Effect of Exercise on Circulating Endothelial Progenitor Cells in Microvascular Angina

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**Background:** Circulating endothelial progenitor cells (EPCs) might limit endothelial dysfunction in patients with microvascular angina (MVA). Endothelial colony-forming cells (ECFCs; displaying the CD34+/KDR+/CD45– phenotype) are currently regarded as true EPCs. The aim of this study was to evaluate exercise-induced ECFC mobilization and platelet reactivity in patients with MVA or with obstructive coronary artery disease (CAD).

**Methods and Results:** Exercise stress test (EST) was performed in 20 MVA patients, 20 CAD patients and 20 controls. Platelet reactivity was assessed before and after EST as formation of monocyte-platelet aggregates (MPAs) and CD41 platelet expression, without and with adenosine diphosphate (ADP) stimulation. ECFC number was measured before and 24 h after EST. At rest, MPAs and CD41 platelet expression increased more with ADP in MVA patients (+71±11.0% and +37±7.5%, respectively), than in CAD patients (+37±8.6% and +19±4.5%, respectively) and controls (+29±3.5% and +21±3.1%, respectively; P<0.001 for both). At rest, ECFCs tended to be lower in CAD patients, compared to MVA patients and controls (4.1±5.0%, 7.2±6.0% and 7.3±7.0% cells/10³, respectively; P<0.056). After EST, ECFCs increased less in MVA patients (+2.8±11) compared to CAD patients (+3.3±15; P<0.05) and controls (+7.4±24; P<0.01).

**Conclusions:** In MVA patients, EST is able to blunt the peculiar increase of platelet reactivity to ADP present at rest; in contrast, no potential protective response of ECFCs to exercise was seen in these patients. (Circ J 2013; 77: 1777 – 1782)

**Key Words:** Endothelial progenitor cells; Exercise stress test; Microvascular angina; Platelet reactivity

Endothelial dysfunction is an early and relevant abnormality in the process that leads to atherosclerosis and its complications. Repair of damaged endothelium by endothelial progenitor cells (EPCs) might be important to prevent or limit vessel dysfunction and injury.

The primary aim of this study was to assess whether, as we showed for platelet reactivity, differences in the level of circulating EPCs exist between patients with MVA and patients with obstructive CAD, which might explain the involvement of different coronary artery districts in the pathogenesis of angina pain.

As in our previous studies we found that the favorable pattern of platelet reactivity in MVA patients was observed after stress stimuli; in this study we assessed the effect of exercise on circulating EPCs in MVA patients compared to patients with obstructive CAD. We also assessed platelet function to evaluate whether any relationship exists between platelet and EPC response to exercise.
Methods

Patients

We studied 3 groups of patients. The first group included 20 patients (mean age, 61±10 years; 10 men) with typical clinical features of MVA, that is, typical effort angina, documented ST-segment depression during exercise stress test (EST), reversible perfusion defects at stress myocardial scintigraphy and smooth coronary arteries at angiography.

The second group included 20 consecutive patients with stable angina (mean age, 63±8 years; 10 men), matched as to age and gender with the MVA patients, who had significant (>50% of the lumen diameter) stenoses in at least one of the major epicardial coronary arteries at angiography and no history of acute coronary syndromes in the previous 12 months.

The third group included 20 age- and sex-matched healthy volunteers (mean age, 59.3±7.8 years; 10 men) without any history of chest pain or evidence of cardiac or systemic diseases, but with evidence of at least 1 cardiovascular risk factor (CVRF; control group).

Exclusion criteria included inability to perform symptom-limited EST, alterations of the ECG that could interfere with ST-segment analysis (eg, bundle branch block, pacemaker rhythm, abnormal ST-segment changes at rest), assumption of anticoagulant drugs. No MVA patient or healthy subject was taking any anti-platelet drug, whereas all stable CAD patients were treated with aspirin (100 mg). Tests were performed under habitual drug therapy, but statins were withdrawn at least 3 weeks before the study protocol, due to their potential effects on EPC mobilization.

All patients and controls gave informed consent for participation in the study, which complied with the Declaration of Helsinki and was approved by the institutional review board.

Study Protocol

All subjects underwent a symptom/sign-limited ECG-EST. Blood samples were collected from a peripheral vein before EST, within 5 min of peak EST and 24 h later.

EST

EST was performed according to standard Bruce protocol. Leads II, V2 and V5 were monitored continuously; a 12-lead ECG was printed at the end of each stage, when clinically indicated, and at 1-min intervals in the recovery phase. Blood pressure was measured at baseline, during the last minute of each stage and at peak exercise. EST was stopped in the case of physical exhaustion, ST-segment depression ≥3 mm, progressive angina (Borg scale >6) or occurrence of relevant clinical events (eg, dyspnea, hypotension, arrhythmias). The test was considered positive for myocardial ischemia when a horizontal or downsloping ST-segment depression ≥1 mm was induced in any ECG lead during exercise.

Platelet Reactivity Analysis

Blood samples were collected through clean, non-traumatic venipunctures and with minimal venous occlusion before and within 5 min from peak exercise; samples were drawn directly into plastic tubes containing 0.106 mol/L trisodium citrate (blood:citrate = 9:1), after discarding the first 2 ml to minimize the formation of platelet aggregates. Blood was kept at room temperature and an aliquot was used within 10 min of collection to prepare samples for flow cytometry. Assessment of platelet reactivity was always performed by the same expert physician who was unaware of patient clinical diagnosis, EST findings and EPC results.

Monocyte-Platelet Aggregates (MPAs)

Blood (100 μl) was labeled within 10 min of collection with a saturating concentration of peridinin chlorophyll protein complex-conjugated CD14 (lipopolysaccharide protein receptor) and fluorescein isothiocyanate (FITC)-conjugated glycoprotein IIb/IIIa (GPIIb CD41) for 15 min at room temperature. Following incubation, erythrocytes were lysed with buffered ammonium chloride and analyzed on FACSscan. MPAs were identified using the logical gating facility by a combination of binding characteristics of anti-CD14 (monocyte marker) and of anti-CD41 (platelet marker) antibodies. A minimum of 3,000 monocytes were counted for each test. MPAs were expressed as percentage of monocyte-binding platelets and as mean fluorescence intensity of CD41 (CD41-m.f.i.) in the monocyte-platelet gate.

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### Table 1. Clinical Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>MVA</th>
<th>CAD</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>61±10</td>
<td>63±8</td>
<td>59±8</td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>10 (50)</td>
<td>10 (50)</td>
<td>10 (50)</td>
<td>0.95</td>
</tr>
<tr>
<td><strong>Cardiovascular risk factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>5 (25)</td>
<td>6 (30)</td>
<td>4 (10)</td>
<td>0.09</td>
</tr>
<tr>
<td>Hypertension</td>
<td>13 (65)</td>
<td>17 (85)</td>
<td>10 (50)</td>
<td>0.4</td>
</tr>
<tr>
<td>Current smoking</td>
<td>9 (45)</td>
<td>10 (50)</td>
<td>8 (40)</td>
<td>0.9</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>13 (65)</td>
<td>16 (80)</td>
<td>8 (40)</td>
<td>0.08</td>
</tr>
<tr>
<td>CAD family history</td>
<td>6 (30)</td>
<td>8 (40)</td>
<td>4 (20)</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>Drug therapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-blockers</td>
<td>9 (45)</td>
<td>11 (55)</td>
<td>7 (35)</td>
<td>0.8</td>
</tr>
<tr>
<td>Calcium-antagonists</td>
<td>5 (25)</td>
<td>4 (10)</td>
<td>4 (10)</td>
<td>0.7</td>
</tr>
<tr>
<td>ACEIs</td>
<td>12 (60)</td>
<td>16 (80)</td>
<td>10 (50)</td>
<td>0.5</td>
</tr>
<tr>
<td>Statins</td>
<td>13 (65)</td>
<td>16 (80)</td>
<td>8 (40)</td>
<td>0.08</td>
</tr>
<tr>
<td>Aspirin</td>
<td>–</td>
<td>20 (100)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Oral anti-diabetic drugs</td>
<td>3 (15)</td>
<td>3 (15)</td>
<td>3 (15)</td>
<td>0.7</td>
</tr>
<tr>
<td>Insulin therapy</td>
<td>2 (10)</td>
<td>3 (15)</td>
<td>1 (5)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Data given as mean±SD or n (%). ACEI, angiotensin-converting enzyme inhibitor; CAD, coronary artery disease; MVA, microvascular angina.
Stimulation With Adenosine Diphosphate (ADP)  The response to ADP stimulation of the percentage of MPAs formation and CD41 expression in the monocyte gate was also assessed. To this aim, blood samples obtained at rest and at peak EST were incubated with ADP (final concentrations $10^{-7}$ mol/L) for 15 min at room temperature and labeled and analyzed as previously described.  

EPC Assessment
We assessed late-outgrowth, or endothelial colony-forming cells (ECFCs), which display the phenotype CD34+/VEGFR2+/CD45−. Venous blood samples were collected before and 24 h after EST, and kept at room temperature until assayed. Analyses were always done within 2 h from collection by an expert technician who was unaware of patient clinical diagnosis, EST findings and results of platelet reactivity tests. A total of 100 μl EDTA-anticoagulated blood was incubated for 15 min in the dark with 5 μl of the following monoclonal antibodies (mAbs): CD34-FITC (Beckman Coulter, Miami, FL, USA); CD45-PC5 (Beckman Coulter); and/or VEGFR2-PE (KDR; R&DSystems, Minneapolis, MN, USA). Appropriate fluochrome-conjugated isotype-matched mAb purchased from different manufacturers was used as a control for background staining. After incubation, cells were processed with the Immuno-Prep reagent system (Beckman Coulter) using Coulter Q-prep (Beckman Coulter) and then the samples were run through an EPIC SXL...
(Beckman Coulter). EPCs were defined as CD34+/KDR+/CD45− cells and were expressed as percentage of cells per total number of cytometry events.15–18

Statistical Analysis
Comparisons among groups of continuous variables were done using analysis of variance or Kruskal-Wallis test, as indicated. Multiple between-group comparisons were done using independent t-test or Mann-Whitney U-test, as indicated, whereas within-group comparisons were done using paired t-test or Wilcoxon test, as indicated. Bonferroni rule was applied for multiple comparisons. Categorical variables were compared using chi-square test. Data are reported as mean±SD or proportions. Two-tailed P<0.05 was considered as statistically significant. Data were analyzed using SPSS version 17.0e (SPSS Italia, Florence, Italy).

Results
Baseline Characteristics
The main clinical characteristics of the study groups are summarized in Table 1. The 3 groups did not differ in age, sex and common CVRFs; in particular, one or more CVRFs were present in all subjects of each group. Furthermore, there was no difference among the MVA and CAD groups in drug therapy, except for aspirin, which was used by CAD patients only.

EST
All patients successfully completed EST without any clinical adverse event. A significant ST-segment depression was induced in all CAD and MVA patients, but in none of the controls. EST duration was similar in CAD (380±104 s) and MVA (433±121 s) patients, but significantly longer in healthy controls (541±135 s) compared to the other groups (P<0.001 vs. both). No significant differences, however, among groups were observed in peak rate-pressure product (CAD, 13,653±4,082; MVA, 14,780±2,970; controls, 15,351±3,119 beats/min×mmHg; P=0.27).

Platelet Reactivity
Cytometry variables of platelet reactivity are summarized in Table 2. Both MPAs and CD41 platelet expression were similar in the 3 groups at baseline, and EST did not induce any significant changes in MPAs and CD41 in any group (Table 2).

ADP stimulation at rest induced a significant increase in MPAs and CD41 expression in all groups (P<0.001 for all), but the increase was significantly higher in MVA patients compared to the other 2 groups (P<0.01; Table 2; Figure 1).

ADP stimulation at peak EST further increased MPAs and CD41 platelet expression, compared to rest, in CAD patients and controls (P<0.001). In contrast, in MVA patients the ADP-induced increase in MPAs and CD41 platelet expression after EST was lower compared to rest (Figure 1), thus resulting in percent changes similar to those observed in CAD patients and controls (Table 2).

Circulating EPCs
The results of circulating EPCs at rest and 24 h after exercise are summarized in Table 3. No significant differences among groups were observed, both at rest and 24 h after EST, although there was a tendency to lower EPC levels in CAD patients at rest, compared to the other 2 groups (P=0.056; Table 3; Figure 2).

A significant increase in circulating EPC levels 24 h after EST was observed in all subjects as a whole (6.1 vs. 10.7±18 cells/105 mononuclear cells; P=0.027). The increase of EPCs was significantly lower both in MVA and in CAD patients compared to controls (P<0.01 for both). Of note, the EPC increase in MVA patients was also lower than that observed in CAD patients (P<0.05). The increase of EPCs in each group, however, did not achieve statistical significance (P>0.05 for all).

No significant correlations were found between the change in platelet variables and in EPC in response to exercise (Table 4).

Discussion
The main objectives of this study were to evaluate whether EPC mobilization by exercise could display some favorable pattern in MVA patients, compared to CAD patients, and whether any relationship existed in these patients between EPC and platelet responses to exercise. No potentially protective behavior of EPCs in MVA patients was identified. Indeed, EPC mobilization by exercise appeared lower in these patients compared to both controls and CAD patients.
At the same time, no significant relationship was found in MVA patients between platelet and EPC responses to EST, while we confirmed the previous observation that exercise in these patients inhibits the increased ADP-induced formation of MPAs observed at rest.6,8,9

The cardiovascular risk profile of MVA patients is largely similar to that of patients with obstructive CAD, except for the larger prevalence of women.3–5 Despite the significant prevalence of common as well as non-conventional CVRFs (eg, inflammatory state, insulin resistance), the clinical outcome of MVA patients was consistently found to be excellent.3–5 We have previously speculated that this favorable prognosis might be related to some phenotypic characteristics that can prevent the development of obstructive CAD and its thrombotic complications.3

In previous studies we consistently found that, in the whole blood, platelet reactivity to collagen/ADP stimulation, is significantly reduced after exercise, compared to rest conditions, in MVA patients, whereas it increases or remains unchanged in those with obstructive CAD or in healthy subjects, respectively.6,8,9 This peculiar platelet response to exercise was associated with differences in MPA formation and in platelet reactivity to monocytes.6 Indeed, at rest, ADP induces formation of a higher number of MPAs in MVA patients, compared to CAD patients and controls, but the difference is abolished by both physical and mental stress stimuli, findings that are confirmed in this study.

The exact mechanisms responsible for the inhibitory effect of stress conditions on platelet reactivity in MVA remain to be defined, but some data suggest that adenosine release likely plays a significant role.6 Independent of the mechanisms, we suggest that this peculiar behavior of platelet reactivity might play some protective role against stress-induced thrombotic events and therefore contribute to the low occurrence of major cardiovascular events in MVA patients.3–5

In this study we also assessed whether MVA patients might have a favorable pattern of circulating EPC levels following exercise. Several previous studies have shown that endothelial function is impaired in MVA patients, and the abnormality may involve not only the coronary microcirculation,19–21 but also large coronary and peripheral arteries.22–25 Given that EPCs contribute to repair damaged endothelial cells,15,16,26 their quantitative and/or qualitative abnormalities might contribute to endothelial dysfunction in MVA.

Two recent studies, in fact, suggested that endothelial dysfunction in MVA patients may also involve EPC abnormalities. Shmilovich et al reported that MVA patients have an increased number of circulating EPCs, compared to healthy subjects, but their proliferative capacity in vitro is significantly reduced, despite a higher adhesiveness to fibronectin.27 In contrast, Huang et al found a reduction in both EPC level and proliferative activity at rest, together with reduced fibronectin adhesiveness, in MVA patients compared to healthy controls, findings that were similar to those in CAD patients.28

The present results are at variance with both previous studies. We, indeed, failed to find any significant difference at rest in the number of circulating EPCs in MVA patients compared to controls and CAD patients, although EPC level tended to be reduced in the latter group. The reasons for these discordant results are not clear, but differences in patient selection, drug therapy, method used for EPC assessment (ie, flow-cytometry vs. culture method) and type of EPC assessed might have played a role.

At variance with most previous studies that investigated early-outgrowth or colony-forming unit (c.f.u.) EPCs,22,29 in this study, for the first time, we assessed the exercise-induced mobilization of ECFCs in MVA and CAD patients. This type of cell is probably related to the replacement of defective endothelial cells and vasculogenesis.17,20,29 In particular, ECFCs, which display the phenotype CD34+/KDR+/CD45–, are currently regarded as prototypical for true EPCs. Indeed, only ECFCs, at variance with c.f.u. cells, display the full properties attributed to EPCs (ie, clonal proliferative status, replacing ability, in vitro tube formation, in vivo de novo vessel formation, homing to ischemic sites in vivo).20,30

Of note, ECFC mobilization in response to EST in MVA patients in this study also did not show any favorable behavior compared to the other groups. The present data, in fact, showed a lower increase of ECFCs after exercise in MVA patients, compared to both healthy controls and CAD patients, although this result should be taken with caution, due to the lack of statistically significant changes in ECFC level within groups, likely related to the low number of patients. It is worth noting that the present data indicating that ECFCs increased following exercise independently of exercise-induced myocardial ischemia are at variance with those of previous reports suggesting that induction of tissue ischemia is a major stimulus for EPC mobilization by exercise.32–34 Other studies, however, have already shown that physical exercise by itself can induce EPC mobilization, independently of tissue ischemia.35–40 Finally, it should be underscored that, in agreement with the different individual behavior, we failed to find any correlation between platelet and ECFC responses to exercise in MVA patients, thus suggesting that the 2 findings are regulated by different mechanisms.

**Study Limitations**

The present study was designed to compare only platelet and ECFC response to exercise in MVA patients, whereas we did not assess any exercise-induced cytokines or other factors that might have influenced ECFCs and/or platelet behavior. Thus, the mechanisms involved in the present findings need to be ascertained in future studies.

It should also be highlighted that in this study we also assessed only blood ECFC concentration; accordingly, the question of whether differences in EPC function, and some relationship with the different platelet behavior among groups, exist cannot be established from the present data and may deserve assessment in further studies.

### Table 4. Changes in Platelet Variables and EPC in Response to Exercise

<table>
<thead>
<tr>
<th></th>
<th>MPA</th>
<th>CD41</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δrest–EST</td>
<td>Δrest–EST + ADP</td>
</tr>
<tr>
<td>MVA</td>
<td>r=0.47, P=0.84</td>
<td>r=0.22, P=0.35</td>
</tr>
<tr>
<td>CAD</td>
<td>r=0.065, P=0.79</td>
<td>r=0.023, P=0.38</td>
</tr>
<tr>
<td>Controls</td>
<td>r=0.098, P=0.67</td>
<td>r=0.10, P=0.66</td>
</tr>
</tbody>
</table>

Abbreviations as in Tables 1–3.
Conclusion
MVA patients are characterized by a basal increased platelet reactivity to monocytes in response to ADP stimulation, compared to CAD patients and healthy subjects, which is abolished by exercise. In contrast, for the first time, we showed that the CD34+/KDR+/CD45−/ECFCs response to exercise in MVA patients does not contribute to a protective role of the endothelial system in these patients.

References