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https://hdl.handle.net/2324/25566

Pigment Epithelium-Derived Factor Gene Therapy Targeting Retinal Ganglion Cell Injuries: Neuroprotection Against Loss of Function in Two Animal Models

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Abstract

Lentiviral vectors are promising tools for the treatment of chronic retinal diseases including glaucoma, as they enable stable transgene expression. We examined whether simian immunodeficiency virus (SIV)-based lentiviral vector-mediated retinal gene transfer of human pigment epithelium-derived factor (hPEDF) can rescue rat retinal ganglion cell injury. Gene transfer was achieved through subretinal injection of an SIV vector expressing human PEDF (SIV-hPEDF) into the eyes of 4-week-old Wistar rats. Two weeks after gene transfer, retinal ganglion cells were damaged by transient ocular hypertension stress (110 mmHg, 60 min) and N-methyl-D-aspartic acid (NMDA) intravitreal injection. One week after damage, retrograde labeling with 4′,6-diamidino-2-phenylindole (DAPI) was done to count the retinal ganglion cells that survived, and eyes were enucleated and processed for morphometric analysis. Electroretinographic (ERG) assessment was also done. The density of DAPI-positive retinal ganglion cells in retinal flat-mounts was significantly higher in SIV-hPEDF-treated rats compared with control groups, in both transient ocular hypertension and NMDA-induced models. Pattern ERG examination demonstrated higher amplitude in SIV-hPEDF-treated rats, indicating the functional rescue of retinal ganglion cells. These findings show that neuroprotective gene therapy using hPEDF can protect against retinal ganglion cell death, and support the potential feasibility of neuroprotective therapy for intractable glaucoma.

Introduction

G LAUCOMA IS the second leading cause of blindness, and affects 70 million people worldwide (Quigley and Broman, 2006). It is recognized as a progressive optic neuropathy, associated with structural change in the optic nerve head. The development and progression of glaucomatous damage result mainly from high intraocular pressure, which is being questioned as many patients continue to demonstrate a downhill clinical course despite controlled intraocular eye pressure (IOP) (Brubaker, 1996). In addition, the prevalence of primary open-angle glaucoma (POAG) was found to be 3.9%, and in 92% patients with POAG the IOP was 21 mmHg or less (Iwase et al., 2004). Research has suggested that several pressure-indepen-

dent mechanisms, such as vascular insufficiency and weakness of retinal ganglion cells, disruption of retrograde transport of neurotrophic factors, glutamate toxicity, and immune system abnormalities, are concerned (Clark and Pang, 2002; Pang et al., 2004; Kuehn et al., 2005). Unfortunately, the exact contribution of any of these factors to the pathogenesis of glaucomatous damage has not been unequivocally determined. It is probable that more than one etiology and multiple mechanisms are responsible in different patients and in different stages of glaucoma, which makes decisions regarding therapy difficult. However, the final common pathological event is the apoptotic death of retinal ganglion cells (RGCs) (Kerrigan et al., 1997; Nickells, 1999). Thus, an approach targeting apoptosis of RGCs is likely to be more useful for glaucoma.

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Among several antiapoptotic and neuroprotective factors, pigment epithelium-derived factor (PEDF) appears to be one of the most effective. It is a 50-kDa secreted glycoprotein, and was first isolated from conditioned medium from both fetal and adult retinal pigment epithelium (RPE) (Tombran-Tink and Johnson, 1989; Ortego *et al.*, 1996). It is contained abundantly in the eye as a physiological factor, and a PEDF-rich condition in the eye, via vector-mediated PEDF over-expression, has been strictly proven to be safe (Miyazaki *et al.*, 2003; Campochiaro *et al.*, 2006; Ikeda *et al.*, 2009b). In addition, the mean level of PEDF in eyes with advanced glaucoma was significantly lower than that in control eyes (Ogata *et al.*, 2004). PEDF receptors exist also on the RGC surface in the neural retina, and PEDF–receptor interactions may serve to localize and direct PEDF activity (Aymerich *et al.*, 2001; Notari *et al.*, 2006). PEDF has broad neuroprotective effects in several neuronal cells and tissues (Taniwaki *et al.*, 1995; Cao *et al.*, 2001; Nomura *et al.*, 2001; Miyazaki *et al.*, 2003, 2008), and also in RGCs *in vitro* and *in vivo* (Ogata *et al.*, 2001; Takita *et al.*, 2003; Pang *et al.*, 2007; Zhou *et al.*, 2009). Moreover, PEDF has strong antiangiogenic ability through the induction of endothelial apoptosis (Dawson *et al.*, 1999). As the unexpected proliferation of neovessels is likely to worsen a patient's vision, PEDF would seem to be a good candidate for retinal gene therapy.

Some experimental studies aimed at neuroprotective gene therapy targeting RGCs have used various vectors, including adenoviral vectors (Takita *et al.*, 2003), adeno-associated viral (AAV) vectors (Martin *et al.*, 2003; Leaver *et al.*, 2006), and lentiviral vectors (van Adel *et al.*, 2003). As an alternative therapy that may be safer for humans and provide long-term gene expression, we previously demonstrated the utility of a lentiviral vector based on simian immunodeficiency virus from African green monkeys (SIVagm) (Nakajima *et al.*, 2000). In previous studies, the SIV vector demonstrated long-term transgene expression in rat eyes and in monkey eyes (Ikeda *et al.*, 2003, 2009a), safety and no toxicity at appropriate concentrations (Miyazaki *et al.*, 2003, 2008; Ikeda *et al.*, 2009b), and significantly neuroprotective effects in several animal models of retinitis pigmentosa (RP) expressing hPEDF and human fibroblast growth factor-2 (hFGF-2) for a long period (Miyazaki *et al.*, 2003, 2008). On the basis of these efficacy studies, we have already completed preclinical studies using nonhuman primates to evaluate the safety of this mode of vector (Ikeda *et al.*, 2009a,b). The results have been sufficient to allow us to make arrangements for a clinical study.

In this study, we assessed, morphologically and functionally, SIV-mediated gene therapy in which hPEDF was expressed in two different RGC-damaged models.

Materials and Methods

SIVagm-based lentiviral vector

A third-generation recombinant SIVagm-based lentiviral vector carrying the human pigment epithelium-derived factor (hPEDF) was prepared as previously described (Miyazaki *et al.*, 2003; Ikeda *et al.*, 2009b). Briefly, human embryonic kidney (HEK) 293T cells were transfected with a packaging vector, a gene transfer vector encoding hPEDF driven by the cytomegalovirus (CMV) promoter, an Rev expression vector, and an envelope vector, pVSVG (Clontech Laboratories, Mountain View, CA), using lipofection. Twelve hours later, the culture medium was replaced to start harvesting viral particles. Harvesting was undertaken at 48 hr, and viral particles were concentrated by ultracentrifugation. The U3 region in the 3′ and 5′ long terminal repeats (LTRs) of SIVagm was deleted to induce self-inactivation. The viral titer was determined by transduction of the HEK 293T cell line and is expressed as transducing units (TU) per milliliter, and the virus was kept at −80°C until just before use. Vector stocks were confirmed to be free of endotoxin, and without extraordinary cytotoxicity as determined by a simultaneous transfection test using HEK 293T cells and human RPE cells (ARPE-19) obtained from the American Type Culture Collection (Manassas, VA).

Animals and subretinal vector injection

Four-week-old male Wistar rats were maintained humanely, with proper institutional approval and in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were done under approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals by the Committee for Animals, Recombinant DNA, and Infectious Pathogen Experiments at Kyushu University (Fukuoka, Japan) and according to Law 105 and Notification 6 of the Japanese government.

Each solution was injected subretinally as previously described with minor modifications. Briefly, the rats were anesthetized by inhalation, and surgical procedures were then performed using an operating microscope. A 30-gauge needle was inserted into the anterior chamber at the peripheral cornea, and the anterior chamber fluid was drained off. A 30-gauge needle was inserted into the subretinal space of the peripheral retina in the nasal hemisphere via an external transscleral, transchoroidal approach. Ten microliters of vector solution (SIV-hPEDF or SIV-empty, 2.5×10^7 TU/ml) was injected, and excess solution from the injection site was washed out with phosphate-buffered saline (PBS). The appearance of a dome-shaped retinal detachment confirmed the subretinal delivery. Eyes that sustained prominent surgical trauma, such as retinal or subretinal hemorrhage or bacterial infection, were excluded from this examination. Moreover, to exclude interanimal variation, each rat received a different solution in the left eye than in the right.

Human PEDF ELISA

The vector-injected eyes were enucleated and homogenized mechanically in lysis buffer. Several eyes were separated into solid (retina, uvea, sclera, etc.) and liquid parts (vitreous body and aqueous humor). After centrifugation at 5000 rpm for 10 min, the supernatants were subjected to human PEDF-specific ELISA according to the instructions of the manufacturer (Chemicon International/Millipore, Temecula, CA). The concentration of each protein was standardized by the concentration of total protein (Miyazaki et al. 2003).

Retinal ganglion cell injury methods

Male Wistar rats, each vector-injected 2 weeks previously, were used in this study. Transient ocular hypertension was

induced in the eye of each rat according to the method of Kawaji and colleagues with slight modifications (Kawaji et al., 2007). Rats were anesthetized with a 1:1 mixture of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). Dilation of the pupil was achieved with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. The anterior chamber of the eye was cannulated with a 30-gauge needle attached to a line for infusion of balanced salt solution. Intraocular pressure (IOP) was raised to 110 mmHg. Complete nonperfusion was confirmed via an operating microscope. After 60 min of ocular hypertension, the needle was withdrawn and the IOP normalized.

N-Methyl-D-aspartic acid (NMDA) was obtained from Sigma-Aldrich (St. Louis, MO). The treatment of retinas with NMDA in this study was similar to that described by Inomata and colleagues (2003). Briefly, rats were anesthetized by intramuscular injection of xylazine (10 mg/kg) and ketamine (20 mg/kg), and the pupil was dilated with phenylephrine hydrochloride and tropicamide. Injection was performed under a microscope, using a microsyringe with a 33-gauge needle inserted approximately 1 mm behind the corneal limbus. A single 5-μl dose of 4 mM NMDA (20 nmol) was administered.

Morphological analysis

The rats were killed, and the eyes were enucleated and fixed with ice-cooled 4% paraformaldehyde in PBS. Twenty-four hours later, the samples were embedded in paraffin, and 5-μm-thick sections along the pupil–optic nerve axis were examined by light microscopy.

Retrograde labeling of RGCs

Four days after RGC injury by transient ocular hypertension and NMDA injection, retrograde labeling of the RGCs was conducted as described by Inomata and colleagues (2003). Briefly, rats were anesthetized and then the heads were fixed in a stereotaxic apparatus. Fluoro-Gold (Fluorochrome, Englewood, CO) was microinjected bilaterally into the superior colliculi of the rats. Three days after Fluoro-Gold injection (7 days after RGC injury), the animals were killed as described and the eyes were enucleated. Eyes were fixed with 4% paraformaldehyde for 1 hr. Retinas were divided by five radial cuts and removed from the sclera and mounted on slides. Analysis of the number of Fluoro-Gold-labeled RGCs was carried out. For this counting procedure, regions were selected from five fields of the central area (1 mm from the optic disk). Thus, in each eye, five fields were examined by counting the labeled RGCs per 1 mm^2.

TUNEL staining

The TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) procedure and quantification of TUNEL-positive cells were performed with an ApopTag fluorescein in situ apoptosis detection kit (Chemicon International/Millipore) for retinal flat-mount according to the instructions of the manufacturer. Two days after RGC injury, the animals were killed as described and the eyes were enucleated. Eyes were fixed with 4% paraformaldehyde for 1 hr. Retinas were divided by five radial cuts and removed from the sclera and mounted on slides. The number of TUNEL-positive cells was counted in a blinded fashion. For this counting procedure, regions were selected from five fields of the central area (1 mm from the optic disk). Thus, in each eye, five fields were examined by counting the labeled TUNEL-positive cells per 1 mm^2.
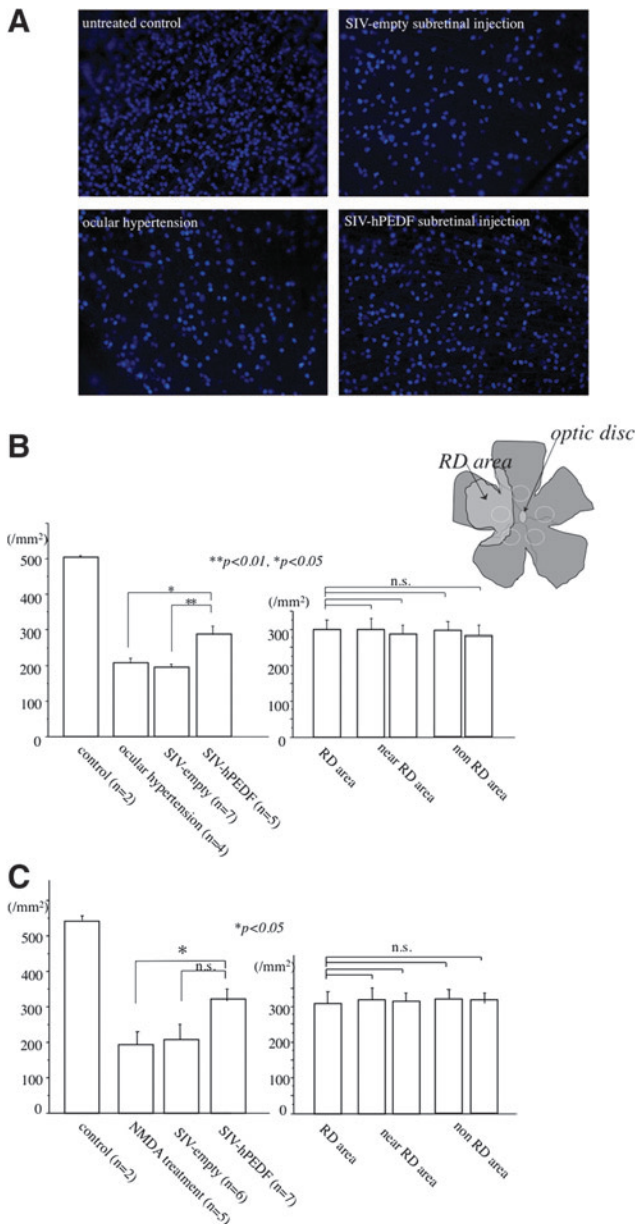
Electroretinograms

Electroretinograms (ERGs) were measured in rats 1 week after RGC injury, and were recorded by an examiner who was blinded concerning whether the eyes were treated or untreated, as previously described (Goto et al., 1999; Miyazaki et al., 2003). The rats were anesthetized with an intraperitoneal injection of saline solution (15 μl/g body weight) containing ketamine (1 mg/ml), pancuronium bromide (0.4 mg/ml), and urethane (40 mg/ml). Both pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride, and the animals were placed on a heating pad to maintain their body temperature. Pattern ERGs were recorded from each eye, using a coiled stainless-steel wire containing the anesthetized (1% proparacaine HCl) corneal surface through a layer of 1% methylcellulose. A similar wire was placed in each of the leads. The responses were differentially amplified (band pass, 0.8 to 1200 Hz) and averaged, and the data were stored in a minicomputer (signal processor 7T17; NEC San-ei Instruments, Tokyo, Japan). We measured the b-wave amplitudes of pattern ERGs for RGC function in this study.

Pattern ERGs were recorded in a dark room. The stimulus used in this study consisted of black–white vertical sinusoidal gratings that were contrast-reversed at 1 Hz. The black–white gratings varied in spatial frequency at 0.5 cycle (c)/degree with 90% contrast. The mean luminance was kept at 50 candelas (cd)/m^2. The area of the display was rectangular at a viewing distance of 57 cm from each eye. Each animal
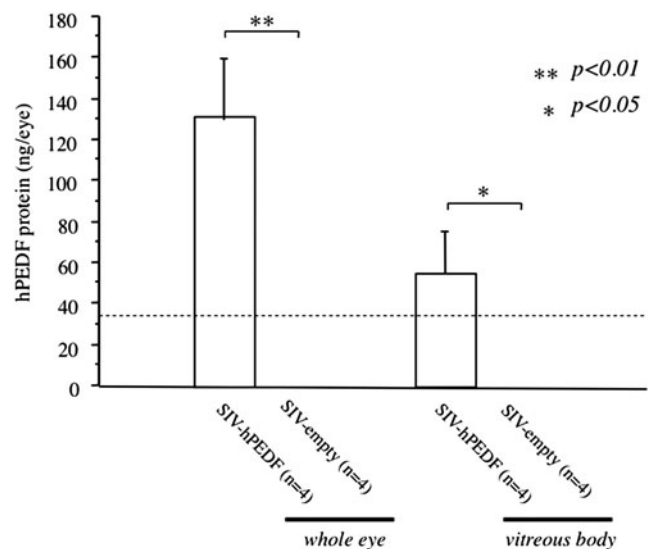
FIG. 1. SIV-mediated human pigment epithelium-derived factor (hPEDF) expression in the eye. Abundant hPEDF protein was expressed in the eye after subretinal injection of SIV-hPEDF, and hPEDF protein secreted from the retinal pigment epithelium (RPE) diffused well into the vitreous body.

kept its eye on the center of display through the corrective lens (+3.0 diopters [D]), and pattern ERGs were recorded for monocular viewing by each eye.

Statistical analyses

All values are expressed as means $\pm$ SEM. Data were analyzed by nonparametric test (Mann–Whitney U test). A p value of less than 0.05 was considered statistically significant.
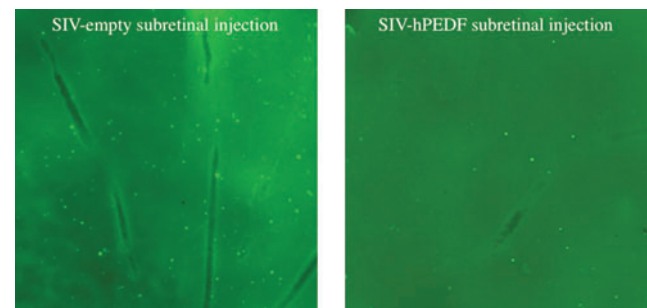
Results

Transgene expression in vivo

We assessed transgene expression *in vivo* (Fig. 1) after subretinal injection of the third-generation SIV (2.5×10^7 TU/ml = 2.5×10^5 TU/10 μl/eye); we also included rats treated with SIV-empty as a control.

Two weeks after gene transfer, eyes infected with SIV-hPEDF significantly expressed hPEDF protein, whereas in the SIV-empty eyes hPEDF protein was not detectable (the ELISA used does not cross-react with rodent PEDF). In addition, to assess whether expressed hPEDF protein spreads widely in the eyeball, we measured the amount of hPEDF in the vitreous body. hPEDF protein, expressed in the RPE of the peripheral retina, diffused well into the vitreous body.

Analysis of retrograde labeling of RGCs

To investigate whether SIV-mediated hPEDF expression protects RGCs from transient ocular hypertension-induced neuronal death, we used retrograde labeling of RGCs with Fluoro-Gold, which allows individual RGCs to be observed in flat-mount retinas (Fig. 2A). The mean density of RGCs was 504 ± 87, 209 ± 56, and 198 ± 49 cells/mm^2 in untreated eyes, transient ocular hypertension control eyes, and transient ocular hypertension eyes treated with SIV-empty, respectively. In contrast, the mean density of RGCs in SIV-hPEDF-treated eyes was 284 ± 65 cells/mm^2, which is significantly higher than that for the control eyes (Fig. 2B).

FIG. 2. Morphological assessment of neuroprotective effects against transient ocular hypertension-induced and *N*-methyl-D-aspartic acid (NMDA)-induced retinal ganglion cell (RGC) injuries. (**A**) Retrograde labeling of RGCs in flat-mount retinas 7 days after RGC injury. The density of RGCs in the SIV-hPEDF-treated eye (*bottom right*) was higher than in the transient ocular hypertension control eye (*bottom left*) or the SIV-empty-treated eye (*top right*). (**B**) Assessment of neuroprotective effects against transient ocular hypertension-induced RGC injury. The mean density of RGCs in SIV-hPEDF-treated eyes was significantly higher than in transient ocular hypertension control eyes or SIV-empty treated eyes. There was no significant difference in labeled RGC density among five measured points in SIV-hPEDF-treated eyes. RD, retinal detachment; n.s., not significant. (**C**) Assessment of neuroprotective effects against NMDA-induced RGC injury. The mean density of RGCs in SIV-hPEDF-treated eyes was significantly higher than in NMDA-treated eyes. There was no significant difference in labeled RGC density among five measured points in SIV-hPEDF-treated eyes.

FIG. 3. TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) staining of retinal flat-mounts of transient ocular hypertension eyes. TUNEL staining of apoptotic RGCs was determined in flat-mount retinas 2 days after RGC injury. The cell density of the TUNEL-positive cells (the apoptotic RGCs) in SIV-empty treated eye (*left*) was higher than that in SIV-hPEDF-treated eye (*right*). The mean cell density of TUNEL-positive cells in SIV-hPEDF-treated eyes was significantly higher than in SIV-empty-treated eyes.

There was no significant difference in labeled RGC density among five measured points, suggesting that neuroprotective effects were observed all over the retina, despite the focal gene transfer. A similar result was observed in NMDA-treated eyes (Fig. 2C).

Analysis of apoptotic RGCs: TUNEL-positive cells

To investigate whether SIV-mediated hPEDF expression prevents apoptosis of RGCs induced by transient ocular hypertension, we conducted TUNEL staining, which detects apoptotic RGCs, in flat-mount retinas 2 days after RGC injury (Fig. 3). The mean cell density of TUNEL-positive cells was 205 ± 44 and 103 ± 18 cells/mm^2 in transient ocular hypertension eyes treated with SIV-empty ($n = 4$) and SIV-hPEDF ($n = 6$), respectively. There was significant difference between these groups ($p < 0.05$).

Functional evaluation using electroretinograms

Last, we examined whether or not the structural rescue of RGCs might actually correspond to retinal electrical function. For this assessment, pattern ERGs were measured in rats 4 weeks after vector injection. Typical wave patterns and quantitative analyses are demonstrated.

A significantly higher b-wave amplitude of pattern ERGs was observed in the SIV-hPEDF-injected eyes (Fig. 4A). Similar results were obtained in the NMDA-treated model (Fig. 4B). These results demonstrated that SIV-hPEDF gene therapy rescued RGC functional damage.

Discussion

In this study, we investigated the efficacy of neuroprotective gene therapy for retinal ganglion cell death, mediated
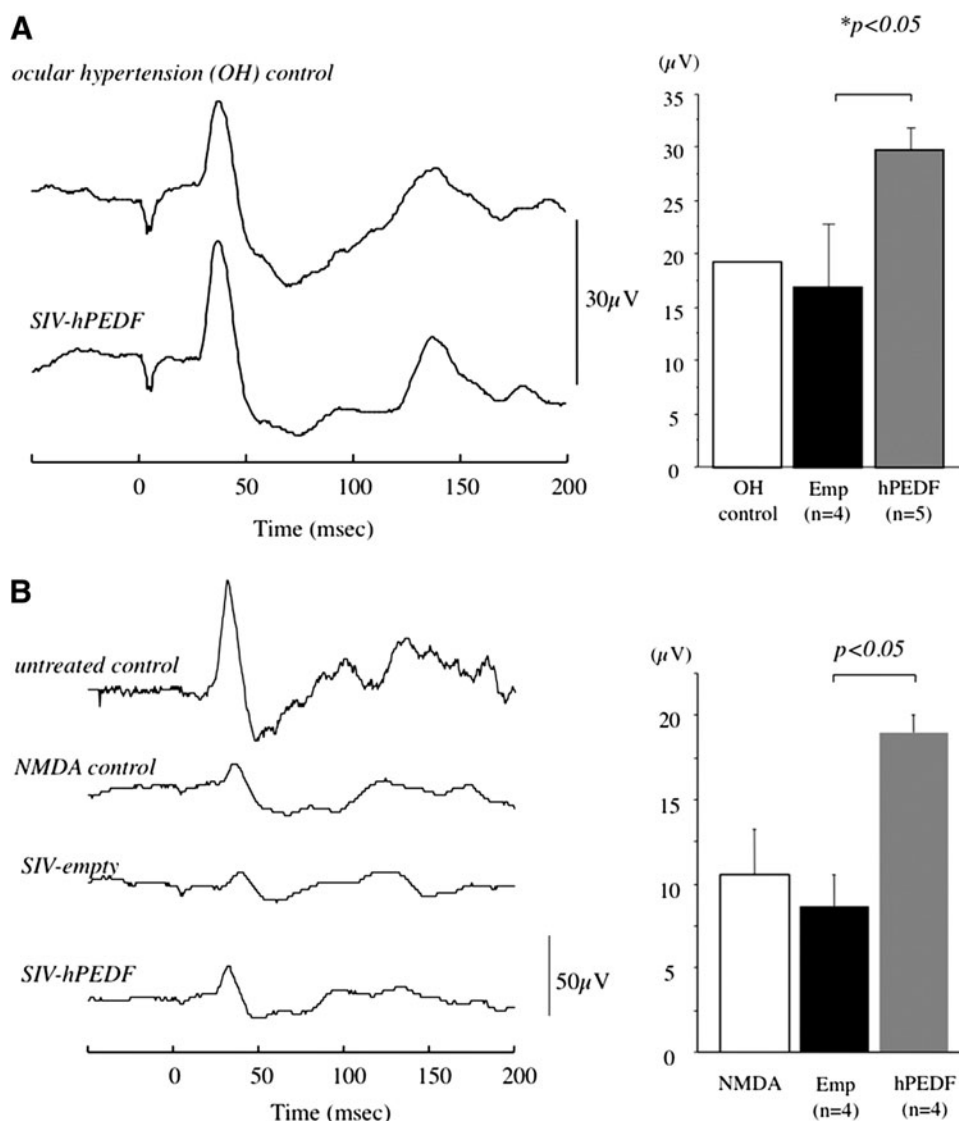
FIG. 4. Functional assessment of neuroprotective effects against transient ocular hypertension-induced and NMDA-induced RGC injuries. (**A**) Typical wave patterns and quantitative analyses of transient ocular hypertension-induced eyes are demonstrated. Significantly higher b-wave amplitudes of pattern ERGs were observed in SIV-hPEDF-injected eyes. (**B**) Typical wave patterns and quantitative analyses of NMDA-induced eyes are demonstrated. Significantly higher b-wave amplitudes of pattern ERGs were observed in SIV-hPEDF-injected eyes.

by subretinal injection of an SIVagm vector carrying the human PEDF gene. The key observations made in this study are as follows: (1) human PEDF protein expressed in retinal pigment epithelium diffused into the vitreous body; (2) hPEDF gene therapy attenuated retinal ganglion cell loss in NMDA-mediated injuries as well as in transient ocular hypertension injuries; and (3) hPEDF gene therapy protected retinal ganglion cell function in NMDA-mediated injuries as well as in transient ocular hypertension injuries.

We previously examined transgene expression via subretinal injection of SIVagm vectors. Expression was detected mainly in the RPE (Ikeda *et al.*, 2003), and the neuroprotective effect against photoreceptor cell death was limited to the area around the point of vector injection in some rodent models (Miyazaki *et al.*, 2003). In this study we have demonstrated neuroprotective efficacy against RGC injuries in the whole retina as well as in the vector-injected area (Fig. 2B and C). As shown in Fig. 1, sufficient human PEDF protein, secreted from the RPE subsequent to subretinal injection of the SIVagm vector, diffused into the vitreous body to protect RGCs at the surface of the retina. Gene transfer efficiency to the retina via vitreous injection of our SIVagm vectors was not good (data not shown). In our preclinical study using nonhuman primates, we demonstrated that SIVagm-mediated subretinal gene transfer neither affected retinal function nor damaged retinal architecture, and that no vector sequence was detected in the serum or urine (Ikeda *et al.*, 2009b). Moreover, only a few RGCs remained in the retina of patients with intractable glaucoma. In the clinical setting of gene therapy for ocular diseases, such as intractable glaucoma, subretinal delivery of SIVagm vectors is more efficient and safer than intravitreal injection.

The therapeutic mechanism for these models seems to be prevention of RGC apoptosis (Takita *et al.*, 2003). Previously, we demonstrated that nuclear translocation of apoptosis-inducible factor (AIF) was also observed in apoptotic photoreceptor cells in an animal model of retinal degeneration, and was dramatically inhibited by retinal gene transfer of PEDF, resulting in significant rescue of their photoreceptors (Murakami *et al.*, 2008). That is to say, the AIF-mediated pathway is an essential target of PEDF during photoreceptor apoptosis in retinal degeneration. In this study, we demonstrated that SIV-mediated PEDF gene transfer to the retina could significantly protect against RGC injuries, and this effect occurred via the inhibition of RGC apoptosis (Fig. 3). However, we could not demonstrate a relationship between therapeutic efficacy and the AIF-mediated pathway (data not shown). Neither has the involvement of this AIF-mediated pathway in RGC apoptosis been demonstrated in previous *in vivo* studies (Tezel and Yang, 2004; Li and Osborne, 2008). One possible explanation is that another pathway, such as the caspase-dependent pathway, contributes to RGC injuries. Further studies will be needed to clarify the mechanism of PEDF neuroprotection in these RGC injuries.

Many previous reports have demonstrated therapeutic efficacy for the treatment of RGC injuries, using morphological assessments of RGCs in flat-mount specimens or histopathological sections (Ogata *et al.*, 2001; Martin *et al.*, 2003; Takita *et al.*, 2003; van Adel *et al.*, 2003; Leaver *et al.*, 2006; Pang *et al.*, 2007). However, studies of the gene therapy of RGC injuries, in which RGC function is assessed, are rare (Zhou *et al.*, 2009). In this study, we demonstrated the neu-

roprotective effect against loss of RGC function, using pattern ERGs in two animal models (Fig. 4A and B).

In conclusion, neuroprotective gene therapy using hPEDF can protect against RGC death; our study supports the potential feasibility of neuroprotective therapy for intractable glaucoma.

Acknowledgments

Conflict of Interest Statement

Dr. Yonemitsu is a member of the Scientific Advisory Board of DNAVEC Corporation.

References

Aymerich, M.S., Alberdi, E.M., Martínez, A., *et al.* (2001). Evidence for pigment epithelium-derived factor receptors in the neural retina. Invest. Ophthalmol. Vis. Sci. 42, 3287–3293.

Brubaker, R.F. (1996). Delayed functional loss in glaucoma: LII Edward Jackson Memorial Lecture. Am. J. Ophthalmol. 121, 473–483.

Campochiaro, P.A., Nguyen, Q.D., Shah, S.M., *et al.* (2006). Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: Results of a phase I clinical trial. Hum. Gene Ther. 17, 167–176.

Cao, W., Tombran-Tink, J., Elias, R., *et al.* (2001). *In vivo* protection of photoreceptors from light damage by pigment epithelium-derived factor. Invest. Ophthalmol. Vis. Sci. 42, 1646–1652.

Clark, A.F., and Pang, I.H. (2002). Advances in glaucoma therapeutics. Expert Opin. Emerging Drugs 7, 141–163.

Dawson, D.W., Volpert, O.V., Gillis, P., *et al.* (1999). Pigment epithelium-derived factor: A potent inhibitor of angiogenesis. Science 285, 245–248.

Goto, Y., Tobimatsu, S., Shigematsu, J., *et al.* (1999). Properties of rat cone-mediated electroretinograms during light adaptation. Curr. Eye Res. 19, 248–253.

Ikeda, Y., Yonemitsu, Y., Miyazaki, M., *et al.* (2003). Simian immunodeficiency virus-based lentivirus vector for retinal gene transfer: A preclinical safety study in adult rats. Gene Ther. 10, 1161–1169.

Ikeda, Y., Yonemitsu, Y., Miyazaki, M., *et al.* (2009a). Stable retinal gene expression in nonhuman primates via subretinal injection of SIVagm-based lentiviral vectors. Hum. Gene Ther. 20, 573–579.

Ikeda, Y., Yonemitsu, Y., Miyazaki, M., *et al.* (2009b). Acute toxicity study of a simian immunodeficiency virus-based lentiviral vector for retinal gene transfer in nonhuman primates. Hum. Gene Ther. 20, 943–954.

Inomata, Y., Hirata, A., Koga, T., *et al.* (2003). Lens epithelium-derived growth factor: Neuroprotection on rat retinal damage induced by *N*-methyl-D-aspartate. Brain Res. 991, 163–170.

Iwase, A., Suzuki, Y., Araie, M., *et al.*; Tajimi Study Group, Japan Glaucoma Society. (2004). The prevalence of primary open-angle glaucoma in Japanese: The Tajimi Study. Ophthalmology 111, 1641–1648.

Kawaji, T., Inomata, Y., Takano, A., *et al.* (2007). Pitavastatin: Protection against neuronal retinal damage induced by ischemia–reperfusion injury in rats. Curr. Eye Res. 32, 991–997.

Kerrigan, L., Zack, D., Quigley, H., *et al.* (1997). TUNEL-positive ganglion cells in human primary open angle glaucoma. Arch. Ophthalmol. 115, 1031–1035.

Kuehn, M.H., Fingert, J.H., and Kwon, Y.H. (2005). Retinal ganglion cell death in glaucoma: Mechanisms and neuroprotective strategies. Ophthalmol. Clin. North Am. 18, 383–395.

Leaver, S.G., Cui, Q., Plant, G.W., *et al.* (2006). AAV-mediated expression of CNTF promotes long-term survival and regeneration of adult rat retinal ganglion cells. Gene Ther. 13, 1328–1341.

Li, G.Y., and Osborne, N.N. (2008). Oxidative-induced apoptosis to an immortalized ganglion cell line is caspase independent but involves the activation of poly(ADP-ribose) polymerase and apoptosis-inducing factor. Brain Res. 1188, 35–43.

Martin, K.R., Quigley, H.A., Zack, D.J., *et al.* (2003). Gene therapy with brain-derived neurotrophic factor as a protection: Retinal ganglion cells in a rat glaucoma model. Invest. Ophthalmol. Vis. Sci. 44, 4357–4365.

Miyazaki, M., Ikeda, Y., Yonemitsu, Y., *et al.* (2003). Simian lentiviral vector-mediated retinal gene transfer of pigment epithelium-derived factor protects retinal degeneration and electrical defect in Royal College of Surgeons rats. Gene Ther. 10, 1503–1511.

Miyazaki, M., Ikeda, Y., Yonemitsu, Y., *et al.* (2008). Synergistic neuroprotective effect via simian lentiviral vector-mediated simultaneous gene transfer of human pigment epithelium-derived factor and human fibroblast growth factor-2 in rodent models of retinitis pigmentosa. J. Gene Med. 10, 1273–1281.

Murakami, Y., Ikeda, Y., Yonemitsu, Y., *et al.* (2008). Inhibition of nuclear translocation of apoptosis-inducing factor is an essential mechanism of the neuroprotective activity of pigment epithelium-derived factor in a rat model of retinal degeneration. Am. J. Pathol. 173, 1326–1338.

Nakajima, T., Nakamaru, K., Ido, E., *et al.* (2000). Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. Hum. Gene Ther. 11, 1863–1874.

Nickells, R.W. (1999). Apoptosis of retinal ganglion cells in glaucoma: An update of the molecular pathways involved in cell death. Surv. Ophthalmol. 43, S151–S161.

Nomura, T., Yabe, T., Mochizuki, H., *et al.* (2001). Survival effects of pigment epithelium-derived factor expressed by a lentiviral vector in rat cerebellar granule cells. Dev. Neurosci. 23, 145–152.

Notari, L., Baladron, V., Aroca-Aguilar, J.D., *et al.* (2006). Identification of a lipase-linked cell membrane receptor for pigment epithelium-derived factor. J. Biol. Chem. 281, 38022–38037.

Ogata, N., Wang, L., Jo, N., *et al.* (2001). Pigment epithelium derived factor as a neuroprotective agent against ischemic retinal injury. Curr. Eye Res. 22, 245–252.

Ogata, N., Matsuoka, M., Imaizumi, M., *et al.* (2004). Decreased levels of pigment epithelium-derived factor in eyes with neuroretinal dystrophic diseases. Am. J. Ophthalmol. 137, 1129–1130.

Ortego, J., Escribano, J., Becerra, S.P., *et al.* (1996). Gene expression of the neurotrophic pigment epithelium-derived factor in the human ciliary epithelium: Synthesis and secretion into the aqueous humor. Invest. Ophthalmol. Vis. Sci. 37, 2759–2767.

Pang, I.-H., Li, B., and Clark, A.F. (2004). The pathogenesis of retinal ganglia cell apoptosis induced by glaucoma. Chin. J. Ophthalmol. 40, 495–499.

Pang, I.-H., Zeng, H., Fleenor, D.L., *et al.* (2007). Pigment epithelium-derived factor protects retinal ganglion cells. BMC Neurosci. 8, 11.

Quigley, H.A., and Broman, A.T. (2006). The number of people with glaucoma worldwide in 2010 and 2020. Br. J. Ophthalmol. 90, 262–267.

Takita, H., Yoneya, S., Gehlbach, P.L., *et al.* (2003). Retinal neuroprotection against ischemic injury mediated by intraocular gene transfer of pigment epithelium-derived factor. Invest. Ophthalmol. Vis. Sci. 44, 4497–4504.

Taniwaki, T., Becerra, S.P., Chader, G.J., *et al.* (1995). Pigment epithelium-derived factor is a survival factor for cerebellar granule cells in culture. J. Neurochem. 64, 2509–2517.

Tezel, G., and Yang, X. (2004). Caspase-independent component of retinal ganglion cell death, *in vitro*. Invest. Ophthalmol. Vis. Sci. 45, 4049–4059.

Tombran-Tink, J., and Johnson, L.V. (1989). Neuronal differentiation of retinoblastoma cells induced by medium conditioned by human RPE cells. Invest. Ophthalmol. Vis. Sci. 30, 1700–1707.

van Adel, B.A., Kostic, C., Déglon, N., *et al.* (2003). Delivery of ciliary neurotrophic factor via lentiviral-mediated transfer protects axotomized retinal ganglion cells for an extended period of time. Hum. Gene Ther. 14, 103–115.

Zhou, X., Li, F., Kong, L., *et al.* (2009). Anti-inflammatory effect of pigment epithelium-derived factor in DBA/2J mice. Mol. Vis. 15, 438–450.

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