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ORIGINAL PAPER

Systemic Gene Delivery Protects the Photoreceptors in the Retinal Degeneration Slow Mouse

Tim Sullivan · Kishore Kodali · Tonia S. Rex

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Abstract The retinal degeneration slow (*rds/rds*) mouse was used to test photoreceptor protection by systemic gene delivery of non-erythropoietic forms of erythropoietin (EPO). Two Epo mutants were generated and packaged into recombinant adeno-associated virus (rAAV) serotype 2/5, controls included rAAV2/5.Epo and rAAV2/5.enhanced green fluorescent protein (eGFP). Mice were injected in the quadriceps at postnatal day seven and analyses were performed at postnatal day 90. Hematocrit, serum EPO levels, and outer nuclear layer (ONL) thickness were quantified. Hematocrit and serum EPO levels in rAAV2/5.eGFP, rAAV2/5.Epo, and rAAV2/5.EpoR103E treated mice were: 46%, 8 mU/ml; 63%, 117 mU/ml; and 52%, 332 mU/ml, respectively. The ONL from *rds/rds* mice treated with the Epo vectors were approximately twice as thick as the negative controls. This demonstrates that the photoreceptors can be protected without performing an intraocular injection and without increasing the hematocrit to unsafe levels. Intramuscular delivery of rAAV.EpoR103E is an attractive treatment for retinal degenerative diseases.

Keywords Erythropoietin · Gene therapy · Retinal degeneration · Neuroprotection

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T. Sullivan · K. Kodali · T. S. Rex (✉)
Department of Ophthalmology, Hamilton Eye Institute,
University of Tennessee Health Science Center,
930 Madison Ave., Ste. 731, Memphis, TN 38163, USA
e-mail: trex@uthsc.edu

Introduction

Over 190 disease genes have been identified that can cause photoreceptor cell death, leading to blindness [1]. As a result of this complexity, generation of gene specific therapies for each form of retinal degenerative disease is a daunting task. An alternative strategy is to block photoreceptor cell death by a gene independent approach, such as treatment with neuroprotective factors. Gene delivery of erythropoietin (EPO) overcomes two major challenges associated with neuroprotective therapy. First, virus mediated gene delivery provides long term gene expression [2], overcoming the need for repeat delivery because of the short half-life of most neuroprotective agents. Second, EPO, unlike other neuroprotective proteins is able to cross the blood retina and blood brain barrier [3–5].

EPO is a secreted cytokine that is produced in the adult kidney and is upregulated under hypoxic conditions. Its classical role is to induce erythropoiesis by activating the EPO receptor homodimer. However, it is also neuroprotective in multiple in vitro and animal models (for review see, [6]). Clinical trials with EPO have been initiated for the treatment of optic neuritis, amyotrophic lateral sclerosis, spinal cord injury, and traumatic brain injury (www.ClinicalTrials.gov). The neuroprotective activity of EPO may be mediated by heterodimers between the EPO receptor and the interleukin beta common receptor and/or the glucocorticoid receptor [7–10].

In the retina, both the retinal ganglion cells and photoreceptors are protected by treatment with EPO. Local delivery of EPO protects the retinal ganglion cells from optic nerve crush induced cell death [11–14]. These cells are also protected from glaucomatous cell death by systemic treatment with EPO [15]. The photoreceptors of the retinal degeneration slow (*rds/rds*) mouse are protected by

subretinal injection of EPO [16]. And, systemic gene delivery of EPO protects the photoreceptors in both *rds/rds* mice and light damaged rats [17].

Our goal remains to treat retinal degenerative diseases without performing an intraocular injection that could disrupt an already fragile retina. Since most of these diseases are slowly progressing, long-term treatment will be needed. However, while continuous systemic delivery of EPO by somatic gene transfer does block photoreceptor cell death, it can also induce polycythemia [17]. Leist et al. 2004 first reported that two mutant forms of EPO (S100E and R103E) were unable to bind the EPO receptor homodimer, but were still neuroprotective in cell culture models of neuronal cell death [18]. We generated and packaged these mutant forms of EPO into recombinant adeno-associated virus (rAAV) and tested neuroprotection in an in vivo model of retinal degeneration. We chose the *rds/rds* mouse model because of its slow rate of retinal degeneration, and the fact that EPO is neuroprotective in this model. We found that the photoreceptors of these mice were protected by a single intramuscular injection of rAAV carrying one mutant form of Epo. This treatment did not induce polycythemia. Therefore, it is both safe and therapeutically beneficial.

Experimental Procedure

Generation and In Vitro Testing of pAAV2/5.CMV.S98E and pAAV2/5.CMV.R103E

The pAAV-TF.rhEpo2.3w (ARIAD Pharmaceuticals, Cambridge, MA) was digested with EcoR1 and Sac1 to release the rhesus Epo sequence that was then ligated into pBluescript11 KS + (Stratagene, La Jolla, CA). The QuickChange multi site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to manufacturer's instruction, using the following primers: CTCAGAAGCT GTCCTGGAGGGCCAGGCCG for R103E and GGGCC TGGCCCTGCTCGAAGAAGCTGTCC for S98E. The mutated Epo sequences were PCR amplified using F-primer GTCATATGCGGCCGCATGGGGGTGCACGAATG and R-primer GATCCAAGCTTTCATCTGTCCCCTCTC CTGCA and Extensor hi-fidelity PCR enzyme mix (Thermo Scientific, Waltham, MA) and cloned into the pAAV2 backbone (gift of Dr. James Wilson, University of Pennsylvania) downstream of the cytomegalovirus promoter (CMV), producing pAAV2.CMV.EpoR103E and pAAV2.CMV.EpoS98E. The mutations were confirmed by sequencing.

The Epo mutant plasmids (1 μg of each) were transfected to human epithelial kidney (HEK) 293 cells (ATCC, Manassas, VA) using PrimeFect1 DNA transfection reagent according to the manufacturers protocol (Cambrex, East Rutherford, NJ). To test the viral vectors, 70% confluent HEK 293 cells were transduced with each vector separately at a MOI of 10. Media from the respective transfections or transductions were collected 72 h later and used for ELISA or Western Blot analysis.

Generation of rAAV

The viral vectors were generated by triple transfection into HEK293 cells, purified by cesium chloride gradient, and titered by real-time quantitative PCR (University of Iowa Vector Core). The final titers were: 1.3×10^{14} vg/ml for rAAV2/5.CMV.EpoS98E; and 8.0×10^{13} vg/ml for rAAV2/5.EpoR103E. The viral vectors were diluted and dialyzed with 7000MWCO Slide-A-Lyzer mini dialysis units (Pierce, Rockford, IL) in lactated Ringers buffer (Baxter Health Care Corp, Deerfield, IL) just prior to use.

Intramuscular Injections

Retinal degeneration slow (*rds/rds*) mice were obtained from Jackson Laboratories (Bar Harbor, ME). A beveled 10 μl Hamilton syringe was used to deliver 1×10^{12} vg in 10 μl into the quadriceps of each mouse at postnatal day (PD) 5. Animal care guidelines as published by the Institute for Laboratory Animal Research were followed.

EPO Elisa

The Quantikine IVD Epo ELISA Kit was used according to manufacturer's protocol to detect EPO and EPO-R103E (R&D Systems, Minneapolis, MN). The absorbance at 450 nm with 600 nm reference was detected on a Bio-Tek—μQuant plate reader (Winooski, VT). In some mice the serum samples were pooled in order to obtain sufficient material for the ELISA.

Optical Coherence Tomography

Mice were anesthetized with ketamine/xylazine/urethane (25/10/1000 μg/g body weight). Eyes were dilated with 1% tropicamide. Imaging was performed using the Bioptigen ultra-high resolution imaging system (Bioptigen, Research Triangle Park, NC).

Histology

Eyes were preserved overnight in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, cryopreserved in 30% sucrose in phosphate buffered saline (PBS) overnight at 4°C, and embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC). Eyes were serially sectioned on a LEICA CM1800 cryostat

(Germany). Ten micron-thick sections were collected such that each slide contained ~20 sections representative of the entire eye. The sections were stained with hematoxylin and eosin and imaged on a Nikon Eclipse 80i microscope using a DXM1200C camera (Nikon, Japan). Outer nuclear layer (ONL) thickness was measured every 0.5 mm from the optic nerve head using NIS-Elements AR 3.0 Nikon software and measurements were analyzed using Prism 4.0 software (GraphPad, San Diego, CA). Two-way ANOVA was performed to determine the effects of treatment on ONL thickness, *P* value of 0.05 was considered significant.

Results

Generation of the Epo Mutants

We performed an amino acid sequence alignment of human, rhesus, and mouse EPO on two separate databases, ENSMBL and genbank. We identified the previously reported arginine at position 103 in all species [18]. However, at position 100, in all cases, there was an alanine at position 100 in contradiction to a previous report of a serine at this position that was converted to a glutamate to generate the non-erythropoietic form of the protein (Fig. 1, [18]). To further confirm this result, we sequenced our mouse and rhesus cDNA clones (data not shown). In both cases there was an alanine at position 100, confirming the results of the database searches. The closest conserved serine residue was at position 98 in all three species and both databases, so we mutated it to a glutamate to generate EPO-S98E.

Production and Detection of EPO Mutants

The plasmids were transfected in vitro to demonstrate production and detection of the EPO mutants from our constructs. The EPO-S98E was undetectable by ELISA, but was detected by Western Blot analysis (data not shown). High levels of EPO-R103E were detected in the media (631 mU/ml; Table 1). Controls included untransfected cells (0 mU/ml) and pAAV2/5.CMV.Epo transfected cells (651 mU/ml).

Intramuscular Gene Delivery Results in High Expression of EPO in the Serum at Postnatal day 90

We detected 8 (±10) mU/ml of EPO in *rds/rds* mice injected intramuscularly with rAAV2/5.CMV.eGFP. In contrast, 117 (±77) mU/ml and 332 (±192) mU/ml were detected in the serum of *rds/rds* mice injected with either rAAV2/5.CMV.Epo or rAAV2/5.CMV.EpoR103E, respectively (Table 2). Serum levels of EPO-S98E were not quantified since it was undetectable by ELISA.

Table 1 In vitro detection of EPO, EPO-S98E, and EPO-R103E in cell culture media after transfection into ARPE-19 cells

Transfection	EPO (mU/ml)
pAAV.CMV.Epo	651
pAAV.CMV.EpoR103E	631
pAAV.CMV.EpoS98E	0
Untransduced	0

Systemic Delivery of rAAV2/5.CMV.R103E does not Result in High Hematocrit Levels

To assess the ability of the EPO mutants to induce erythropoiesis, the hematocrit was measured at postnatal day 90 (Table 3). The hematocrit in the rAAV2/5.CMV.eGFP treated mice was in the normal range at 46%. In contrast, the hematocrit in the rAAV2/5.CMV.Epo and rAAV2/5.CMV.S98E treated mice was increased to 63, and 64%, respectively. The rAAV2/5.CMV.R103E treated mice had hematocrit levels in the normal range, 52%. To confirm that over-expression of a non-erythropoietic form of EPO does not suppress production of endogenous EPO, we performed real-time quantitative PCR of endogenous mouse EPO in the kidney. There was no difference in mouse Epo message levels in rAAV2/5.CMV.eGFP treated mice and rAAV2/5.CMV.EpoR103E treated mice (data not shown).

All Forms of EPO Tested Protect the Photoreceptors in the *rds/rds* Mouse

Optical coherence tomography was performed in treated and control mice at postnatal day 90 (Fig. 2). In wild-type mice, the ONL is ~52 μm thick (Fig. 2a). In contrast the retinas of age-matched rAAV2/5.CMV.eGFP treated *rds/rds* mice were 29 μm thick (Fig. 2b). Both rAAV2/5.CMV.Epo and rAAV.CMV.Epo-R103E treated mice had a thicker ONL, 42 and 38 μm (Fig. 2c, d).

Histological analysis confirmed the optical coherence tomography results, showing a thicker ONL in the retinas of rAAV2/5.CMV.Epo and rAAV2/5.CMV.Epo-R103E

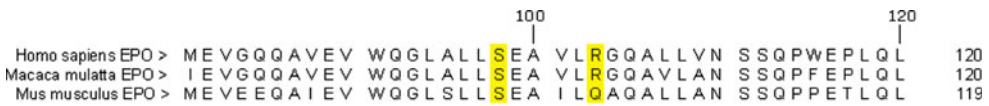

Fig. 1 Amino acid sequence alignment of mouse, rhesus, and human Epo. Sequences were obtained from NCBI and Ensembl. The relevant amino acids (S98 and R103) are highlighted

Table 2 Serum levels of EPO and EPO-R103E in transduced *rds/rds* mice

Treatment	*N*	EPO (mU/ml)
rAAV.2/5.CMV.eGFP	6	8 ± 10
rAAV2/5.CMV.Epo	4	117 ± 77
rAAV2/5.CMV.EpoR103E	4	332 ± 192

N number of mice; plus or minus the standard deviation

Table 3 Hematocrit levels in transduced *rds/rds* mice

Treatment	*N*	Hematocrit (%)
rAAV.2/5.CMV.eGFP	22	46
rAAV2/5.CMV.Epo	29	63
rAAV2/5.CMV.EpoS98E	15	64
rAAV2/5.CMV.EpoR103E	10	52

N number of mice

treated mice when compared to rAAV2/5.CMV.eGFP treated *rds/rds* mice (Fig. 2e–h). The outer segment defect of the *rds/rds* mouse was uncorrected by treatment with any vector (Fig. 2f–h). ONL thickness was measured at 0.5 mm intervals on either side of the optic nerve head (Fig. 3). A significant difference in ONL thickness was observed in the central portion of the retina. At 0.5 and 1.0 mm from the optic nerve head all treatment groups showed a minimum of 21 and 24% increase in ONL thickness ($P < 0.01$ and $P < 0.001$, respectively) when compared to the control group. At 1.5 mm rAAV2/5.CMV.Epo and rAAV2/5.CMV.S98E treatment resulted in a 23 and 30% increase in ONL thickness ($P < 0.01$ and $P < 0.001$, respectively). No significant difference was observed at the peripheral sections of the retina (2.0 mm) in any of the treatment groups.

Discussion

Two single amino acid EPO mutants were reported to be neuroprotective without inducing erythropoiesis [18]. One of the amino acids was a serine at position 100, however, we were unable to identify this amino acid despite searching two databases for the Epo sequence in three species, mouse, rhesus, and human, and sequencing Epo cDNA from mouse and rhesus. In all cases there was no serine residue at position 100. The closest serine in all

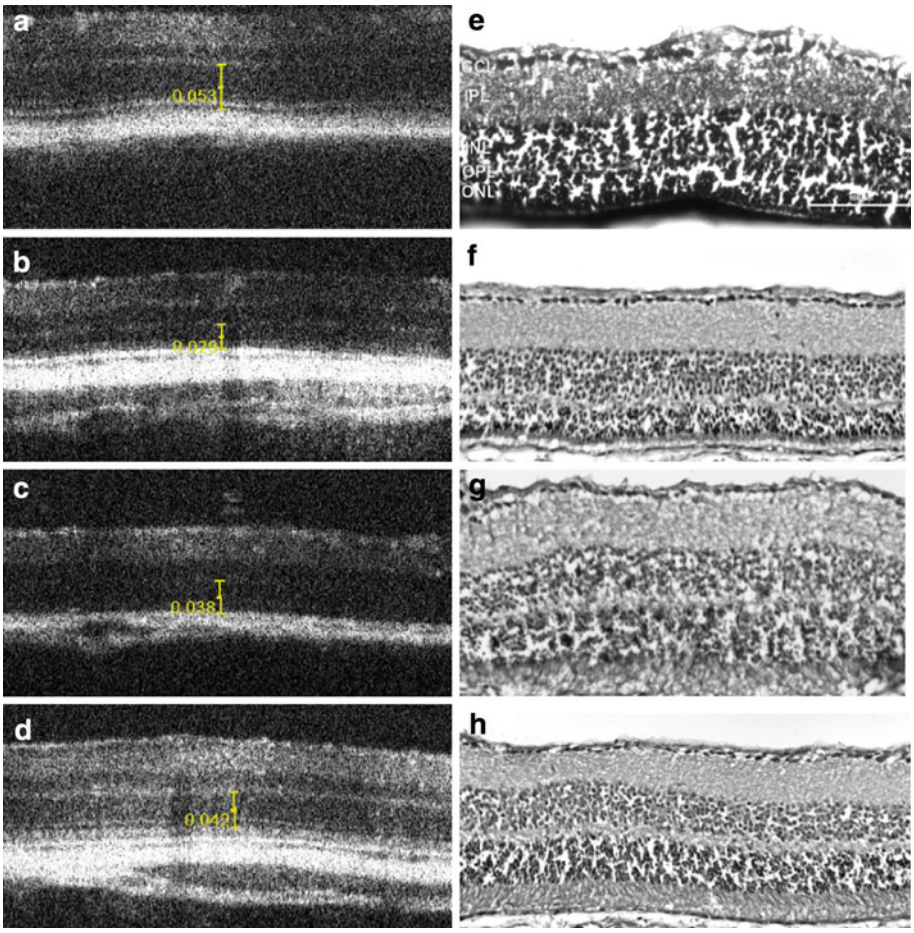

Fig. 2 Treatment with either rAAV2/5.CMV.Epo or rAAV2/5.CMV.Epo-R103E protects the photoreceptors from cell death. Optical coherence tomography (**a**–**d**) and histological cross sections (**e**–**h**) images of wild-type (**a**, **e**) and *rds/rds* (**b**–**d**, **f**–**h**) mice treated with rAAV2/5.CMV.eGFP (**b**, **f**), rAAV2/5.CMV.Epo (**c**, **g**), or rAAV2/5.CMV.EpoR103E (**d**, **h**). The calipers in **a**–**d** indicate the micron thickness of the ONL, 0.038, 0.020, 0.053, and 0.042, respectively. (*GCL* ganglion cell layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *OPL* outer plexiform layer, *ONL* outer nuclear layer)

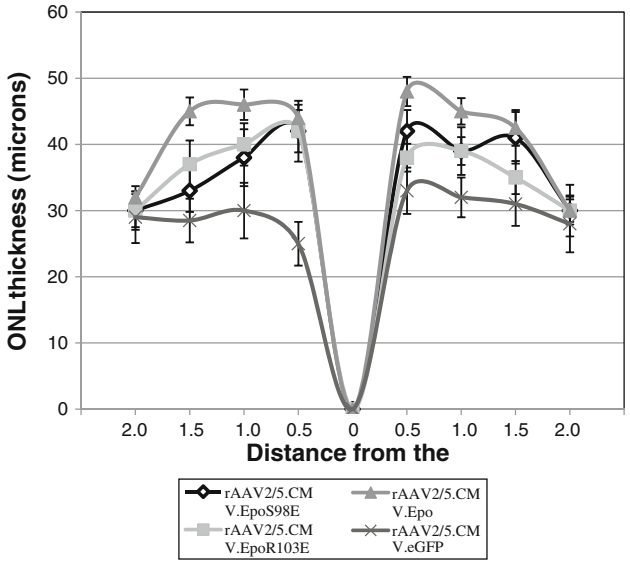

Fig. 3 Treatment with either rAAV2/5.CMV.Epo or rAAV2/5.CMV.Epo-R103E in *rds/rds* mice preserves ONL thickness at postnatal day 90. Measurements of the ONL from histological sections from each treatment group were recorded at 0.5, 1.0, 1.5, and 2 mm from the optic nerve head. The *error bars* represent S.E.M

cases was at position 98. However, conversion of this serine to a glutamate did not alter the hematopoietic characteristics from wild-type EPO. It remains unclear how the reported S100E mutant was generated.

In contrast, we were able to produce the other mutant, EPO-R103E. To provide long-term therapy, we packaged the mutant Epo into rAAV. Intramuscular delivery of the rAAV2/5.CMV.EpoR103E resulted in very high levels of EPO-R103E in the serum for the entire length of the study, 90 days. Despite the over-expression of EPO-R103E, hematocrit levels were not significantly altered. The photoreceptors were protected by rAAV-mediated systemic delivery of EPO-R103E in the *rds/rds* mouse. The level of protection appeared to be lower than was achieved by treatment with rAAV2/5.CMV.Epo. It is unclear why EPO-R103E was not as effective. One possibility is differences in the receptors and other signaling molecules that are activated by wild-type and mutant EPO. It is known that EPO-R103E does not bind to the EPO receptor homodimer efficiently [18]. Some have reported that the neuroprotective activity of EPO is enacted through an EPO receptor heterodimer or a different receptor altogether [7–10, 19]. This would explain why EPO-R103E is able to protect the photoreceptors without causing high levels of erythropoiesis. It is unknown how the mutation in EPO would affect the efficiency of binding to an EPO receptor heterodimer. However, the results of this study would indicate that EPO-R103E is less effective than the wild-type form of EPO at activating the neuroprotective receptor. Further studies would need to be performed to determine if this is the case.

The extent of neuroprotection may also be affected by the amount of EPO or EPO-R103E that enters the eye. Twice as much EPO-R103E as compared to EPO was detected in the serum after transduction of the quadriceps with the appropriate vector. This likely also translates into higher levels of EPO-R103E in the eye. High levels of EPO are not neuroprotective in the eye [16, 17]. So, it may be that the levels of EPO-R103E were at the upper end of the therapeutic range for EPO in the eye. The high variability in serum levels of EPO and EPO-R103E detected might also have caused variability in the level of neuroprotection if levels were above or below the therapeutic range in some animals. In future studies, we will perform a dose study to determine the therapeutic range for EPO-R103E. Finally, these results confirm our previous findings that the ability of EPO to protect the photoreceptors is independent of its effect on erythropoiesis [17]. In addition, this study demonstrates that intramuscular gene delivery of EPO-R103E is a safe and effective means of protecting photoreceptors from cell death, long-term, without performing an intraocular injection.

The maximal protection achieved by intramuscular injection of rAAV2/5.CMV.Epo or rAAV2/5.CMV.EpoR103E was preservation of seven rows of photoreceptors (as compared to 13 rows in wild-type retina) regardless of serotype tested (rAAV2/2; 17) or rAAV2/5 (this study). Both of these serotypes take $\sim$2 weeks to reach high levels of transgene expression. Since gene delivery was performed at PD 7, this means that peak transgene expression was reached at about the same time as peak cell death (PD 20; 17). Therefore, it is likely that the limiting factor to maximal neuroprotection is the lag time in transgene expression from the rAAV vector. In future studies we will test vectors with faster onsets of transgene expression, and earlier treatment. We will also test the efficacy of this therapy in other models of retinal degenerative disease.

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