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Non-Invasive Gene Transfer by Iontophoresis for Therapy of an Inherited Retinal Degeneration

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Abstract

Despite extensive research on many of the genes responsible for inherited retinal degenerations leading to blindness, no effective treatment is currently available for patients affected with these diseases. Among the therapeutic approaches tested on animal models of human retinal degeneration, gene therapy using different types of viral vectors as delivery agents has yielded promising results. We report here our results on a non-invasive, non-viral delivery approach using transscleral iontophoresis for transfer of plasmid DNA into mouse retina. Proof of principle experiments were carried out using plasmid containing GFP cDNA to demonstrate expression of the transferred gene in the retina after single applications of iontophoresis. Various parameters for multiple applications of iontophoresis were optimized to sustain GFP gene expression in mouse photoreceptors. Subsequently, repeated iontophoresis of plasmid containing normal β -phosphodiesterase (β -PDE) cDNA was performed in the *rd1* mouse, an animal model of autosomal recessive retinitis pigmentosa caused by a mutant β -PDE gene.

In normal mice, transscleral iontophoresis of the GFP plasmid provided a significant increase in fluorescence of the retina in the treated versus non-treated eyes. In *rd1* mice, repeated iontophoresis of β -PDE cDNA plasmid partially rescued photoreceptors morphologically, as observed by microscopy, and functionally, as recorded on ERG measurements, without adverse effects. Therefore, transscleral iontophoresis of plasmid DNA containing therapeutic genes may be an efficient, safe and non-invasive method for the treatment of retinal degenerations.

Keywords

gene transfer; gene therapy; iontophoresis; retina; retinitis pigmentosa; *rd1* mouse

INTRODUCTION

Retinitis pigmentosa (RP), a group of hereditary degenerative retinal diseases characterized by loss of photoreceptor function, is recognized as one of the most common causes of blindness in persons between the ages of 20 and 60 (Dryja and Berson, 1995). Over the past few years, more than 140 chromosomal loci have been mapped and more than 80 of the corresponding

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mutant genes have been identified for various forms of retinitis pigmentosa and inherited retinal degenerations (Hims et al., 2003). Currently, no alternative therapeutic procedure for the treatment of these diseases is available. Diverse therapeutic strategies, including diet supplementation, surgical transplantation of retinal pigment epithelium and photoreceptor cells, application of trophic growth factors, cell-based delivery of neurotrophic factors and ribozyme therapy have been attempted for these blinding disorders (Berson et al., 1993; Frasson et al., 1999; Kumar-Singh and Farber, 1998; LaVail et al., 2000). Gene therapy trials conducted in animal models of RP employed different types of viral vectors delivered by an intraocular injection into the subretinal space or vitreous cavity (Acland et al., 2001; Dejneka et al., 2003; Mashhour et al., 1994; Miyoshi et al., 1997; Takahashi et al., 1999). Most of these studies demonstrated successful morphological and functional rescue of photoreceptor cells. However, direct needle injections into the eye have the inherent risk of visually debilitating trauma, such as retinal detachment and intraocular infection or hemorrhage. In addition, the issue of safety of viral vectors remains challenging for the treatment of human patients affected with ocular disorders (Dejneka et al., 2003).

The goal of the current study was to investigate a safe, efficient, non-invasive and non-viral approach for delivering appropriate genetic material to the retina that could be used for the treatment of retinal degenerations. To avoid injections of viral vectors, we investigated whether we could transfer 'naked' plasmid DNA carrying gene therapy material into the mouse retina using transscleral iontophoresis. Iontophoresis - the enhanced permeation of ionized molecules across biological membranes under the influence of an electrical field - had been safely applied for administration of small molecules to several tissues, including the eye (Sarraf and Lee, 1994; Guy, 1995; Hirvonen et al., 1996; Behar-Cohen et al., 2002; Voigt et al., 2002; Parkinson et al., 2003; Davies et al., 2003; Eljarrat-Binstock and Domb, 2006; Hsu, 2007; Andrieu-Soler et al., 2007). In this paper, we show that transscleral iontophoresis can be successfully used to deliver therapeutic genes to retinal photoreceptor cells.

MATERIALS and METHODS

Plasmid Constructs

Three plasmids, pCMV-GFP, pPDE-GFP and pPDE-PDE, were constructed for iontophoretic gene transfer. The pCMV-GFP plasmid contained the cytomegalovirus (CMV) promoter and the coding region of enhanced green fluorescence protein (GFP). It was obtained by deleting the IRES region of pIRES-EGFP (Clontech, Palo Alto, CA) yielding a 4.7 kb plasmid.

The second plasmid, pPDE-GFP, included the promoter region (bp -93 to +53) of the human β -PDE gene (*PDE6B*) and the coding region of enhanced GFP. The promoter was obtained by PCR using p β PDE as template (Lerner et al., 2002) and primers ATAAGAATGCGGCCGAGCGCAGGCCCCCCATTTGTAGG and GCTCTAGAGGTGGCTGCCTGTCCCTGGAGA. The PCR product was digested with Not I and Xba I and subcloned into pBlueScript II SK yielding plasmid pHBP. Enhanced GFP and the SV40 early polyA signal were cut out from pIRES2-EGFP (Clontech, Palo Alto, CA) with BamH I and Xho I, and ligated into pHBP yielding pPDE-GFP.

For constructing pPDE-PDE, the mouse β -PDE cDNA and the SV40 early polyA signal were released from pCISB7 (Piriev et al., 1993) by digestion with Xba I and BamH I and subcloned into pHBP yielding pPDE-PDE. Thus, *Pde6b* expression was driven by human *PDE6B* promoter.

All three plasmids were diluted in a balanced salt solution (BSS, Alcon Laboratory, Ft. Worth, TX) and kept at 4°C.

Iontophoresis

Normal C57BL/6J pups and adult mice (2 to 6-months of age), and *rd1* pups were used in this study. The Animal Ethics Committee approved all animal procedures. Before iontophoresis, adult mice were anesthetized with an intraperitoneal injection of 15 mg/kg of ketamine and 7 mg/kg of xylazine in saline. Cold anesthesia was used for pups 8 and 13 days of age. In addition, two drops of 0.5% proparacaine hydrochloride (Alcon Laboratory) were applied to the cornea. Since the eyes of 8-day-old pups were not yet opened, a slit was made on the eyelid to allow the placement of the iontophoresis applicator.

The iontophoresis procedure was performed with the aid of a Coulomb-controlled device (Eyegate; Optis, Paris, France). The positive electrode was placed on the back of the mouse and the negative electrode was inserted into the applicator that was filled with plasmid solution maintained at 4°C (Fig 1). The applicator was constructed so that one of its ends opened wide enough for the plasmid solution to bathe the cornea, the limbus and the adjacent sclera. The cross section area of the applicator was approximately 3 mm for adult mice and smaller for pups. The plasmid concentration was maintained by creating a flow of the solution in the reservoir connected with the applicator during iontophoresis and 2 minutes after turning off the instrument. The current was switched on and off at the 8th minute of iontophoresis in order to simulate a brief electroporation.

Various iontophoretic parameters were tested; a total of 148 eyes were used to optimize conditions. For the experiments carried out to evaluate the feasibility of transscleral iontophoretic delivery of plasmid into the retina and the efficiency of *PDE6B* promoter in driving genes into the photoreceptors, single iontophoresis was carried out in normal adult mice at 600 μ A for 15 minutes with a plasmid concentration of 1000 μ g/ml (64 eyes). Single iontophoresis with different parameters (300 μ A, 15 min) was also performed in normal adult eyes with pCMV-GFP at 200 μ g/ml (28 eyes). These new parameters were used in a paradigm designed to yield a steady level of protein expression by repeating the application of iontophoresis every 6 days, 4 times, to the same eyes of normal adult mice (6 eyes). For analysis of kinetics, the iontophoresis procedure was performed on 6 eyes of normal adult mice.

Before conducting the gene therapy studies, preliminary experiments were carried out to optimize the iontophoretic parameters on very young animals. Various current intensities and duration of iontophoresis were tested on normal (10 eyes) and *rd1* (8 eyes) 8 to 17 days of age. A protocol of repetitive iontophoresis for the rescue of the photoreceptor cells in *rd1* pups was finally established: 3 repetitive iontophoretic applications of pPDE-PDE (1000 μ g/ml) at ages 8 days (200 μ A, 5 min), 13 days (300 μ A, 5 min) and 17 days (400 μ A, 10 min). The pups underwent eyelid opening at day 8.

Histology

The mouse eyes were enucleated after euthanasia, fixed in 4% paraformaldehyde, frozen on dry ice and sectioned at 10 or 30 μ m in a cryostat. We took special care in embedding the tissues in the same orientation. We compared separately sections from the peripheral retina and the posterior pole since the thickness of the different retinal layer varies across the retina. The 10 μ m sections were used for general histological examination and immunostaining, whereas the thicker sections were used for confocal microscopic analysis of green fluorescence. These sections were collected and allowed to adhere onto Superfrost Plus glass slides (Fisher Scientific, St Louis, MO). Sections were stained with toluidine blue or ethidium bromide to evaluate the general morphology of retinas and the number of rows of nuclei in the outer nuclear layer.

Quantification of Green Fluorescence

Confocal microscopic evaluation of green fluorescence was carried out for pCMV-GFP (92 eyes) and pPDE-GFP (8 eyes) treated and 24 non-treated control eyes. To avoid bleaching and to standardize the scanning conditions, single confocal optical sections of the periphery and of the posterior pole of the retina were acquired per frozen section under identical settings using a Zeiss LSM 110 confocal microscope. Confocal images were collected under the excitation of blue (488 nm) line from an argon laser for the detection of green fluorescence. The photoreceptor outer segments (OS) in each optical section were selected by the Magic Wand Tool of Adobe PhotoShop, and the level of fluorescence was measured as previously described (Reid et al., 2003). In brief, the averaged fluorescence intensity per pixel was calculated on a scale of 0 to 255 using the Luminosity channel of the Histogram function in Adobe PhotoShop. The fluorescence level in the adjacent blank area was also measured for the evaluation of background fluorescence. Pixels that were saturated at 255 were less than 5%. All results are expressed as percent of averaged green fluorescence level of control, non-treated eyes.

Thickness measurement

Cross sections (30 μ m) from 2 eyes of *rd1* mice treated with pPDE-GFP, 8 eyes of *rd1* mice treated with pPDE-PDE and 3 eyes of normal mice treated with pPDE-GFP were cut perpendicular to the photoreceptor-nerve fiber layer axis of the retina. Images of randomly chosen retinal sections were captured with a digital camera mounted on a Zeiss microscope through a 40X objective lens. For each eye, 2 images of the posterior retina and 2 images of the peripheral retina were used for ONL thickness measurements. These measurements were made at three different locations on each image.

Immunostaining

Frozen sections were treated with 2% fish gelatin and 0.5% Triton X-100 and were then incubated overnight at 4°C with a 1/100 dilution of a rabbit polyclonal antibody against GFP (Living Colors™ A.v. peptide antibody, Clontech Laboratories, Inc., Palo Alto, CA). After washing with PBS, sections were reacted with a biotin-conjugated goat anti-rabbit antibody (1/200; Jackson Immuno Laboratories, West Grove, PA) for 2 hours at room temperature, and treated with Vectastain ABC reagent (Vectastain ABC elite standard kit; Vector Laboratories, Burlingame, CA). 3', 3'-diaminobenzidine (1 mg/ml) was used as chromogen in the final visualization step. Both antibodies were diluted in 2% fish gelatin/0.1% Triton X-100 and other reaction solutions were made in 10 mM PBS.

Immunofluorescent staining for the detection of rhodopsin was carried out by first blocking the non-specific background with a 5% normal rabbit serum/0.5% Triton X-100 solution. Sections were then incubated with a rabbit polyclonal antibody against rhodopsin³³ (1/200) overnight at 4°C. The protein-antibody complex was finally detected with a fluorescein-isothiocyanate (FITC)-conjugated donkey anti-rabbit secondary antibody (1/200; Jackson Immuno Labs). The antibodies were diluted in 1% normal serum/0.1% Triton X-100 (normal rabbit serum for the rabbit antibody and normal donkey serum for the donkey antibody).

Western blot analysis

The remaining retina from 5 blocks that had been sectioned to examine GFP fluorescence was used to carry out Westerns. Briefly, the extra OCT surrounding the tissue was trimmed on dry ice and protein was extracted using the procedure of Ikeda et al., 1998, incubating in 200 μ l RIPA buffer (1.0 M sodium phosphate buffer, pH 7.6, 154 mM NaCl, 1% Triton X-100, 12 mM sodium deoxycholate, 0.2 % sodium azide, 2 mM phenylmethylsulfonyl fluoride, 50 mg/ml aprotinin) for 20 min at 100 °C, followed by 2 hrs at 60 °C. The sample was then homogenized and centrifuged for 5 min at 1,000 $\times$ g. Protein concentration in the supernatants

from wild-type, untreated *rd1* mouse and β -PDE-treated *rd1* mouse retinas was determined, and equal amounts of protein were loaded in each lane for Western blot analysis. Proteins were separated on an inverted 9.0 % to 6.5 %/1.5 % cross-linked polyacrylamide gel (modified from Zardoya et al; 34], using a tris/tricine buffer system (Piriev et al., 2003; Schagger and von Jagow, 1987) for 20 hr at 22 mA constant current; they were then transferred to a PVDF membrane. The transblotted proteins were incubated with a 1:1000 dilution of polyclonal antibody against PDE α and β subunits (MOE, a generous gift from Dr. Bernard Fung) and visualized using alkaline phosphatase coupled to anti-rabbit secondary antibody and the ECL chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Electroretinograms

Electroretinographic measurements (ERGs) were recorded using previously described methods (Nusinowitz et al., 2001). Our protocol was designed to maximize the contribution from the rod-mediated system (eg. dark-adapted, relatively dim short-wavelength stimuli). After overnight dark adaptation, mice were anesthetized with intraperitoneal injection of 15 mg/kg ketamine and 7 mg/kg xylazine in saline. ERGs were recorded from the corneal surface of both eyes after pupil dilation (1% atropine sulfate) using a gold loop corneal electrode together with a mouth reference and tail ground electrode. Stimuli were produced with a Grass Photoc Stimulator (PS33 Plus, Grass Instruments, Quincy, MA) affixed to the outside of a highly reflective Ganzfeld dome. The mouse eye was positioned orthogonal to an opening cut into the wall of the dome. Response signals were amplified (CP511 AC amplifier, Grass Instruments), digitized (PCI-1200, National Instruments, Austin, TX) and computer-averaged with at least 50 to 100 responses recorded to the weakest stimuli. Rod-mediated ERGs were recorded to short-wavelength (Wratten 47A; $\lambda_{\text{max}} = 470$ nm) flashes of light over a broad range of flash intensities where responses were clearly recordable.

Statistical Analyses

ANOVA was used to analyze the relative level of green fluorescence and Tukey HSD multiple comparison was used in the post hoc analyses. T-tests with pooled variances were also used in individual comparisons.

RESULTS

GFP gene transfer into the retina using iontophoresis

Initially, we evaluated whether a 4.7 kb plasmid, pCMV-GFP, containing the coding region of enhanced green fluorescent protein (GFP) driven by the cytomegalovirus (CMV) promoter could be transferred into the retina of normal adult mice. After a single application of transscleral iontophoresis (1000 $\mu\text{g/ml}$ plasmid, 600 μA , 15 minutes; Fig 1), green fluorescence was detected by confocal microscopy all over the retina (Fig. 2a, left panel). The intensity of green fluorescence in the retina reflected the level of GFP expression, with the highest level observed in the inner (IS) and outer segment of the photoreceptors (OS), followed by that in the outer and inner plexiform layers. Quantitative analyses showed a significant increase in fluorescence from the retina in the treated versus non treated eyes (ANOVA, $F_{[1, 715]} = 110.089$, $P < 0.001$). The level of fluorescence was more intense in the periphery than in the posterior pole of the retina (ANOVA, $F_{[1, 704]} = 67.578$, $P < 0.001$), corresponding with the location of the iontophoresis applicator (covering cornea and including the anterior part of the sclera) and with the anterior to posterior direction of the current (Fig 2b). GFP expression was subsequently confirmed by immunostaining with an anti-GFP antibody (Fig. 2a, right panel).

Kinetics of GFP expression after iontophoresis

In order to determine the time-course of GFP expression, the fluorescence in the OS was quantified over a 4-week period after a single iontophoresis application. GFP expression in the treated eyes was maximal at day 6 post-iontophoresis and decreased by day 9 post-iontophoresis ($P < 0.001$; Fig. 2c). At day 14 and day 28 post-iontophoresis, the fluorescence level was no longer statistically different in treated and control eyes (Fig. 2c). The decrease in fluorescence after day 6 post-iontophoresis suggested the need for repeated iontophoretic applications to sustain the level of GFP in photoreceptors (half-life, 2.1 days). Therefore, iontophoresis was carried out on the same eyes every 6 days, for 4 consecutive applications, under optimized conditions, using lower plasmid concentration (200 $\mu\text{g/ml}$) and current (300 μA). One single iontophoresis application using these new parameters yielded a similar level of GFP expression in the OS as using 1000 $\mu\text{g/ml}$ of plasmid at 600 μA ($t_{[70]} = 0.516$, $P = 0.608$). The level of gene expression 3 days after the fourth iontophoretic application of plasmid DNA was comparable to that observed 3 days after a single iontophoretic treatment ($t_{[30]} = 1.417$, $P = 0.167$) (Fig. 2d). This level was similar to that of day 6 ($t_{[57]} = 0.012$, $P = 0.991$) but higher than that at day 9 ($t_{[39]} = 2.874$, $P < 0.01$) after the single application of 1000 $\mu\text{g/ml}$ of plasmid at 600 μA . No corneal lesions, lens opacities, retinal damage or scleral burns were observed even after 4 repeated applications. These results indicate that the multiple application paradigm could be safely used to sustain a steady level of gene expression under the optimized conditions.

β -PDE gene promoter directs GFP gene transfer into mouse photoreceptors

In humans, mutations in the cGMP- β -phosphodiesterase subunit β -PDE gene lead to autosomal recessive RP (Danciger et al., 1995; McLaughlin et al., 1993). We wanted to investigate whether the iontophoretic transfer of the normal β -PDE gene to the photoreceptors could be a potential strategy to treat this hereditary disorder. We had previously shown that the β -PDE promoter directs expression to the photoreceptors, and that multiple elements in this promoter regulate this expression (Lerner, 2001, 2002; Ogueta et al., 2000;). To examine if a plasmid containing the human β -PDE promoter and the coding region of enhanced GFP cDNA (pPDE-GFP), transferred by iontophoresis, could direct efficient and specific gene expression to mouse photoreceptors, a single transscleral procedure (600 μA , 15 minutes, 1000 $\mu\text{g/ml}$ plasmid) was performed on 6 eyes of adult mice. Confocal microscopy detected green fluorescence in the plexiform and photoreceptor layers of retinas of plasmid-treated eyes, most prominent in the photoreceptor OS (Fig. 3a). Quantitative analyses showed a significantly higher level of green fluorescence in the OS of treated eyes than in control eyes (190%; Fig. 3b). More GFP gene expression in the OS of the photoreceptors was observed when the plasmid containing the GFP gene under the β -PDE promoter was iontophoresed into the mouse eye (Fig 3b) than when the similar plasmid with the CMV promoter was used (Fig 2a). This comparison was statistically significant ($t_{[72]} = 2.08$, $p < 0.05$). The localization of the β -PDE promoter-driven GFP to OS is an interesting phenomenon that is difficult to explain based on our existing knowledge. GFP expression from the rhodopsin kinase (GRK1) promoter was also shown to be localized to photoreceptor OS (Young et al., 2005, Mol Vision).

Iontophoresis of pPDE-GFP into the retina of *rd1* mouse

The *rd1* mouse, an animal model of autosomal recessive retinal degeneration caused by a mutation of the β -PDE gene (Bowes et al., 1990), has been widely used for testing different gene therapy approaches. In the *rd1* mouse, rapid degeneration of photoreceptors is observed from postnatal day 8 and no rod photoreceptors are detectable by postnatal day 20 (Farber et al., 19994). Therefore, any treatment for *rd1* mice would need to be started at the latest by 8 days of age. We verified if the previously used conditions for application of iontophoresis in adult mouse eyes were safe for the small eyes of young pups. For this, we performed repetitive applications at 8, 13 and 17 days of age subjecting normal (C57BL/6J) pups to transscleral

iontophoresis (pPDE-GFP plasmid at 1000 $\mu\text{g/ml}$). All mice were sacrificed at day 23. When 600 μA current was used for 10 minutes on the small eyes of pups, some lesions were observed, including burns on the cornea and sclera, and corneal vascularization. Therefore, lower current intensities of shorter duration were used for 8 eyes of normal pups: 200 μA for 5 min at postnatal day 8, 300 μA for 5 min at postnatal day 13, and 400 μA for 10 min at postnatal day 17. Under these optimized conditions, no signs of inflammation, necrosis or trauma were observed by clinical examination, no cellular infiltrates in the retina that would suggest an ongoing inflammatory response were seen in the histopathological evaluations, and no electroretinographic (ERG) defects were detected.

In order to investigate whether iontophoresis per-se would have any positive effect slowing the retinal degeneration of *rd1* mice, we conducted the same experiment on 8 eyes of *rd1* mice: iontophoresis at 8, 13 and 17 days of age with DNA that is biologically irrelevant for treatment (pPDE-GFP plasmid). No morphological or ERG improvements were observed in these negative control mice. Retinas of these *rd1* mice did not show GFP expression in the OS because all photoreceptors had degenerated. The remaining outer nuclear layer (ONL) of these *rd1* mice was only $4.7 \pm 0.1 \mu\text{m}$ thick at the posterior retina and $4.4 \pm 0.3 \mu\text{m}$ thick at the peripheral retina, compared to 54.7 ± 2.6 and $40.9 \pm 2.6 \mu\text{m}$ at the respective locations of the normal mouse retinas treated with the same plasmid (Fig. 4a). Thus, iontophoresis of the pPDE-GFP plasmid does not prevent the *rd1* retinal degeneration.

Iontophoresis of pPDE-PDE into the *rd1* mouse retina

A plasmid containing the coding region of mouse β -PDE driven by the human β -PDE promoter (pPDE-PDE) was transferred by 3 consecutive iontophoretic applications into 8 eyes of *rd1* pups at 8, 13 and 17 days of age. The ONL thickness is considered a reliable index for comparing the number of photoreceptors between normal and degenerative retinas that suffer from environmental or genetic insults. Morphological examination of retinas of *rd1* mice treated with 3 consecutive iontophoretic applications of pPDE-PDE showed substantial rescue of the photoreceptors. In these mice, the ONL was thicker by $9.7 \pm 0.5 \mu\text{m}$ (not statistically significant) in the posterior retina and by $18.7 \pm 0.6 \mu\text{m}$ ($p < 0.001$) at the peripheral retina than in *rd1* mice treated with the control plasmid pPDE-GFP (Fig. 4a). Up to 6 rows of photoreceptor nuclei were seen at the periphery of the retina (Fig. 4b, middle panel), in the vicinity of the iontophoresis application, that decreased in number forming a gradient towards the posterior pole of the eye. This corresponds to 46% of the photoreceptor nuclear layer of the normal mouse retina that has 8–10 rows of photoreceptor nuclei at this location (Fig. 4b, right panel). Many of these photoreceptors had inner and outer segments. In contrast, non-treated *rd1* mice at age 23 days showed a retina totally devoid of rod photoreceptors (Fig. 4b, left panel). It is well known that the rod photoreceptor specific protein rhodopsin, the most abundant protein in the photoreceptor OS of normal mice, is absent in *rd1* mice at day 23²⁴. Detection of rhodopsin by immunostaining corroborated some degree of rescue of visual cells in the treated *rd1* eyes (Fig. 4c). Furthermore, detection of the α and β subunits of cGMP-PDE on a Western blot containing proteins from *rd1* retinas after iontophoretic treatment (lane +, Fig. 4d) constitutes proof of scientific principle about the effective delivery of β -PDE to the photoreceptor cells. Most important, ERGs at day 23 were recordable, showing that the rescued rod photoreceptors were able to function producing visual responses in the treated *rd1* mice (Fig. 5). The recorded ERG responses were limited, in accordance with the partial morphological rescue of photoreceptors. Our protocol was designed to maximize the contribution from the rod-mediated system (e.g., we dark-adapted the mice overnight and used relatively dim short-wavelength stimuli). On the recorded ERGs, the implicit time for the peak of the *b*-wave decreased as stimulus intensity increased (Fig. 5). This is characteristic of rod ERG responses. To further evaluate a possible interfering cone-mediated function, we presented our brightest blue flashes on a rod suppressing background (30 cd/m^2). There was

no recordable cone response. We also tested these mice with stimuli that were white with a retinal illuminance approximately 1.0 log unit higher than the blue flashes; again no cone signal was detectable. From this pattern of responses we can infer that the ERG signals that we recorded were rod-mediated. In contrast, non-treated *rd1* animals at this age always showed complete absence of electrophysiological retinal activity (Fig. 5). The electrical response of the rescued photoreceptors was however lower than that of photoreceptors of normal mice (Nusinowitz et al., 2001). Therefore, iontophoretic delivery of the β -PDE gene under the conditions used in the present study partially rescues photoreceptors both structurally and functionally in *rd1* mice.

DISCUSSION

We have investigated a gene therapy approach without viral vectors or invasive intraocular injections and achieved encouraging partial rescue of photoreceptors in the *rd1* mice retinas. We showed that repeated applications of transscleral iontophoresis using genetic material cause no adverse effects on ocular tissues once the iontophoresis conditions have been optimized. In addition, transscleral iontophoresis of non-genetic material such as antibiotics using very low electrical current and adapted electrodes has been previously shown to be safe and very well tolerated in animal models and humans (Asahara et al., 2001; Behar-Cohen, 1997, 2002; Halhal et al., 2003; Parkinson et al., 2003). In the current approach we used non-viral plasmid DNA that does not integrate into the host genome and has no reactivation potential. Nevertheless, potential hazards linked to long term or to numerous repeated applications of iontophoresis cannot totally be ruled out.

One of the advantages of the current method is that with a multiple treatment regimen the level of gene expression can be regulated so that the duration of expression of the transferred gene after one single iontophoresis procedure is not a limiting factor for the structural and functional rescue of photoreceptors.

For iontophoresis of plasmid β -PDE to prevent the degeneration of photoreceptors that occurs in *rd* retina as a consequence of lack of the β -PDE protein, β -PDE has to be expressed at the outer segments where it is needed for its function. Rescue of the photoreceptors is not possible without the delivery of the β -PDE cDNA to these cells. We had effectively shown this with the generation of transgenic mice that integrated into their genome the β -PDE transgene (Lem et al., 1992) and when we partially rescued the photoreceptors of *rd1* mice using modified adenoviruses to deliver the β -PDE cDNA (Kumar-Singh and Farber, 1998). We performed iontophoresis of irrelevant DNA (pPDE-GFP) using exactly the same conditions, concentration of DNA, and animal ages as with pPDE-PDE, and demonstrated that it did not have any effect on the course of retinal degeneration in *rd* mice. Additional proof that β -PDE reached the photoreceptors and prevented their degeneration was obtained with the Western blot that showed the presence of both α and β subunits of cGMP-PDE in the sections of retinas subjected to pPDE-PDE iontophoretic treatment. These subunits were not observed in sections of 23-day-old *rd1* mouse retinas that had no photoreceptors because they did not receive the pPDE-PDE plasmid.

In this study, the ocular route of gene transfer to the retina by iontophoresis is likely to be transscleral rather than anterior to posterior through the cornea. Several lines of evidence support this hypothesis. Diffusion of high molecular weight compounds through the sclera has been previously demonstrated (Ambati et al., 2000). In our experiments, after iontophoresis-assisted transfer of plasmid DNA to the retina we observed the brightest GFP fluorescence and highest photoreceptor rescue in the periphery, in the region of the retina covered by the applicator. GFP fluorescence as well as photoreceptor rescue progressively decreased away from this area, which indicates that DNA was preferentially transferred directly through the

sclera. Furthermore, plasmid DNA has been shown to be unstable in the vitreous, with a very short half-life (Pitkanen et al., 2003). However, we observed some evidence of gene transfer to the posterior retina away from the applicator, suggesting that the therapeutic DNA material may have also diffused in the vitreous cavity and then reached all areas of the retina. On the whole, it is likely that the prevention of photoreceptor degeneration observed in our study is the result of predominantly transscleral delivery of pPDE-PDE.

In summary, gene transfer to the retina using transscleral iontophoresis and non-viral DNA is a non-invasive and safe procedure that can be performed repetitively without any adverse effects. Since the many parameters applied in these studies were optimized on an empirical basis, different combinations of them should be tried to adapt this procedure to other animal models and to increase the rescue of photoreceptors in the posterior pole of the retina. From the encouraging results described in this paper, we can infer that transscleral iontophoresis has the potential of becoming a method of choice for the treatment of a variety of inherited retinal disorders.

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Figure 1.

Iontophoresis set-up. Bottom left: a photograph of the localization of the applicator on the eye of the mouse. A negative electrode is in the applicator. The positive electrode is placed on the back of the animal. The applicator is filled with the plasmid solution (in grey) supplied from a reservoir. The solution is continuously renewed with a constant flow. The current is controlled by the iontophoresis device.

Figure 2.

Expression of GFP after iontophoresis of a GFP-encoding plasmid, pCMV-GFP. Iontophoresis (600 μ A, 15 minutes) of pCMV-GFP (1000 μ g/ml) was performed on the eyes of adult mice. (a) GFP green fluorescence (left panels) and immunohistochemistry (right panels). On the posterior pole of mice, higher level of fluorescence is observed by confocal microscopy in the plasmid-treated (GFP) than in non-treated eyes (Control). Retinal layers are seen under bright field (middle panels). Immunohistochemistry using anti-GFP antibodies corroborates the localization of expressed GFP in treated but not in control retinas. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL/NFL, ganglion cell and nerve fiber layers. (b) Low magnification of transduced and untransduced retinas. On the top panel, GFP fluorescence in

photoreceptor OS decreases from the periphery (intense fluorescence on the left side) toward the posterior pole (on the right side) of the retina. On the bottom panel, non-treated retina (control) express weak homogeneous autofluorescence **(c)** Time course of gene expression after iontophoresis. Results are expressed as percent of averaged green fluorescence of non-treated eyes (first column, 0). Total number of optical sections analyzed is indicated inside the bars (6 eyes in each group). P values are from comparisons between treated and non-treated eyes. ANOVA ($F_{[5,231]}=20.903$, $P<0.001$) and Tukey HSD multiple comparisons show significantly higher gene expression in treated than control eyes at days 3, 6, and 9 post-iontophoresis, but not at days 14 and 28 ($P=0.096$ and $P=0.430$, respectively). **(d)** Repetitive iontophoresis (4 applications) sustained the level of GFP expression in the photoreceptors. Three days after the last application of pCMV-GFP (200 μ g/ml, 300 μ A, 15 min), OS of treated animals (5 eyes) showed fluorescence significantly higher than that in non-treated controls; this level is similar to that of the 11 eyes that received a single iontophoresis with these new parameters (ANOVA, $F_{[3,60]}=21.765$, $P<0.001$; sample size is in the bars and P values denote comparison with controls). Scale bar: 30 μ m.

Figure 3.

Gene expression driven by the human β -PDE promoter in the mouse retina after single iontophoresis of pPDE-GFP (1000 μ g/ml, 600 μ A, 15 min). **(a)** Confocal images detected green fluorescence only in retinas subjected to iontophoresis of pPDE-GFP, mainly in the OS of photoreceptors (right panel). Left panel: control, untreated retina. See Figure 2 for abbreviations. **(b)** Quantification of gene expression at day 3 after a single iontophoresis application indicates that the OS of 6 plasmid-treated eyes have significantly higher level of green fluorescence than those of 12 control, non-treated eyes (ANOVA, $F_{[1,67]} = 88.321$, $P < 0.001$). Total number of optical sections analyzed is indicated inside the bars. Scale bar: 30 μ m.

Figure 4.

Iontophoretic-enhanced transfer of pPDE-PDE to the eyes of *rd1* mice rescues their photoreceptor cells from degeneration. **(a)** Photoreceptor cells at the peripheral and posterior retina. Two-way ANOVA revealed that the ONL thickness differs among pPDE-GFP-treated *rd1*, pPDE-PDE-treated *rd1* and pPDE-GFP-treated normal mouse retinas ($F_{[2,150]}=422.061$, $p<0.001$) and that the rescuing effect of pPDE-PDE is significant at the peripheral retina (two factor interaction $F_{[2,150]}=39.591$, $p<0.001$) but not at the posterior retina. The P values denote the individual comparisons by Tukey HSD multiple comparisons. Sample size is noted inside the bars. **(b)** Vertical sections of toluidine blue-stained peripheral retinas from non-treated homozygous *rd1/rd1* (left panel), pPDE-PDE-treated *rd1/rd1* (middle panel) and normal mice

(right panel) at postnatal day 23. Note the absence of an ONL in the non-treated *rd1* mouse retina and the substantial number of photoreceptor nuclei (in the ONL), IS and OS in *rd1/rd1* mice treated with 3 consecutive iontophoretic applications of pPDE-PDE (middle panel). See Figure 2 for abbreviations. **(c)** Expression of rhodopsin in the rod photoreceptors of a 23-day old *rd1* mouse after repetitive iontophoresis of pPDE-PDE. Immunofluorescence microscopy detected mild expression of rhodopsin at the periphery of the pPDE-PDE-treated retinas of *rd1* mice (upper panel). The lower panel is an overlay of bright field and fluorescent images. PR, photoreceptors. **(d)** Western blot demonstrating the presence of α - and β -PDE subunits in a 23-day old *rd1* mouse retina after iontophoretic-enhanced delivery of pPDE-PDE.. Proteins were separated as described in Methods in a gradient polyacrylamide gel, transblotted, incubated with a polyclonal antibody recognizing both subunits of cGMP-PDE and visualized with alkaline phosphatase coupled to anti-rabbit secondary antibody and the ECL chemiluminescence system. Lane 1, α - and β -PDE from normal mouse retina. Lane 2, treated 23 day-old *rd1* retina. Although the bands are weak, both PDE subunits are observed. Lane 3, non-treated 23 day-old *rd1* retina. Notice the absence of the PDE doublet. Scale bar: 30 μ m.

Figure 5.

Electroretinograms (ERGs) recorded from non-treated (left panel) and treated (right panel) eyes of *rd1* mice. Each record shows the response to a different intensity stimulus, from the lowest intensity at which a response was detectable (bottom tracing) to the highest intensity provided by our stimulator (top tracing). Each tracing is the average of 50–100 sequential flash presentations for a single eye. Without any treatment, visual stimuli elicited no recordable ERGs from the *rd1* mouse retina. After 3 consecutive iontophoretic applications of pPDE-PDE, clearly detectable responses were observed indicating partial rescue of visual function.