Preclinical research

Erythropoietin improves myocardial performance in doxorubicin-induced cardiomyopathy

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Aims Doxorubicin (Dox) is a potent chemotherapeutic agent associated with severe cardiotoxicity. Erythropoietin (Epo) has recently been shown to exhibit proangiogenic properties related to endothelial progenitor cell (EPC) mobilization. We tested the hypothesis that EPC are compromised in rats with Dox-induced cardiotoxicity and correction of this functional impairment by treatment with Epo could result in attenuation of myocardial dysfunction.

Methods and results Wistar rats were either treated with two different doses of Epo (20U or 200U) or PBS (n = 40 in each group) for four consecutive weeks, followed by Dox administration. In a second study, EPC obtained from healthy rats were transfused intravenously (n = 20/group) prior to induction of Dox cardiomyopathy. EPC from healthy subjects were evaluated for their proliferative and migratory properties in the presence or absence of Dox and Epo pre-treatment.

Echocardiography demonstrated an improvement in fractional shortening (FS) in Epo-treated rats. Epo treatment was associated with a reduced mortality in both Epo-treated groups. Circulating EPC numbers were three times higher in Epo-treated compared with non-treated animals. Adhesive properties, migration, and tube formation capacity in matrigel of EPCs from both Epo-treated groups as compared with controls were significantly enhanced. EPC transfer to Dox-treated rats led to functional myocardial improvement equivalent to the protection afforded by treatment with Epo. In EPC obtained from humans, pre-incubation with Epo significantly attenuated the anti-proliferative and anti-migratory effects of treatment with Dox.

Conclusion Epo treatment is potentially protective against myocardial dysfunction induced by Dox. These effects are partially mediated by enhancement in the number of EPC and their functional properties.

KEYWORDS
EPC; Epo; Cardiomyopathy; Stem cell; Cell therapy

Introduction

Doxorubicin (Dox) is a commonly employed anti-neoplastic agent for treatment of solid and haematologic tumors. Dox-induced cardiomyopathy may result in progressive heart failure after anti-neoplastic therapy, thus limiting the application of this potent chemotherapeutic agent. The mechanisms of cardiac toxicity are not fully understood and are thought to include heightened oxidative stress status and apoptosis of endothelial cells and cardiomyocytes.

To avoid cardiac toxicity from Dox, a total cumulative dose of 500 mg m−2 body surface area should not be exceeded. Several agents have been employed for the attenuation of cardiomyopathic damage induced by Dox and include anti-oxidants, angiotensin converting enzyme inhibitors, and dexrazoxane, all of which have not yet gained sufficient proof of efficacy to justify a routine use.

Erythropoietin (Epo) is an endogenous protein produced by the adult kidney that regulates the production of red blood cells. Epo is induced by hypoxia via the hypoxia-inducible factor family of transcription factors that mediate a series of events culminating in global adaptation to tissue hypoxia. Epo has been shown to exhibit several properties that are associated with its tissue protective effects, when applied therapeutically. Several recent studies demonstrated that Epo is capable of significantly reducing the extent of experimentally induced myocardial infarct as well as ischaemic-reperfusion injury, although the mechanisms mediating the protective effects have not yet been elucidated. Endothelial progenitor cells (EPCs) are bone marrow-derived cells that are mobilized to the peripheral circulation and contribute to post-natal angiogenesis and vasculogenesis. Transfer of these cells was found to attenuate tissue damage in models of myocardial and hindlimb ischaemia (reviewed by Rafii and Lyden). Recent reports suggest that Epo is a significant promoter...
of EPC mobilization from the bone marrow.\textsuperscript{13} Also, it plays an important role in the differentiation and maturation towards endothelial lineage.\textsuperscript{3–15} Thus, Epo is a potent proangiogenic factor and its protective effects could result from provision of improved blood supply to damaged tissues through the establishment of a richer capillary network.

In the current study, we tested the hypothesis that Epo treatment could improve myocardial performance and mortality in rats with Dox-induced cardiomyopathy. To provide a possible insight into the mechanisms leading to the beneficial effects, we studied the influence of Epo on EPC number and function in \textit{vivo} and \textit{in vitro}.

\section*{Methods}

\subsection*{Treatment of rats with Dox cardiomyopathy with Epo}

Dox cardiomyopathy was induced in 8-week-old male Wistar rats (Harlan) by intraperitoneal injection of Dox as previously described.\textsuperscript{16} A total dose of 15 mg/kg was administered over a 3-week period. In the first study, rats were divided to four groups. Group A ($n = 40$) received subcutaneous Epo 20 U/kg, three times weekly, starting one week before initial Dox injection and continued for an additional week after final Dox administration. Group B ($n = 40$) received 200 U/kg of Epo, employing a similar schedule. Control group ($n = 40$; Group C) received PBS. Fourth group ($n = 10$, group D) received subcutaneous Epo 20 U/kg, three times weekly, similar to Group A with no subsequent Dox injections. The animals were allocated randomly to each of the study groups, after recording their weights. The number of animals was selected based on the estimated high mortality in this model and the need to complete the study with at least seven rats for the echocardiographic study (aiming to detect a meaningful change in FS).

The doses of Epo were selected on the basis of the equivalent regimen used in humans with anaemia (the lower dose) and the higher dose group was one order of magnitude higher, assuming that conventional doses would be insufficient to negate the potent effect of Dox. Animals were maintained for 7 weeks after the last Dox injection, and during that period, death was recorded as well as body weight and general health assessment. At the end of the study, \textit{in vivo} echocardiographic analysis of the heart was performed\textsuperscript{17} after which the animals were sacrificed. Upon sacrifice, blood was obtained for evaluation of haematological parameters, heart was divided into three sections for histological analysis, and spleen and bone marrow were obtained for the assessment of EPC number and function.

\subsection*{Transfer of EPC to rats with Dox-induced cardiomyopathy}

In the second \textit{in vivo} study, rats were induced to develop Dox cardiomyopathy in a similar manner. The animals were allocated randomly to each of the study groups, after obtaining their weights. At the day of initial Dox administration, EPC obtained from healthy syngenic wistar rats were transferred intravenously (10\textsuperscript{7}/rat) to 40 rats and the procedure was repeated twice at weekly intervals. A second group of Dox administered rats ($n = 40$) received five weekly injection of PBS and served as control. The study was conducted similar to the first \textit{in vivo} study and the same parameters were evaluated by histology and echocardiography in addition to observation of mortality.

\subsection*{Assessment of vascular density in sections from rats with Dox-induced cardiomyopathy}

Vascular density was assessed in four groups in each group. The number of vessels was counted in 48 sections (two sections per slide, two slides per heart) after staining with an antibody anti-CD31 using a light microscope at a $\times 400$ magnification. Five high-power fields in each section were randomly selected, and the number of vessels in each field was averaged and expressed as the number of vessels per high-power field (0.2 mm\textsuperscript{2}).

\subsection*{8-Isoprostane analysis}

Urinary levels of 8-isoprostane levels in five animals/group were measured using enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer’s instructions. Urine samples were frozen immediately after collection and stored at $-80\,\text{C}$ till the performance of the assay. Briefly, urine was spiked with $[^3\text{H}]-8$-ISO, treated with ethanol followed by 15% potassium hydroxide, incubated for 1 h at 40°C, and acidified to pH 4.0 with hydrochloric acid. The sample was extracted using a polyboronic acid column, eluted with ethyl acetate containing 1% methanol, and evaporated under nitrogen. 8-isoprostane was assayed using competitive binding with mouse anti-rabbit IgG monoclonal antibody in a 96-well plate.

\subsection*{Isolation of peripheral human EPCs}

\paragraph*{Preparation of spleen derived and circulating}

Peripheral blood mononuclear cells were isolated from healthy subjects (mean age of 32 ± 6, three males and seven females) by Ficoll (Amersham Biosciences) density-gradient centrifugation (1200 rpm, 12 min, 23°C) for the recovery of mononuclear cells. Pellets were washed with PBS and resuspended in M199 with 10% FCS and antibiotics (penicillin/streptomycin), and identical cell numbers (1 × 10\textsuperscript{6} cells/well) were plated onto fibronectin coated 24-well plates. Media was changed the first day after the seeding, and every three days thereafter. These cells were used for \textit{in vitro} testing of the effects of Dox and Epo.

\section*{Preparation of human EPC for \textit{in vitro} assays}

All studies were approved by the local institutional Ethics Committee. Peripheral blood mononuclear cells were isolated from healthy subjects (mean age of 32 ± 6), three males and seven females) by Ficoll density-gradient centrifugation (Sigma) from 20 ml blood samples and EPC-CFU was assayed after two plateings and a 9-day culture on fibronectin-coated, 24-well plates for 7 days, as described.\textsuperscript{18} These cells were used for \textit{in vitro} testing of the effects of Dox and Epo.

Endothelial phenotype was verified by indirect immunostaining with the use of 1, 1'-dioctadecyl-3, 3', 3'-tetramethyl-indocarbocyanine-labeled acetylated low-density lipoprotein (DiI-acLDL) (Molecular Probes) and co-staining with FITC-labeled Ulex Europaeus BS-1 lectin (Sigma). A blinded investigator viewed at least three randomly selected high-power fields with a fluorescent microscope and counted double-stained cells as EPCs.

\subsection*{Colony forming unit assay for assessment of EPC numbers}

In order to evaluate the quantity of EPCs, we measured the ability of isolated EPCs to form colony forming unit (CFU) \textit{in vitro}. A colony of EPCs consisted of multiple thin, flat cells emanating from a central cluster of rounded cells. After 5 days of EPC culture, colonies were counted manually using a light microscope in a minimum of three wells per rat. Results were expressed as CFU/well and were counted by two different observers.
Endothelial phenotype of CFU was confirmed by indirect fluorescence employing the following antibodies: 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. Secondary FITC-conjugated antibodies were from Jackson.

Fibronectin adhesion assay
EPCs 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 (10^5/well) were placed onto fibronectin-coated culture dishes and incubated for 30 min at 37°C. Adherent cells were counted by two independent blinded investigators.

Adhesion of EPC to bovine aortic endothelial cell monolayer
EPCs (day 5) were detached and labeled with the fluorescence marker (DiI, Molecular Probes) according to the manufacturer’s instruction. Identical numbers of DiI-labeled EPCs (1 x 10^5) were cultured on a bovine aortic endothelial cells (BAECs) monolayer plated on fibronectin-coated 96 well plates for 30 min and then washed to remove non-adherent cells. The total number of adherent EPCs in each well was counted by a fluorescent microscope in a blinded manner.

Matrigel tube formation assay
To analyse the capillary tube formation, 50 μL of matrigel per well was laid into a 96-well plate and incubated overnight at 37°C to solidify. DiI-labeled EPCs (10^5) were resuspended in 200 μL M199 medium with identical cell number of BAECs and plated onto matrigel. Capillary tube formation in matrigel was observed by fluorescent microscope after 24 h incubation in 37°C and evaluated by scoring. Scores were given from 0 to 5 to every well in the plate then results were averaged as mean ± SD. Scores are, 0, individual cells, well separated; 1, cells begin to migrate and align themselves; 2, capillary tubes visible, no sprouting; 3, sprouting of new capillary tubes visible; 4, closed polygons begin to form; 5, complex mesh-like structure develop.

Migration assay
EPC were suspended 50 μL medium and placed in the upper chamber of a modified Boyden chamber (Transwell, 6.5 mm, 5 μm pore size, uncoated, polycarbonate membrane). An amount of 10 ng/mL of VEGF in 30 μL medium was placed into the bottom chamber wells. After 24 h of incubation at 37°C, cells that migrated through the pores of the membrane to the bottom chamber were counted manually by light microscope. Data are expressed as the number of migrating cells.

Statistical analysis
Comparison between the groups was performed employing the one-way ANOVA test or the two-sided Student’s t-test. Level of significance was set at 0.05. For the first in vivo study, adjustments for the inflation of the type I error were made employing the Dunnett adjustment test.

Results

Effect of Epo treatment on the functional properties of human EPC
We hypothesized that EPC from humans and from rats will be reduced in number and functionally compromised in the presence of Dox in vitro and these properties could be partially rescued by Epo pre-treatment. Incubation of EPCs from healthy volunteers with Dox induced a dose dependent reduction in the number of CFU that was partially reversed by the pre-treatment with Epo. Employing the boyden chamber, we have also observed that incubation with Dox significantly impaired the migratory capacity of human EPC and this effect was partially reversed by pre-treatment with Epo (Figure 1).

Effect of Epo treatment on the functional properties of rat EPC
We then investigated the effects of Epo pre-treatment on the functional properties of EPC obtained from bone marrow or spleens of healthy rats. Dox significantly depleted the number of CFU after a 7-day culture (Figure 2). Epo treatment initiated prior to Dox incubation significantly rescued impaired proliferative capacity by partially maintaining CFU numbers (Figure 2).

Incubation of rat spleen cell-derived EPCs with Dox significantly hampered their ability to form tube-like structures in the matrigel assay. Epo pre-treatment partially preserved...
the capacity of EPC to form tube-like structures with BAEC as evident by higher scores (Figure 2).

Effect of treatment with Epo on Dox-induced cardiomyopathy in rat

To test the hypothesis that protection afforded by Epo on EPC function may have a role in vivo, we conducted a study with rats. Wistar rats were treated with Dox according to the acceptable protocol and two groups of rats were pre-treated with either 20 or 200 IU of Epo, one week prior to Dox injections. The general health of the rats was monitored as well as mortality and assessment of cardiac performance by echocardiography prior to sacrifice, 6 weeks after termination of Dox injections. Animals in the Dox group developed scruffy, yellowish fur and red exudates around the eyes with diarrhea within 1 week of the last Dox injection. Animals in both Epo groups did not exhibit these changes.

Of the 40 animals in Group C, five animals died during the initial 2 weeks of Dox initiation and were not included in the survival calculations, as it is considered that mortality at this stage is not a result of cardiac dysfunction, but rather relates to peritonitis or toxic systemic effects. Of the remaining 35 animals, 30 died in the 6-week period following the completion of the treatment with Dox, yielding a cumulative mortality of 85% (Figure 3). In both Epo (A and B) groups, no animal died during the 2 weeks of Dox injections. However, 16 animals died in Group A and 24 animals in Group B during the 6-week follow-up period, resulting in a cumulative mortality of 40% and 65%, respectively ($P = 0.038$ for both, compared with Group C) (Figure 3). No death was evident in Group D. It should be mentioned that due to the high mortality in Group C, a selection bias may have occurred that should be taken into account when interpreting the results.

By echocardiography performed prior to sacrifice, left ventricular (LV) diameter in systole (LVIDs) was significantly increased in Group C in comparison with both Epo groups ($P = 0.041$ for the Epo 20 and $P = 0.039$ for the Epo 200 group) (Table 1). FS was significantly reduced in Group C compared with group A (47.1 ± 1.3 vs. 59.2%; $P = 0.043$) but a trend towards reduction compared with Group B was evident. LV posterior wall thickness in diastole (LVPWd) was significantly decreased in Group C compared with Groups A and B.

Blood counts obtained at sacrifice exhibited marked bone marrow suppression evident by reduced erythrocyte, white blood cell, and platelet counts in Group C (Table 2). Treatment with Epo was associated with normalization of haemoglobin levels and with raised levels of all cell counts (Table 2).

Histological analysis of H&E sections revealed a significantly preserved LV wall thickness (LWWT) in both Epo pre-treated groups (2.9 ± 0.1 mm in the Epo 20 and 3.2 ± 0.1 mm in the Epo 200) (Figure 4) as compared with Group C (2.3 ± 0.1 mm; $P < 0.0001$ for both comparisons). Masson’s trichrome and TUNEL staining exhibited no significant evidence of fibrosis or apoptosis in all three groups (data not shown). Urinary 8-isoprostane levels were significantly reduced in Groups A (47.1 ± 1.3 vs. 59.2%; $P = 0.043$) but a trend towards reduction compared with Group B was evident. LV posterior wall thickness in diastole (LVPWd) was significantly decreased in Group C compared with Groups A and B.

Vascular density was significantly enhanced in both Epo-treated groups (3.9 ± 1.1 vessels/high power field for Epo 20 and 3.4 ± 0.9 vessels/high power field in the Epo 200) following the completion of the treatment with Dox, yielding a cumulative mortality of 85% (Figure 3). In both Epo (A and B) groups, no animal died during the 2 weeks of Dox injections. However, 16 animals died in Group A and 24 animals in Group B during the 6-week follow-up period, resulting in a cumulative mortality of 40% and 65%, respectively ($P = 0.038$ for both, compared with Group C) (Figure 3). No death was evident in Group D. It should be mentioned that due to the high mortality in Group C, a selection bias may have occurred that should be taken into account when interpreting the results.

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Both Groups A and B exhibited a significant increase in the cell CFU obtained from spleens of the rats upon sacrifice. P 0.009; Figure 5). Adhesion of EPC to cultured BAEC was significantly reduced in rats from Group C (18.5 ± 7.2 cells/field) as compared with rats from Groups A (48.3 ± 6.8 cells/field, P < 0.0001) and B (57.2 ± 5.2 cells/field, P < 0.0001) (Figure 5).

We tested the effects of in vivo treatment with Epo on the ability of EPCs to form tubes in vitro and to migrate. EPC from both Epo-treated rats were significantly more potent in achieving in vitro tube formation when combined with BAEC as evident by higher scores. Furthermore, EPC from Groups A and B had a significantly better preserved migratory capacity as compared with EPC obtained from rats of Group C employing the modified boyden chamber.

Effect of transfer of EPC on Dox-induced cardiomyopathy in rat
To investigate whether Epo-mediated protection of Dox cardiomyopathy is mediated partially by the favorable effects on EPC number and functions, we conducted an additional in vivo study, wherein rats were induced to develop Dox cardiomyopathy and treated with either EPC or saline as control. We found that EPC transfer was not associated with improved survival (data not shown). However, myocardial performance in the EPC-transferred rats was significantly improved as compared with Dox-treated rats evident by a higher FS (Figure 6).

Discussion
The late consequences of treatment with Adriamycin are associated with the development of myocardial dysfunction...
and clinically overt heart failure that precludes a more intensive use of this effective chemotherapeutic agent.\textsuperscript{1,2} Although several preventive modalities have been tested, no effective treatment is approved for the attenuation of Dox-mediated myocardial toxicity to date.\textsuperscript{2}

The mechanisms of myocardial dysfunction induced by Dox remain elusive. It has been suggested that production of free radicals is associated with myocardial toxicity.\textsuperscript{1,20} Several recent studies suggest that Dox is toxic to the endothelium and it has been proposed that apoptosis of myocardial vasculature results in enhancement of cardiomyocyte ischaemia and free radical formation.\textsuperscript{21,22}

Other than its well-recognized haematopoietic properties, Epo has recently been shown to have potent proangiogenic properties. These effects are partially mediated by its ability to facilitate mobilization of endothelial precursors.\textsuperscript{13-15} Based on these observations, we tested the hypothesis that Epo will be effective in attenuating Dox-mediated myocardial damage. Indeed, Epo treatment was associated with an improved myocardial function evident by echocardiography and importantly, this was obtained employing the lower Epo dosages that are equivalent to those given to human subjects with anaemia. Treatment with Epo also resulted in a preserved myocardial mass as observed by thicker myocardial walls by H&E staining. EPC numbers were increased in Epo protected animals as compared with controls. Epo treatment was also associated with improved functional properties of EPC namely, increased ability to adhere to fibronectin, and cultured endothelial cells as well as enhanced tube formation and migration in-vitro. Importantly, hearts from Epo-treated rats exhibited significantly increased capillary density indicating that the effects on EPC translated into increased vascularization in vivo.

Figure 5 Treatment with Epo enhances number and functional properties of EPC from Dox-administered rats. EPC were obtained from Epo- and PBS-treated Dox rats and assessed for CFU numbers (A, EPC from spleens; B, EPC from bone marrows), adhesion (C, EPC from spleens; D, EPC from bone marrows); in vitro tube formation by matrigel (E); and migration (F). Results were scored by two independent observers and a representative experiment of three is shown. Results are represented as mean ± SD. *P < 0.05; **P < 0.01.
Epo may lead to a significant increase in blood viscosity and blood pressure. These effects may partially offset some of the beneficial influences with regard to mortality improvement and myocardial protection.

To confirm the direct role of Epo on EPC protection, we conducted in vitro assays employing spleen cell- and bone marrow-derived EPCs from healthy rats and peripheral blood of humans. Indeed, we have found that incubation with Dox was associated with a reduction in CFU numbers that was partially restored by prior addition of Epo. Dox treatment of EPC also resulted in compromised adhesion, migration, and ability to form tubes in the matrigel assay. These compromised properties were partially reversed by prior incubation with Epo.

To obtain further support to the hypothesis that the effect of Epo on EPCs was associated with the observed functional myocardial improvement, we conducted an additional in vivo study. We have found that EPC obtained from healthy syngenic rats and transferred to Dox-treated animals provided a similar extent of myocardial protection as treatment with Epo. Interestingly, mortality in rats transferred with EPC was not significantly reduced as compared to PBS-treated animals. This finding may suggest that myocardial protection by Epo could be preferentially mediated by EPCs whereas salvage of other organ systems by Epo could have contributed to the beneficial effect on mortality.

Several reports have documented beneficial effects of anti-oxidants and free radical scavenging molecules in the rat model of Dox-induced cardiomyopathy. These effects were largely related to the preventive effect of these compounds on the generation of free radicals and subsequent oxidative stress. Epo treatment, to our current experience, provides a combined impact of a potential anti-oxidant agent with the added value of inducing proangiogenic properties through facilitation of enhanced EPC survival and improved functional properties.

In conclusion, we have found that Epo treatment was effective in attenuating myocardial damage induced by Dox, with a possible role for EPC in mediating this effect. If further confirmed, Epo treatment could be considered as a protective agent in patients treated with Dox.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

**Conflict of interest:** none declared.

**References**


