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Inhibition of Choroidal Neovascularization via Brief Subretinal Exposure to a Newly Developed Lentiviral Vector Pseudotyped with Sendai Viral Envelope Proteins

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Abstract

Lentiviral vectors are promising tools for the treatment of chronic retinal diseases, including age-related macular degeneration (AMD), as they enable stable transgene expression. On the other hand, Sendai virus (SeV) vectors provide the unique advantage of rapid gene transfer. Here we show that novel simian immunodeficiency viral vectors pseudotyped with SeV envelope proteins (SeV-F/HN-SIV) achieved rapid, efficient, and long-lasting gene transfer in the mouse retina. Subretinal exposure to SeV-F/HN-SIV vectors for only a few minutes resulted in high-level gene transfer to the retinal pigment epithelium, whereas several hours were required for gene transfer by standard vesicular stomatitis virus G-pseudotyped SIV vectors. Transgene expression continued over a 1-year period. SeV-F/HN-SIV vector-mediated retinal overexpression of soluble Fms-like tyrosine kinase-1 (sFlt-1) or pigment epithelium-derived factor (PEDF) significantly suppressed laser-induced choroidal neovascularization (CNV). Histologically, 6-month-long sustained overexpression of PEDF did not adversely affect the retina; however, that with sFlt-1 resulted in photoreceptor degeneration associated with choroidal circulation defects. These data demonstrate that brief subretinal administration of SeV-F/HN-SIV vectors may facilitate safe and efficient retinal gene transfer, and suggest the therapeutic potential of PEDF with a higher safety profile for treating CNV in AMD patients.

Introduction

THE DEVELOPMENT OF CHOROIDAL NEOVASCULARIZATION (CNV) is a principal pathological feature of age-related macular degeneration (AMD), the leading cause of adult blindness in industrialized countries (Lopez *et al.*, 1991; Friedman *et al.*, 2004; Miyazaki *et al.*, 2005). One of the major factors that induce CNV is vascular endothelial growth factor-A (VEGF-A), a diffusible cytokine that promotes angiogenesis and vascular permeability (Ishibashi *et al.*, 1997; Krzystolik *et al.*, 2002). Clinical data analyses have revealed that the intravitreal administration of VEGF-A antagonists such as ranibizumab, bevacizumab, and pegaptanib arrests CNV progression and leakage from CNV, and improves visual acuity (Gragoudas *et al.*, 2004; Avery *et al.*, 2006; Brown *et al.*, 2006; Rosenfeld *et al.*, 2006). However, these drugs need to be used repeatedly at 4- to 6-week intervals, which raises concerns about injection-related adverse events including ocular inflammation, retinal injury, and endophthalmitis.

Advances in gene therapy technologies have been introduced as components of novel therapeutic approaches to the treatment of retinal diseases. Among several vector systems, recombinant adeno-associated viral (rAAV) and lentiviral vectors are likely to be useful for patients with chronic progressive retinal diseases, as these vectors can achieve gene transfer in nondividing cells and yield long-term transgene expression (Miyoshi *et al.*, 1997; Acland *et al.*, 2001; Lai *et al.*, 2002; Ikeda *et al.*, 2003). Clinical studies demonstrated the safety and efficacy of the subretinal delivery of rAAV carrying the *RPE65* gene for patients with Leber's congenital amaurosis, thereby providing the proof of concept for retinal gene therapy strategies (Bainbridge *et al.*, 2008; Hauswirth *et al.*,

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2008; Maguire *et al.*, 2008; Cideciyan *et al.*, 2009). The clinical study has shown that the viral vector solution left in the subretinal space is almost fully absorbed by 24 hr after injection (Bainbridge *et al.*, 2008); however, it is more preferable to remove the vector solution and resolve the retinal detachment (RD) during gene transfer surgery, because the outer retinal cells are deprived of trophic and metabolic support from the retinal pigment epithelium (RPE) and choroid vessels during RD. Hauswirth and colleagues reported that retinal thinning was observed in one of three patients after subretinal injection of rAAV-RPE65 (Hauswirth *et al.*, 2008). We developed novel simian lentiviral vectors pseudotyped with Sendai virus (SeV) hemagglutinin–neuraminidase (HN) and fusion (F) envelope proteins (SeV-F/HN-SIV) (Kobayashi *et al.*, 2003). The F and HN proteins of SeV mediate viral attachment and penetration (Lamb and Kolakofsky, 1996), and SeV-derived vectors have shown efficient gene transfer with only a few minutes of vector–cell interaction (Yonemitsu *et al.*, 2000; Masaki *et al.*, 2001). Therefore, SeV-F/HN-SIV vectors are expected to facilitate rapid transfection, and they may enable the removal of the vector solution shortly after injection.

Neovascularization is thought to be regulated by the balance between angiogenesis inducers and inhibitors (Folkman, 1995). Soluble Fms-like tyrosine kinase-1 (sFlt-1)/VEGF receptor (VEGFR)-1, a splice variant of the VEGF receptor Flt-1, is an endogenous inhibitor of VEGF-A (Kendall *et al.*, 1996), and has been used successfully to attenuate CNV in experimental models (Honda *et al.*, 2000; Bainbridge *et al.*, 2002; Gehlbach *et al.*, 2003). However, studies have indicated that VEGF-A acts as a survival factor in normal vascular endothelial cells and neural cells (Darland *et al.*, 2003; Lee *et al.*, 2007; Maharaj *et al.*, 2008), and some investigators have warned that a long-term blockade of VEGF signaling may lead to vascular and tissue dysfunction in the retina, as observed in the case of other systemic organs (Maynard *et al.*, 2003; Levine *et al.*, 2004; Hurwitz and Saini, 2006). Pigment epithelium-derived factor (PEDF) is a secreted glycoprotein isolated from the conditioned medium of human RPE (Tombran-Tink and Johnson, 1989; Steele *et al.*, 1993), and it has been shown to exhibit both antiangiogenic and neuroprotective properties (Taniwaki *et al.*, 1995; Dawson *et al.*, 1999). Retinal gene transfer of PEDF by adenoviral vectors substantially inhibited experimental CNV by inducing apoptosis in activated endothelial cells (Mori *et al.*, 2002); and this approach is currently under evaluation in a clinical trial investigating its efficacy in AMD patients (Campochiaro *et al.*, 2006). However, the effects of long-term PEDF overexpression on the normal retinal vasculature and neurons remain unknown.

In the present study, we aimed to evaluate the availability of SeV-F/HN-SIV vectors for retinal gene transfer. We demonstrated the rapid and efficient transduction ability of these vectors, both *in vitro* and *in vivo*. Using this new vector system, we assessed the efficacy and safety of long-term overexpression of the antiangiogenic agents sFlt-1 and PEDF in a mouse model of laser-induced CNV.

Material and Methods

SIV vectors

To produce third-generation recombinant SIV-based lentiviral vectors, HEK 293T cells were transfected with the packaging vector; gene transfer vectors encoding enhanced green fluorescent protein (EGFP), hPEDF, or hsFlt-1 driven by the cytomegalovirus promoter; the ReV expression vector; and the envelope vector (vesicular stomatitis virus G glycoprotein [pVSV-G] [Clontech Laboratories, Mountain View, CA] or pSeV-F/HN with a truncation of the cytoplasmic tail of F and the addition of the cytoplasmic tail of the SIV transmembrane envelope protein to the N terminus of HN) (Kobayashi *et al.*, 2003). The SIV vector lacking a transgene cassette (SeV-F/HN-SIV-Empty) was used as the control vector. The U3 region in the 3′ and 5′ long terminal repeat of SIV was deleted to induce self-inactivation. The viral titer was determined by the transduction of HEK 293T cells and expressed as transducing units (TU) per milliliter, and the viruses were kept at −80°C until just before use.

Cell culture and in vitro *gene transfer*

ARPE-19 cells, a human RPE-derived cell line, were purchased from the American Type Culture Collection (Manassas, VA). ARPE-19 cells were seeded in 24-well plates at 1×10^5 cells per well in serum-free Dulbecco's modified Eagle's medium (DMEM)–F12. Twelve hours later, either SeV-F/HN-SIV-luciferase or VSV-G-SIV-luciferase was added to each well at a multiplicity of infection (MOI) of 10. After various times of incubation with each vector solution, the culture medium was removed, the cells were washed twice with phosphate-buffered saline (PBS), and fresh medium was added. At 48 hr after gene transfer, the cells were harvested and subjected to luciferase assay.

Animals and in vivo *gene transfer*

Adult C57BL/6 mice were maintained humanely, with proper institutional approval, and in accordance with the statement of the Association for Research in Vision and Ophthalmology. All animal experiments were carried out according to 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 and according to the Law (No. 105) and Notification (No. 6) of the Japanese Government. The subretinal injection of each solution was performed as previously described (Murakami *et al.*, 2008b). Briefly, mice were anesthetized by inhalation of ether. A 30-gauge needle was inserted into the subretinal space of the peripheral retina in the nasal hemisphere via an external transscleral transchoroidal approach. Subretinal injection of vector solution (SeV-F/HN-SIV or VSV-G-SIV vector; 2.5×10^7 TU/ml $\times 2\,\mu$l) resulted in a dome-shaped detachment of about half the retina. To assess the effects of vector–cell interaction time on retinal gene transfer, we used two different subretinal administration techniques: either simple injection (*leave*) or removal 5 min after gene transfer by draining the subretinal vector solution and injecting balanced salt solution (BSS) after drainage (*remove*). The following assessments for duration of transgene expression, the efficacy of antiangiogenic factors for laser-induced CNV, and their retinal toxicity were performed by the *remove* procedure. Eyes that sustained marked surgical trauma (e.g., retinal or subretinal hemorrhage, bacterial infection) were excluded from further analyses.

Luciferase assay

Procedures used for the luciferase assay have been described previously (Ikeda *et al.*, 2002). ARPE-19 cells and enucleated mouse eyes were treated with 1× lysis buffer (Promega, Madison, WI) with a protease inhibitor cocktail and centrifuged, and then 20-μl samples of the supernatants were mixed with 100 μl of luciferase assay buffer. Light intensity was measured with a luminometer (model LB 9507; Berthold Technologies, Bad Wildbad, Germany) with 10-sec integration.

Detection of GFP expression in vivo *and indocyanine green angiography*

GFP expression in the mouse retina was examined with a scanning laser ophthalmoscope (Heidelberg retinal angiograph; Heidelberg Engineering, Heidelberg, Germany), using a 488-nm excitation laser light and a 500-nm barrier filter. Indocyanine green (ICG) angiography was performed 7 min after intraperitoneal injection of 0.2 ml of 10% ICG, using a 795-nm excitation diode laser light and an 810-nm barrier filter, as previously described (Janssen *et al.*, 2008).

Histological examination

Mouse eyes were enucleated, and both paraffin and cryosections were prepared. To prepare the paraffin sections, the eyes were fixed with 4% paraformaldehyde in PBS for 24 hr, and were then mounted in paraffin. For the cryosections, the eyes were frozen in liquid nitrogen, and 5-μm-thick sections were prepared along the horizontal meridian. The sections were subsequently stained with hematoxylin and eosin. The number of cells in the outer nuclear layer was counted per 250 μm at six points around the retinal section (A1–A3, from the ora serrata to the optic nerve of the temporal hemisphere; A4–A6, from the optic nerve to the ora serrata of the nasal hemisphere).

Immunohistochemistry

Five-micrometer-thick cryosections were cut, air dried, and fixed in cold acetone for 10 min. The sections were blocked with 3% nonfat dried milk and labeled with rabbit anti-GFP polyclonal antibody (diluted 1:300; Invitrogen Molecular Probes, Eugene, OR) at 4°C for 24 hr. After biotinylated goat anti-rabbit IgG (H+L) (diluted 1:200; Vector Laboratories, Burlingame, CA) was applied as a secondary antibody, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (diluted 1:100; BD Biosciences, San Jose, CA). After labeling, 4′,6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei. Immunofluorescence images were acquired with an Olympus BX51 microscope with a fluorescence attachment (Olympus, Tokyo, Japan). For negative controls, the primary antibody was omitted.

Enzyme-linked immunosorbent assay

The protein content of mouse eyes was determined with an enzyme-linked immunosorbent assay (ELISA) kit for human PEDF (not available for mouse PEDF; Chemicon International, Temecula, CA) and human sFlt-1 (not available for mouse sFlt-1; R&D Systems, Minneapolis, MN). For the preparation of ocular tissue, conjunctival and muscular tissues were removed from enucleated eyes. The eyes were washed with PBS, minced with scissors in 500 μl of 1× lysis buffer with a protease inhibitor cocktail, and centrifuged at 15,000 rpm for 5 min at 4°C. The supernatants were subjected to ELISA according to the manufacturer's instructions.

Laser-induced CNV

Laser photocoagulation (630 nm, 150 mW, 0.1 sec, 75 μm) was performed on one eye (four spots per eye) of each animal, using a slit-lamp delivery system (NIDEK, Aichi, Japan), as previously described (Tsutsumi *et al.*, 2003). Burns were performed at the 2, 5, 7, and 10 o'clock positions around the optic disk such that two laser-treated sites were within the vector-injected area, and the other two laser-treated sites were located outside of that area. Laser injury disrupted the RPE, Bruch's membrane, and choroid, and induced the subsequent proliferation and migration of choroidal endothelial cells, resulting in CNV. On day 14 after the laser induction of injury, the eyes were enucleated and fixed with 4% paraformaldehyde. The eyecups, obtained by removing the anterior segments and the entire neural retina, were incubated with FITC-conjugated isolectin B4 (Vector Laboratories) at 4°C for 24 hr. Then, CNV was visualized by Olympus BX51 fluorescence microscopy, and the area was measured with National Institutes of Health (Bethesda, MD) ImageJ software.

Transmission electron microscopy

The eyes were enucleated, and the posterior segments were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 *M* cacodylate buffer with 0.08 *M* $CaCl_2$ at 4°C. Sections of retina, RPE, and choroid complex in the vector-injected area were postfixed for 1.5 hr in 2% aqueous OsO_4, dehydrated in ethanol and water, and embedded in Epon. Ultrathin sections were cut from blocks and stained with saturated, aqueous uranyl acetate and Sato's lead stain. The specimens were observed with a Hitachi H-7650 electron microscope (Hitachi High-Technologies, Tokyo, Japan).

Statistical analyses

All values are expressed as means ± SD. Statistical differences were assessed by analysis of variance (ANOVA) followed by Tukey–Kramer adjustments for multiple comparisons. Numbers per group are as indicated. A p value of less than 0.05 was considered statistically significant.

Results

SeV-F/HN-SIV vectors require only a brief vector–cell interaction to achieve efficient gene transfer

We previously demonstrated that recombinant Sendai viral (rSeV) vectors exhibited efficient gene transfer into the RPE of the rat retina with a brief exposure time (Ikeda *et al.*, 2002). To determine whether SeV F- and HN-pseudotyped SIV vectors would be capable of rapid and efficient transfection, we first investigated the vector–cell interaction time-dependent transgene expression level. In a human RPE-derived cell line, ARPE-19, conventional VSV-G-pseudotyped SIV (VSV-G-SIV) vectors encoding luciferase showed an interaction time-dependent increase in luciferase expression, and more than 24 hr of exposure was required to achieve maximal gene transfer (Fig. 1A). In contrast, with SeV-F/HN-SIV-luciferase, only 1 min of exposure yielded efficient luciferase expression at

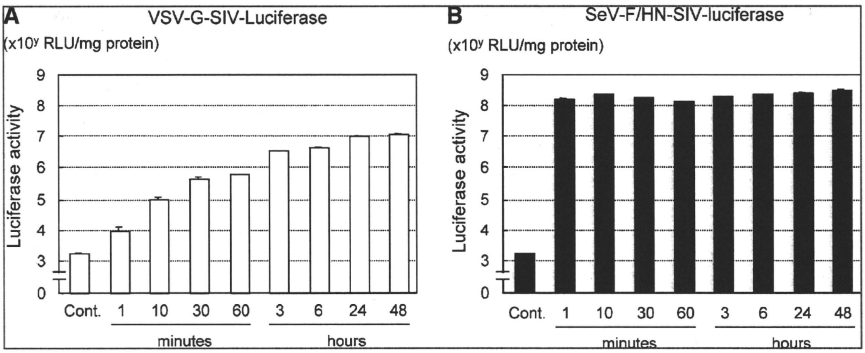


FIG. 1. Effect of vector–cell interaction time on gene transfer to ARPE-19 cells. (**A** and **B**) ARPE-19 cells were exposed to (**A**) VSV-G-SIV-luciferase or (**B**) SeV-F/HN-SIV-luciferase at a multiplicity of infection (MOI) of 10 for each time period, and the cells were washed three times with PBS ($n = 4$ each). Forty-eight hours later, the cells were subjected to luciferase assay. VSV-G-SIV vectors required more than 24 hr of exposure time to achieve maximal gene transfer, whereas only 1 min of exposure was sufficient for SeV-F/HN-SIV vectors to reach the maximal expression level. (Note the log scale.) Cont., control; RLU, relative light units.

levels similar to those observed with 48 hr of exposure (Fig. 1B). Moreover, the luciferase expression levels achieved with SeV-F/HN-SIV vectors were more than 10-fold higher than the peak value obtained with VSV-G-SIV vectors (Fig. 1A and B).

Next, we assessed the *in vivo* transduction efficiency of SeV-F/HN-SIV vectors in the mouse retina. To assess the effects of vector–cell interaction time on retinal gene transfer, we subretinally injected the SIV vectors by one of two different techniques, that is, either the vector solution was left in the subretinal space (*leave*), or the solution was removed 5 min after vector injection (*remove*). In eyes treated with VSV-G-SIV-luciferase, transgene expression in the *remove*

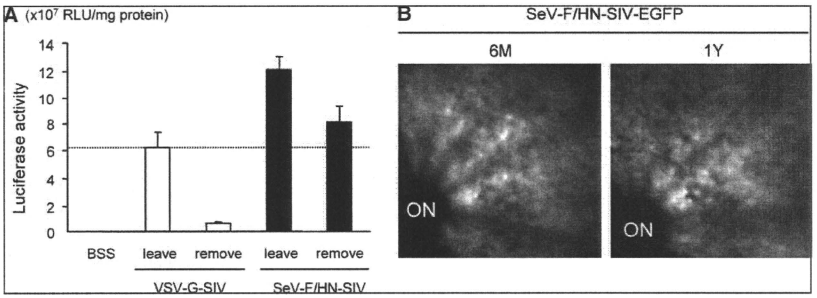
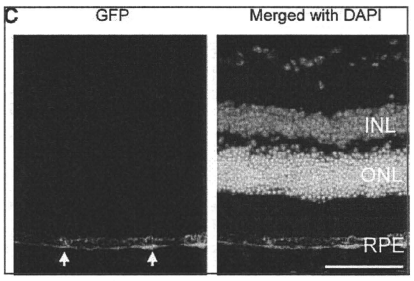


FIG. 2. SeV-F/HN-SIV vector-mediated retinal gene transfer with brief exposure time. (**A**) Effect of vector exposure time *in vivo*. VSV-G-SIV-luciferase or SeV-F/HN-SIV-luciferase was injected into the subretinal space of mice by two different techniques: simple injection (*leave*), or removal 5 min after injection (*remove*). The eyes were subjected to luciferase assay 2 weeks after gene transfer ($n = 5$ or 6). (**B**) Retinal GFP expression in eyes treated with SeV-F/HN-SIV-EGFP. GFP fluorescence was assessed by scanning laser ophthalmoscopy 6 months (6M) and 1 year (1Y) after vector injection. ON, optic nerve. (**C**) Immunohistochemical staining for GFP in retina treated with SeV-F/HN-SIV-EGFP. Arrows indicate staining in the retinal pigment epithelium (RPE). DAPI, 4′,6-diamidino-2-phenylindole; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar, 100 μm.



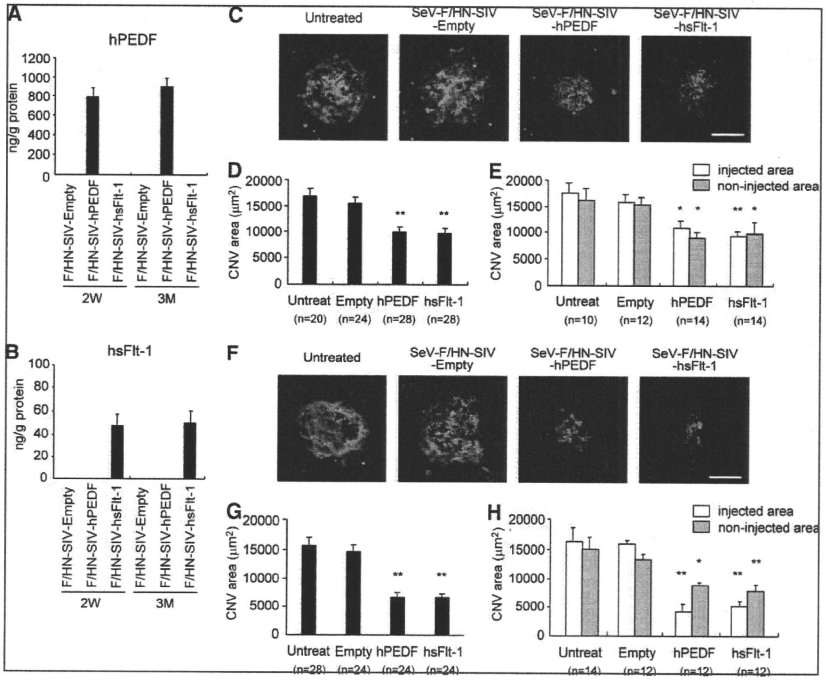


FIG. 3. Suppression of laser-induced choroidal neovascularization (CNV) by subretinal injection of SeV-F/HN-SIV-hPEDF or SeV-F/HN-SIV-hsFlt-1. (**A** and **B**) ELISA to detect (**A**) human pigment epithelium-derived factor (hPEDF) and (**B**) human soluble Fms-like tyrosine kinase-1 (hsFlt-1) protein 2 weeks and 3 months after vector injection ($n = 5$ or 6). (**C–H**) Representative CNV lesions of choroidal flat mounts and quantitative analysis of the CNV area. Laser photocoagulation was performed (**C–E**) 2 weeks or (**F–H**) 3 months after each vector injection ($n = 20$–28). Eyes left untreated and those treated with SeV-F/HN-SIV-Empty were used as controls. The graphs show CNV size in the total retinal area (solid columns; **D** and **G**) and in vector-injected areas (open columns) and noninjected areas (shaded columns; **E** and **H**). $^{*}p < 0.05$, $^{**}p < 0.01$ versus untreated eyes. Scale bars, 100 μm.

group was markedly reduced, by approximately one-tenth, in comparison with that of the *leave* group (Fig. 2A). In contrast, eyes treated with SeV-F/HN-SIV-luciferase showed efficient transgene expression even in the *remove* group, and the luciferase expression levels in the SeV/F/HN-SIV-vector *remove* group were 1.3-fold higher than those of the VSV-G-SIV-vector *leave* group (Fig. 2A). These data indicate that brief vector–cell contact is sufficient for SeV-F/HN-SIV vectors to achieve efficient retinal gene transfer.

Stable long-term transgene expression in RPE by SeV-F/HN-SIV vectors

Our previous study demonstrated that subretinal injection of VSV-G-SIV vector at 2.5×10^7 TU/ml resulted in sustained transgene expression over a 1-year period in the rat retina (Ikeda *et al.*, 2003). To determine the longevity of SeV-F/HN-SIV vector-mediated retinal gene transfer, we monitored the time course of transgene expression using GFP as a reporter. Mouse retinas treated with SeV-F/HN-SIV-EGFP by the *remove* procedure showed intense GFP fluorescence in an area corresponding to the vector-injected area, and the extent of GFP fluorescence was maintained for at least 1 year (Fig. 2B). Histological examination revealed that GFP expression was located in the RPE layer (Fig. 2C), as previously observed in the case of rSeV-mediated retinal gene transfer (Ikeda *et al.*, 2002; Murakami *et al.*, 2008b). Taken together, these findings indicate that SeV-F/HN-SIV vectors exhibit the advantageous features of both SeV vectors and SIV vectors, that is, rapid transduction and long-term transgene expression ability, in retinal tissue.

SeV-F/HN-SIV vector-mediated retinal delivery of antiangiogenic factors suppresses laser-induced CNV

Next, we sought to investigate the effects of SeV-F/HN-SIV vector-mediated PEDF or sFlt-1 gene transfer on

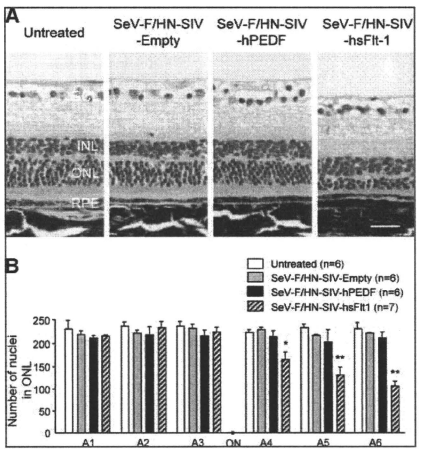


FIG. 4. Assessment of potential retinal toxicity resulting from gene transfer of PEDF or sFlt-1 in normal adult mice. (**A**) Histological findings of the vector-injected area in the retina of untreated mice, and of mice treated with SeV-F/HN-SIV-Empty, SeV-F/HN-SIV-hPEDF, or SeV-F/HN-SIV-hsFlt-1, 6 months after vector injection. GCL, ganglion cell layer. Scale bar, 25 μm. (**B**) Quantitative analysis of cells in the outer nuclear layer (ONL). The number of nuclei in the ONL per 250 μm was counted at six points along the horizontal meridian of the eye ($n = 6$ or 7). Region A4–A6 corresponds to the nasal hemisphere of the eye. $^*p < 0.05$, $^{**}p < 0.01$ versus untreated eyes.

laser-induced CNV. Mouse eyes were treated with SeV-F/HN-SIV-Empty, -hPEDF or -hsFlt-1 at 2.5×10^7 TU/ml by the *remove* procedure. ELISA revealed that sufficient levels of hPEDF (790.3 ± 96.1 ng/g protein) and hsFlt-1 (47.6 ± 10.0 ng/g protein) were obtained 2 weeks after vector injection, and were sustained for at least 3 months postinjection (Fig. 3A and B). We performed laser photocoagulation 2 weeks after vector injection, and assessed the area of CNV 2 weeks after the laser treatment. CNV size was significantly reduced with SeV-F/HN-SIV vector-mediated retinal delivery of hPEDF or hsFlt-1, compared with CNV size in the eyes untreated or treated with SeV-F/HN-SIV-Empty ($p < 0.01$; Fig. 3C and D). Treatment with SeV-F/HN-SIV-hPEDF or hsFlt-1 also showed potent suppression of CNV when laser photocoagulation was performed 3 months after vector injection ($p < 0.01$; Fig. 3F and G), suggesting potential long-term inhibitory effects on CNV. Next, we compared CNV size within and outside of the vector-injected area. Treatment with SeV-F/HN-SIV-hPEDF or -hsFlt-1 was associated with significant CNV suppression in both areas ($p < 0.05$; Fig. 3E and H).

Assessment of retinal toxicity with long-term overexpression of antiangiogenic factors

To determine any potential adverse effects of long-term overexpression of antiangiogenic factors on the retina, we next performed a histological examination of SeV-F/HN-SIV-treated retinas 6 months after vector injection by the *remove* procedure. Eyes that had undergone 6 months of sustained overexpression of hPEDF showed no significant changes in retinal structure; however, 6 months of sustained overexpression of hsFlt-1 resulted in a significant loss of photoreceptors in the vector-injected area ($p < 0.05$; Fig. 4A and B). These findings suggest that long-term blockade of VEGF signaling may be deleterious for maintaining retinal homeostasis in adult mice.

In the adult mouse retina, VEGF-A is secreted basally from the RPE, and fetal liver kinase-1 (Flk-1)/VEGFR-2 is expressed on the endothelium of the choriocapillaris facing the RPE layer, which is suggestive of a paracrine interaction between the two tissues (Saint-Geniez *et al.*, 2006). To analyze the changes in choroidal vasculature observed with long-term overexpression of sFlt-1, we performed ICG angiography by *in vivo* scanning laser ophthalmoscopy. Retinas treated with SeV-F/HN-SIV-hPEDF or -Empty showed no significant alteration of ICG fluorescence, compared with that of untreated retinas. In contrast, fluorescein-filling defects of choroidal vessels were observed in those retinas treated with SeV-F/HN-SIV-hsFlt-1 in the area corresponding to the vector-injected site (Fig. 5). Ultrastructural analysis by transmission electron microscopy revealed that there were choriocapillaris vessels filled with densely packed, malformed erythrocytes with adjoining thrombocytes 1 month after SeV-F/HN-SIV-hsFlt-1 treatment (Fig. 6A and B). These findings were not observed in retinas untreated or treated with SeV-F/HN-SIV-Empty or -hPEDF (Fig. 6C and data not shown). No alteration of choriocapillaris endothelial cell fenestrations was observed between the groups (Fig. 6D). Ultrastructural signs

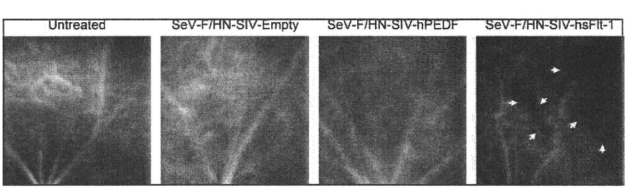


FIG. 5. *In vivo* imaging of choroidal vessels by indocyanine green (ICG) angiography. Shown are ICG angiograms of the vector-injected area of mouse retinas 6 months after treatment with SeV-F/HN-SIV-Empty, SeV-F/HN-SIV-hPEDF, or SeV-F/HN-SIV-hsFlt-1 ($n = 4$ each). Eyes of untreated mice were used as a control. Arrows indicate defects in choroidal circulation in a retina treated with SeV-F/HN-SIV-hsFlt-1.

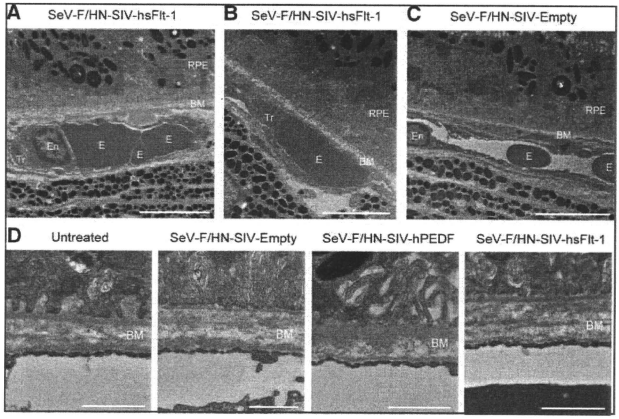


FIG. 6. Ultrastructural analysis of the choriocapillaris by transmission electron microscopy. (**A–C**) Electron microscopy of the choriocapillaris in vector-injected areas 1 month after treatment with (**A** and **B**) SeV-F/HN-SIV-hsFlt-1 or (**C**) SeV-F/HN-SIV-Empty. Erythrocytes (*E*) and thrombocytes (*Tr*) were densely packed in the choriocapillaris lumen (**A** and **B**). BM, Bruch's membrane; En, endothelial cell. Scale bars, 5 μm. (**D**) Endothelial cell fenestrations of the choriocapillaris in retina untreated or treated with SeV-F/HN-SIV-Empty, SeV-F/HN-SIV-hPEDF, or SeV-F/HN-SIV-hsFlt-1. No alteration of choriocapillaris fenestrations was observed between the groups. Scale bars, 1 μm.

of apoptosis of endothelial cells or RPE cells were not evident (data not shown). These findings indicate that long-term retinal delivery of PEDF is a safe and effective approach to suppressing CNV, but that long-term sFlt-1 expression may disturb the homeostatic balance of the retina, resulting in a disruption of the choroidal circulation and photoreceptor degeneration.

Discussion

In the present study, we characterized novel SIV-based lentiviral vectors pseudotyped with SeV-F and SeV-HN for retinal gene transfer. The key observations made in this study are as follows: (1) a brief vector–cell interaction period was sufficient for the SeV-F/HN-SIV vectors to achieve efficient gene transfer into RPE cells; (2) transgene expression mediated by SeV-F/HN-SIV vectors was stable and sustained over a 1-year period; (3) SeV-F/HN-SIV vector-mediated retinal gene transfer of hPEDF or hsFlt-1 substantially suppressed experimental CNV in mice; and (4) long-term overexpression of hPEDF did not exert any significant deleterious effects on the retinal tissue, whereas the long-term overexpression of hsFlt-1 resulted in photoreceptor degeneration in association with choroidal circulation defects. The rapid transduction ability of SeV-F/HN-SIV vectors is in clear contrast to reported findings obtained with conventional VSV-G-pseudotyped lentiviral vectors, rAAV, or adenoviral vectors (Teramoto *et al.*, 1998; Masaki *et al.*, 2001). To the best of our knowledge, this is the first report to demonstrate a limitation of the use of sFlt-1 for retinal gene therapy.

In this study, we demonstrated that the conventional VSV-G-pseudotyped SIV vectors required more than 24 hr of interaction time to achieve maximal gene transfer. rAAV and adenoviral vectors, which have been used in clinical trials of gene therapy for retinal diseases, also showed an interaction time-dependent increase in transgene expression and required more than 12 hr to reach the maximal expression level (Maeda *et al.*, 1998; Teramoto *et al.*, 1998). In contrast, the novel SIV vectors pseudotyped with SeV-F and SeV-HN achieved high-level gene transfer within several minutes, both *in vitro* and *in vivo*, as seen in previous reports using rSeV vectors (Ikeda *et al.*, 2002). This unique feature of SeV-F/HN-SIV vectors enables the removal of subretinal vector solution and the resolution of RD during gene transfer surgery. Although subretinal injection of rAAV-RPE65 at the doses used in clinical studies has been shown to be safe in dogs and nonhuman primates (Jacobson *et al.*, 2006a,b), retinal thinning was observed after subretinal injection of rAAV-RPE65 in one of three patients (Hauswirth *et al.*, 2008). Of note, optical coherence tomography showed that the thickness of the outer nuclear layer was especially reduced in this patient. Retinal neurons, which are chronically stressed by degenerative retinopathy, may be more vulnerable to environmental changes. It is premature to assess the complications associated with subretinal viral vector injection, due to the small number of patients treated; however, we believe that the *remove* technique may reduce the nutrient starvation in outer retinal cells caused by RD and may provide safer retinal gene transfer. In addition, SeV-F/HN-SIV vectors exhibited stable and long-term transgene expression in the RPE over a 1-year period. In the clinical setting, most cases of AMD progress over several years, and long-lasting therapeutic effects are required for treatment. One limitation of the currently available anti-VEGF drugs is their short half-life in the eye and the need for repeated injections (Bakri *et al.*, 2007). Therefore, SeV-F/HN-SIV vector-mediated continuous delivery of therapeutic

proteins would be an attractive approach for the long-term inhibition of CNV in AMD patients.

Clinical studies have demonstrated the beneficial therapeutic effects of intravitreal injection of anti-VEGF-A drugs such as pegaptanib (an aptamer specific for VEGF-A_{165}) and ranibizumab (an Fab fragment of a humanized monoclonal pan-VEGF-A antibody) (Gragoudas *et al.*, 2004; Brown *et al.*, 2006; Rosenfeld *et al.*, 2006). However, some investigators have raised concerns about the safety of long-term intraocular VEGF-A neutralization (Sang and D'Amore, 2008). In this study, we delivered the *sFlt-1* gene into the RPE of the mouse retina via subretinal injection of SeV-F/HN-SIV-hsFlt-1, and we demonstrated that 6-month sustained overexpression of sFlt-1 resulted in photoreceptor degeneration. Although we previously demonstrated that subretinal injection of high-titer VSV-G-SIV vector (2.5×10^8 TU/ml) induced sustained inflammation around the RPE and caused retinal degeneration (Ikeda *et al.*, 2003), such inflammatory reaction and retinal damage were not observed in retinas treated with SeV-F/HN-SIV-Empty or -hPEDF at 2.5×10^7 TU/ml, or in retinas treated with VSV-G-SIV vector at this dose (Ikeda *et al.*, 2003), suggesting that overexpression of sFlt-1 is a major mediator of photoreceptor degeneration after SeV-F/HN-sFlt-1 treatment. In line with our data, Saint-Geniez and colleagues demonstrated that systemic VEGF neutralization by adenoviral vector-mediated overexpression of sFlt-1 induced the apoptosis of retinal neuronal cells, including photoreceptors (Saint-Geniez *et al.*, 2008). However, there are several contradictory reports on the retinal effect of sFlt-1. Lai and colleagues and Pechan and colleagues demonstrated the long-term safety of rAAV vector-mediated retinal gene transfer of sFlt-1 (Lai *et al.*, 2005; Pechan *et al.*, 2009). One reason for these differences might involve differences in the levels of sFlt expression, because hsFlt-1 protein levels achieved with rAAV vectors were substantially lower than those observed in our study and in the study by Saint-Geniez and colleagues. In another study, Ueno and colleagues generated transgenic mice with doxycycline-inducible expression of sFlt-1 in their photoreceptors, and no significant changes in retinal structure or function were observed after a 7-month period of doxycycline treatment (Ueno *et al.*, 2008). The reasons for these discrepant results remain unclear, but differences between cells expressing sFlt-1 may account for such discrepancies. Because VEGF-A is secreted basally from the RPE (Blaauwgeers *et al.*, 1999), sFlt-1 expressed by the RPE may more effectively sequester VEGF-A and disrupt the homeostatic balance of the RPE–choriocapillary complex. These retinal effects of sFlt-1 are not necessarily identical to those of anti-VEGF-A drugs, because sFlt-1 binds not only to VEGF-A, but also to other VEGF family members such as VEGF-B, which exerts a potent neuroprotective effect on retinal neurons (Takahashi and Shibuya, 2005; Li *et al.*, 2008). However, long-term follow-up of patients administered treatment with anti-VEGF-A drugs is required to monitor the eyes for retinal toxicity, as intravitreal injection of bevacizumab is known to cause mitochondrial swelling and disruption of the cristae in the photoreceptors of rabbit eyes (Inan *et al.*, 2007).

The mechanisms of photoreceptor loss after VEGF neutralization remain unknown. One possible explanation would be that sFlt-1 may block the neuroprotective signaling of VEGF-A in photoreceptors. Nishijima and colleagues reported that VEGF-A directly protected retinal neurons in the ganglion cell layer and inner nuclear layer after ischemic reperfusion injury via the activation of Flk-1 (Nishijima *et al.*, 2007). However, it remains unclear whether or not VEGF-A also provides direct neuroprotection to photoreceptors, because the levels of expression of VEGF receptors in photoreceptors were significantly lower than those in inner retinal neurons (Stitt *et al.*, 1998; Nishijima *et al.*, 2007; Li *et al.*, 2008). As an alternative possibility, the impairment of choroidal circulation, which provides vascular support for photoreceptors and RPE cells, might induce photoreceptor degeneration. Flk-1 is strongly expressed in the endothelium of the choriocapillaris facing the RPE layer in the adult mouse retina (Saint-Geniez *et al.*, 2006). In this study, we showed that persistent expression of sFlt-1 in the RPE resulted in a disruption of choroidal circulation, which suggests that VEGF signaling plays an essential role in maintaining the choroidal circulation. This explanation may also be supported by the finding that conditional inactivation of VEGF-A expression in the RPE layer resulted in an absence of choroidal vessels, disorganization of photoreceptors, and loss of visual function (Marneros *et al.*, 2005). In ultrastructural analysis, we found choriocapillaris vessels filled with packed erythrocytes and thrombocytes after sFlt-1 gene transfer. These findings were similar to those made after intravitreal bevacizumab treatment in nonhuman primates (Peters *et al.*, 2007), and suggest that these obstructions of the choriocapillaris might be a cause leading to choroidal circulation defects. No alteration of choriocapillaris endothelial cell fenestrations was observed in this study. Although Peters and colleagues reported early reduction of fenestrations after bevacizumab treatment, the reduction was recovered transiently (Peters *et al.*, 2007). These changes in fenestrations were not observed after sustained systemic or local overexpression of sFlt-1 (Saint-Geniez *et al.*, 2008; Ueno *et al.*, 2008), suggesting that the loss of fenestrations might be a transient effect and would not be detectable at later time points.

Retinal gene transfer of PEDF by SeV-F/HN-SIV vectors efficiently inhibited experimental CNV at a level equivalent to that seen with SeV-F/HN-SIV-hsFlt-1, and did not exert any toxic effects on the normal retinal tissue. It has been demonstrated that PEDF induces the apoptosis of stimulated endothelial cells, but not that of quiescent endothelial cells, by targeting Fas/CD95 and the nuclear factor of activated T cells upregulated or activated by VEGF-A (Volpert *et al.*, 2002; Zaichuk *et al.*, 2004). Using the same mechanism, PEDF also induces the apoptosis of endothelial cells stimulated by other angiogenic factors such as basic fibroblast growth factor. Taken together, our findings and those of previous reports indicate that PEDF may specifically target activated endothelial cells during neovascularization, without affecting mature existing vessels. In addition to its antiangiogenic effects, PEDF has a neuroprotective effect on retinal neuronal cells (Miyazaki *et al.*, 2003; Takita *et al.*, 2003). We reported that PEDF directly inhibited photoreceptor apoptosis by regulating the mitochondrial release of apoptosis-inducing factor via Bcl-2 upregulation (Murakami *et al.*, 2008a). These results suggest that gene therapy strategies using PEDF, which possesses both antiangiogenic and neuroprotective abilities, may be safe and effective for the treatment of retinal neovascular and degenerative diseases.

In conclusion, we have demonstrated that novel SIV vectors pseudotyped with SeV-F and SeV-HN showed rapid and

efficient gene transfer to the RPE; thus, this system would enable the removal of subretinal vector solution shortly after vector injection. The long-term retinal delivery of PEDF by SeV-F/HN-SIV vectors was safe and effective at suppressing experimental CNV, whereas long-term delivery of sFlt-1 led to a disruption of the choroidal circulation and photoreceptor degeneration. These findings indicate that retinal gene therapy using PEDF may be a useful therapeutic strategy for the long-term management of CNV in AMD patients with a higher safety profile.

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Author Disclosure Statement

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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

GLAUCOMA 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-independent 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 m*M* 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

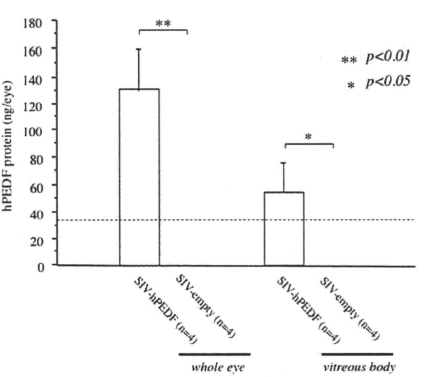

FIG. 1. SIV-mediated human pigment epithelium-derived factor (hPEDF) expression in the eye. Abundant hPEDF protein was expressed in the eye after subretinal injection of SIV-hPEDF, and hPEDF protein secreted from the retinal pigment epithelium (RPE) diffused well into the vitreous body.

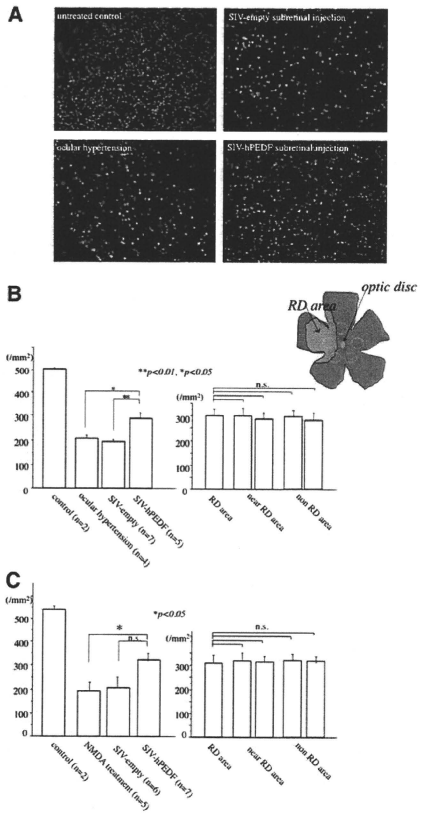

FIG. 2. Morphological assessment of neuroprotective effects against transient ocular hypertension-induced and *N*-methyl-D-aspartic acid (NMDA)-induced retinal ganglion cell (RGC) injuries. (**A**) Retrograde labeling of RGCs in flat-mount retinas 7 days after RGC injury. The density of RGCs in the SIV-hPEDF-treated 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.

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 ± 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).

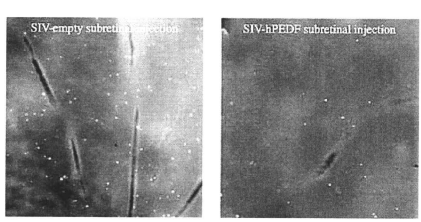

FIG. 3. TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) staining of retinal flat-mounts of transient ocular hypertension eyes. TUNEL staining of apoptotic RGCs was determined in flat-mount retinas 2 days after RGC injury. The cell density of the TUNEL-positive cells (the apoptotic 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

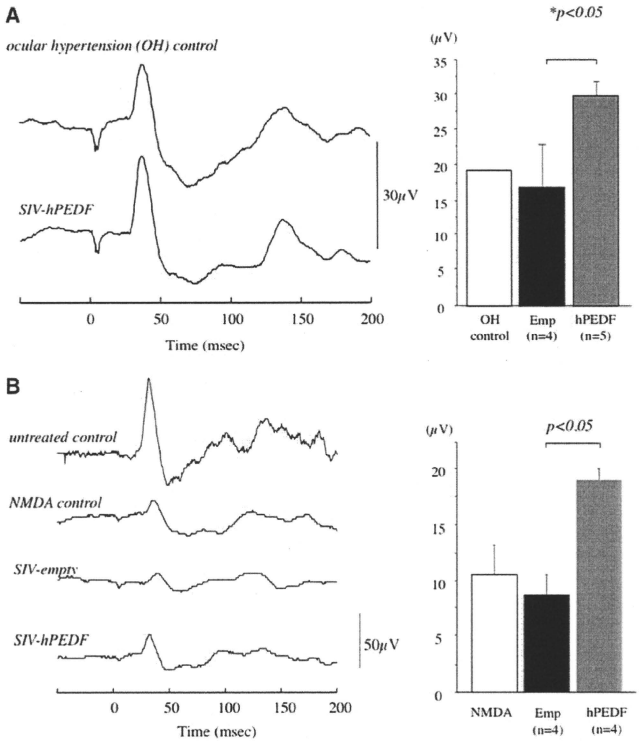

FIG. 4. Functional assessment of neuroprotective effects against transient ocular hypertension-induced and NMDA-induced RGC injuries. (**A**) Typical wave patterns and quantitative analyses of transient ocular hypertension-induced eyes are demonstrated. Significantly higher b-wave amplitudes of pattern 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 neuroprotective 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.

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Conflict of Interest Statement

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

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