determine whether the increase in EPC counts could predict adverse events during follow-up. In patients with AMI, future studies could evaluate the prognostic value of serum biomarkers such as VCAM-1.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2015.01.005.

Funding sources

This work was supported by grants from Spain’s Ministry of Economy and Competitiveness through the State Plan for Research and Innovation, and co-financed by the ISCIII-Subdirectoratge General for Research Assessment and the European Regional Development Fund - FEDER- (grants Red Cardiovascular RD12/0042/0006, RD12/0042/0010, RD12/0042/0014, RD12/0042/0020, RD12/0042/0052, PI08/0272, PI08/0634, PI13/00517, PI13/00617), SAF2011-28214 and SAF2012-38140.

Disclosures

None.

Acknowledgments

All of the co-authors wish to acknowledge our dear friend and colleague, Magda Heras, recently deceased. She was the driving force of this line of research and none of the present work would have been possible without her leadership. Our memories of her will remain with us always.

We want to thank Susanna Tello and her collaborators in recruiting the control group, and to Marta Palomo for her help with setting up the cell counting technique. Their work has been instrumental to the conduct of this trial, and we are very grateful for their commitment. We also appreciate the English language revision by Elaine Lilly, Ph.D.

References


