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Summary

No data are available about the possible role of endothelial progenitor cells (EPCs), cytochemokines and N-terminal pro-brain natriuretic peptide (NT-proBNP) in determining a different response to short period of cardiologic rehabilitation (CR), as measured by the improvement of exercise capacity. In a population of 86 cardiac surgery patients, we evaluated the numbers of EPCs, pro- and anti-inflammatory cytokines (IL-6,IL-8, IL-10, IL-1ra), hs-C-reactive protein (CRP), vascular endothelial growth factor (VEGF) and NT-proBNP before (T1), and after 15 days of CR (T2). EPCs were measured by flow cytometry, and the exercise capacity was measured at T1 and T2 by using the six-minute walk test (6MWT). At T2, a significant increase of 6MWT ($p<0.0001$) was detected. No significant increase of EPCs was observed, while a significant (at least $p<0.05$) decrease in cytochemokines, CRP and NT-ProBNP levels was evidenced. By analyzing the median improvement of 6MWT, only patients with a median improvement ≥23% showed a significant ($p<0.05$) increase of EPCs at T2, with significant correlations between EPCs, VEGF and IL-10. On the contrary, in patients with a median improvement <23% a negative correlation between CRP and EPCs was observed. Finally, CD34+/KDR+ EPCs showed significant correlation with IL-8 at T1. In conclusion, a short period of CR intervention determines a different pattern of modifications for EPCs in relation to the improvement of exercise capacity.

Keywords

Endothelial progenitor cells, cytochemokines, cardiac rehabilitation, exercise capacity

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Introduction

During the last years, increasing evidence showing that vascular function depends not only on cells that reside within the vessel but also on circulating cells derived from bone marrow, has been reported (1). Indeed, a specific subset of these stem cells, named endothelial progenitor cells (EPCs), has been shown to enhance angiogenesis, promote vascular repair and improve endothelial function (2–3).

Physical exercise has recently been demonstrated to be capable to mobilise EPCs from the bone marrow (4–7). However, this phenomenon may be transient and the specific grade of exercise required to mobilise EPCs has not been yet determined.

Upregulation of EPCs by exercise is dependent, at least in part, from the increased activity of endothelial nitric oxide synthase and from the subsequently increase in nitric oxide (NO) bioavailability (8–9); however, despite recent advances in EPCs' research, the mechanisms stimulating the increase of circulating EPCs' numbers are not completely known. At present, a various number of cytokines and chemokines, such as vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor (G-CSF) and interleukin-8 (IL-8), able to enhance the mobi-

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lization of EPCs, can be involved in exercise-induced release of EPCs from the bone-marrow (10–12).

Actually, EPCs have been analysed in different clinical settings in relation to physical training and exercise and recently, in a report by Paul et al. (13), an increase of circulating EPCs in 46 patients after a cardiac rehabilitation program lasting for three months has been reported. To the best of our knowledge, no data are available on the effect of a shorter period of cardiac rehabilitation intervention, and on the improvement of exercise capacity in relation to EPCs' number.

Thus, we aimed at evaluating, in patients who underwent cardiac surgery, the possible role of EPCs, cytochemokines and NT-proBNP in determining a different response to short period of cardiologic rehabilitation (CR), as measured by the improvement of exercise capacity.

Materials and methods

Study population

The study population comprised 86 patients admitted to the Units of Cardiac Rehabilitation "Ulivella and Glicini" and "Don Carlo Gnocchi Foundation" of Florence, Italy, included in a cohort of patients previously investigated (14). All patients underwent a cardiac surgery at the Department of Heart and Vessels of the University of Florence, Italy.

Thirty-seven (28 male; 9 female) patients with a median age of 73 (58–84) years underwent coronary artery bypass grafting, and 49 (23 male; 26 female) patients with a median age of 72 (47–88) years underwent a valve replacement.

The subjects were classified as having hypertension according to the guidelines of European Society of Hypertension/European Society of Cardiology (15) or if they reported taking antihypertensive medications, as verified by the physician. Diabetic subjects were defined in agreement with the American Diabetes Association (16) or on the basis of self-report data (if confirmed by medication or chart review). Dyslipidemia was defined according to the Third report of the National Cholesterol Education Program (NCEP-III) or if they reported taking antidyslipidemic drugs, as verified by the physician (17). A positive family history was defined as the presence of at least one first-degree relative who had developed coronary artery disease before the age of 55 years for men and 65 years for women.

All subjects gave informed consent; the study complies with the Declaration of Helsinki and was approved by the local ethic committee.

Cardiac rehabilitation (CR)

All patients were included in the CR protocol that lasted for 15 days. The rehabilitation program began between 10–15 days after the cardiac surgery, and comprised optimal medication adjustment according to the available guidelines (18), physical training prescription based on the result of the six-minute walk test (6MWT) and serial clinical and instrumental examinations. In detail, physical training included aerobic exercise at cycle ergometer, based on the maximal heart rate recorded during the 6MWT performed on admission; gentle, low-level (25 W), and short lasting (1–2 minutes [min]) calisthenic exercises, with the resistance sequentially provided by the weight of single body segments and gentle, passive stretching involving all the main joints. The training frequency was six times per week for a total of 12 training sessions.

6MWT

All subjects performed the 6MWT at the onset and at the end of the cardiac rehabilitation intervention according to the American Thoracic Society (19) protocol with electrocardiographic monitoring by telemetry.

Blood collection

Blood samples were obtained in the morning after an overnight fasting at two time points for each patient: at the onset of the rehabilitation program and at the end of the rehabilitation program at least 48 hours after the last program of exercise in the cardiac rehabilitation protocols. Blood samples were collected from the antecubital vein into evacuated plastic tubes (Vacutainer) containing ethylenediaminotetracetate (EDTA) 0.17 M for obtaining plasma samples and for EPCs' evaluation and with no anticoagulant for obtaining sera samples. The samples were centrifuged at 2,000 g for 10 min a 4°C and then stored in aliquots at –80°C until analysis.

Flow cytometric analysis

EPCs were evaluated by flow cytometry as previously described (14). Circulating EPCs were identified through their expression of CD34, KDR, and CD133 and were considered as EPCs cells CD34+/KDR+; CD133+/KDR+ and CD34/CD133+/KDR+.

The intra- and inter-observer variations of our method showed an intraclass correlation coefficient of 0.97 and 0.92, respectively (n=20 determinations).

Cytochemokines' analysis

Quantitative determination of sera levels of IL-6, IL-8, IL-10, VEGF and IL-1 receptor antagonist (IL-1ra) were performed in duplicate by using the Bio-Plex cytokine assay (Bio-Rad Laboratories Inc, Hercules, CA, USA) according to manufacturer's instructions.

High sensitivity C-reactive protein (hsCRP) determination

CRP was assessed with a high-sensitivity assay on a BN II nephelometer (Dade Behring, Marburg, Germany).

NT-pro-BNP determination

NT-pro-BNP was measured with a chemiluminescent immunoassay kit (Roche Diagnostic Laboratory, Indianapolis, IN, USA) on an Elecsys 2010 analyser.

Statistical analysis

Statistical analysis was performed using the SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) software for Windows (Version 13.0). Values are presented as median and range.

The Mann-Whitney test for unpaired data was used for comparison between groups. The Wilcoxon test for related data was used to evaluate differences between two time-points.

In order to analyse EPCs' number in relation to the exercise capacity we calculated the delta (Δ) of the 6MWT improvement (end-onset of the rehabilitation) for each patient and we divided our study population according to the median percentage of improvement (23%).

Post-hoc sample size calculation indicated that the number of 43 patients for group has a sufficient statistical power (Beta=0.80) to detect a significant difference in EPC number between groups according to the improvement of exercise capacity, with an alpha coefficient of 0.05.

Results

Demographic and clinical characteristics of the study population are summarised in Table 1.

The baseline median value of the 6MWT in the whole study population was 304 (53–560) m, with a significant difference according to age: in fact, 6MWT was significantly reduced in older (≥72 years) than in younger patients (<72 years) [289 (53–380) vs. 320 (66–560) m; p<0.0001]. In addition, baseline 6WTC showed a significant negative correlation with baseline levels of NT-ProBNP (r=- 0.31 p=0.006), whereas no relationships were observed between 6MWT, cytochemokines and EPCs.

Similar to 6MWT, circulating EPCs were also significantly (p<0.01) influenced by age; in fact, lower EPCs' number was observed in older (≥72 years) than in younger patients (<72 years) [CD34+/KDR: 0.21 (0–1.65) vs. 0.35 (0–1.34) cells/μl, p=0.02; CD133+/KDR+: 0.19 (0–0.75) vs. 0.30 (0–1.18) cells/μl, p=0.03; CD34+/CD133+/KDR+: 0.14 (0–1.58) vs. 0.22 (0–1.13) cells/μl, p=0.03].

No significant differences of EPCs in relation to other traditional cardiovascular risk factors or by medications were observed.

Patients with POAD showed lower number of EPCs than patients without [CD34+/KDR: 0.033 (0–1.65) vs. 0.28 (0–1.34) cells/μl, p=0.01; CD133+/KDR+: 0.082 (0.03–0.20) vs. 0.25 (0–1.18) cells/μl, p=0.01, respectively].

Among pro and anti-inflammatory cytochemokines, no significant difference according to age, comorbidities or cardiovascular risk factors was observed. Nonetheless, in relation to medications, IL-10, VEGF and IL-8 showed higher levels in patients under ACE-inhibitors treatment (p=0.02; p=0.009; and p<0.01; respectively). A significant (p=0.001) difference for circulating levels of NT-ProBNP according to age was found [NT-ProBNP: 1970 (16–12240) vs 823.7 (331–4606) pg/ml, for patients older and younger than 72 years, respectively].

At baseline, a positive association between all the three types of EPCs and IL-10 and VEGF was observed [CD34+/KDR and IL-10: r=0.24, p<0.01; CD133+/KDR+ and IL-10: r=0.28, p=0.009; CD34+/CD133+/KDR+ and IL-10: r=0.28, p=0.009; CD34+/KDR and VEGF: r=0.21, p<0.05; CD133+/KDR+ and VEGF: r=0.22, p<0.01; CD34+/CD133+/KDR+ and VEGF: r=0.26, p<0.01]. Moreover, CD34+/KDR+ EPCs showed a significant correlation with plasma levels of IL-8 (r=0.33, p=0.002).

Table 1: Demographic and clinical characteristics of the study population.

Variable	
Age (years) *	72.5 (47–88)
Males, n (%)	51 (59.3)
Cardiovascular risk factors	
Hypertension, n (%)	50 (58.1)
Smoking habit, n (%)	39 (45.3)
Dyslipidemia, n (%)	41 (47.7)
Diabetes, n (%)	17 (19.8)
Familial history of CVD, n (%)	33 (38.4)
BMI > 25 (kg/m^2), n (%)	55 (63.9)
Comorbidities	
POAD n (%)	7 (8.1)
TIA/Stroke, n (%)	4 (4.6)
COPD, n (%)	7 (8.1)
Atrial fibrillation, n (%)	25 (29.1)
LVEF < 50%, n (%)	10 (11.6)
Medications	
Statins, n (%)	24 (27.9)
ACE inhibitors, n (%)	24 (27.9)
Antiplatelet drugs, n (%)	33 (38.4)
β-blockers, n (%)	23 (26.7)

*Median and range. ACE, angiotensin converting enzyme; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular diseases; LVEF, left ventricular ejection fraction; POAD, peripheral occlusive arterial disease; TIA, transient ischaemic attack.

Modifications of 6MWT, EPC number, cytochemokines, hsCRP and NT-ProBNP after CR and in relation to exercise capacity

A significant increase of 6MWT was detected at the end of the CR program [375 (54–580) m vs. 304 (53–560) m, p<0.0001], in both old and young patients. In older patients (≥72 years) the final 6MWT was significant lower than in younger patients (<72 years) [345 (54–468) m vs. 400 (105–580) m; p<0.0001, respectively] and a negative association between the 6MWT and NT-ProBNP levels was detected (r=- 0.24, p=0.03).

After 15 days of rehabilitation period, the number of circulating EPCs were similar, while a significant decrease in cytochemokines, hsCRP and NT-ProBNP levels was evidenced (Table 2).

When data were analysed in relation to the improvement of exercise capacity, a significant difference of EPCs between patients with an improvement ≥ 23% in the 6MWT (median percentage of improvement among the whole study population) with respect to patients with a median improvement <23%, was demonstrated (Table 3). Furthermore, patients with a median improvement of 6MWT≥23% showed significantly lower levels of IL1ra with respect to patients with a median improvement <23% (Table 3). Moreover, by dividing patients according to the tertiles of improvement in the 6MWT [1st tertile: <20.43%, 2nd tertile:

Variable	T1	T2	P-value
CD34+/ KDR+ (cells/μl)	0.27 (0–1.65)	0.26(0–3.92)	n.s.
CD133+/ KDR+ (cells/μl)	0.24 (0–1.18)	0.25(0–1.66)	n.s.
CD34+/CD133+/ KDR+ (cells/μl)	0.16 (0–1.58)	0.16(0–1.43)	n.s.
IL1ra, pg/ml	191.7 (54.5–1910.4)	151.9 (51.9 –1105)	<0.0001
IL-10, pg/ml	35.9 (6.2–122.5)	29.3 (3.4–136.9)	<0.0001
VEGF, pg/ml	166.5 (5.8–858.1)	138.5 (6.8 –776.2)	<0.001
IL-8, pg/ml	23.9 (7.6–231.2)	20.0 (6.9–317)	<0.05
IL-6, pg/ml	28.5 (11.6–100)	18.7 (10.4–152.1)	<0.0001
CRP, mg/L	46 (4.50–187.0)	10 (1.4–75)	<0.0001
NT-ProBNP, pg/ml	1354 (16–12240)	826.9 (103.7–8967)	<0.0001

Values are reported as median and (range). CRP, C-reactive protein; IL-6, interleukin-6; IL-8, interleukin 8; IL-10, interleukin 10; IL1ra, interleukin 1 receptor antagonist; NT-ProBNP, N-terminal pro-brain natriuretic peptide; VEGF, vascular endothelial growth factor.

Table 2: Modifications of cytokines, NT-ProBNP and C-reactive protein between T1 and T2.

Variable	<23%	≥23%	P-value
CD34+/ KDR+ (cells/μl)	0.17 (0–3.92)	0.32 (0–2.71)	<0.05
CD133+/ KDR+ (cells/μl)	0.20 (0–0.87)	0.32 (0–1.66)	<0.05
CD34+/CD133+/ KDR+ (cells/μl)	0.13 (0–0.81)	0.21 (0–1.43)	<0.05
hs-CRP, mg/l	10 (3.2–74)	10 (1.4–75)	n.s.
IL-6, pg/ml	17.9 (10.4–152.1)	19.1 (11.5–33.7)	n.s.
VEGF, pg/ml	174.7 (15.3–730.6)	131.4 (17.5–776.2)	n.s.
IL-8, pg/ml	20.2 (6.9–80.8)	19.7 (8–317.0)	n.s.
IL-10, pg/ml	32.7 (5.4–98.3)	28.0 (3.4–136.9)	n.s.
IL-1ra, pg/ml	183.5 (77.5–1105.0)	147.5 (59.8–898.8)	<0.05
NT-ProBNP, pg/ml	711 (248.9–5439.0)	811.0 (126.4–8967.0)	n.s.

Values are reported as median and (range), CRP, C-reactive protein; IL-6, interleukin-6; IL-8, interleukin 8; IL-10, interleukin 10; IL1ra, interleukin 1 receptor antagonist; NT-ProBNP, N-terminal pro-brain natriuretic peptide; VEGF, vascular endothelial growth factor.

Table 3: Differences in EPC number, cytochemokines, hs-CRP and NT-ProBNP according to the median improvement in 6MWT at T2.

20.44–25.71%, 3rd tertile: ≥25.72] a trend of increase in EPCs' number at the end of the rehabilitation program was observed (data not shown).

No differences for the cytochemokines, NT-ProBNP and hsCRP levels were observed in relation to the improvement in exercise capacity (Table 3).

Significant correlations between EPCs, VEGF and IL-10 were detected in patients with a median improvement of 6MWT>23% [CD34+/KDR and IL-10: r=0.42, p=0.007; CD133+/KDR+ and IL-10:r=0.41, p=0.009; CD34+/CD133+/ KDR+ and IL-10: r=0.40, p=0.01; CD34+/KDR and VEGF: r=0.37, p=0.01; CD133+/KDR+ and VEGF: r=0.39, p=0.01 CD34+/CD133+/KDR+ and VEGF: r=0.39, p=0.01]. In patients with a median improvement in 6WTC <23% negative correlations between CRP and EPCs were observed [CD34+/KDR and hsCRP: r=- 0.34, p=0.03; CD133+/KDR+ and hsCRP: r= – 0.35, p=0.02; CD34+/CD133+/KDR+ and hsCRP: r= – 0.40, p=0.01

Discussion

Cardiac rehabilitation (CR) is indicated for patients undergoing cardiac surgery and is associated with a decrease of cardiovascular morbidity and mortality and a significant improvement of exercise tolerance and functional capacity.

In this study we report that after a 15-day rehabilitation period, even if there is a lack of changes in EPCs' number in the whole group of patients, a higher number of circulating EPCs is observed in relation to the improvement of exercise capacity at the end of the rehabilitation intervention, as measured by the 6MWT.

Furthermore, at the end of the rehabilitation only in patients with a better improvement of 6MWT (>23%) significant associations between EPCs, proangiogenic factors and anti-inflammatory cytokines, were found.

With their characterization in vascular biology, bone marrow-derived EPCs appear to form a natural system for maintaining vascular function, enhancing endothelial repair and neovascularization. After the incorporation into the vasculature, they

are able to differentiate into mature endothelial cells and to release angiogenic growth factors such as VEGF and stromal derived factor-1, which act in a paracrine manner to support local angiogenesis and mobilise tissue residing progenitor cells (20).

At present, different methods have been carried out to determine EPCs (flow cytometry or in-vitro cell culture assays) and there is a considerable debate on which set of markers is sufficient to identify these types of cells. The use of markers as CD34, CD133 and KDR for EPCs' identification is one of the most widely used system that allow to analyse at the same time three (CD34+/KDR+; CD133+/KDR+; CD34+/CD133+/KDR+) possible EPCs' populations.

The effects of physical exercise on EPCs' levels and function has been previously described in both patients with vascular disease and healthy subjects, showing an increase of circulating EPCs (4–7). This may represent an important beneficial outcome of physical exercise, probably mediated by a shear stress-induced upregulation of endothelial nitric oxide synthase (8). However, only one study is available on patients who underwent a CR intervention after cardiac surgery and/or PCI: Paul et al. (13) recently showed that, after an exercise protocol lasting for three months, an increase of circulating EPCs' number, survival and differentiation in coronary artery disease patients after PCI or coronary artery bypass grafting, takes place. Our study reports the effect of a shorter period of rehabilitation program, extending findings on cytochemokines' profile and on the improvement of exercise capacity. The lack of a significant EPCs' increase at the end of the rehabilitation program observed in the whole patients group can be due to several different mechanisms. From one side, the duration of CR protocol could be insufficient to determine a significant mobilization of EPCs from the bone marrow to peripheral circulation.

On the other side, our study population comprised only patients that have previously experienced a cardiac surgery (CABG or valve replacement) and it's possible that the surgical intervention, through the release of pro-inflammatory cytokines, determined a different baseline value of EPCs with respect to the patients of Paul et al. In addition, patients described by Paul et al., were on optimal statin therapy for al least one month, and it has been previously demonstrated that lipid-lowering therapy can determine an increase of EPCs in the circulation.

Actually, a various number of mediators has been reported to mobilise EPCs from the bone marrow, and in particular an important role seems to be ascribed to the angiogenic cytokines VEGF that is demonstrated to be able to mobilise circulating progenitor cells in animal models (21) and in patients with acute myocardial infarction (22). We have recently demonstrated a positive association between EPCs numbers and IL-10 levels and in this study we confirm our previous findings (14).

The precise mechanisms involved in this relation remained to be defined, but the inflammatory balance might play a relevant role in the modulation of EPCs' mobilisation. We can speculate that the anti-inflammatory cytokine IL-10 is able to modulate EPCs' mobilization probably through the inhibition of the CRP, which is known to cause a significant reduction of EPCs' differentiation, survival and function (23). In fact, in our patients with a modest improvement of the 6MWT<23%, a negative correlation between EPCs and hsCRP has been reported.

What is known about this topic?

- Endothelial progenitor cells (EPCs) contribute to endothelial regeneration and postnatal neovascularization.
- Physical exercise is able to mobilize EPCs from the bone marrow.
- Only one study reported an increase of circulating EPCs after a three-month cardiac rehabilitation program.

What does this paper add?

- Even after a short (15-days) rehabilitation period, a higher number of circulating EPCs is observed in relation to the improvement of exercise capacity as measured by the six-minute walk test.
- After a cardiac rehabilitation period a significant decrease of cytochemokines, C-reactive protein and NT-ProBNP was oserved.
- The association between VEGF, IL-10 and IL-8 with EPCs' number, suggests their role in favouring the mobilisation of these particular type of progenitor cells.

Moreover, at baseline, in the whole study population a significant association between EPCs and IL-8 has been observed. IL-8 is a cytochemokine that exerts pro-angiogenic effects, and various studies showed that is able to induce stem cell mobilisation through the activation of matrix metalloproteinases and the degradation of extracellular matrix in the stem cells niche (12).

At the end of the rehabilitation program a significant decrease of pro and anti-inflammatory cytokines, CRP and NT-ProBNP was observed, without any substantial difference in cytochemokines profile between patients with a median improvement of 6MWT≥23% with respect to patients with a median improvement <23%.

For this reason, the differences in EPCs' number that we observed between the two groups cannot be ascribed to a different pattern of cytochemokines at the end of rehabilitation. Therefore, it's rather possible to hypothesise that, despite of similar levels of circulating cytochemokines, a different bone marrow response to the stimulus is a determinant for the differences between the two groups of patients.

Despite no significant differences in the levels of pro-inflammatory cytokines between the two groups, the difference detected for the IL1ra levels can reflect an up-regulation of this cytokine due to a more pronounced inflammatory state present in the group without an important improvement of the 6MWT.

A possible limitation of this study is the observational design that does not allow us to determine if the different number of EPCs in the two groups of patients after the rehabilitation intervention is a cause or effect of the better improvement in exercise capacity; however, this is the first study that reported EPCs' number in relation to the improvement of exercise capacity and cytochemokines in this type of clinical setting. Another limitation can be identified in the use of the 6MWT that is a simple functional test possibly influenced by several parameters. However, 6MWT is a widely-used tool, reliable to assess physical

performance, even in patients who are not able to perform standard maximal symptom-limited exercise tests, such as elderly and severely limited patients.

In conclusion, in this study we observed that the improvement of exercise capacity after a short period of rehabilitation intervention is associated to modifications of EPCs' number in patients who underwent cardiac surgery. Furthermore, a decrease of cytochemokines, CRP and NT-ProBNP and an improvement of exercise capacity was detected. However we were not able to demonstrate if the decrease of cytochemokines and neuro-humoral factors was related to a beneficial effect of cardiac rehabilitation "per se" or represents only the effect of the natural course of the postoperative period.

In addition, the association between VEGF, IL-10 and IL-8 with EPCs' number, both at baseline and in the patients group with an improvement in 6MWT≥23%, suggests a role in favouring the mobilisation of these particular type of progenitor cells.

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