Biochemical and Biophysical Research Communications, Vol. 344(3), 987-994, 2006 Cardioprotective effects of recombinant human erythropoietin in rats with experimental autoimmune myocarditis.

Wataru Mitsuma^a, Masahiro Ito^{a,*}, Makoto Kodama^a, Koichi Fuse^a, Kazuki Okamura^a, Shiro Minagawa^a, Kiminori Kato^a, Haruo Hanawa^a, Ken Toba^a, Mikio Nakazawa^b, Yoshifusa Aizawa^a.

^aDivision of Cardiology, Niigata University Graduate School of Medical and Dental Sciences, Japan. ^bDepartment of Medical Technology, School of Health Sciences, Faculty of Medicine, Niigata University, Japan. *Corresponding author. Fax:+81252270774. E-mailaddress:pigtailef45@yahoo.com (M. Ito).

Abstract Erythropoietin (EPO) has been known to have cytoprotective effects on several types of tissues, presumably through modulation of apoptosis and inflammation. The effect of EPO on myocardial inflammation, however, has not yet been clarified. We investigated the cardioprotective effects of EPO in rats with experimental autoimmune myocarditis (EAM). Seven-week-old Lewis rats immunized with cardiac myosin were treated either with EPO or vehicle and were examined on day22. EPO attenuated the functional and histological severity of EAM along with suppression of mRNAs of tumor necrosis factor (TNF)-a and interleukin(IL)-6 in the hearts as well as a reduction of apoptotic cardiomyocytes. The EPO receptor (EPO-R) was upregulated in the myocardium of EAM compared with that of healthy rats. These results may suggest that EPO ameliorated the progression of EAM by modulating myocardial inflammation and apoptosis.

Keywords: Erythropoietin; Experimental autoimmune myocarditis; Erythropoietin receptor; Apoptosis.

Introduction

Inflammation and autoimmunity are involved in many cardiac diseases. Myocarditis is an inflammatory heart disease, and causes both acute and chronic heart failure as a result [1]. Some patients with myocarditis show a fluminant course and die of intractable cardiogenic shock, and the treatment strategy for the disease is still unresolved [2]. One of the fatal subtypes of myocarditis is giant cell myocarditis, and its etiology is considered to be related to autoimmunity [3,4]. A model of rat experimental autoimmune myocarditis (EAM) resembles human giant cell myocarditis, and the recurrent form of EAM leads to dilated cardiomyopathy [3,4]. EAM is induced by T cell activation and the peak of inflammation is observed in the heart around day21 after immunization [5,6]. Neurohumoral factors such as cytokines and chemokines, and myocardial remodeling including myocardial apoptosis play important roles in the progression of EAM [7-9].

Erythropoietin (EPO) is a hematopoietic cytokine that stimulates the production of red blood cells in bone marrow [10], and has been recently shown to have broader activities outside of bone marrow beyond hematopoiesis [11–14]. EPO and its receptor (EPO-R) have been shown to be present in tissue outside the hematopoietic system, including the heart [15,16]. Recently, the biological effects of EPO on proliferation and cell

survival in various cells have been well characterized. Several reports have revealed the cardioprotective effects by EPO against myocardial ischemia-reperfusion injury and heart failure [17–20]. The mechanisms of these effects have been explained mainly by modulation of either inflammatory responses or apoptosis [21–23]. At present considerable evidence supports that EPO is a cytoprotective agent, including cardioprotection.

These observations have suggested the beneficial effects of EPO on myocarditis, but which have not yet been clarified. Accordingly, the goal of this study was to investigate the effect of recombinant human EPO (rhEPO), which is widely used in the clinical field, on rat EAM.

Materials and methods

Animals.

Seven-week-old male Lewis rats were purchased from Charles-River Laboratories, Japan (Atsugi, Kanagawa, Japan), and were maintained in our animal facilities. Throughout the studies, all the animals were treated in accordance with the guidelines for animal experiments as laid out by our institute.

Erythropoietin.

Recombinant human erythropoietin (rhEPO) was provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

Induction of EAM.

Cardiac myosin was prepared from the ventricular muscle of porcine hearts as previously described [3]. It was dissolved in PBS at a concentration of 10mg/ml and emulsified with an equal volume of complete Freund's adjuvant supplemented with 10mg/ml of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, Michigan). On day 0, the rats received a single immunization at two subcutaneous sites (both foodpads) with a total of 0.2ml of emulsion for each rat.

Experimental protocol.

A total of 20 rats were immunized in this study. Immunized rats were divided into two groups: Group C (given only vehicles) and Group E (rhEPO, 500 IU/kg/day). They were administered intraperitoneally once a day from day 0 to day 21.

Evaluation of echo cardiography and hemodynamic parameters.

On day 22, the peak of the inflammatory phase [6,7], echocardiography was performed with a 7.5-MHz probe (SSD-630, Aloka ECHO camera). Left ventricular (LV) internal diameter in end diastole and end systole, interventricular septal thickness, LV posterior wall thickness, pericardial effusion, and LV fractional shortening were calculated from M-mode echocardiograms over three consecutive cardiac cycles. The hemodynamic parameters were measured after echocardiography. Mean arterial pressure was recorded through a catheter introduced into the right femoral artery. Heart rate was calculated from electrocardiograms. These hemodynamic parameters were recorded on a thermostylus recorder after a stabilizing period of 10 min.

Histopathology and sampling.

All immunized rats were humanely killed under anesthesia on day 22 after recording hemodynamic parameters. Thoracotomy was performed and then macroscopic score was classified into four grades as described [3]. Blood samples were then obtained from the inferior vena Hematopoietic parameters cava. including hemoglobin concentration, and white blood cell and platelet counts were measured. Subsequently, heart and body weights were measured, and the ratio of heart weight to bodyweight was calculated. Several transverse sections were cut from the midventricle slice, fixed in 10% formalin, and then stained with Azan-Mallory and

Hematoxylin–eosin. The myocarditis area of each specimen was determined with a color image analyzer (MacSCOPE version 2.6, Mitani Corp.) as previously described [24].

QuantitativeRT-PCR.

Total RNA was isolated from one-third (apex side) of the rats' ventricles on day 22 and reverse transcription (RT) was performed [25]. Real time polymerase chain reaction (PCR) using a Light-Cycler-Fast Start DNA Master SYBR Green I (Roche, Indianapolis IN) was performed. Primer sequences were as follows: interferon (IFN)-γ, 50-tcatggccctctctggctgttac-30 (sense) and 50-caagaggaggctctttccttccatag-30 (antisense); interleukin (IL)-1β, 50-cttcaaatctcacagcagcatctcg -30 (sense) and 50-tccacggcaagacataggtagc-30 (antisense); tumor necrosis factor (TNF)-a, 50ctgtgcctcagcctcttctcattc-30 (sense) and 50- ttggga acttctcctccttgttgg-30 (antisense); monocyte chemo attractant protein-1 (MCP-1), 50-ctgtctc agccagatgcagttaat-30 (sense) and 50-tatgggtca agttcacxattcaaag-30 (antisense); IL6, 50-ccagtt gccttcttgggactgatg-30 (sense) and 50-attttctgacc acagtgaggaatg-30 (antisense); IL-10, 50-tgccttcag tcaagtgaagact-30 (sense) and 50-aaactcattcatg gccttgta-30 (antisense); γ-actin, 50-agccttccttccgg gcatggagt-30 (sense) and 50-tggaggggcctgactcg tcatact-30 (antisense). The absolute copy numbers of particular transcripts were calculated by LightCycler soft ware using a standard curve approach. The levels of y -actin mRNA were used as the internal control.

Immunohistochemistry.

Immunohistochemistry was performed on paraffin-embedded sections included hearts from EAM rats and age-matched healthy rats. The sections were deparaffinized, rehydrated, and subjected to endogenous peroxidase block in 3% H₂O₂. Slides were incubated overnight at 4°C with a rabbit anti-erythropoietin receptor (EPO-R) polyclonal antibody (1:250 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), a mouse anti-a-actinin (1:800 dilution, Sigma Saint Louis, Missouri, USA), or isotype-matched control IgG as primary antibodies. After overnight incubation, slides were washed and incubated using HISTFINE Simple Stain rat MAX-PO (MULTI) kit (Nichirei Bioscience, Japan), which was an amino acid polymer combined with peroxidase and a secondary antibody, for 30 min. The sections were developed with diaminobenzidine tetrahydrochloride chromogen and counterstained with hematoxylin.

Detection of apoptosis.

Terminal transferase-mediated DNA nick end labeling (TUNEL) assay was performed [9] using a Cardio TACS kit (TrevigenInc., Gaithersburg, MD), as described. In brief, paraffin sections were affixed to slides. After deparaffinization, the sections were washed in PBS, incubated with 20 mg/dl proteinase K for 25 min at room temperature, and then washed in deionized water for 2 min. After blocking of endogenous speroxidase by covering sections with 3% H₂O₂ for 5 min at room temperature, sections were rinsed with deionized water and incubated with TUNEL reaction mixture for 1 h at 37°C in a humidified chamber and slides were counterstained. TUNEL-positive cells per high -power field (400 x) were counted in 10 randomly chosen fields, and counts were averaged.

Statistics.

Statistical measurements were performed by unpaired Student's *t* test. Fisher's exact test was used for the evaluation of pericardial effusion on echocardiography. The differences were considered significant at P<0.05. All data were expressed as means \pm standard error of mean (SEM).

Results

Effect of EPO on general characteristics

Of the 20 rats, 3 died during the active phase, 2 in Group C and 1 in Group E. The remaining 17 rats were utilized for evaluation (Group C, n=8; Group E, n=9). Baseline characteristics on day 22 are shown in Table 1. The macroscopic score in the EPO-treated rats was significantly lower compared with the control rats (P<0.01). The hemoglobin level in Group E was significantly elevated compared to Group C (P<0.01). On the other hand, the white blood cell count of Group E was significantly lower than that of Group C (P<0.05).

Echocardiography

On day 22, fractional shortening (FS) in Group C was $33.4\pm2.5\%$. EPO improved FS up to $48\pm3.2\%$ (P<0.01) (Fig.1A, B). There was no significant difference in LV end diastolic diameter and wall thickness between the two groups. Massive pericardial effusion was not observed in Group E, while five of eight rats in

Group C showed moderate to severe pericardial effusion (P < 0.05).

Histology

Severe inflammatory lesions were observed in the hearts in Group C (Fig. 2A). These lesions were composed of extensive myocardial necrosis and showed infiltration of mononuclear cells, polymorphonuclear neutrophils, and multi -nucleated giant cells. In contrast, the hearts in Group E showed only slight myocardial necrosis (Fig. 2B). The ratio of the myocarditis area was significantly smaller in Group E than that in Group C. The area ratio was $40.5\pm4.1\%$ in Group C and $26.6\pm4.1\%$ in Group E (P<0.05; Fig. 2C).

EPO effect on cytokine production in hearts

EPO reduced the expression of TNF- α mRNA and IL-6 mRNA in the heart (*P*<0.05 for both; Fig. 3A, B). The mRNA level of MCP-1 showed a tendency to decrease in Group E (*P*=0.10; Fig. 3C). However, there was no significant difference in the level of IL1- β (*P*=0.11; Fig. 3D), IFN- γ (*P*=0.28; Fig. 3E), and IL-10 (*P*=0.77; Fig. 3F) between the two groups.

Expression of EPO-R in the hearts

Immunohistochemical staining for EPO-R revealed that cardiomyocytes were stained slightly in healthy age-matched rats (Fig. 4A), and strongly stained in rats with EAM (Fig. 4B), while inflammatory cells, such as macrophages, lymphocytes, and neutrophils, were not stained with the antibody.

EPO effects on apoptosis of cardiomyocytes

By using the TUNEL assay, some of the nuclei were stained positively in Group C, while a smaller number of nuclei were stained in Group E (P<0.05) (Fig. 5C, D, E). The TUNEL-positive cells were mainly cardiomyocytes in consecutive sections with immunohistochemical staining by the anti- α -actinin antibody (Fig. 5A, B).

Discussion

In the present study, we demonstrated that rhEPO ameliorated the progression of EAM in rats. EPO attenuated myocardial inflammation and heart failure, at least in part, by modulating the inflammatory cytokine profiles and myocyte apoptosis.

EAM is a T cell-mediated disease. Cytokines are important in controlling T cell response and play a major role in the pathogenesis of EAM [5–7]. The expressions of inflammatory cytokines, such as TNF- α and IL-6, were significantly reduced by treatment with rhEPO. Another inflammatory cytokine, IL-1ß, tended to be lower in the treated groups than in the non-treated groups. We previously showed that Th1/Th2 balance regulates the clinical course of myocarditis [6], and serum levels of Th1 and Th2 cytokines increased in parallel with its mRNA expression in the heart during the clinical course of EAM [6,7]. mRNA expression of Th1 cytokines increased from the onset of the disease, and that of Th2 cytokines could be subsequently detected in the recovery phase. We also demonstrated that modulation of inflammatory cytokines suppressed myocardial inflammation [24,26]. MCP-1 also showed a tendency to be reduced by rhEPO treatment. MCP-1 is a member of the C-C chemokine family that has been shown to play a major role in the migration of monocytes and T cells to an inflammatory focus [27]. We previously found that increased MCP-1 was observed in EAM from the acute phase and that MCP-1 plays an important role in the early progression of EAM [8]. Furthermore, MCP-1 levels of fatal patients with myocarditis were significantly higher than those of patients with myocarditis who survived [8]. Taken together with the previous studies, we concluded that rhEPO attenuates EAM, at least in part, by modulation of the inflammatory cytokine axis. The mechanisms of how rhEPO attenuated the expression of these molecules are still unclear from this study. Recently, we have shown that most immunological molecules, including Th1 cytokines and proinflammatory cytokines, were expressed in infiltrated inflammatory cells [28]. MCP-1 was produced by infiltrated monocytes in EAM. EPO exerts its physiological effects by binding to EPO-R. EPO-R is, so far, considered not to exist on peripheral matured hematopoietic cells [15,16]. No infiltrated inflammatory cells were stained with an anti-EPO-R antibody at least on day 22 in this study (data not shown). Further study for the precise mechanisms of EPO on reducing the inflammatory cytokine axis in the heart is needed.

The number of TUNEL-positive myocytes was significantly lower in rhEPO-treated rats than in non-treated rats. Several studies have shown that cardiac myocyte apoptosis is one of the mechanisms of the progression of EAM [9,29]. Recently, Tramontano et al. found that EPO protects cardiacmyocytes from hypoxia-induced apoptosis through an Akt-dependent pathway both in vivo and in vitro [21]. Interestingly, the expression of EPO-R was higher in the myocardium of EAM rats than in the myocardium of normal rats by immunohistochemistry in the present study. These results lead to the suggestion that rhEPO prevents myocyte apoptosis through increased EPO-R expression on myocytes. In this regard, it is noteworthy that the expression of EPO-R is hypoxia dependent and there are localized hypoxic areas in the myocardium of EAM [30]. Given the well-described role of EPO to prevent cell apoptosis [19, 21, 23], it is possible that improved cardiac function on echo -cardiography reflects a decrease in myocyte loss. Of note, myocyte apoptosis itself is causal component in the pathogenesis of heart failure, and inhibition of myocyte apoptosis markedly inhibits its development [31].

We cannot exclude the possibility that the reduction in levels of cytokines and in myocyte apoptosis in the EPO-treated groups is an indirect response of rhEPO. It is possible that the beneficial effects depend on the increased oxygen-carrying capacity through elevation of the hemoglobin level by EPO. The level of hemoglobin in age-matched healthy rat was 16.93±0.03 g/dl. We observed hemoglobin reduction in the non-treated myocarditis group (13.44±0.47 g/dl) and the levels of hemoglobin had recovered significantly (19.21±0.27 g/dl) in the group treated with rhEPO. In clinical studies, anemia was found to be an independent risk factor for de novo and recurrent congestive heart failure [32]. Normalization of hemoglobin levels in anemic patients with heart failure has a positive effect on LV dysfunction [33, 34]. Fiordaliso et al. reported a non-erythropoietic derivative of EPO; carbamylated EPO (CEPO), and its biological effects on the cardiovascular system [35]. CEPO binds not to EPO-R homodimer expressed in hematopoietic system, but to heterodimer EPO-R/CD131 expressed in other organs such as the central nervous and cardiovascular system, and acts through JAK/STAT pathway. CEPO prevented apoptosis of cardiomyocytes in the absence of erythropoietic activity. Therefore, the cardioprotective effects of EPO were at least in part, one of the direct effects of EPO on the cardiovascular system, but not an indirect effect through improvement of anemia. In the present study, both direct effects of EPO, surviving activity for cardiomyocytes, and indirect effects, anti-inflammatory effect and erythropoietic activity, might work in concert beneficially in the

rat EAM model.

In summary, the present study demonstrated that rhEPO treatment ameliorated the progression of rat EAM. These results may have particular clinical relevance because rhEPO is safely used in the clinical field. The EPO dose used in this study is clinically relevant. There is increasing evidence that rhEPO may exert beneficial effects on the clinical course of patients with heart failure. Further study is needed, but the present study suggests the beneficial effect of rhEPO for patients with inflammation-related cardiac disease.

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Table 1.

Baseline characteristics on day 22

	Group C $(n = 8)$	Group E $(n = 9)$
Hemodynamics		
Heart Rate (beat/min)	336.3 ± 16.5	319.9 ± 8.6
MBP (mmHg)	70.68 ± 5.27	81.57 ± 2.19
HW/BW (mg/g)	5.11 ± 0.28	5.231 ± 0.27
Histopathology		
Macroscopic score ^a	3.25 ± 0.25	$2.44\pm0.17^{\dagger}$
Echocardiography		
FS (%)	33.45 ± 2.54	$48.41\pm3.24^{\dagger}$
LVEDd (cm)	0.645 ± 0.017	0.614 ± 0.024
LVEDs (cm)	0.428 ± 0.020	$0.317\pm0.025^{\dagger}$
IVSd (cm)	0.146 ± 0.009	0.158 ± 0.006
LVPWd (cm)	0.203 ± 0.009	0.194 ± 0.010
PE	62.5% (5/8)	11.1% (1/9) [†]
Hematopoietic parameters		
Hb (g/dl)	13.44 ± 0.47	$19.21\pm0.27^{\dagger}$
WBC (×10 ³ /µl)	16.9 ± 0.9	$13.9\pm0.8^{\ddagger}$
Plt (×10 ³ / μ l)	1067 ± 51	862 ± 80

MBP, mean blood pressure; EDP, left ventricular end diastolic pressure; HW/BW, ratio of heart weight to bodyweight; FS, fractional shortening; LVEDd, left ventricular end diastolic diameter; LVEDs, left ventricular end systolic diameter; IVSd, diastolic intraventricular septum thickness; LVPWd, diastolic left ventricular posterior wall thickness; PE, pericardial effusion; Hb, hemoglobin; WBC, white blood cell; Plt, platelet. Data are mean ± SEM.

^a0, no inflammation; 1, presence of small discolored focus; 2, presence of multiple discolored foci; 3, diffuse discolored areas not exceeding a total of one-third of the cardiac surface; and 4, diffuse discolored areas totaling more than one-third of the cardiac surface.

** P<0.05 vs Group C.

* P<0.01 vs Group C.



Fig.1. Improvement of cardiac function on echocardiography on day 22. (A) M-mode echocardiograms in Group C and Group E. Besides impaired left ventricular contractility, severe right ventricular dilatation was observed in Group C. (B) Effect of EPO on left ventricular fractional shortening (%FS). LV, left ventricle; RV, right ventricle. *P<0.01 vs Group C.



Fig.2. Azan-Mallory staining. Representative cases of myocardial lesions on day 22 in Groups C (A) and E (B). Severe myocarditis lesions were observed in the hearts of all Group C rats. These lesions showed extensive necrosis and infiltration by inflammatory cells and the degree of these findings was mild in Group E. (C) Myocarditis area ratios in the respective groups. Original magnification in (A) and (B) is 100 x. *P<0.05 vs Group C.



Fig.3. Copy numbers of cytokines and chemokines mRNA/copy numbers of γ -actin mRNA in rat hearts with EAM. (A) TNF- α , (B) IL-6, (C) MCP-1, (D) IL-1 β , (E) IFN- γ and (F) IL-10. EPO significantly reduced TNF- α and IL-6 production in the heart. **P*<0.05 vs Group C.



Fig.4. Immunohistochemical staining of EPO-R in rat hearts. (A) An age-matched healthy Lewis rat. Cardiomyocytes were slightly stained with the anti-EPO-R antibody. (B) A rat with EAM. Inflammatory cell infiltration and fragment of degenerated myocardial fibers were observed. Cardiomyocytes were strongly stained, while inflammatory cells were not stained with the anti-EPO-R antibody. (C) Cardiomyocytes were not stained with the control antibody. Original magnificationis 600 x.



Fig.5. TUNEL assay. (A) TUNEL-positive cell in EAM hearts on day 22. TUNEL-positive cells are visible as dark brown color in the nuclei (arrows in A). (B) The same cardiomyocyte (arrow) in a consecutive section stained by the immunohistochemical method with α -actinin. TUNEL staining of Group C (C) and Group E (D). Arrows of (C) and (D) showed TUNEL-positive cardiomyocytes. The number of TUNEL-positive cells was significantly lower in rats treated with EPO (E). Original magnification is 600 x. **P*<0.05 vs Group C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)