Erythropoietin promotes endothelial progenitor cell proliferative and adhesive properties in a PI 3-kinase-dependent manner

Jacob George\textsuperscript{a,c,*}, Emil Goldstein\textsuperscript{a,c}, Anastasia Abashidze\textsuperscript{a,c}, Dov Wexler\textsuperscript{a,c}, Saher Hamed\textsuperscript{a,c}, Haim Shmilovich\textsuperscript{a,c}, Varda Deutsch\textsuperscript{b}, Hylton Miller\textsuperscript{a,c}, Gad Keren\textsuperscript{a,c}, Arie Roth\textsuperscript{a,c}

\textsuperscript{a}The Department of Cardiology, Tel Aviv Sourasky Medical Center, Tel Aviv University, Israel
\textsuperscript{b}Hematology Institute, Tel Aviv Sourasky Medical Center, Tel Aviv University, Israel
\textsuperscript{c}The Sackler Faculty of Medicine, Tel Aviv University, Israel

Received 25 April 2005; received in revised form 6 June 2005; accepted 17 June 2005
Available online 26 July 2005

Time for primary review 28 days

Abstract

Objectives: Patients with congestive heart failure (CHF) suffer considerable morbidity and mortality despite advances in therapy. Treatment with erythropoietin (Epo) has shown promise in CHF patients, yet its mechanisms of action remain elusive. Endothelial progenitor cells (EPC) contribute to postnatal angiogenesis and vasculogenesis, and Epo was shown to promote EPC mobilization. We explored the effect of chronic treatment with Epo on the numbers and functional properties of EPC in CHF patients.

Methods and results: Twenty-eight patients with CHF treated with Epo for a mean period of 28 months were compared to a matched group (n = 28) with regard to the number of circulating hematopoietic and endothelial stem cells (either CD34+, CD34+/CD45+, CD34+/CD133+, CD34+/VEGF-R2+ or CD34+/CD133+/VEGF-R2+) as well as their proliferative and adhesive capacity. In vitro, Epo was added to cultured EPC from healthy subjects to test proliferation and adhesion. No differences were observed in circulating numbers of hematopoietic and endothelial stem cells between CHF patients chronically treated with Epo or untreated. EPC from Epo-treated patients exhibited enhanced proliferation as well as a trend towards adhesion to cultured endothelial cells prior to and following stimulation with TNF-alpha. Addition of Epo to EPC from healthy subjects dose-dependently increased their proliferation and adhesion to fibronectin, cultured endothelial cells, and cardiomyocytes. These effects were significantly reduced in the presence of phosphatidylinositol (PI) 3-kinase inhibitors.

Conclusions: Chronic Epo treatment is associated with an increase in the adhesive and proliferative properties of circulating EPC in patients with CHF.

© 2005 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Epo; EPC; CHF; Angiogenesis; VEGF; Stem cell

1. Introduction

Erythropoietin (Epo) is an endogenous protein that controls production of red blood cells produced principally by the adult kidney \cite{1}. Epo is induced by hypoxia via the hypoxia-inducible factor (HIF) family of transcription factors, which mediate a series of events culminating in general adaptation to tissue hypoxia. It appears that Epo possesses several properties that are associated with its tissue protective effects, when applied as a therapeutic protein in vivo \cite{2,3}. A recently reported mechanism of action of Epo relates to its promoting effect on angiogenesis \cite{4,5} that led to a considerable interest in the possible application of this protein for cardiovascular protection.

Endothelial progenitor cells (EPC) are present in the circulation and have the capacity to transform to mature endothelial cells thereby promoting postnatal angiogenesis and vasculogenesis \cite{6–8}. Circulating numbers of EPC has
been shown to negatively correlate with risk factors for atherosclerosis [9,10] and with disorders associated with vascular dysfunction [11,12], whereas a positive association was found in the presence of myocardial ischemia [13,14]. These initial observations were soon followed by cell transfer studies showing promising data with regard to the ability to attenuate myocardial dysfunction upon intracoronary provision of progenitor cells [15,16]. Interestingly, several studies have demonstrated that Epo is a powerful mobilizer of bone marrow cells to the peripheral circulation, partially accounting for its pro-angiogenic properties [5,17,18].

Patients with congestive heart failure (CHF) still suffer considerable morbidity and mortality despite rapidly advancing pharmacological and non-pharmacological measures [19]. Treatment with Epo has been found to be of clinical benefit in a subgroup of these patients [20], and although anemia correction has been suggested as the mediating mechanism, it is probable that other mechanisms are also operable.

In the current study, we evaluated the effects of chronic treatment with Epo in patients with CHF, on EPC number and functional properties. As we attribute considerable importance to the adhesive properties of EPC to matrix proteins as well as to mature endothelial cells and cardiomyocytes, we supplemented our findings by in vitro studies to assess the mechanisms responsible for the favorable effects on adhesion.

2. Materials and methods

2.1. Patients

All patients were recruited, upon provision of informed consent in accord with the institutional Ethics Committee, from the outpatient clinic of the Tel Aviv Sourasky Medical Center. All patients with CHF were clinically controlled with no hospital admissions, 6 months prior to blood withdrawal. Two groups of patients (n = 28 each) were enrolled: the first group consisted of patients with CHF, treated with Epo for a mean duration of 28 ± 5 months. Epo was given once weekly at a starting dose of 4000–5000 IU/week and the dose was increased to up to 10,000 IU/week or decreased to once every 2–3 weeks or discontinued entirely to achieve and maintain a target Hb of 12.5 g/dl. The second group consisted of CHF patients matched for the Epo-treated group with regard to clinical parameters as well as medications, creatinine clearance and hemoglobin levels. This was achieved by selecting CHF patients based on the extended use of Epo. After selecting this group, a retrospective matching of the control CHF patients, not treated with Epo, was performed and both groups were then recruited for EPC retrieval.

The study was approved by the local institutional committee conforming to the declaration of Helsinki.

2.2. Assessment of EPC numbers by flow cytometry

Twenty millimeters of heparinized blood were obtained and subjected to ficoll for recovery of peripheral mononuclear cells. Cells, 5 × 10⁶, were stained for 4 color-FACS analysis employing the following monoclonal antibodies: Cy-Q anti-CD45 (IQ products), phycocerythrin-anti-CD34 (IQ products), allophtocyanin-anti VEGF-receptor 2 (KDR, R and D systems) and FITC-anti-CD133 (R and D systems).

2.2.1. Evaluation of EPC proliferation by the colony forming unit (CFU) assay

Ficoll-eluted cells were cultured on fibronectin-coated plates, replated 48 h later and grown in the presence of M-199 as described previously [10] for assessment of colony forming unit (CFU) numbers at day 7. Colonies were assessed for expression of endothelial cell markers on day 7. Colonies were counted manually in a minimum of 3 wells by observers who were unaware of the patients’ clinical data. Results were expressed as CFU/well. For phenotyping of endothelial characteristics of CFU, the following antibodies

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EPO Tx (n = 28)</th>
<th>No EPO (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men/women</td>
<td>21/7</td>
<td>21/7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>74.25 ± 6.5</td>
<td>71.8 ± 10.3</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>42.2 ± 13.7</td>
<td>37.3 ± 11.0</td>
</tr>
<tr>
<td>CAD extent (n × vessels)</td>
<td>1.71 ± 1.2</td>
<td>1.85 ± 1.2</td>
</tr>
<tr>
<td>NYHA</td>
<td>2.67 ± 0.61</td>
<td>2.57 ± 0.57</td>
</tr>
</tbody>
</table>

Risk factors

<table>
<thead>
<tr>
<th>Smoking</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, n (%)</td>
<td>21 (75)</td>
<td>20 (71)</td>
</tr>
<tr>
<td>Treated Diabetes, n (%)</td>
<td>14 (50)</td>
<td>14 (50)</td>
</tr>
</tbody>
</table>

Medications

<table>
<thead>
<tr>
<th>Drug</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin, n (%)</td>
<td>11 (39)</td>
<td>7 (25)</td>
</tr>
<tr>
<td>Digoxin, n (%)</td>
<td>4 (14)</td>
<td>7 (25)</td>
</tr>
<tr>
<td>Carvedilol, n (%)</td>
<td>10 (35)</td>
<td>15 (53)</td>
</tr>
<tr>
<td>Other Beta-blockers, n (%)</td>
<td>6 (21)</td>
<td>4 (14)</td>
</tr>
<tr>
<td>ACEI, n (%)</td>
<td>10 (35)</td>
<td>15 (53)</td>
</tr>
<tr>
<td>ARB, n (%)</td>
<td>9 (32)</td>
<td>7 (25)</td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>12 (42)</td>
<td>17 (60)</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>19 (67)</td>
<td>16 (57)</td>
</tr>
<tr>
<td>Calcium blocker, n (%)</td>
<td>8 (28)</td>
<td>4 (14)</td>
</tr>
<tr>
<td>Nitrates, n (%)</td>
<td>14 (50)</td>
<td>8 (28)</td>
</tr>
<tr>
<td>Diuretics, n (%)</td>
<td>27 (96)</td>
<td>26 (92)</td>
</tr>
<tr>
<td>Clopidogrel, n (%)</td>
<td>1 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hypoglycemics, n (%)</td>
<td>14 (50)</td>
<td>14 (50)</td>
</tr>
</tbody>
</table>

LVEF—left ventricular ejection fraction; CAD—coronary artery disease; NYHA New York heart association; ACEI angiotensin convetin enzyme inhibitors; ARB angiotensinogen receptor blockers.
were used for immunofluorescent and flow-cytometric analysis: Rabbit polyclonal anti-Tie-2 (C-20), mouse monoclonal anti-flk-1 (A-3) and goat polyclonal anti-CD31 (PECAM-1, M-20); all from Santa-Cruz. Endothelial-cell lineage was further confirmed by indirect immunostaining with the use of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate–acetylated low-density lipoprotein (DiI–acLDL) and co-staining with BS-1 lectin (Sigma).

2.3. Assessment of EPC proliferation by thymidine incorporation

Day 7 Epc were incubated in the presence of different concentrations of Epo for 5 days and subsequently pulsed with radioactive thymidine for an additional 24 h. Thymidine incorporation was measured using a β-counter.

2.3.1. Fibronectin and HUVEC adhesion assays

EPCs (day 7) were washed with PBS and gently detached with 0.5 mmol/L EDTA in PBS. After centrifugation and resuspension in basal complete medium supplemented with 5% FCS, identical cell numbers were placed onto fibronectin-coated culture dishes of HUVECs with and without prior treatment with 10 ng of TNF-alpha, and incubated for 30 min at 37 °C [21]. Adherent cells were counted by two blinded investigators.

2.3.2. Determination of serum VEGF levels

VEGF serum levels were determined by ELISA in accord with the manufacture’s instructions (R and D systems).

2.3.3. In vitro assays

Ficoll eluted mononuclear cells obtained from healthy volunteers were replated after 48 h and subjected to treatment with different concentrations of Epo for 5 days. After centrifugation and resuspension in basal complete medium supplemented with 5% FCS, identical cell numbers were labeled with DiI and placed onto fibronectin-coated culture dishes or on plates with human umbilical vein endothelial cells (HUVEC) or cultured rat cardiomyocytes.

Fig. 1. Chronic Epo treatment does not alter circulating hematopoietic or endothelial stem cell numbers. Ficoll-eluted mononuclear cells were stained with labeled antibodies to CD34, CD45, CD133 and VEGF-R2 and the number of circulating hematopoietic and endothelial stem cells evaluated between both groups.
and incubated for 30 min at 37 °C. Adherent cells were counted by two blinded investigators. To test for the possible dependence of EPC adhesion and proliferation on Akt, the same assays were conducted with addition of the PI 3-kinase inhibitors LY294002 (LY) (10 μmol/L) and Wortmannin (Wort) (100 nmol/L).

2.3.4. Statistical analysis

Categorical variables were compared between the two groups by use of the Chi square test. The double-sided Student’s t-test was employed for comparison of continuous variables. EPC/CFU numbers were evaluated by the Mann–Whitney test. One-way ANOVA was used to compare the different treatment groups in the in vitro experiments. The Tukey–Kramer post hoc test was chosen for the statistical analysis. p < 0.05 was set to determine statistical significance.

3. Results

To be able to evaluate the effects of chronic Epo treatment in CHF patients, the two patient groups were matched for all clinical data. Indeed, both patient groups were preselected so that no differences were evident with regard to the mean age, ejection fraction by echocardiography, the extent of coronary artery disease affliction, and the severity of CHF as measured by the New York Heart Association (NYHA) class (Table 1). Importantly, both groups did not differ with regard to atherosclerotic risk factors and creatinine clearance, both of which are known to influence the number and properties of EPC [9,10,23]. Medication use was similar between groups also indicating that the clinical status was similar in both groups.

Chronic treatment with Epo was associated with an increase of Hb levels from a mean of 10.7±1.7 g/dl to 12.3±2.2 g/dl and a concomitant reduction in mean NYHA class from 3.3±0.5 to 2.7±0.6. Epo treated patients had significantly increased platelet counts (295,000±69,000 mm³) as compared with control CHF subjects (218,000±59,000 mm³; p<0.05).

Chronic treatment with Epo was not associated with alteration in the number of circulating number of total progenitor CD34+ cells (0.22±0.07% in the Epo treated as compared with 0.25±0.08% in the non-treated group, p=0.78). No differences were evident between both groups with regard to the number of circulating hematopoietic precursors (CD34+/CD45+).

We have tested three populations of circulating EPC by FACS (Fig. 1). No differences were found between both groups with respect to the total number of CD34+/CD133+ cells (0.14±0.06% in Epo-treated versus 0.15±0.06% in non-treated patients, p=0.89). Similarly, no differences were evident between groups with regard to circulating CD34+/VEGF-R2+ cells (0.013±0.006% versus 0.15±0.008, in Epo treated and non-treated, respectively; p=0.78). Assessment of circulating EPC positive for triple marker staining (CD34, CD133 and VEGF-R2) also did not disclose differences between the groups (Fig. 1). Statin treatment was not associated with an increase in any of the progenitor cell populations within Epo treated and non-treated groups (data not shown), possibly because of complex additional other factors and medications that influence EPC numbers in this group of patients. Multivariate analysis considering age, risk factors, medications and use of Epo use were not found to be independent predictors of EPC numbers.

Fig. 2. Chronic Epo treatment increases proliferative and adhesive capacity of EPC from CHF patients. EPC obtained from Epo treated or non-treated CHF patients were evaluated for their ability to proliferate by the CFU assay and by the ability to adhere to fibronectin and TNF-stimulated and non-stimulated endothelial cells (EC). *p<0.01; **p<0.5.
The proliferative capacity of EPC from patients chronically treated with Epo, evident by the CFU assay was significantly enhanced (101.5 ± 16.8 CFU/well) as compared with the non-treated group (65.4 ± 9.6 CFU/well; p < 0.05, Fig. 2). Adhesion of EPC from Epo-treated as compared to non-treated, to immobilized fibronectin, was enhanced, yet did not reach statistical significance (Fig. 2). Adhesion of EPC from Epo-treated patients to cultured mature HUVEC with and without prior stimulation with TNF-alpha was increased as compared with non-treated subjects.

Levels of serum VEGF were higher in patients treated with Epo (62.4 ± 4.1 pg/ml) as compared with non-treated subjects (49.0 ± 3.3 pg/ml; p = 0.007).

To confirm the effects of Epo in vitro, we tested its effects on EPC from healthy subjects. Treatment with Epo dose dependently increased CFU number determined at day
Epo treatment for 5 days, significantly and dose dependently increased adhesion of EPC to fibronectin, HUVEC and cultured rat cardiomyocytes. A significant inhibition of the increase in Epo-induced EPC adherence to fibronectin, HUVEC and cultured cardiomyocytes was achieved by prior treatment with the PI3 kinase inhibitors Wort and LY (Fig. 4).

4. Discussion

Patients with CHF suffer considerable morbidity and mortality despite intensive pharmacological therapy and an evolving spectrum of non-pharmacological devices [19]. Treatment strategies in CHF patients are aimed at blocking principal neurohormonal axes that result in clinical deterioration. However, heart muscle protection is not considered a major mechanism of currently employed therapeutics. Recent studies employing cell therapy in patients undergoing myocardial infarction suggest that progenitor cells are capable of attenuating negative remodeling with subsequent decline in ejection fraction in patients after myocardial infarction [15,16]. Treatment of CHF patients with Epo is a relatively novel strategy that is currently applied to patients with renal failure [24] and anemia (reviewed in Ref. [25]). In this context, it appears particularly appealing as preliminary studies suggest that it significantly improves functional status [20,21]. However, in patients without anemia, Epo and Iron treatment could potentially exacerbate CHF progression due to the elevation of hematocrit. Treatment with Epo and iron should thus be reserved, at this stage, to patients with Hb levels below 12 g/dl who are not adequately controlled by standard treatment modalities.

Epo exerts a variety of pleiotropic effects irrespective of its hemoglobin elevating properties [25]. Accordingly, Epo has been shown to protect cardiomyocytes from ischemic reperfusion damage and to reduce the size of experimental myocardial infarction [26–28].

Recently, several studies have shown that Epo mobilizes endothelial progenitor cells from the bone marrow to the periphery and enhances progenitor cell differentiation and proliferation after a short period of administration [5,17,18]. These properties could potentially be associated with the clinical benefit observed in patients treated with Epo. In the present study, we studied the effects of prolonged treatment of EPO in patients with CHF. We have found that patients with CHF treated with Epo for approximately 2.5 years did not exhibit a significant mobilization of EPC or hematopoietic cells to the periphery, unlike the short-term effects shown with in vivo Epo treatment. These observations may result from the initially increased number of EPC present in this subset of patients as has previously been reported [29]. It could also reflect the heterogeneous population of studied CHF patients with several risk factors and medications which may all influence EPC mobilization.

It appears from recent literature, that not only the absolute number of EPC is of importance in assessment of patients with vascular disorders, but also their functional properties [6]. Among these, migration of EPC [15], resistance to apoptotic cell death [30], secretion of angiogenic cytokines, capacity to support tube formation [17,18], proliferation [17] and adhesion [14,22] are thought to be important. Indeed, for an EPC to be functional, it should be capable of adhering to the damaged tissue with subsequent proliferation. We have thus tested the effects of chronic Epo treatment on proliferation and adhesion of EPC. Interestingly, whereas circulating numbers of EPC were not different, proliferative capacity of EPC from CHF patients chronically treated with Epo were significantly enhanced. Moreover, we observed a trend towards increased adhesion of EPC to TNF stimulated and nonstimulated cultured endothelial cells. These results may suggest that Epo could be beneficial to patients with CHF also due to its proangiogenic properties that may improve myocardial perfusion and reduce myocardial and peripheral ischemia thus contributing to improved well being.

Interestingly, serum levels of VEGF—a potent promoter of angiogenesis and mobilizer of EPC [31] were significantly increased in Epo-treated patients, unlike that lack of rise observed in short term treated subjects [17,18] and did not correlate with numbers of EPC. Elevated VEGF levels in Epo treated patients could be the result of increased synthesis of this angiogenic factor by endothelial cells [32]. Furthermore, these authors have shown that Epo–Epo-receptor interactions are responsible for the increased expression of these angiogenic proteins. Interestingly, Wang et al. [33] have also demonstrated that treatment with Epo of rats induced to develop stroke was associated with a significant increase in VEGF levels. An additional source of increased VEGF could be the higher platelet counts that were found in Epo treated patients and previously found to be producers of this angiogenic protein [34].

Aiming to provide in vitro support of our findings in the patients, we studied the effects of Epo added to cultured EPC from healthy subjects. We have found that Epo dose dependently enhanced CFU numbers indicating EPC proliferation. This effect was evident at levels expected to be in the therapeutic range (1 U/ml), whereas interestingly, in concentrations of 25 U/ml the effect was lost. This may be related to an early proapoptotic effect of Epo that was not detected by conventional viability tests we employed. Moreover, addition of Epo, dose dependently increased EPC adhesion to fibronectin, cultured endothelial cells and cardiomyocytes. The more pronounced effects of Epo on EPC from healthy subjects compared with CHF patients with regard to the proliferation and adhesion argue for a possible blunted response to EPC mediated myocardial protection conferred by Epo in the latter group.
Several studies suggest that Epo induces activation of protein kinase B (Akt) in mature endothelial cells [35] and cultured EPCs [5,17,18]. We thus evaluated the dependence of Epo-induced EPC proliferation and adhesion on the PI 3-kinase pathway of signal transduction. Indeed, we have found that prior treatment with the PI 3-kinase inhibitors Wort and LY significantly attenuated Epo mediated increase in proliferation and adhesion to fibronectin, cultured endothelial cells and cardiomyocytes.

In conclusion, we have found that EPC numbers from CHF patients chronically treated with Epo are not increased, yet their adhesive and proliferative properties are enhanced as compared to non-treated CHF subjects. These findings suggest that Epo-mediated promotion of EPC proliferation and adhesion could be associated in part with the beneficial effects observed in treated patients, irrespective of the rise in hemoglobin levels.

References

