Biochemical and Biophysical Research Communications 371 (2008) 75–78

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Erythropoietin has an antiapoptotic effect after myocardial infarction and stimulates in vitro aortic ring sprouting

Agneta Månsson Broberg [a], Karl-Henrik Grinnemo [b], Rami Genead [a], Christian Danielsson [a], Agneta B. Andersson [a], Eva Wärdell [a], Christer Sylvén [a,*]

[a] Department of Cardiology, M52 Karolinska University Hospital, S14186 Stockholm, Sweden
[b] Department of Cardiothoracic Surgery, Karolinska University Hospital, Sweden

ARTICLE INFO

Article history:
Received 31 March 2008
Available online 14 April 2008

Keywords:
Erythropoietin
Mouse
Myocardial infarction
Angiogenesis
Apoptosis

ABSTRACT

Aims were to explore if darbepoietin-α in mouse can induce angiogenesis and if moderate doses after myocardial infarction stimulates periinfarct capillary and arteriolar densities, cell proliferation, and apoptosis. Myocardial infarction was induced by ligation of LAD. Mouse aortic rings (0.8 mm) were cultured in matrigel and the angiogenic sprouting was studied after addition of darbepoietin-α with and without VEGF-165. After 12 days the hemoglobin concentration was 25% higher in the darbepoietin-α treated mice than in the control group. No difference in capillary densities in the periinfarct or noninfarcted areas was seen with darbepoietin-α. Cell proliferation was about 10 times higher in the periinfarct area than in the noninfarcted wall. Darbepoietin-α treatment led to a decrease of cell proliferation (BrdU, ($p < 0.02$)) and apoptosis (TUNEL, $p < 0.005$) with about 30% in the periinfarct area. Darbepoietin-α and VEGF-165 both independently induced sprouting from aortic rings. The results suggest that darbepoietin-α can induce angiogenesis but that moderate doses after myocardial infarction are not angiogenic but antiapoptotic.

Erythropoietin has been reported that in addition to its hematopoietic stimulating effect it also may have nonhematopoietic and protective effects following ischemic insult with decreased infarct size in a variety of organs: kidney, brain, and heart [1]. In the heart erythropoietin receptors were shown to be expressed on endothelial cells and to a lesser extent on cardiomyocytes [2]. Treatment with recombinant erythropoietin has thus been shown to have such effects dependent on antiapoptotic effects as well as a direct angiogenic effect on endothelial cells or a vasculogenic effect with recruitment of endothelial progenitor cells. The dose range of erythropoietin used in these experiments has been generally high, 5–10 times the dose recommended for human use [3–6].

Angiogenesis stimulating effects following experimental myocardial infarction have been demonstrated for a variety of substances such as VEGF, PDGF-BB, FGF-1, and angiopoietin-1 [7–9]. In a rat nephrotoxicity model, erythropoietin had a similar degree of angiogenic effect as fibroblast growth factor-1 [10]. We therefore thought it would be interesting to explore if therapeutically relevant doses of erythropoietin, that did not effect the hemoglobin concentration, could have angiogenic effects of similar order as VEGF-165 as well as protective antiapoptotic effects in a mouse myocardial infarction model. In order to mimic a clinical situation, erythropoietin (darbepoietin-α) was administered within 10 min after permanent ligation of the left anterior descending artery in a mouse myocardial infarction model.

Methods

Animals. C57BL/6 mice weighing around 25 g were used (Taconic, Denmark). The study was approved by the Stockholm Southern Ethics Review Board for Animal Experiments. The animals were treated in accordance with the Institutional Guidelines for care of laboratory animals.

Myocardial infarction model. The mice were anaesthetized with a intraperitoneal injection of Midazolam (Dormicum, 5 mg/kg) (F. Hoffmann-La Roche Ltd., Switzerland), Medetomidin hydrochloride (Domitor vet, 0.1 mg/kg) (Orion Corp., Espoo, Finland), Fentanyl (0.3 mg/kg) (B. Braun Medical AG, Seesatz, Switzerland) and subsequently endotracheally intubated, ventilated using a Zoovent ventilator (Model CWC600AP, BK Universal, UK). The heart was exposed via a thoracotomy, and the left anterior descending artery (LAD) was ligated with a 7.0 polypropylene suture. Myocardial infarction was confirmed by a color change and dyskinesia of the anterior wall. The anesthesia was reversed by an intraperitoneal injection of flumazenil (Lanexat, 0.1 mg/kg) (F. Hoffmann-La Roche Ltd., Switzerland), atipamezol hydrochloride (Antisedan vet 5 mg/kg) (Orion Corp., Espoo, Finland) and buprenorphin hydrochloride (Temgesic, 0.1 mg/kg) (Schering-Plough Corp., Kenilworth, UK). Using parallel group design animals were randomly allocated to either of three groups ($n = 6$, each group): group 1: Darbepoietin-α 7.5 μg/kg given ip at day 5; group 2: Darbepoietin-α 7.5 μg/kg given ip at day 0 within 10 min from ligation of LAD and at day 5 and group 3: control. This dose of darbepoietin-α was previously reported to increase hemoglobin by 10–20 g/L/week when given once weekly [11]. Animals were sacrificed after 12 days and the hearts harvested.

* Corresponding author.
E-mail address: christer.sylven@ki.se (C. Sylvén).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.04.025

Capillary and arteriolar densities. The harvested hearts were divided in two halves and the apical parts were embedded in OCT (Histolab, Sweden) and frozen in liquid nitrogen, and kept in −70 °C. Five micrometer thick sections were prepared using a cryostat. Staining with eosin/hematoxylin verified that the samples chosen came from the periinfarct area or from the noninfarcted area. For the analysis of capillary density, the sections were incubated with Griffonia Badeiraea Simplicifolia Isolectin B4 (GSL-I-B4, Vector Laboratories, USA) followed by a second incubation with ABC Complex. Capillaries were visualized by DAB with supplementation of 0.03% hydrogen peroxide. The capillaries were counted from images obtained at a magnification of 400× taken with a LCD camera (Olympus BX60, Olympus Optical CO., Japan) connected to a microscope. Photomicrographs of eight fields around the injection site were taken, and the capillary count was analyzed using a blinded procedure with an image analysis program (Micro Image, Olympus). For the analysis of density of vessels associated with smooth muscle cells, namely arterioli, the lectin-stained sections were incubated with primary antibodies against α-actin (Sigma–Aldrich Corp., USA). Rabbit anti-mouse secondary antibody (FITC-labeled, Dako Cytomation, Denmark) was used to visualize the blood vessels. Positively stained blood vessels around the injection site were counted under 200× magnification using a fluorescent microscope. All analysis were performed in a strictly blinded manner. Densities obtained for each individual mouse is expressed as the mean count of eight fields.

Bromodeoxyuridine labeling. The mice were given 5-bromo-2′-deoxyrubicine (Sigma–Aldrich, Germany), (BrdU) 100 mg/kg/daily, ip day 6–9 in order to assess mitotic activity. The same sections as used in the lectin staining were double stained with BrdU antibody. Unmasking of proteins was done by boiling the sections in a citrate buffer, followed by the incubation with the primary antibody mouse anti-BrdU (Dako Cytomation, Denmark, Clone Bu20a) overnight. As secondary antibody we used polyclonal rabbit anti-mouse Ig/FITC (Dako Cytomation), dilution 1:10–1:20. The slides were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). The amount of BrdU positive cells were counted manually in the same areas as the lectin staining. The counting was performed in a strictly blinded manner.

Apoptosis assay. TUNEL assay was performed on frozen sections with an ApopTag Fluorescein in situ Apoptosis Detection Kit (CHEMICON International, CA, USA) according to the manufacturer's specifications. Sections of heart tissue were treated with digoxigenin-dNTP and terminal deoxynucleotidyl transferase followed by incubation with anti-digoxigenin conjugated with fluorescein. Counterstaining with DAPI was performed to visualize nuclei. Five fields of apoptotic positive cells were counted under 400× magnification in a blinded fashion.

Aortic ring culture. C57/BL6 mice were euthanized and the thoracic aortae were dissected and cut into 0.8 mm pieces. An average of five rings per well were cultured in Growth Factor Reduced Matrigel TM Matrix (BD Biosciences) in Human Endothelial-SMF Basal Growth Medium (GIBCO, Invitrogen Corporation, cat no 11111-044), with and without the supplement of VEGF-165 (20 ng/ml) and darbepoietin-α 0, 1, and 5 ng/mL. Each point of determination represents the mean of five experiments. The number of endothelial sprouts were assessed by visual counting at days 5 and 12.

Statistical analysis. Values are expressed as means ± SEM. Statistical nonhomogeneity was tested with Student's unpaired *t*-test or with one or two-way Analysis of Variance (ANOVA). A p value of <0.05 was considered significant.

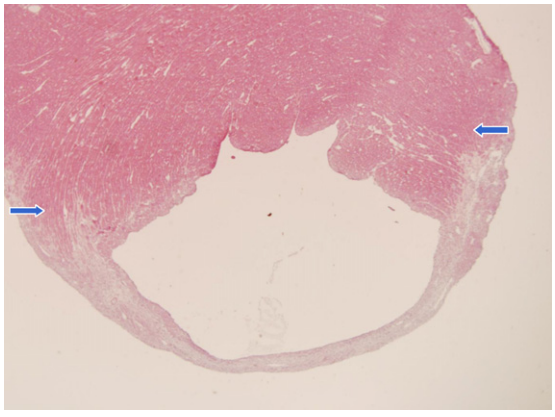

Fig. 1. Mouse heart (left ventricle) 12 days after LAD ligation. Arrows indicate the periinfarct area.

Results

The LAD ligation induced a transmural infarction in a reproducible manner (Fig. 1). Analysis were done in specimens from the periinfarct and noninfarcted areas. In the control group venous hemoglobin concentration decreased about 20% 12 days after myocardial infarction. Darbepoietin-α effected increased hemoglobin concentration compared to control (Table 1). Capillary ($p < 0.01$) and arteriolar ($p < 0.03$) densities were about 80% lower in the periinfarct area (Fig. 2) with no difference between the groups. The cell proliferation was about 10 times higher in the periinfarct area compared to the noninfarcted with a darbepoietin-α depen-

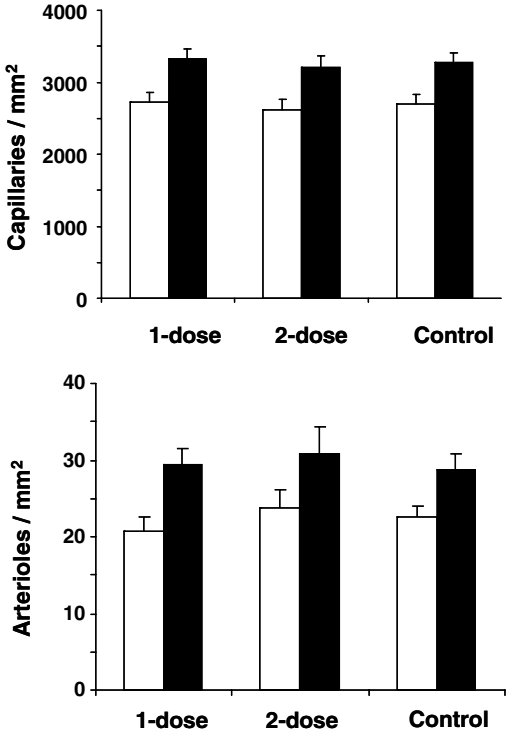

Fig. 2. Capillary and arteriolar densities in periinfarct (open columns) and noninfarcted (filled columns) areas 12 days after infarction. Values are given for the 3 groups: 1-dose: ip darbepoietin-α 7.5 μg/kg day 5; 2-dose: ip darbepoietin-α day 0 (within 10 min after ligation of LAD) + at day 5 and group 3: control. Compared to the noninfarcted area capillary and arteriolar densities were lower in the periinfarct area ($p < 0.01$ and $p < 0.03$, respectively). Darbepoietin-α did not have any effect on the vascular densities. Values are means ± SEM.

Table 1
Hemoglobin concentration before LAD ligation and after 12 days

	Day 5		Day 0 + 5		Control	
	Before	12 d	Before	12 d	Before	12 d
Hb g/L	154 + 2.2	150 + 2.1	148 + 2.5	155 + 4.4	154 + 2.5	123 + 2.4
Delta g/L		−19 + 5.6		29 + 2.6		−31 + 2.1
p vs control		<0.0001		<0.0001		

In the three different groups darbepoietin-α (7.5 μg/kg day 5) or 2 dose darbepoietin-α (day 0 + 5) were given and the effect was compared to the control mice. $N = 4$ at each time point. ANOVA for the three groups $p < 0.0001$. Values are means ± SEM.

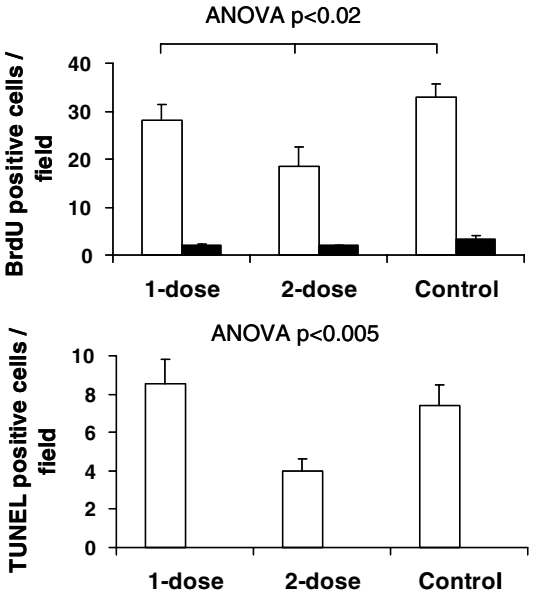

Fig. 3. BrdU determined cell proliferation per field (above) in the periinfarct (open columns) and noninfarcted (filled columns) areas and frequency of TUNEL positive cells per field (below) 12 days after infarction. No TUNEL staining was present in nonfarcted area. Values are given for the 3 groups: 1-dose: ip darbepoietin-α 7.5 μg/kg day 5; 2-dose: ip darbepoietin-α day 0 (within 10 min after ligation of LAD) + at day 5 and group 3: control. Values are means ± SEM.

dent decrease ($p < 0.02$) of about 30% for the 2-dose regimen (Fig. 3). No TUNEL staining was observed in the remote noninfarcted myocardium. TUNEL stained cells were present in the periinfarct area and the frequency of these decreased ($p < 0.005$) with darbepoietin-α (Fig. 3).

In vitro sprouting from mouse aortic rings was stimulated to a similar degree by VEGF-165 and darbepoietin-α (Fig. 4).

Discussion

Therapeutically relevant doses of darbepoietin-α caused moderate increase of hemoglobin concentration and it had no effect on the periinfarct capillary and arteriolar densities in this mouse permanent occlusion myocardial infarction model. At the same time cell division and apoptosis was decreased in the periinfarct area. In vitro darbepoietin-α induced sprouting from aortic rings similar to VEGF-165.

As previous investigations have been made with considerably higher doses of erythropoietins [3–6] it thus appears that darbepoietin-α stimulation of angiogenesis in vivo is dose dependent. From a clinical point of view high doses of erythropoietin may not be relevant since it causes an increase in hemoglobin and hematocrit which might have adverse effects such as loading the heart, hypertension, retinopathy, neurotoxicity, and thromboembolism.

Interestingly cell division as determined by BrdU covaried with the degree of apoptosis (TUNEL staining) with decreased activities following darbepoietin-α administration. The cause of this is unclear. The hypothesis that this might be dependent on an antiinflammatory effect was explored with CD3 and CD11 stainings (data not shown). However, no effect of darbepoietin-α was observed on these expressions. Another cause might be decreased connective tissue formation. This could, however, not be evaluated as fibroblasts cannot be specifically stained in the mouse.

In vitro in mouse aortic ring matrigel culture with increasing doses of darbepoietin-α, did have an angiogenic sprouting effect of similar order as VEGF-165. Although any angiogenic effect could not be confirmed in the used mouse permanent occlusion myocardial infarction model, the moderate dose of darbepoietin-α used did counteract cell proliferation and apoptosis. Our findings are in accordance to the work of Bahlmann et al. [12] who reported

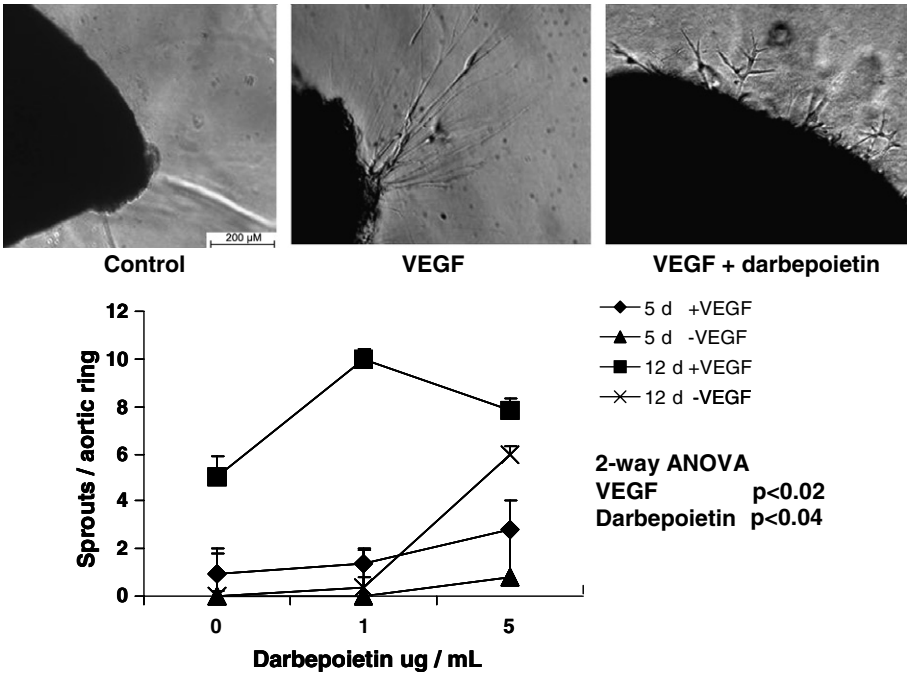

Fig. 4. Sprouts from mouse aortic rings in matrigel (above) control, after addition of VEGF-165 (20 ng/mL) and VEGF-165 + darbepoietin-α after 10 days. Below quantitative results without and with VEGF-165 and darbepoietin-α 0, 1, and 5 ng/mL. $N = 5$ at each time point. The number of sprouts were assessed by visual counting on days 5 and 12. Values are given as means ± SEM. Two-way ANOVA showed similar and independent effects of VEGF-165, $p < 0.02$ and darbepoietin-α, $p < 0.04$.

that a similar dose (0.1 μg/kg/week) had tissue protective effects in the kidney without the induction of an increased hematocrit.

In conclusion, although in vitro darbepoietin-α was shown to induce angiogenesis similar to VEGF-165, in relevant doses it did not induce any angiogenesis or arteriogenesis in a mouse permanent occlusion myocardial infarction model. The antiapoptotic effects of darbepoietin-α might be useful to inhibit the cell loss after myocardial infarction and this together with the synergistic angiogenic effect of VEGF-165 might also inhibit the remodeling process. Further studies need to be performed to explore this hypothesis.

Acknowledgments

This work was supported by the Swedisk Research Council and the Karolinska Institute.

References

[1] E. Lipsic, R. Schoemaker, P. van der Meer, A. Voors, D. van Veldhuisen, W. van Gilst, Protective effects of erythropoietin in cardiac ischemia, J. Am. Coll. Cardiol. 48 (2006) 2161–2167.
[2] P. van der Meer, E. Lipsic, R.H. Henning, et al., Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia-reperfusion injury, Eur. J. Heart Fail. 6 (2004) 853–859.
[3] C.J. Parsa, A. Matsumoto, J. Kim, et al., A novel protective effect of erythropoietin in the infarcted heart, J. Clin. Invest. 112 (2003) 999–1007.
[4] A.F. Tramontano, R. Muniyappa, A.D. Black, et al., Erythropoietin protects cardiac myocytes from hypoxia-induced apoptosis through an Akt-dependent pathway, Biochem. Biophys. Res. Commun. 308 (2003) 990–994.
[5] C. Moon, M. Krawzyk, D. Paik, E.G. Lakatta, M.I. Talan, Cardioprotection by recombinant human erythropoietin following acute experimental myocardial infarction: dose response and therapeutic window, Cardiovasc. Drugs Ther. 19 (2005) 243–250.
[6] P. van der Meer, E. Lipsic, R.H. Henning, et al., Erythropoietin induces neovascularization and improves cardiac function in rats with heart failure after myocardial infarction, J. Am. Coll. Cardiol. 46 (2005) 125–133.
[7] S. Yla-Herttuala, K. Alitalo, Gene transfer as a tool to induce therapeutic vascular growth, Nat. Med. 9 (2003) 694–701.
[8] X. Hao, A. Månsson-Broberg, T. Gustafsson, K.H. Grinnemo, P. Blomberg, A.J. Siddiqui, E. Wärdell, C. Sylvén, Angiogenic effects of dual gene transfer of bFGF and PDGF-BB after myocardial infarction, Biochem. Biophys. Res. Commun. 315 (2004) 1058–1063.
[9] A.J. Siddiqui, P. Blomberg, E. Wärdell, I. Hellgren, M. Eskandarpour, K.B. Islam, C. Sylvén, Combination of angiopoietin-1 and vascular endothelial growth factor gene therapy enhances arteriogenesis in the ischemic myocardium, Biochem. Biophys. Res. Commun. 310 (2003) 1002–1009.
[10] A. Efthimiadou, O. Pagonopoulou, M. Lambropoulou, N.K. Nikolettos, Erythropoietin enhances angiogenesis in an experimental cyclosporine A-induced nephrotoxicity model in the rat, Clin. Exp. Pharmacol. Physiol. 34 (2007) 866–869.
[11] K.R. LaMontagne, J. Butler, D.J. Marshall, J. Tullai, Z. Gechtman, C. Hall, A. Meshaw, F.X. Farrell, Recombinant epoietins do not stimulate tumor growth in erythropoietin receptor–positive breast carcinoma models, Mol. Cancer Ther. 5 (2006) 347–355.
[12] F.H. Bahlmann, R. Song, S.M. Boehm, et al., Low-dose therapy with the long-acting erythropoietin analogue darbepoietin alpha persistently activates endothelial Akt and attenuates progressive organ failure, Circulation 110 (2004) 1006–1012.