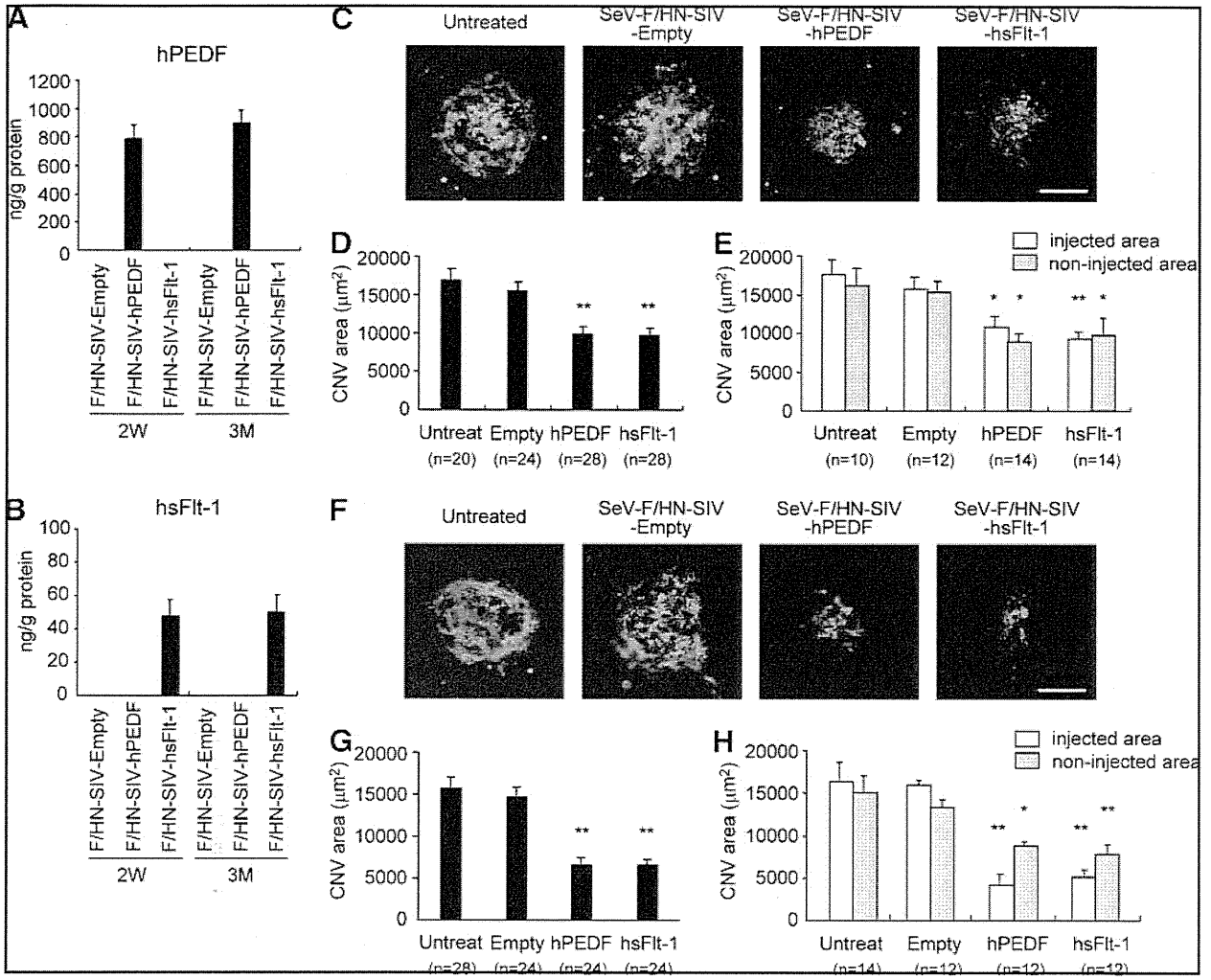

FIG. 3. Suppression of laser-induced choroidal neovascularization (CNV) by subretinal injection of SeV-F/HN-SIV-hPEDF or SeV-F/HN-SIV-hsFlt-1. (**A** and **B**) ELISA to detect (**A**) human pigment epithelium-derived factor (hPEDF) and (**B**) human soluble Fms-like tyrosine kinase-1 (hsFlt-1) protein 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

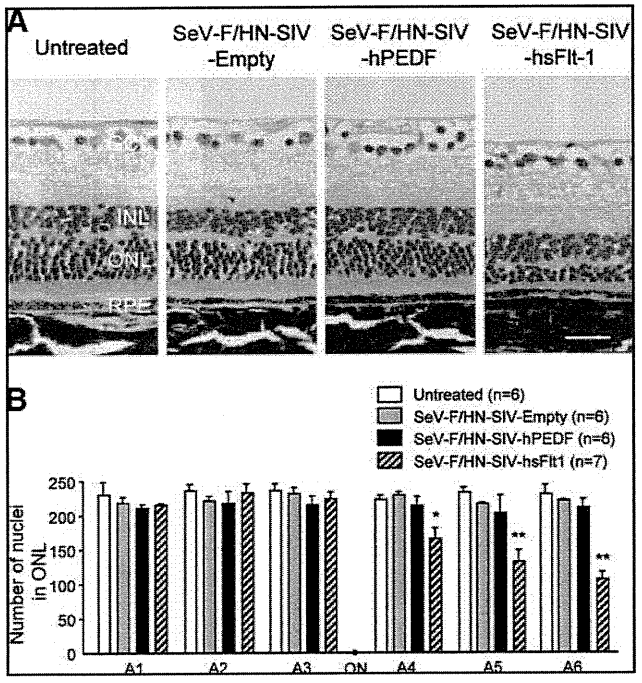

FIG. 4. Assessment of potential retinal toxicity resulting from gene transfer of PEDF or sFlt-1 in normal adult mice. (**A**) Histological findings of the vector-injected area in the retina of untreated mice, and of mice treated with SeV-F/HN-SIV-Empty, SeV-F/HN-SIV-hPEDF, or SeV-F/HN-SIV-hsFlt-1, 6 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

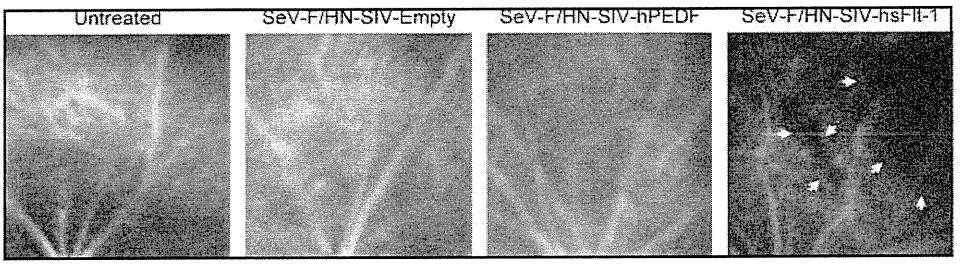

FIG. 5. *In vivo* imaging of choroidal vessels by indocyanine green (ICG) angiography. Shown are ICG angiograms of the vector-injected area of mouse retinas 6 months after treatment with SeV-F/HN-SIV-Empty, SeV-F/HN-SIV-hPEDF, or SeV-F/HN-SIV-hsFlt-1 ($n = 4$ each). Eyes of untreated mice were used as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

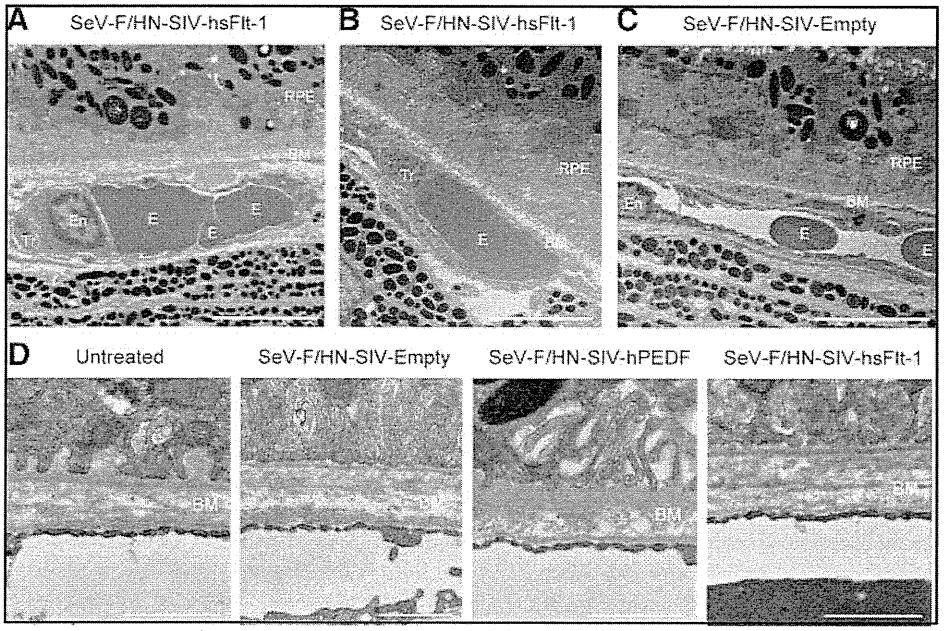

FIG. 6. Ultrastructural analysis of the choriocapillaris by transmission electron microscopy. (**A–C**) Electron microscopy of the choriocapillaris in vector-injected areas 1 month after treatment with (**A** and **B**) SeV-F/HN-SIV-hsFlt-1 or (**C**) SeV-F/HN-SIV-Empty. Erythrocytes (*E*) and thrombocytes (*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

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

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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 overexpression, 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

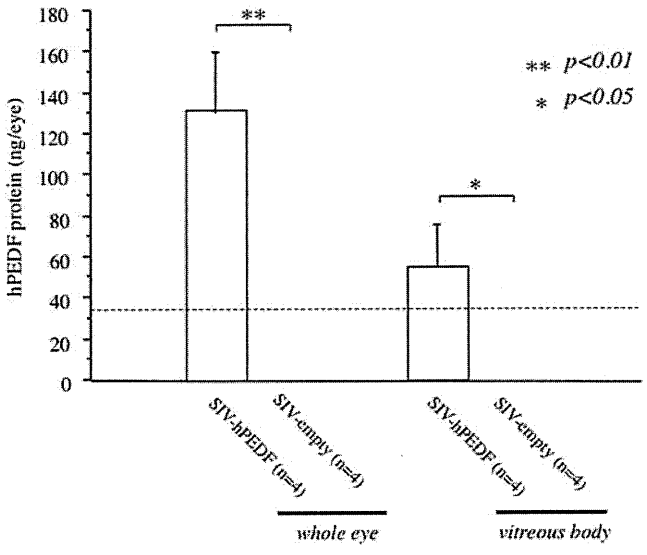

FIG. 1. SIV-mediated human pigment epithelium-derived factor (hPEDF) expression in the eye. Abundant hPEDF protein was expressed in the eye after subretinal injection of SIV-hPEDF, and hPEDF protein secreted from the retinal pigment epithelium (RPE) diffused well into the vitreous body.

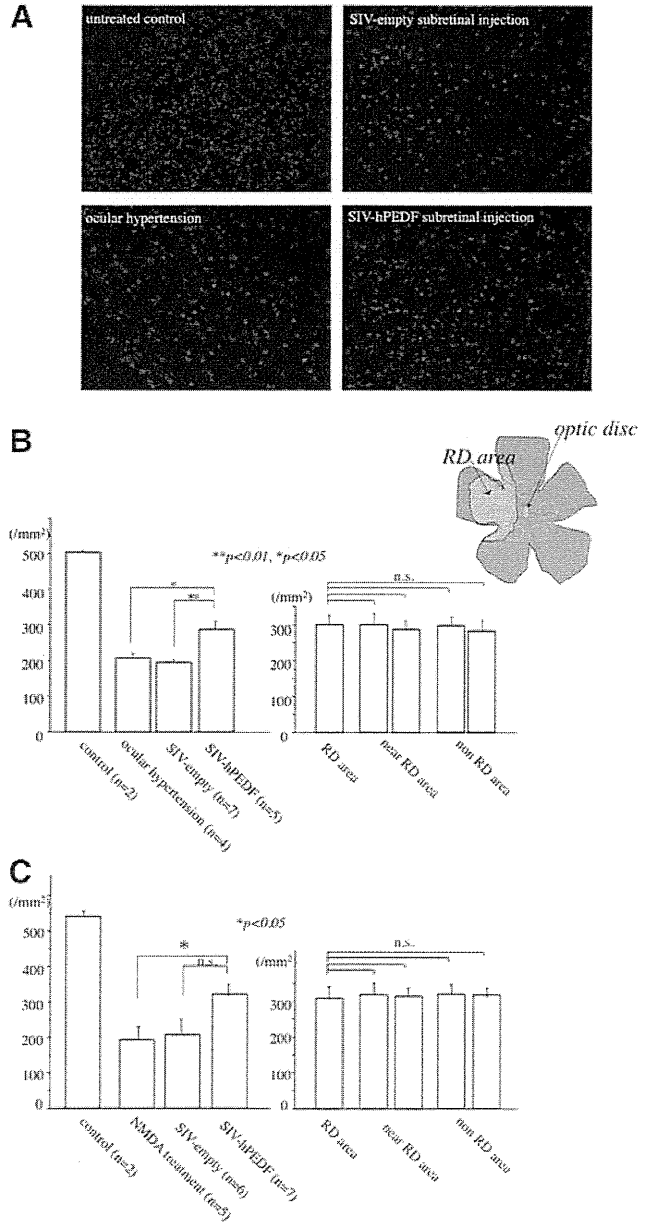

FIG. 2. Morphological assessment of neuroprotective effects against transient ocular hypertension-induced and *N*-methyl-D-aspartic acid (NMDA)-induced retinal ganglion cell (RGC) injuries. (**A**) Retrograde labeling of RGCs in flat-mount retinas 7 days after RGC injury. The density of RGCs in the SIV-hPEDF-treated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

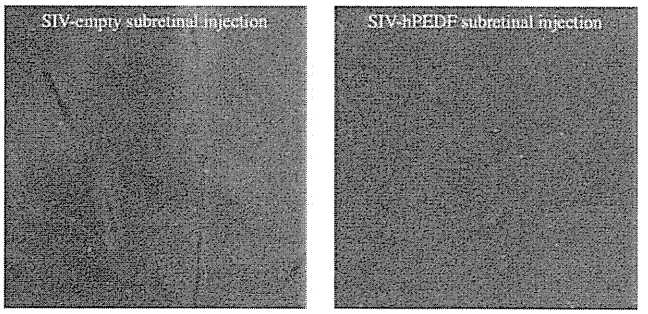

FIG. 3. TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) staining of retinal flat-mounts of transient ocular hypertension eyes. TUNEL staining of apoptotic RGCs was determined in flat-mount retinas 2 days after RGC injury. The cell density of the TUNEL-positive cells (the apoptotic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

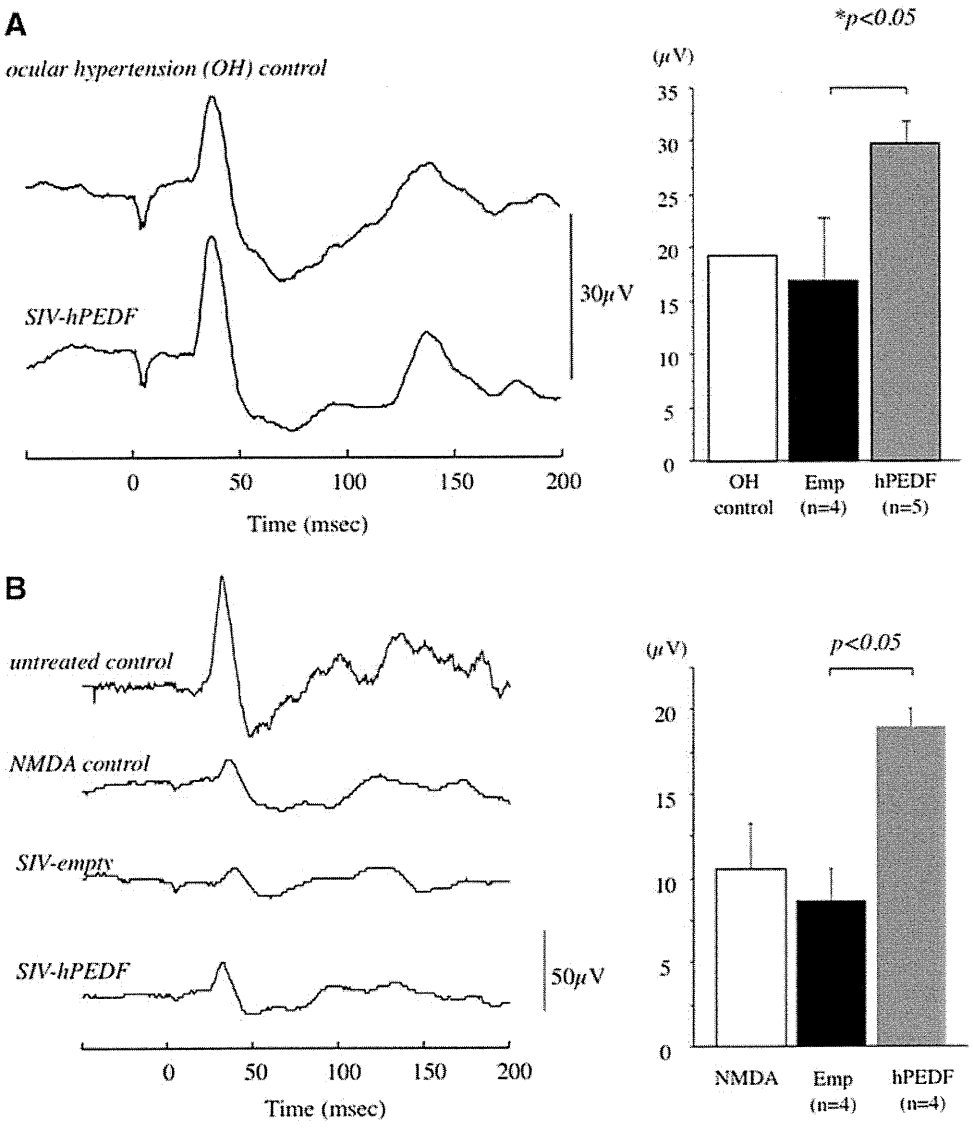

FIG. 4. Functional assessment of neuroprotective effects against transient ocular hypertension-induced and NMDA-induced RGC injuries. (**A**) Typical wave patterns and quantitative analyses of transient ocular hypertension-induced eyes are demonstrated. Significantly higher b-wave amplitudes of pattern 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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網膜色素変性に対する視細胞保護遺伝子治療

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総　　説

網膜色素変性に対する視細胞保護遺伝子治療

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はじめに

我々が取得する外界情報の約80％を得るために必要な視覚を失うこと，すなわち失明は，患者のQOLを著しく低下させ，社会活動を大幅に制限させる．世界の中途失明原因の上位を占める疾患のうち，白内障や緑内障は，手術療法の進歩や点眼薬などの充実により，治療することができる疾患である．一方，我々が遺伝子治療の対象疾患として選択した網膜色素変性（retinitis pigmentosa：RP）は，現時点で有効な治療法が確立されておらず，この疾患が原因で失明する患者は増加しており，早期の治療法開発が望まれている．

1．網膜色素変性（RP）

RPは進行性の夜盲，求心性の視野狭窄，視力低下を主な症状とする遺伝性の網膜変性疾患で，“視細胞と網膜色素上皮細胞の機能を原発性，びまん性に傷害する遺伝性かつ進行性の疾患群”と定義されている．すなわち，網膜の外層にある視細胞や網膜色素上皮細胞に特異的に発現している遺伝子の異常により，一般には若年期に発症して緩徐に進行し，中年ないし老年で高度な視力障害に至る疾患の総称である（図1）．遺伝性疾患としては比較的頻度が高く，我が国での有病率は3,400～8,000人に1人とされており，患者数は約3万人と推定されている．本疾患は我が国における先天盲の第一位で，中途失明原因でも第三位である．

Dryjaらにより，1990年に視細胞に特異的に発現しているロドプシン遺伝子が常染色体優性RPの原因遺伝子であることが報告されて以来[1)]，これまでに約40種類の原因遺伝子が報告されている．RPは，異なる遺伝子異常が共通する表現型を示す，非対立遺伝子異質性という性質を有するが，病態に共通するメカニズムは視細胞のアポトーシスである．眼底所見は特徴的で，網膜色素上皮の粗造化，網膜血管の狭細化，骨小体様色素沈着などが認められる（図2）．

この疾患に対する臨床的に明確な効果を有する治療法は確立されておらず，予後は不良である．従って，患者にとっては現状の視力を有効に利用するための情報提供，すなわち補装具の紹介・処方，特定疾患の認定とそれによるサービスの情報提供，診断書（身体障害者手帳・障害年金）の交付，リハビリテーションの紹介などといった，QOLを高めるための総合的な支援が現時点では重要である．一方，新しい治療法開発も盛んに進められており，今回紹介する遺伝子治療の他に，他家網膜移植や人工網膜移植，さらには幹細胞を利用した網膜再生療法などが将来の臨床応用を期待されている．

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Neuroprotective Gene Therapy to Treat Patients with Retinitis Pigmentosa

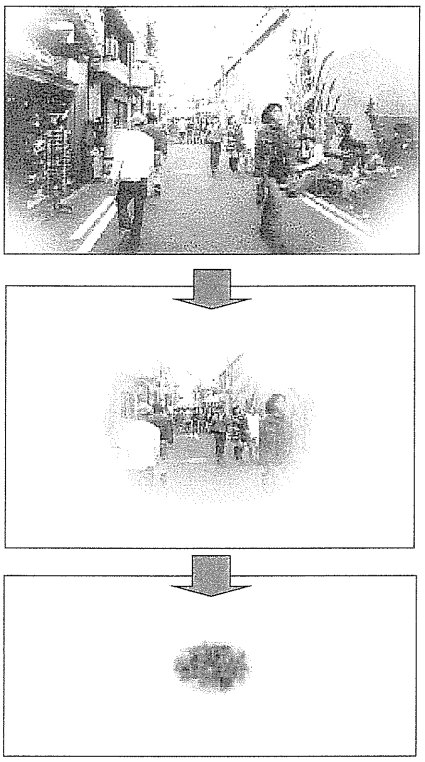

図1　網膜色素変性（RP）患者の見え方
見える範囲が少しずつ周辺部から狭くなり，50円玉の穴から覗いているような見え方になっていく（中段），さらにその見えている部分もはっきり見えなくなってくる（下段）．また，幼少時より暗いところでは見えにくいため，「鳥目」と言われた経験を持つ患者が多い．

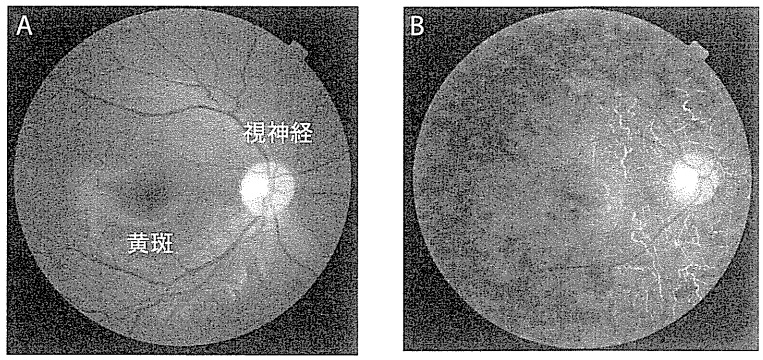

図2　網膜色素変性(RP)の眼底所見
(A) 正常眼底写真（右眼）．(B) RPに特徴的な色素を伴う網膜変性が認められる．

2．眼科領域の遺伝子治療

（1）歴史

遺伝子治療における世界初の臨床応用は，1990年に米国においてアデノシンデアミナーゼ欠損症の女児に対して行われたのは有名な話であるが[2]，眼科領域の遺伝子治療研究は当時大きく遅れていた．実験動物の網膜への遺伝子導入がはじめて報告されたのが1994年[3]，疾患モデル動物への治療効果のはじめての報告が1995年であった[4]．

眼科領域での遺伝子治療の臨床応用は，網膜芽細胞腫[5]，加齢黄斑変性[6]，レーバー先天黒内障[7)~9)]に対してこれまでに行われている．

（2）レーバー先天黒内障に対する遺伝子治療

レーバー先天黒内障（Leber's congenital amaurosis：LCA）に対する遺伝子治療は，疾患モデル動物など用いた基礎研究に裏打ちされた新しい治療法開発の素晴らしい一例であるので，ここで紹介する．

LCAは，生後早期より高度に視力が障害されるRP類縁疾患で，その原因遺伝子として*RPE65*や*CRX*などが知られている．*RPE65*は網膜色素上皮細胞（RPE）に発現し11-cis-retinalの産生に関わるが，*RPE65*遺伝子に変異があると11-cis-retinalが産生されず，視細胞（桿体）が光に反応できなくなり，最終的に視細胞は死に至ってしまう．Aclandらは，このLCAに対する遺伝子治療法として，AAVベクターを用いたRPEへの正常*RPE65*遺伝子導入という方法を試み，イヌのLCAモデルにおいて著明な治療効果が得られることを報告した[10]．さらに，小型・中型動物を用いてAAVベクター網膜下投与の安全性を確認した[11]．2007年2月より英国のAliらのグループによって，また2007年9月より米国のBennettらのグループによって，ヒトLCA患者に対する遺伝子治療臨床研究が開始されており，その途中経過が報告された[7)8)]．

英国での臨床研究では，17-23歳のLCA患者3名に対して遺伝子が投与された．その結果，1名では，投与部位に一致した感度の改善を認め，さらに暗所下での行動の著しい改善を認めたと報告されている．

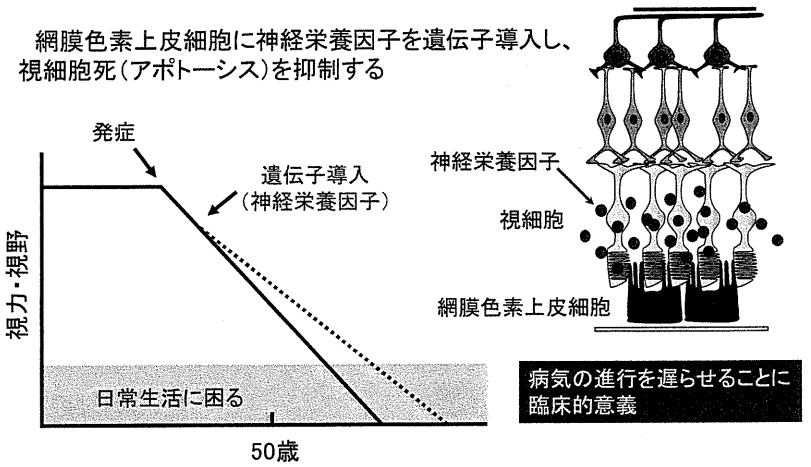

図3　視細胞保護遺伝子治療のコンセプト
網膜内の細胞（網膜色素上皮細胞）に神経栄養因子を遺伝子導入し，そこから分泌されたタンパクにより視細胞のアポトーシスを抑制する．視機能低下のスピードを抑えることで，日常生活に困らないレベルを保つことを目標としている．

また米国の臨床研究でも同様に，19-26歳の3名の患者を対象に遺伝子治療が行われ，治療を受けた3名とも視野に改善を認め，うち2名では視力の改善も認めたと報告されている．

3．網膜色素変性（RP）に対する視細胞保護遺伝子治療

（1）視細胞保護遺伝子治療のコンセプト

遺伝子治療の当初の発想は「遺伝子の異常を直す」，すなわち病気を根本的に治療しようというもので，異常のある遺伝子を正常遺伝子と置換することができれば理想的である．しかしながら，現時点では技術的に難しいので，現実的には遺伝子異常を有する細胞に単に正常な遺伝子を補充する（異常遺伝子はそのまま残る）方法が取られていることが多い．この成功例が先ほどのLCAに対する遺伝子治療である．

一方，「遺伝子を用いて治療する」という方法も遺伝子治療のひとつの治療戦略である．RPは網膜に発現する分子の遺伝子異常によって最終的には視細胞が死に至るが，その共通するメカニズムは，視細胞のアポトーシスである．九州大学眼科で計画中の視細胞保護遺伝子治療のコンセプトを図3に示す．神経栄養因子と称されるタンパクは，神経細胞に対し保護作用を有するが，筆者らはこれらの中から色素上皮由来因子（pigment epithelium-derived factor：PEDF）を選択した．網膜を構成する細胞に対する神経保護効果に加え，血管新生抑制効果を併せ持つことから，眼にとって好都合なタンパクと考えられた．このPEDF遺伝子を搭載したウイルスベクター（サル由来レンチウイルスベクター：SIVベクター）をRP患者の網膜下に投与し，患者の眼内をPEDFタンパクでいっぱいにしようと考えている．PEDFの視細胞保護作用により視細胞の喪失を防ぎ，患者の視機能低下を防ぐことを目的としている．

（2）SIVベクターの網膜への遺伝子導入特性

ヒト免疫不全ウイルス（human immunodeficiency virus：HIV）に代表されるレンチウイルスはレトロウイルス科に属するRNAウイルスである．宿主細胞の染色体に遺伝子が組み込まれるため，安定した長期間の遺伝子発現が得られること，神経細胞などの終末分化細胞や造血幹細胞などの休止期細胞といった非分裂細胞にも遺伝子を効率的に導入できるという特徴を持っており，慢性疾患であるRPに対する治療用ベクターとして注目されている．

筆者らは，アフリカミドリサル由来免疫不全ウイルス（SIVagm：simian immunodeficiency virus from African green monkey）を基本骨格としたSIVベクターを選択した．自然宿主であるサルにも病原性がな

いため安全性に優れており，また我が国において開発された独自性の高いベクターであったからである[12]．このSIVベクターによる網膜への遺伝子導入には以下の特性があることがわかった[13)14)]．

①ベクター注入部位に一致した網膜色素上皮細胞への遺伝子導入が可能であること
②長期間（少なくとも3年間）の安定した遺伝子発現が持続すること
③遺伝子導入部の網膜に機能的な影響を生じないこと

（3）SIV-hPEDFによる網膜変性モデル動物への治療効果

SIVベクターにより網膜への安定した遺伝子導入が可能であることが確認できたので，次にPEDFを遺伝子導入することによる，網膜変性モデル動物であるRCSラットに対する治療効果の検討を行った（図4）[15)16)]．その結果，

①無治療群では7週齢で多くの視細胞が消失してしまうのに対し，PEDF遺伝子導入群では15週齢（投与後12週）まで組織学的に視細胞数が保たれること，
②視細胞死の抑制にはPEDFによるアポトーシス抑制が関与していること，
③網膜電図（ERG）を用いた電気生理学的検討において，機能的な保護効果が得られること，
④神経栄養因子である塩基性線維芽細胞増殖因子（FGF-2）を同時に発現させることでより高い保護効果が得られること，

が明らかとなった．さらに，他の網膜変性モデル動物においても同様の検討を行い，有効な治療効果が確認できた．

（4）PEDFによる視細胞保護のメカニズム

さらに，PEDFの視細胞死に対する保護効果の詳細なメカニズムを知るために，in vitroならびにin vivoでの検討を行った．その結果，アポトーシス誘導因子（apoptosis-inducing factor：AIF）というひとつの因子にたどり着いた[17]．網膜変性モデル動物において，このAIFがミトコンドリアから放出され，核内に移行することによって視細胞にアポトーシス死を誘導すること，およびPEDFはミトコンドリアの膜を安定させることによりAIFの放出を抑制することで，視細胞死を防いでいることがわかった．

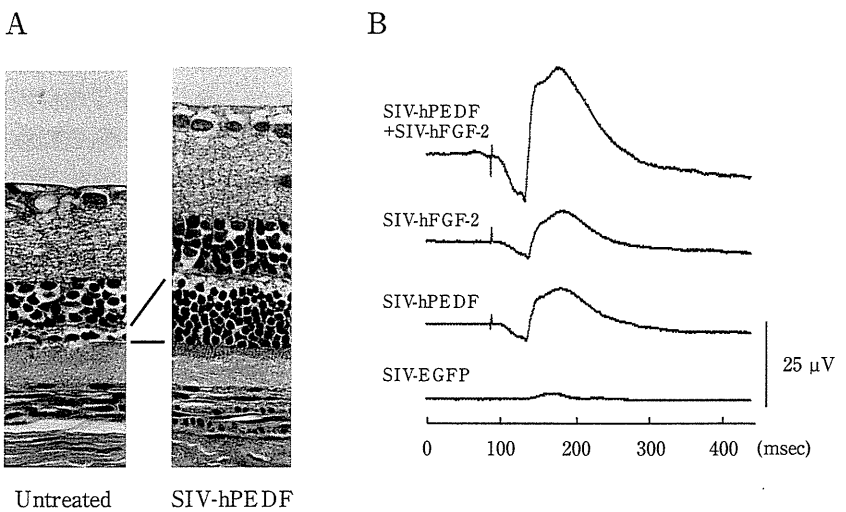

図4　SIV-hPEDFによる網膜変性モデル動物への治療効果
（A）RCSラット7週齢の網膜組織．治療群（SIV-hPEDF）では，視細胞の変性が抑制されているため，外顆粒層が厚い（文献15を改変）．（B）RCSラット7週齢の網膜電図．PEDF，FGF-2それぞれの遺伝子導入により電位が高くなっているが，同時に発現させることによってさらに高い電位が得られている（文献16を改変）．

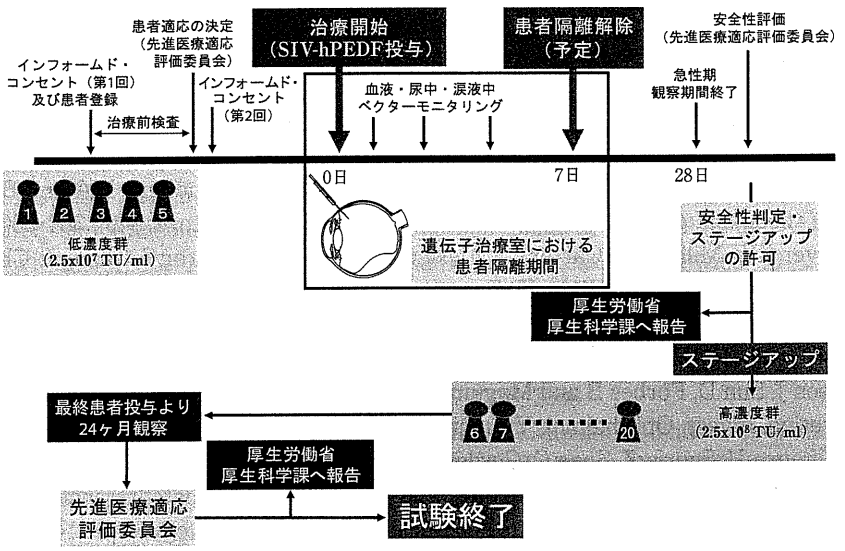

図5　視細胞保護遺伝子治療臨床研究のおおまかな流れ

（5）大型動物を用いた安全性試験

眼科領域の遺伝子治療臨床研究としてレンチウイルスベクターが用いられた前例がないことより，SIVベクター投与による安全性（局所ならびに全身）を詳細に検証することは必須であった．筆者らは，大型動物であるカニクイザルを用いた安全性試験を計画した．長期的な安全性に関しては現在も検討中であるが，急性期においては，

①ベクター投与に伴う眼局所および全身における重大な有害事象がないこと，

② PEDF の過剰発現による眼局所での副作用がないこと，

が確認できている[18]．

（6）臨床試験実施計画書

九州大学眼科で計画中の視細胞保護遺伝子治療実施計画の大まかな流れを図５に示す．まず第１ステージとして５名の被験者に低濃度のベクター溶液を投与し各々４週間観察する．急性期の異常が認められないことを確認した後，第２ステージで 15 名の被験者に有効濃度と考えられる量のベクターを投与する計画となっている．安全性を見極める第Ｉ相臨床研究として位置付けており，最終被験者の投与終了後２年間経過観察するが，副作用の発生については終生追跡する予定になっている．

おわりに

現在，「神経栄養因子（ヒト色素上皮由来因子：hPEDF）遺伝子搭載第３世代組換えアフリカミドリザル由来サル免疫不全ウイルスベクターの網膜下投与による網膜色素変性に対する視細胞保護遺伝子治療臨床研究，（英文標記）The Clinical Study for Neuroprotective Gene Therapy to Treat Patients with Retinitis Pigmentosa via Subretinal Injection of The 3rd Generation of Recombinant Simian Immunodeficiency Virus (SIVagm) Vector Expressing Human Pigment Epithelium-Derived Factor Gene.」という臨床研究プロトコールが完成しており，学内倫理委員会での審議・承認を得ることができた．さらに，平成 22 年 10 月に厚生労働省への実施計画の申請が完了し，今年度中に厚生科学審議会において実施計画に対する審議が開始される見込みである．近い将来，この新しい治療法が臨床応用される可能性がある．

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