Available online at www.sciencedirect.com
ScienceDirect
Experimental Neurology 204 (2007) 260–263
Experimental Neurology
www.elsevier.com/locate/yexnr

Erythropoietin delays disease onset in an amyotrophic lateral sclerosis model

Jonathan F. Grunfeld [a,*], Yael Barhum [b], Netta Blondheim [b], Jose-Martin Rabey [a], Eldad Melamed [b], Daniel Offen [b]

[a] Department of Neurology, Asaf HaRofeh Medical Center, Tel Aviv University, Sackler School of Medicine, Israel
[b] Felsenstein Medical Research Center, Department of Neurology, Rabin Medical Center, Tel Aviv University, Sackler School of Medicine, Israel

Received 28 July 2006; revised 1 November 2006; accepted 5 November 2006
Available online 15 December 2006

Abstract

Erythropoietin (Epo) has been shown in the recent years to have neuroprotective activity in a variety of settings. In this study, we investigated its impact on the progression of paralysis in a mouse model simulating the human disorder amyotrophic lateral sclerosis (ALS). We found that Epo can delay the onset of motor deterioration in transgenic SOD G93A mice without prolonging their survival. Notably this effect was selective for the females only. These initial findings encourage further investigation of this biological avenue in the search for improved remedies for this fatal disease.

Keywords: Amyotrophic lateral sclerosis; Animal model; Neuroprotection; Erythropoietin

Amyotrophic lateral sclerosis (ALS) is the most common of neurodegenerative disorders primarily involving motor neurons. It occurs worldwide with an annual incidence ranging between 1 and 2.5 per 100,000 (2001), primarily affecting adults, with a mean age of onset between 60 and 65 (2001). Progressive motor paralysis is the dominant clinical feature, which most commonly leads to respiratory failure related death (Sejvar et al., 2005). Despite ongoing research efforts, no effective therapy for this fatal disease has been discovered to date, and median survival with best care does not extend beyond 3 years from onset of symptoms (del Aguila et al., 2003).

Several clinical variants of ALS are known, with 10% representing hereditary forms commonly referred to as familial ALS (fALS). Around 20% of fALS have been tracked to mutations in the cytosolic superoxide dismutase (SOD-1) gene. Over expression of these abnormal genes in mice causes a progressive clinical disorder that closely simulates central features of the human disease, making them useful as models to be used in research (Gurney et al., 1994).

Contemporary literature suggests that erythropoietin (Epo) is capable of counteracting a variety of neurodegenerative processes caused, for example, by excitotoxic injury, oxidative stress, hypoxia or inflammation (Brines and Cerami, 2005). As these include mechanisms implicated in the pathogenesis of ALS (Bruijn et al., 2004), we initiated a study to assess the potential of Epo to ameliorate the clinical manifestations of the disease in a relevant animal model.

The animals used for this purpose were transgenic mice expressing human cytosolic superoxide dismutase (SOD-1) with a mutation characteristic of a familial form of ALS in humans. The colony of well-characterized TgN (SOD1-G93A) Gur transgenic mice (Gurney et al., 1994) obtained from the Jackson Laboratory (USA) was bred in C57bl mice. At 1 month of age, offspring were genotyped by PCR analysis to confirm their transgenic status. The study was approved by the Tel-Aviv University ethical committee, and the mice were housed and handled in compliance with local and international regulations.

Four treatment groups were used. Group 1 ($n=6$) was composed of transgenic females treated with placebo. Group 2 ($n=11$) consisted of transgenic females treated with Epo. Group 3 ($n=8$) was formed of transgenic males treated with placebo.

* Corresponding author. Fax: +972 8 977 9714.
E-mail addresses: jonathangrunfeld@yahoo.com (J.F. Grunfeld), yaelba3@clalit.org.il (Y. Barhum), netrb@yahoo.com (N. Blondheim), mrabey@asaf.health.gov.il (J.-M. Rabey), emelamed@clalit.org.il (E. Melamed), emelamed@clalit.org.il (D. Offen).

0014-4886/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2006.11.002

Group 4 ($n=9$) contained transgenic males treated with Epo. Subsequently, two additional groups of females ($n=3$ each) treated with Epo and placebo, respectively, were evaluated histologically.

The treatment consisted of intraperitoneally injected albumin free erythropoietin beta (Recormon, Roche), which was administered twice weekly at 1000 U/kg body weight (8.3 μg/kg). Intraperitoneal injections of saline served as placebo in the control group. Treatment was started at the age of 47 days.

The treatment regimen was chosen with the intention of using a dose higher than the minimal 450 U/kg that had already demonstrated CNS effects after peripheral administration (Brines et al., 2000; Erbayraktar et al., 2003), but low enough to avoid persistent increase of hematocrit with chronic treatment, to levels liable of causing additional morbidity (Erbayraktar et al., 2003).

The effect of Epo on the motor function was assessed in terms of performance on a rotarod task. This was tested prior to treatment initiation and on the average every 2 weeks thereafter (range 9–15 days). Care was taken to avoid uneven distribution of the models into the groups according to their initial performance. The task consisted of three consecutive trials in which the mice were placed on the rod and challenged to keep from falling off as it rotated at 6 revolutions per minute (RPM). Each trial was continued up to a maximum of 2 min. The interval between trials was 30 s. This was followed by testing in an identical manner at 16 RPM. Additional data recorded include mice body weight, hematocrit level and survival.

For histological evaluation, spinal cords from the last two female groups ($n=3$ each) were extracted at the age of 80 days, aligned and sectioned transversely at corresponding thoracic levels. The average number of motor neurons per slice was calculated based on 10 hematoxylin-and-eosin-stained sections per mouse.

Statistical analysis of the results was performed on data regarding the duration of time the mice succeeded to stay on the rotating cylinder. For each mouse, the average times on the initial three 16 RPM trials were recorded as 100%, with subsequent averages represented as percentages of the initial results. These are presented as mean±standard errors of the mean (SEM); p values were calculated using the two-tailed unpaired Student t-test. In all tests, significance was assigned when $p<0.05$.

It was found that the performance of the Epo-treated females was significantly better than the placebo-treated controls 29 and 38 days after initiation of treatment (see Fig. 1). The performance of the two groups converged on the following test 53 days after initiation of treatment. The performance of both male treatment groups was indistinguishable throughout the experiment (see Fig. 1). Survival data were comparable in all groups, with females in the Epo-treated group surviving 131 days on the average (range 120–141), 142 days (131–153) in the control group and the male mice surviving 135 days in the Epo-treated group (122–141) and 137 days in the control group (129–149). Testing of a subgroup of mice after 4 weeks of treatment revealed a significant increase of hematocrit in the

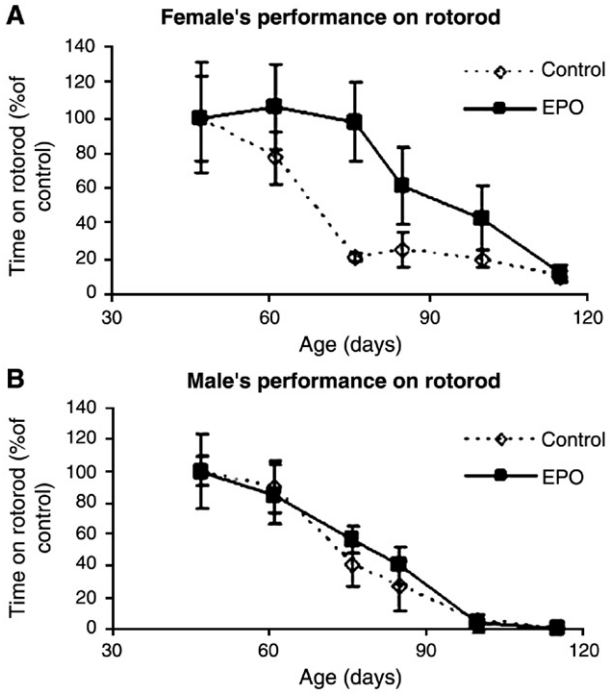

Fig. 1. Data are charted relative to the baseline performance on the rotarod at 16 RPM. (A) Females treated with erythropoietin continued to perform on the rotarod significantly better than controls until 53 days after initiation of treatment. Epo-treated group $n=11$; control $n=6$. (B) No impact of erythropoietin was observed on the performance of male models on the rotarod. Epo-treated group $n=9$; control $n=8$.

Epo-treated mice as compared to the controls with both males (control $n=3$, Epo $n=4$) and females (control $n=3$, Epo $n=4$) attaining similar levels (50.8% and 51.2%, respectively). No effect of treatment on body weight was observed.

The average number of motor neurons per section in thoracic spinal cord was 3.8±1.22 and 2.4±1.44 in Epo ($n=3$)- and placebo ($n=3$)-treated female mice, respectively. This represents a significant difference ($p=0.026$) between the two groups sacrificed at the age of 80 days, at the time corresponding differences were apparent in the larger group undergoing functional evaluation.

In summary, the following remarks may be made. The pathogenesis of neurodegeneration in ALS remains an enigma. Multiple mechanisms have been implicated by ongoing research, but their relative importance in the overall process remains to be determined. Among these may be found oxidative stress, excitotoxic injury, inflammatory response, mitochondrial disregulation and apoptosis (Bruijn et al., 2004). Altered activity of certain cytokines has also been proposed (Lambrechts et al., 2003).

Following the discovery of Epo in the hematopoietic system, it was identified in the central nervous system along with associated signal transduction systems (Brines and Cerami, 2005). Further investigations have produced an ever growing body of evidence in support of Epo's neuroprotective activity, which is effective in countering many of the processes thought to contribute to the pathogenesis of ALS (Brines and Cerami, 2005).

Thus, Epo has been demonstrated to initiate antiapoptotic signaling, to modulate nitric oxide (NO) production, to protect neural components from excitotoxic and oxidative radical species and to possess anti-inflammatory activity in the CNS (Brines and Cerami, 2005). Furthermore, both the necessary receptors for interaction with Epo, as well as it's production of cytoprotective effects, have been demonstrated in spinal motor neurons and adjacent elements in animal and human samples (Celik et al., 2002; Siren et al., 2001).

It should be noted that erythropoietin can enhance muscular performance, primarily with regard to endurance, independently of any neuroprotective activity (Jelkmann, 2003). This could provide an alternative or additional explanation of the better performance observed in the Epo-treated mice.

There are, however, considerations supportive of the claim that the delay of functional deterioration associated with the Epo treatment was primarily effected by it's neuroprotective activity. Notably, the motor deterioration in our model is caused by neurodegeneration rather than loss of stamina, which may be influenced by doping with Epo. The marked difference in the impact of Epo treatment in the actively treated male and female groups despite the similarity of their hematocrit levels also argues against the hematopoietic effect being a significant performance determining factor.

Our histological evaluation of spinal motor neuron count is insufficient to make a claim regarding the neuroprotective activity of Epo in our models due to the small size of the sample and lack of well-correlated data on performance but does justify entertaining this idea.

With these reservations, we propose that the current study supports an extension of the range of neuroprotective effects attributed to Epo to yet another model simulating a human neurodegenerative disorder. Consolidation of this hypothesis would mean that once again the systemic administration of Epo has demonstrated a significant biologic impact on the CNS, countering concerns regarding its obstruction by the blood brain barrier (BBB) (Brines and Cerami, 2005).

The differential response of males and females to Epo in our experiment is remarkable. Similar sex-related variation in the response of transgenic mutant SOD mice to other neuroprotective interventions has already been reported (Veldink et al., 2003), and epidemiologic data suggest an impact of gender on the frequency and course of ALS in humans as well (Haverkamp et al., 1995). It is possible that this represents a private case of a more general gender-related difference in the regulation of survival and death of neurons (Li et al., 2005) that has also been observed in the context of other spinal cord injuries (Farooque et al., 2006).

The translation of the neuroprotective effects of Epo to the clinical reality of ALS requires further investigation and depends primarily on the consolidation of these initial results, production of evidence in favor of efficacy in humans and lack of adverse effects.

Epo has already demonstrated clinical efficacy as a neuroprotective agent in the only study to evaluate this aspect of its activity so far, which tested its use in humans suffering from acute ischemic stroke (Ehrenreich et al., 2002). It is a widely available pharmaceutical product with an excellent safety profile based on extensive experience of use in human subjects (Sowade et al., 1998).

The apparent constraint on chronic treatment with Epo imposed by excessive erythropoiesis may have several solutions. For one, the frequency and dosage of treatment may be adjusted to minimize the rise of the hematocrit. Such an approach may be supported by identification of concomitant interventions designed to achieve more neuroprotection. Alternatively, phlebotomy is an effective intervention to remove excesses of RBC from the circulation should the need arise and could possibly contribute to the removal of iron from the system with theoretical therapeutic effects (Carri et al., 2003). In addition, novel neuroprotective Epo derivatives have been identified recently, which are devoid of erythropoietic activity (Brines and Cerami, 2005; Leist et al., 2004). These represent a source for eventual agents to provide chronic Epo-related neuroprotection.

Based on our preliminary findings, further studies probing alternative treatment schedules and combinations with additional neuroprotective interventions are warranted. These could lead to the development of therapeutic protocols with potential for clinical application.

References

Brines, M., Cerami, A., 2005. Emerging biological roles for erythropoietin in the nervous system. Nat. Rev., Neurosci. 6, 484–494.

Brines, M.L., Ghezzi, P., Keenan, S., Agnello, D., de Lanerolle, N.C., Cerami, C., Itri, L.M., Cerami, A., 2000. Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury. Proc. Natl. Acad. Sci. U. S. A. 97, 10526–10531.

Bruijn, L.I., Miller, T.M., Cleveland, D.W., 2004. Unraveling the mechanisms involved in motor neuron degeneration in ALS. Annu. Rev. Neurosci. 27, 723–749.

Carri, M.T., Ferri, A., Cozzolino, M., Calabrese, L., Rotilio, G., 2003. Neurodegeneration in amyotrophic lateral sclerosis: the role of oxidative stress and altered homeostasis of metals. Brain Res. Bull. 61, 365–374.

Celik, M., Gokmen, N., Erbayraktar, S., Akhisaroglu, M., Konakc, S., Ulukus, C., Genc, S., Genc, K., Sagiroglu, E., Cerami, A., Brines, M., 2002. Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. Proc. Natl. Acad. Sci. U. S. A. 99, 2258–2263.

del Aguila, M.A., Longstreth, W.T., McGuire, V., Koepsell, T.D., van Belle, G., 2003. Prognosis in amyotrophic lateral sclerosis: a population-based study. Neurology 60, 813–819.

Ehrenreich, H., Hasselblatt, M., Dembowski, C., Cepek, L., Lewczuk, P., Stiefel, M., Rustenbeck, H.H., Breiter, N., Jacob, S., Knerlich, F., Bohn, M., Poser, W., Ruther, E., Kochen, M., Gefeller, O., Gleiter, C., Wessel, T.C., De Ryck, M., Itri, L., Prange, H., Cerami, A., Brines, M., Siren, A.L., 2002. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol. Med. 8, 495–505.

Erbayraktar, S., Grasso, G., Sfacteria, A., Xie, Q.W., Coleman, T., Kreilgaard, M., Torup, L., Sager, T., Erbayraktar, Z., Gokmen, N., Yilmaz, O., Ghezzi, P., Villa, P., Fratelli, M., Casagrande, S., Leist, M., Helboe, L., Gerwein, J., Christensen, S., Geist, M.A., Pedersen, L.O., Cerami-Hand, C., Wuerth, J.P., Cerami, A., Brines, M., 2003. Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. Proc. Natl. Acad. Sci. U. S. A. 100, 6741–6746.

Farooque, M., Suo, Z., Arnold, P.M., Wulser, M.J., Chou, C.T., Vancura, R.W., Fowler, S., Festoff, B.W., 2006. Gender-related differences in recovery of locomotor function after spinal cord injury in mice. Spinal Cord 44, 182–187.

Gurney, M.E., Pu, H., Chiu, A.Y., Dal Canto, M.C., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y.W., Deng, H.X., et al., 1994. Motor

neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 264, 1772–1775.

Haverkamp, L.J., Appel, V., Appel, S.H., 1995. Natural history of amyotrophic lateral sclerosis in a database population. Validation of a scoring system and a model for survival prediction. Brain 118 (Pt 3), 707–719.

Incidence of ALS in Italy: evidence for a uniform frequency in Western countries, 2001. Neurology 56, 239–244.

Jelkmann, W., 2003. Erythropoietin. J. Endocrinol. Invest. 26, 832–837.

Lambrechts, D., Storkebaum, E., Morimoto, M., Del-Favero, J., Desmet, F., Marklund, S.L., Wyns, S., Thijs, V., Andersson, J., van Marion, I., Al-Chalabi, A., Bornes, S., Musson, R., Hansen, V., Beckman, L., Adolfsson, R., Pall, H.S., Prats, H., Vermeire, S., Rutgeerts, P., Katayama, S., Awata, T., Leigh, N., Lang-Lazdunski, L., Dewerchin, M., Shaw, C., Moons, L., Vlietinck, R., Morrison, K.E., Robberecht, W., Van Broeckhoven, C., Collen, D., Andersen, P.M., Carmeliet, P., 2003. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat. Genet. 34, 383–394.

Leist, M., Ghezzi, P., Grasso, G., Bianchi, R., Villa, P., Fratelli, M., Savino, C., Bianchi, M., Nielsen, J., Gerwien, J., Kallunki, P., Larsen, A.K., Helboe, L., Christensen, S., Pedersen, L.O., Nielsen, M., Torup, L., Sager, T., Sfacteria, A., Erbayraktar, S., Erbayraktar, Z., Gokmen, N., Yilmaz, O., Cerami-Hand, C., Xie, Q.W., Coleman, T., Cerami, A., Brines, M., 2004. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science 305, 239–242.

Li, H., Pin, S., Zeng, Z., Wang, M.M., Andreasson, K.A., McCullough, L.D., 2005. Sex differences in cell death. Ann. Neurol. 58, 317–321.

Sejvar, J.J., Holman, R.C., Bresee, J.S., Kochanek, K.D., Schonberger, L.B., 2005. Amyotrophic lateral sclerosis mortality in the United States, 1979–2001. Neuroepidemiology 25, 144–152.

Siren, A.L., Fratelli, M., Brines, M., Goemans, C., Casagrande, S., Lewczuk, P., Keenan, S., Gleiter, C., Pasquali, C., Capobianco, A., Mennini, T., Heumann, R., Cerami, A., Ehrenreich, H., Ghezzi, P., 2001. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc. Natl. Acad. Sci. U. S. A. 98, 4044–4049.

Sowade, B., Sowade, O., Mocks, J., Franke, W., Warnke, H., 1998. The safety of treatment with recombinant human erythropoietin in clinical use: a review of controlled studies. Int. J. Mol. Med. 1, 303–314.

Veldink, J.H., Bar, P.R., Joosten, E.A., Otten, M., Wokke, J.H., van den Berg, L.H., 2003. Sexual differences in onset of disease and response to exercise in a transgenic model of ALS. Neuromuscul. Disord. 13, 737–743.