Table 2 Clinical and pathological information of LB positive cases. Values for age, duration and brain weight are given as mean ±SD, and those for LB score and Braak stage as median (*LB* Lewy bodies, *nd-LB* non-demented subjects with LB, *DLB* dementia with LB)

Group	*n* (male/female)	Age at onset (years)	Age at death (years)	Duration (years)	Brain weight (g)	LB score (range)	Braak and Braak stage (range)
nd-LB	11 (5/6)		84.5±6.9		1199.5±96.2	1 (0–10)	4 (0–6)[a]
Brain stem type	8 (5/3)		84.5±8.2		1215.0±77.9	0.5 (0–2)	3.5 (0–6)
Neocortical type	3 (0/3)		84.3±2.1		1158.3±146.8	10 (8–10)	4 (0–4)[b]
DLB	12 (3/9)	82.2±9.5	88.2±8.2	5.8±5.1	1149.6±128.8	6 (1–10)	5 (0–6)[a]
Brain stem type	2 (1/1)	86.5±7.8	91.0±11.3	4.4±3.6	1195.0±233.3	1 (0–2)	4.5 (4–5)
Limbic type	5 (2/3)	83.4±10.3	87.6±9.9	4.2±4.6	1203.0±109.0	5 (3–6)	5 (0–6)
Neocortical type	5 (0/5)	79.2±10.2	87.6±6.9	8.0±6.0	1078.0±95.8	8 (8–10)	6 (5–6)[b]

Significant difference between the two values [a]$P<0.01$, [b]$P<0.05$

0.06). Mean age at death and mean brain weight were not significantly different between DLB cases and nd-LB subjects. The distribution between genders in the LB-positive cases was more weighted to females (15 females/8 males) and this tendency was clearly evident in the DLB group.

LB pathology in non-demented subjects and in demented cases

An age-specific prevalence of LB pathology was seen (Fig. 1). In particular, an age-related increase in the percentage of DLB cases was identified, increasing from 7.4% to 21.7% between the eight and tenth decades. The prevalence increased on average by 7% per decade. In demented subjects, however, the percentage of DLB cases was similar between the eight and tenth decades (50.0% in eighth decade, 38.5% in the ninth decade, and 41.7% in the tenth decade). We categorized LB cases into three subgroups (brain stem, limbic, and neocortical types) according to LB score. The clinical courses (age at onset, age at death, and duration), brain weight, and Braak stage between each subgroup were not significantly different in nd-LB and DLB cases (Table 2). All the DLB cases showed widespread distribution of LBs in all of the three regions of the brain stem investigated (SN, LC, and DVN). In addition, most DLB cases displayed extensive and widely distributed LBs throughout the limbic cortex to the neocortex, in other words, many DLB cases belonged to the limbic or neocortical types. Moreover, almost all DLB cases showed neuronal loss in LC and/or SN (10 cases), Lewy-related neurites in hippocampal CA2 region (9 cases), and spongiform changes involving at least the mesial temporal region (11 cases). In contrast, most of nd-LB subjects showed minimal or absent cortical LBs and classical spherical intracytoplasmic inclusions were mostly restricted to three regions of brain stem. In 2 nd-LB subjects, LBs were found only in DVN or in DVN and LC. Additionally, most of nd-LB subjects did not present regional neuronal loss, Lewy-related neurites, or spongiform changes. Three nd-LB subjects, however, showed a remarkable LB pathology accumulation throughout the brain stem to the neocortex (Fig. 2). One of these three subjects presented neuronal loss in substantia nigra and spongiform changes in the mesial temporal region. These

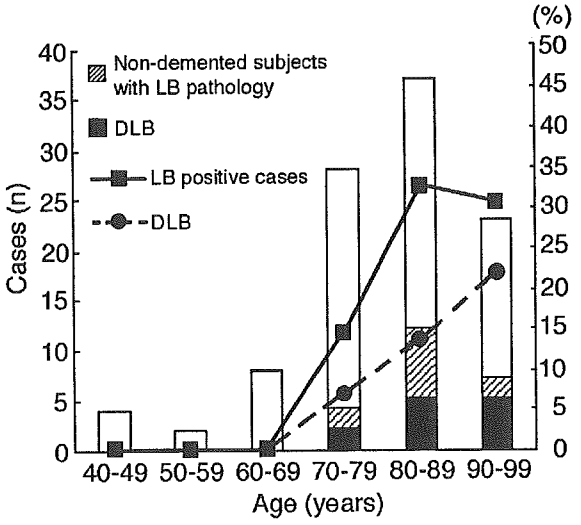

Fig. 1 Influence of age on LB pathology. *Bars* show the number of the deceased subjects in each decade. *Solid area* of the *bars* represensts the numbers of DLB cases; *hatched area* the numbers of non-demented subjects with LB pathology; *solid line* the percentage of LB-positive subjects; *broken line* the percentage of DLB cases (*LB* Lewy bodies, *DLB* dementia with LBs)

three cases showed neither cognitive decline clinically nor did they present any significant neuropathological change corresponding to ATD or VD.

Relation between LB and ATD pathology

We found a significant difference among the LB-positive cases relating to LB scores when categorized according to the degree of ATD pathology described in the NIA-RI criteria or in the CERAD guidelines (Fig. 3). The group that showed severe ATD pathology had a significantly higher LB score than the group with minimal ATD pathology (Mann-Whitney U-test, $P<0.05$). However, we did not find any significant correlation between LB scores and the amount of neurofibrillary tangles proposed in Braak stage (Spearmans's rank correlation test, $P=0.17$).

Among the DLB subjects, 7 cases presented severe ATD pathology. The severity of their ATD pathology corresponded to "definite" as defined by the CERAD guide-

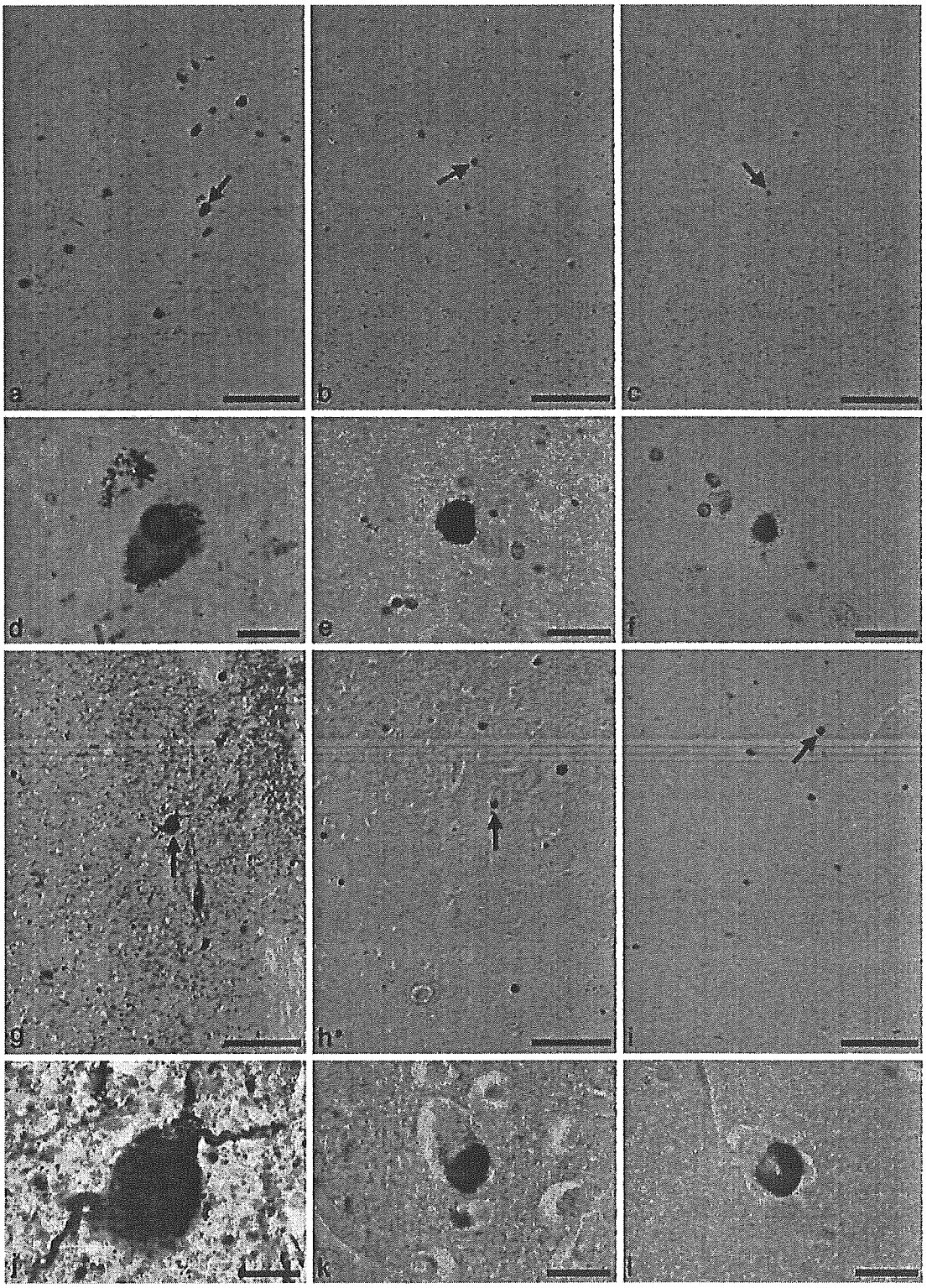

Fig. 2 LBs detected by α-synuclein immunohistochemistry in substantia nigra (**a**, **d**, **g**, **j**), in cingulate gyrus (**b**, **e**, **h**, **k**), and in middle temporal gyrus (**c**, **f**, **i**, **l**). **a–f** A demented 79-year-old woman who died with aspiration pneumonia. Her brain weighed 1,010 g and presented LBs through the brain stem to the neocortex (LB score 8). **g–l** A non-demented 85-year-old woman who died from pneumonia. Her brain weighed 1,185 g and also showed LB formation in the brain stem, limbic system, and neocortex (LB score 10). No ATD changes or VD pathology is demonstrated in either case. LBs (*arrows*) in **a–c**, **g–i** are shown at high magnifications in **d–f**, **j–l**, respectively (*ATD* Alzheimer-type dementia, *VD* vascular dementia). *Bars* **a–c**, **g–i** 100 μm; **d–f**, **j–l** 20 μm

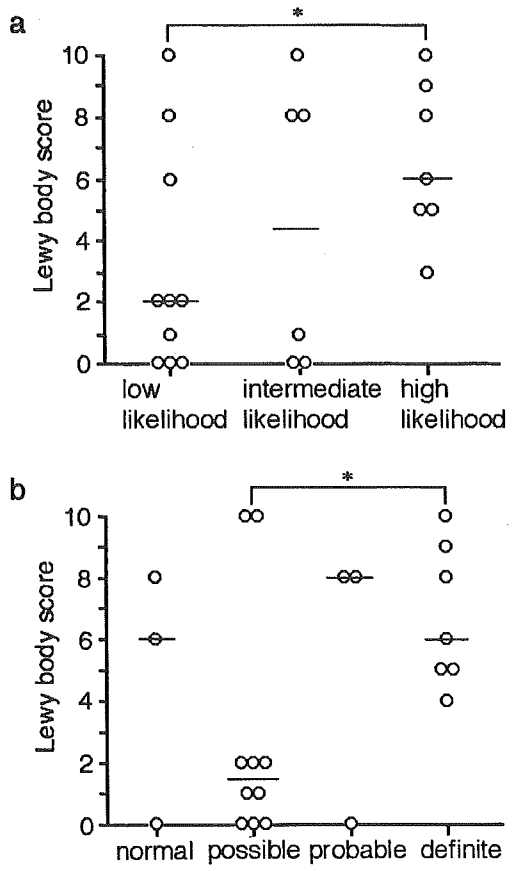

Fig. 3 Correlation between ATD pathology and LB score. LB scores were plotted against the degree of ATD pathology described by NIA-RI criteria (**a**) or CERAD guidelines (**b**). The *horizontal lines* represent the medians. *Asterisks* show statistical difference found using non-parametric Mann-Whitney U-test ($P<0.05$)

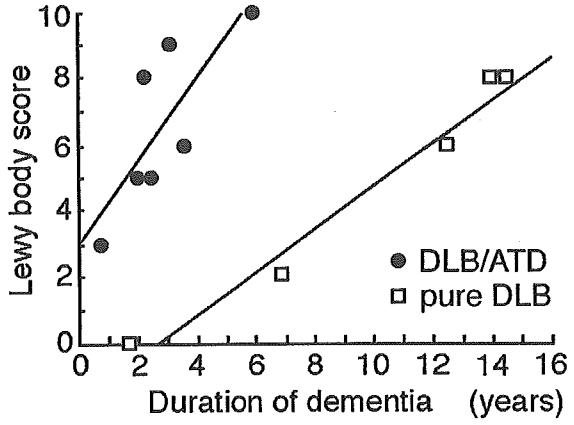

Fig. 4 Influence of duration on the proliferation of LB pathology. In each DLB subgroup, LB scores are plotted against the duration of dementia. Any significant correlation between the duration of dementia and the LB score was evaluated by Spearman's rank correlation test ($P<0.05$)

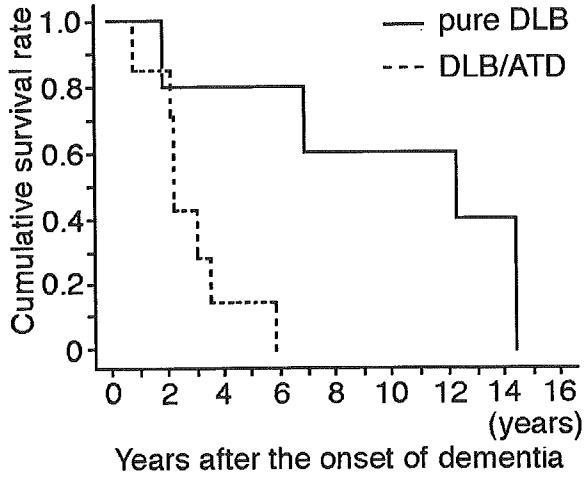

Fig. 5 Survival rates in DLB subgroups. Cumulative survival rates between DLB subgroups were evaluated using a Kaplan-Meier test. The onset age of dementia is not significantly different between two groups. The survival rate at 5 years after the onset of dementia for pure DLB is 80.0%, whereas it is 14.3% for DLB/ATD

lines and also to "high-likelihood" as described in the NIA-RI criteria. There was no significant difference between the DLB cases and other dementia subgroups in relation to age at onset of dementia, age at death, duration of dementia, or brain weight. In pure DLB cases, the age at onset appeared to be younger compared with the DLB/ATD group, but there was no significant difference (t-test, $P=0.29$) (Table 1). There was also no significant difference between pure DLB group and other dementia subgroups in relation to age at death, brain weight, LB score, and Braak stage. However, pure DLB cases had a significantly longer duration of dementia than did DLB/ATD and VD cases. Although the duration of dementia was not significantly different between pure DLB cases and ATD cases, pure DLB cases tended to show a longer duration than ATD cases (t-test, $P=0.07$) (Table 1). In each DLB subgroup, any significant correlation between the duration of dementia and the LB score was found (Spearmans's rank correlation test, $P<0.05$). Additionally, the DLB/ATD group showed a more rapid increase of LB score than did the pure DLB group (Fig. 4). Moreover, survival rates between the two groups were significantly different (log rank test, $P<0.05$) (Fig. 5). The cumulative survival rate at 5 years after the onset of dementia for DLB/ATD group and pure DLB group were 14.3% and 80.0% respectively.

Discussion

Our study had four major findings. First, we documented the frequency of LB pathology in a community-based population; 22.5% of 102 consecutive autopsies. Second, aging was shown to be one of the risk factors causing the prevalence of LB pathology; we found an increase in the prevalence of LB pathology between the eighth and tenth decades. Third, severe ATD pathology can have a strong effect of accumulating LB pathology through the brain

stem to the neocortex. Fourth, a concomitant severe ATD pathology may be critical for the prognosis of DLB patients; DLB cases with severe ATD pathology showed about a one fifth the survival rate of DLB cases without severe ATD pathology.

Several studies regarding the prevalence of LB pathology based on clinico-pathological methods have been carried out in various regions of the world. The reported prevalences range widely, from 2.3% to 60.7%. There are at least two reasons for the differences among the results. First, the reported prevalences of LB pathology are highly dependent on the source of the material. Although a community-based study is important to precisely estimate the prevalences of LB pathology, most of the previous results are from hospital-based postmortem studies, or investigations of demented subjects. Only two prospective longitudinal studies randomly selected their cases from a community-based population [30, 32]; however, it was not clear how far their results reflect the general population because they did not mention the autopsy rate. Second, because many previous studies used nonspecific LB markers to identify LB pathology [23], an accurate assessment of the number of LBs would be difficult. In our study, although the number of cases is relatively small compared with previous studies, the autopsy rate for the subjects is high enough to consider the result as representative of the population. In addition, we used α-synuclein IHC, which is a very sensitive and specific method for the detection of LBs, to determine LB pathology [4, 17, 37]. Thus, our results should reflect the true prevalence of LB pathology in a general population, with LB pathology was found in 41.4% of demented cases, but only 15.1% of non-demented subjects. Our higher prevalence of LB pathology in demented compared to non-demented subjects supports the role of LBs in the pathogenesis of dementia, as has previously been suggested [10, 32]. Moreover, DLB is considered to be a distinct clinicopathological entity, and is believed, after ATD, to be the second most common cause of dementia in adults. DLB cases presenting ATD pathology were reported to show fewer NFT and lower Braak stage compared with those of ATD [10, 11, 16]. In our study, we also found a tendency for DLB cases to show a lower Braak stage than ATD cases. This distinction is considered to be one of the key features that discriminate DLB from ATD. Yet, some still subscribe to the view that DLB is largely a variant of ATD [13, 31]. In the current study, we tentatively defined ATD as those demented cases with ATD pathology, but without LB pathology. As a result, DLB became the most common form of dementia. The number of demented patients with severe ATD pathology was 14 (pure ATD: 4 cases, DLB with severe ATD pathology: 7 cases, MD: 3 cases), which was almost same as the number of DLB (12 cases). We are presently extending our observations to more cases to draw a better conclusion about the most frequent demented illnesses, and the most significant pathological findings for cognitive dysfunction.

The British CFA study showed that the presence of LBs was not related to age [30]; however, in our study, an age-related increase in the prevalence of LB pathology was found between the eighth and tenth decades, a result comparable to other studies [8, 32]. Thus, we concluded that aging plays a role in the evolution of LB pathology. In our study, all LB-positive cases showed classical LBs in the brain stem. In particular, we found two nd-LB subjects presenting LBs only in the LC and/or DVN. These findings corresponded to the previous report that extranigral sites in the lower brain stem are affected with LB pathology prior to SN involvement [7], although SN has until now been considered to be the region primary affected [33]. In addition, almost all DLB cases showed neuronal loss in SN, while most of nd-LB subjects did not present regional neuronal loss. These findings also agree with previous reports that neuronal loss in the SN was significantly associated with clinical symptoms, including dementia, in patients with PD and DLB [14, 35, 39]. Moreover, we found three non-demented subjects presenting LB pathology not only in the brain stem but also in the limbic system and neocortex. These findings suggest that LB pathology may exist not only in demented patients but also in a normal elderly population, although LB pathology may contribute to the pathogenesis of dementia. This is similar to senile plaques, which exist in ATD patients and also in some non-demented individuals [6]. Further investigation is needed to clarify the reasons for the discrepancies between clinical and pathological findings.

In our study, about 60% of DLB cases presented severe ATD pathology. Previous studies also demonstrated that LB pathology frequently accompanied ATD pathology in genetic and sporadic cases [10, 24]. However, the relationship between LB and ATD pathologies is complex and remains unresolved. In ATD, there is evidence that in most patients the disease follows a hierarchic evolution from medial temporal lobe regions to other limbic areas and then to temporal, parietal, frontal and occipital neocortices [3, 5]. In DLB, there has been no obvious evidence for such a hierarchic spread [9, 16]. In this study, we showed that the greater the intensity of the degree of ATD pathology, when semiquantitatively assessed according to CERAD and NIA-RI criteria, the greater the LB score increase. We also found an increase in LB scores as the duration of dementia lengthened. In addition, the DLB/ATD group showed a more rapid increase of LB score than the pure DLB group. This does not present direct evidence that LB pathology followed a hierarchic pattern of progression, but we consider that severe ATD pathology contributed to the accumulation and distribution of LB pathology through the brain stem to the limbic system or neocortex.

The clinical significance of ATD pathology for DLB is not clear. It has been reported that the clinical symptoms of DLB patients are not significantly different between those with or without concomitant ATD pathology [9]. On the other hand, a pure DLB group has been reported to have a longer disease duration than a DLB/ATD group because of the early onset in the pure DLB cases [22]. In our study, the DLB/ATD group had significantly shorter durations compared to the pure DLB group, although there

was no significant difference in the age of disease onset between these two groups. In addition, DLB/ATD patients had a lower survival rate than did pure DLB subjects. Previous studies have shown that ATD pathology substantially increased the risk of dementia in DLB patients and that the severity of dementia correlated significantly and positively with the spread of LBs [12, 31]. Therefore, concomitant ATD pathology may accelerate the accumulation and progression of LB pathology to increase the severity of dementia. Moreover, co-existent ATD pathology was reported to be significantly associated with dementia and poor survival in patients with PD [18]. Thus, in DLB patients, accompanying severe ATD pathology may worsen the general condition, leading to poorer prognosis.

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Prevalence and risk factors for epiretinal membranes in a Japanese population: the Hisayama study

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Abstract *Purpose:* To examine the prevalence and risk factors for epiretinal membranes (ERMs) in a sample Japanese population. *Methods:* In 1998 a cross-sectional community survey was conducted among residents of Hisayama. A total of 688 men and 1087 women living in Hisayama, Japan, aged 40 years or older consented to participate in the study. Each participant underwent a comprehensive physical examination that included an ophthalmic examination. The presence of ERMs was judged from grading based on fundus examination using indirect ophthalmoscopy, slit lamp examination, and color fundus photographs. This study used non-stereoscopic 45° fundus photographs to grade ERMs, whereas the other population-based studies used 30° stereoscopic fundus photographs, which might explain some differences in prevalence of ERMs. Multiple logistic regression analysis was performed on the cross-sectional data to determine the risk factors for ERMs. The following ten possible risk factors were used: age; gender; hypertension; diabetes; serum cholesterol; serum HDL cholesterol; serum triglycerides; smoking habits; alcohol intake; and body mass index. *Results:* The prevalence of ERMs was 4.0%, and increased with age. The ERMs were more prevalent in women (4.3%) than in men (3.5%). Multiple logistic regression analysis showed that age and serum cholesterol significantly associated with ERMs. *Conclusions:* This study suggests that ERMs are less common in the Japanese population than in similar populations in Western countries, and that hypercholesterolemia is a relevant risk factor for ERMs.

Introduction

Epiretinal membranes (ERMs) are associated with a variety of ocular diseases [1], particularly diabetic retinopathy and retinal vein occlusion, or after surgery for cataracts [1], retinal detachment [7, 13], or posterior vitreous detachment, or other eye problems. In most cases, however, ERMs occur independently of these specific diseases and are termed "idiopathic" [14]. The pathogenesis of idiopathic ERMs has been explored in detailed histopathologic and immunohistochemical studies [6, 9, 20] but is still tentative. In most cases, ERMs have small or no effect on vision, although in some cases membrane contraction may exert tangential traction on the macular retina, causing severe vision loss [22]; therefore, it is crucial to identify the risk factors for idiopathic ERMs to enhance understanding of the disease. Several risk factors for idiopathic ERMs have been investigated in population-based [12, 14] and case-control studies [8, 25]. The risk factors examined include arteriovenous nicking [12], hypertension [8], atherosclerosis [25], and diabetes [6, 12, 14]. To the best of our knowledge, however, no population-based studies have assessed the prevalence and risk factors for ERMs in Japan. The only two popu-

lation-based reports are the Beaver Dam Eye Study that was conducted in the United States [12], and the Blue Mountains Eye Study in Australia [14]. To examine the prevalence and risk factors for ERMs, we conducted a cross-sectional community survey of the residents of Hisayama, Kyushu Island, Japan.

Patients and methods

Study population

A prospective population-based follow-up study has been carried out since 1961 in Hisayama [16, 24]. The population of the town is approximately 7500, and it has been shown to be demographically representative of Japan based on the national census [17]. As part of the follow-up survey, we performed a physical examination, including an eye examination, of Hisayama residents aged 40 years or older in 1998. Of the 4187 residents aged 40 years or older, 2211 (52.8%) consented to participate in the study; of these, 349 (15.8%) underwent the physical examination at home, so they did not get an eye examination, whereas 3 (0.1%) refused to participate in the ophthalmic examination. Ultimately, 1775 (80.3%) individuals (688 men and 1087 women) underwent an ophthalmic examination. Of the 1775 subjects examined, 10 subjects with late age-related macular degeneration in one or both eyes were excluded because this disease was considered likely to confound the grading of ERMs. Thirty-eight subjects with retinal vein occlusion and 46 subjects with diabetic retinopathy were excluded.

Ophthalmic examination

The methods used in the ophthalmic examination have been described in detail previously [15]. Briefly, each participant underwent a comprehensive ophthalmic examination, including lens grading using a slit lamp, stereoscopic fundus examination using indirect ophthalmoscopy, and examination with a slit-lamp biomicroscope with a "superfield lens" (Volk, Mentor, Ohio) after pupil dilatation with 1.0% tropicamide and 10% phenylephrine. Forty-five-degree fundus photographs were taken using a Topcon "non-mydriatic" TRC NW-5 fundus camera (Topcon, Tokyo, Japan) and Fujichrome slide film (Sensia, Fuji Film, Tokyo, Japan). The photographs were taken of a field centered horizontally and vertically on a point midway between the temporal edge of the optic disc and the fovea. The photographic image included the area above and below the temporal arcades, and areas just nasal to the disc and temporal to the macula. The 35-mm slide transparencies were mounted in clear plastic sheets and graded at Kyushu University by two experienced graders (M.M. and T.I.).

Classification of epiretinal membranes and subjects included

The presence of ERMs was based on the grading of fundus examinations by indirect ophthalmoscopy, slit lamp, and color fundus photographs. The site of each ERM was judged using a grid developed for grading age-related maculopathy [11]. The grid radius was 3000 μm, corresponding to the anatomic macula, and the grid was placed over fundus photographs when grading. The ERMs wholly outside the grid were not graded as present. Two experienced ophthalmologists examined all the participants, without knowledge of clinical information. The classification of Mitchell et al. [14] was adopted for this study. Briefly, ERMs were classified as either early or late stage. The earlier stage was defined by the presence of a thin layer of preretinal cells, also termed cellophane macular reflex (CMR), within the grid in the absence of the later stage in either eye. The later stage was defined by the presence of superficial retinal folds or traction lines, also termed preretinal macular fibrosis (PMF). Subjects with both CMR and PMF present were classified in the PMF group. Two experienced graders, masked to subject information, assessed the ERMs. The analysis included people with gradable photographs of either eye providing the clearest macular characteristics detected by stereoscopic eye examination. Inter- and intra-observer variability were analyzed using kappa statistics. The level of agreement was moderate to substantial for most features.

Data collection

Blood pressure was measured three times after resting for at least 5 min in the sitting position. The average of three measurements was used for the analysis. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or current use of antihypertensive medication. Blood samples were collected from the antecubital vein after an overnight fast. After taking the fasting blood specimen, a 75-g oral glucose tolerance test was performed with a 75-g glucose equivalent carbohydrate load (Trelan G, Shimizu Pharmaceutical, Shimizu, Japan). Diabetes was defined as a fasting plasma glucose level ≥7.0 mmol/l or a 2-h postloading glucose level ≥11.1 mmol/l, in addition to a medical history of diabetes. The serum cholesterol and serum triglycerides were determined enzymatically using an autoanalyzer (TBA-80S, Toshiba, Tokyo, Japan). Information on smoking habits and alcohol intake was obtained using a standard questionnaire, and these factors were classified into either current habitual use or nonuser. Body height and weight were measured in light clothing without shoes, and the body mass index (BMI) was calculated as the weight in kilograms divided by the height in square meters.

Statistical methods

We defined a subject as having ERMs if the subject had CMR or PMF in at least one eye. We considered the following ten possible risk factors for ERMs: age; gender; hypertension; diabetes; serum cholesterol; serum HDL cholesterol; serum triglycerides; smoking habits; alcohol intake; and BMI. Age, BMI, serum cholesterol, serum LDL cholesterol, serum HDL cholesterol, and serum triglycerides were treated as continuous variables, and the others as categorical variables. Each categorical variable was coded as either 1 or 0 depending on the presence or absence of the factor, respectively. The association of the variables with ERMs was assessed using Student's *t*-test for the continuous variables and the Pearson chi-square test for the categorical variables. Multiple logistic regression analysis was performed to determine risk factors for ERMs, using odds ratio estimates with 95% confidence intervals. The SAS software package (SAS Institute, Cary, N.C.) was used to perform the statistical analyses [21]. A two-sided *p*-value less than 0.05 was considered statistically significant.

Results

Of the 1765 subjects examined, 71 had ERMs (4.0%). The ERMs were more prevalent in women (4.3%) than in men (3.5%) [odds ratio (OR), 1.36; 95% confidence interval (CI), 0.82–2.26]. Table 1 shows the age-specific prevalence of ERMs by gender. For all subjects, the prevalence was 1.4, 0.5, 6.3, 6.8, and 4.7% for persons in their fifth through ninth-plus decades of life, respective-

Table 1 Age-specific prevalence of epiretinal membranes by gender, the Hisayama Study (1998). *CMR* cellophane macular reflex, *PMF* preretinal macular fibrosis, *ERM* epiretinal membrane

Age range (years)	Men				Women				Both genders
	Population at risk (*n*)	CMR *N*	PMF *N*	All ERM *N*	Population at risk (*n*)	CMR *N*	PMF *N*	All ERM *N*	All ERM *N*
40–49	92	0 (0.0)	0 (0.0)	0 (0.0)	201	4 (2.0)	0 (0.0)	4 (2.0)	4 (1.4)
50–59	152	0 (0.0)	0 (0.0)	0 (0.0)	284	1 (1.4)	1 (0.4)	2 (0.7)	2 (0.5)
60–69	227	9 (4.0)	1 (0.4)	10 (4.4)	335	18 (5.4)	7 (2.1)	25 (7.5)	35 (6.3)
70–79	176	10 (5.7)	2 (1.1)	12 (6.8)	211	11 (5.2)	3 (1.4)	14 (6.7)	26 (6.8)
80+	32	2 (6.3)	0 (0.0)	2 (6.3)	55	1 (1.8)	1 (1.8)	2 (3.6)	4 (4.7)
Total	679	21 (3.1)	3 (0.4)	24 (3.5)	1086	35 (3.2)	12 (1.1)	47 (4.3)	71 (4.0)

Numbers in parentheses are percentages

Table 2 Mean values or frequencies of risk factors for epiretinal membranes, the Hisayama Study (1998)

Risk factor	Non-ERM (*n*=1694)	ERM (*n*=71)
Age (years)	62±11	68±8**
Gender (men/women)	658/1037	24/47
Drusen (%)	10.1	8.5
Retinal pigment epithelial abnormality (%)	5.9	4.2
Hypertension (%)	56.0	56.3
Diabetes (%)	12.0	15.5
Serum cholesterol (mmol/l)	5.3±0.9	5.5±0.4
Serum HDL cholesterol (mmol/l)	1.4±0.9	1.5±1.0
Serum triglycerides (mmol/l)	1.5±0.2	1.5±0.4
Smoking habits (%)	34.3	29.6
Alcohol intake (%)	37.0	29.6
Body mass index (kg/m^2)	23.1±3.3	22.9±3.3

Values are expressed as the mean±SD or percent
**p<0.01, ERM vs non-ERM; *p<0.05, ERM vs non-ERM

Table 3 Age-adjusted odds ratios (OR) associated with epiretinal membranes, the Hisayama Study (1998). *CI* confidence interval

Risk factor	OR	95% CI
Gender (men/women)	1.36	0.82–2.26
Drusen (%)	0.57	0.24–1.37
Retinal pigment epithelial abnormality (%)	0.64	0.20–2.07
Hypertension (%)	1.16	0.70–1.91
Diabetes (%)	1.14	0.59–2.21
Serum cholesterol (mmol/l)	1.29*	1.00–1.68
Serum HDL cholesterol (mmol/l)	1.01	052–1.99
Serum triglycerides (mmol/l)	1.14	0.93–1.39
Smoking habits (%)	0.75	0.45–1.27
Alcohol intake (%)	0.81	0.48–1.37
Body mass index (kg/m^2)	1.01	0.94–1.09

**p<0.01, *p<0.05

ly. This age-related increase was significant (Cochran-Armitage Trend Test; p<0.01). The increased odds of ERMs for persons over 59 years of age compared with those younger than 60 years of age were 8.5. Early epiretinal membranes, termed cellophane macular reflex (CMR), and advanced epiretinal membranes, termed preretinal macular fibrosis (PMF), are considered separately in Table 1. The CMR was found in 0.0% of men and 2.0% of women in the 40- to 49-year age group, and increased significantly with age in both genders (Cochran-Armitage Trend Test; p<0.01). The PMF was found in a small number of subjects, and was not significantly related to age in either gender.

We compared the distribution of possible risk factors in subjects with and without ERMs (Table 2). The subjects with ERMs were on average 6 years older than those without ERMs (p<0.01). Cataracts were more frequent in those with ERMs (p<0.05).

The result of the multiple logistic regression analysis of risk factors for ERMs is shown in Table 3. Age (OR, 1.06; 95%CI, 1.03–1.08), and serum cholesterol (OR, 1.29; 95%CI, 1.00–1.68) were significantly associated with ERMs (p<0.05).

Discussion

To our knowledge, this is the first study to investigate the prevalence and risk factors of ERMs in Japan, using a population-based sample. The results show that ERMs are more prevalent in women than in men in Japan, and that age and hypercholesterolemia are significantly associated with ERMs.

Only two previous population-based studies of ERMs have been reported: the Beaver Dam Eye Study that was conducted in the United States [14] and the Blue Mountains Eye Study in Australia [12]. Both studies used similar methods of case ascertainment and assessment of the prevalence of ERMs. The results of this study can be compared with those two population-based studies, but some methodological issues should be mentioned. This study used non-stereoscopic 45° fundus photographs to

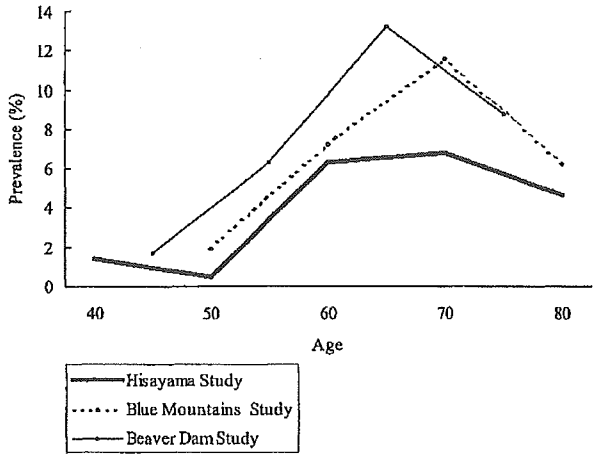

Fig. 1 Comparison of the prevalence of epiretinal membranes in population-based studies

grade ERMs, whereas the other studies used 30° stereoscopic fundus photographs. Figure 1 compares the age-specific prevalence of ERMs in the Hisayama Study and the population-based studies conducted in the United States and Australia. The Beaver Dam Eye Study found ERMs in 11.8% of 4802 participants 43–86 years of age. The Blue Mountains Eye Study found ERMs in 7.0% of 3490 participants aged 49 years or older. Our study produced the lowest estimates (4.0%). It is conceivable that part of the difference resulted from our use of non-stereoscopic photographs, although a real difference must also be considered. This study suggests that ERMs are less common in the Japanese population than in similar populations in Western countries.

On the other hand, Roth and Foos reported that the incidence of ERM is 5.4% in their large, consecutive series of eyes enucleated at autopsy [18]. That is much closer to that of the present study than either the Beaver Dam or Blue Mountains Eye Studies. The age distribution in the Roth and Foos study was also similar to the present study.

Our study demonstrated that hypercholesterolemia is a risk factor for ERMs. The mechanism of the formation of idiopathic ERMs is not well known; however, glial cells, fibrocytes, retinal pigment epithelial (RPE) cells, and myofibrocytes are frequently implicated in idiopathic ERMs [2, 6, 10, 23]. The migration of glial cells or RPE cells may be mediated by both biochemical and physiological factors, such as chemoattractants in serum and substances released from vascular endothelial cells or RPE cells themselves [3, 4, 5,19]. Smiddy et al. speculated that those mediators and other biochemical interactions mediate cell migration and proliferation and consequently form ERMs [23]. In hypercholesterolemic rabbits, vascular endothelial cells release chemoattractants in the area of ischemic choroid caused by occluding a short posterior ciliary artery [19]. Furthermore, the permeability of endothelial cells is increased in hypercholesterolemia [26]; therefore, in patients with hyperlipidemia, chemoattractants from the serum or vascular endothelial cells may mediate cell migration and proliferation, thereby promoting the development of idiopathic ERMs.

The well-known clinical association between diabetic retinopathy and ERMs was seen in the Beaver Dam population [12]. In the Blue Mountains Eye Study, there was no association between diabetes and CMR, but there was a significant association between diabetes and PMF, after subjects with any signs of diabetic retinopathy were excluded; however, we failed to find a significant association between diabetes and ERMs. Diabetes might promote the development and progression of ERMs, and different stages of ERMs might have different risk factors. In our study, diabetes was not associated with ERMs, possibly because there were too few subjects with PMF.

Several factors limit the interpretation of the results of this study. Firstly, this study used non-stereoscopic 45° fundus photographs to grade ERMs, whereas the other studies used 30° stereoscopic fundus photographs. Non-stereoscopic photographs might have resulted in an underestimation of the prevalence of ERMs by missing subtle early macular changes, especially CMR; however, we sought to minimize this potential shortcoming by having trained ophthalmologists evaluate the patients. Secondly, the rate of participation in the examination was low. As a result of the participation rate, the study is likely subject to selection bias, which could have influenced the results. Hypercholesterolemia and ERMs resulting in impaired vision would have been major motivations to participate. Consequently, the study might have examined a higher proportion of hypercholesterolemia and visually impaired participants than it would have done from a representative sample.

Conclusion

In conclusion, our population-based study suggests that ERMs are less common in a sample Japanese population than in similar populations in Western countries, and that hyperlipidemia is a significantly relevant factor. Due to the cross-sectional design of this study, it is still unclear whether hypercholesterolemia is a cause of ERMs. Further prospective studies will help to clarify the causal relationships between hypercholesterolemia and ERMs.

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Simian lentiviral vector-mediated retinal gene transfer of pigment epithelium-derived factor protects retinal degeneration and electrical defect in Royal College of Surgeons rats

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Retinitis pigmentosa (RP) is a heterogenous group of inherited retinal diseases resulting in adult blindness caused by mutations of various genes. Although it is difficult to cure the blindness that results from these diseases, delaying the disease progression may be of great benefit, since the majority of RP diseases are seen in middle age or later. To test a gene therapy strategy for RP using a neurotrophic factor gene, we assessed the effect of simian lentivirus (SIV)-mediated subretinal gene transfer of pigment epithelium-derived factor (PEDF), a potent neurotrophic factor, during the disease progression in Royal College of Surgeons (RCS) rats, a well-accepted animal model of RP. Regional gene transfer via SIV into the peripheral subretinal space at the nasal hemisphere was performed in all animals to monitor site-specific transgene expression as well as the therapeutic effect in each retina. Gene transfer of lacZ and PEDF was observed in the regional pigment epithelium corresponding to the regional gene transfer. Histologically, PEDF gene transfer significantly protected the loss of photoreceptor cells (PCs) corresponding to the regions of the gene transfer, compared to those of control groups during the course of the experiment. The antiapoptotic effect of PEDF on PCs is likely to be a related mechanism, because a significant reduction of terminal dUTP-nicked end labeling-positive PC numbers was found in PEDF-treated eyes compared to those of the control group ($P < 0.05$). PEDF-treated eyes also retained a significant sensitivity to light flash during the experimental course. These findings clearly show that neuroprotective gene therapy using PEDF can protect retinal degeneration and functional defects in individuals with RP.
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Keywords: *retinitis pigmentosa; simian immunodeficiency virus; pigment epithelium-derived factor; Royal College of Surgeons rats; apoptosis*

Introduction

Retinitis pigmentosa (RP) is a group of inherited retinal degenerative diseases characterized by a progressive degeneration of rod and cone photoreceptor cells (PCs). RP is a major cause of blindness in adults, affecting approximately one in 3,500.[1,2] This group of diseases is caused by mutations in various genes, including rhodopsin, cGMP phosphodiesterase β-subunit (PDE-β), and rds/peripherin.[3–5] Although extensive efforts have been made throughout the world to treat or cure RP, these diseases remain intractable.

Recently, advent gene therapy technology has been introduced as a possible novel therapeutic strategy for treating RP. Gene transfer vectors including recombinant adenovirus, adeno-associated virus (rAAV), and human immunodeficient virus (HIV)-based lentivirus have been employed and have shown significant therapeutic effects in animal models of RP.[6–11] In the clinical setting, rAAV and lentivirus are likely to be better for RP than adenovirus, because both gene transfer to nondividing cells and long-lasting therapeutic effects are required to treat RP.[12–14]

Several experimental approaches have been suggested by these studies, including (1) supplementation of a normal gene to PCs directly,[6,9,10,15–17] (2) prevention of the accumulation of abnormal proteins by enhanced degradation using ribozyme in PCs,[8,18] (3) inhibition of apoptotic loss of PCs via cellular antiapoptotic genes,[19] and (4) suppression of apoptotic loss of PCs via overexpression of neurotrophic factors.[7,11,20] Clearly, the tropism of each vector is a critical piece of information, because the former three strategies require highly efficient gene transfer to PCs.

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We recently developed a novel lentiviral vector based on the simian immunodeficiency virus (SIV) from African green monkeys[21] and showed safe, stable, and long-term transgene expression over 1 year in the retinal tissue of adult rats under suitable titer.[22] According to our findings via repeated experiments, transgene expression using SIV was limited to the retinal pigment epithelium (RPE) via subretinal injection in young adult rats with the exception of accidental gene transfer to PCs.[22] This tropism of SIV suggests that gene transfer of the neurotrophic factor may be a better choice for treating RP.

Pigment epithelium-derived factor (PEDF) is a 50-kDa secreted glycoprotein, and was first isolated from conditioned medium from both fetal and adult RPE.[23,24] PEDF binds to the glycosaminoglycans of the interphotoreceptor matrix, placing it in a prime physical location to affect the underlying neural retina.[25,26] PEDF functions as a survival factor for cultured cerebellar granule cells,[27–29] spinal motor neurons,[30,31] and hippocampal neurons[32] *in vitro* and delays the death of photoreceptor cells in the rd mouse *in vivo*.[33] In addition to its neurotrophic action, PEDF is antiangiogenic, inducing endothelial apoptosis via the Fas–Fas ligand system.[34–36]

Our current study attempted to determine whether SIV-mediated PEDF gene transfer to the retina might correct the histological and functional deficits of PCs in a well-accepted animal model of RP, Royal College of Surgeons (RCS) rats, in which these deficits are caused by a mutation of the receptor tyrosine kinase *Mertk*,[37] which corresponds to the same kinase in human populations.[38]

Results

Histological assessment of transgene expression

We previously determined that SIV-mediated gene transfer was frequently seen in the RPE lasting more than 1 year stably, but was also occasionally detected in the PCs via the subretinal injection technique.[22] To examine whether these findings were also representative in RCS rats, we repeated nls-lacZ and human PEDF gene transfer. Transgene expressions were assessed by X-gal histochemistry for lacZ and by immunohistochemistry for PEDF at various time points (4, 8, 12, and 24 weeks).

At 4 weeks after subretinal injection of SIV-nls-lacZ, frequent and widespread X-gal-positive blue spots were detected in the retina, almost coinciding with the area of vector injection, and especially in the RPE (Figures 1c and d). These positive reactions were observed up to at least 24 weeks after gene transfer (Figure 1e).

Immunohistochemical analysis showed that PEDF expression was observed in RPE only in the SIV-PEDF-treated retinas 4 weeks after injection (Figure 1f), and the positive reaction was also found at all time points after gene transfer (4, 8, 12, and 24 weeks after injection; data not shown). No positive reaction was observed in the retinas, either in the temporal hemisphere (Figure 1g) or in the retinas treated with SIV-nls-lacZ (Figure 1h), suggesting that the antibody used may not crossreact with rat PEDF.

Histological evaluation of photoreceptor cell degeneration

At various time points after the gene transfer (4, 8, 12, and 24 weeks), the number of nuclei of PCs in each retinal area was counted to determine the effect of PEDF gene transfer on PC degeneration. At 4 weeks after injection, the retinas treated with PEDF demonstrated significant rescue of PCs in the nasal hemisphere ($P<0.01$), compared to the retinas that were not treated and to those treated with BSS or SIV-nls-lacZ (Figures 2a–e). As shown in Figure 3a, the region nearest the injection site showed the greatest prevention of PC death, and in the three regions nearest to the injection site, PCs were significantly preserved by PEDF gene transfer, compared to the levels in controls. Similar and significant PC protections were also obtained at 8 and 12 weeks after injection. (Figures 3b–d). Throughout the study period, neither severe inflammatory infiltration nor morphological change of RPE was seen in any of the retinas.

Evaluation of PC apoptosis by TUNEL staining

To confirm the frequency of PC apoptosis, we administered terminal dUTP-nicked end labeling (TUNEL) stain to the retinas 4 weeks after injection. The retinas of BSS and SIV-lacZ-injected eyes demonstrated scattered positive reactions against apoptosis in the thinned outer nuclear layer, as well as in the untreated control eyes (Figures 4a–c). On the other hand, the number of apoptotic nuclei was significantly smaller in the retinas treated with SIV-PEDF (Figures 4a–c). The density of TUNEL-positive photoreceptor nuclei was lower in the SIV-PEDF-treated retina than in the other control eyes (Figure 4d).

Evaluation of retinal electrical function using electroretinograms

Dark-adapted electroretimograms (ERGs) were recorded at 4 and 8 weeks after subretinal injection to determine whether the histopathological protective effect of PEDF gene transfer on retinal degeneration might reflect retinal functions. A blinded examiner performed the ERG recording. At 4 weeks after the injection, the ERG responses had disappeared to a significant extent in the groups of untreated, balanced salt solution (BSS)-treated, and SIV-nls-lacZ-treated RCS rats, while significant amplitudes of a- and b-waves were observed in the group of SIV-PEDF-injected rats. In two of the six rats treated with lacZ, small but significant electrical responses were detected. Although the amplitudes gradually decreased in a time-dependent manner, a decline of the mean amplitudes of the a- and b-waves was significantly prevented by PEDF at 4 and 8 weeks after the gene transfer (Table 1).

Discussion

In the present study, we clearly demonstrated that gene transfer of human PEDF, a neurotrophic factor, into RPE via our novel lentiviral vector SIV significantly delayed photoreceptor degeneration and electrophysiological defects in RCS rats. These findings suggest that there exists a neuroprotective effect of PEDF gene transfer in photoreceptor degenerative diseases.

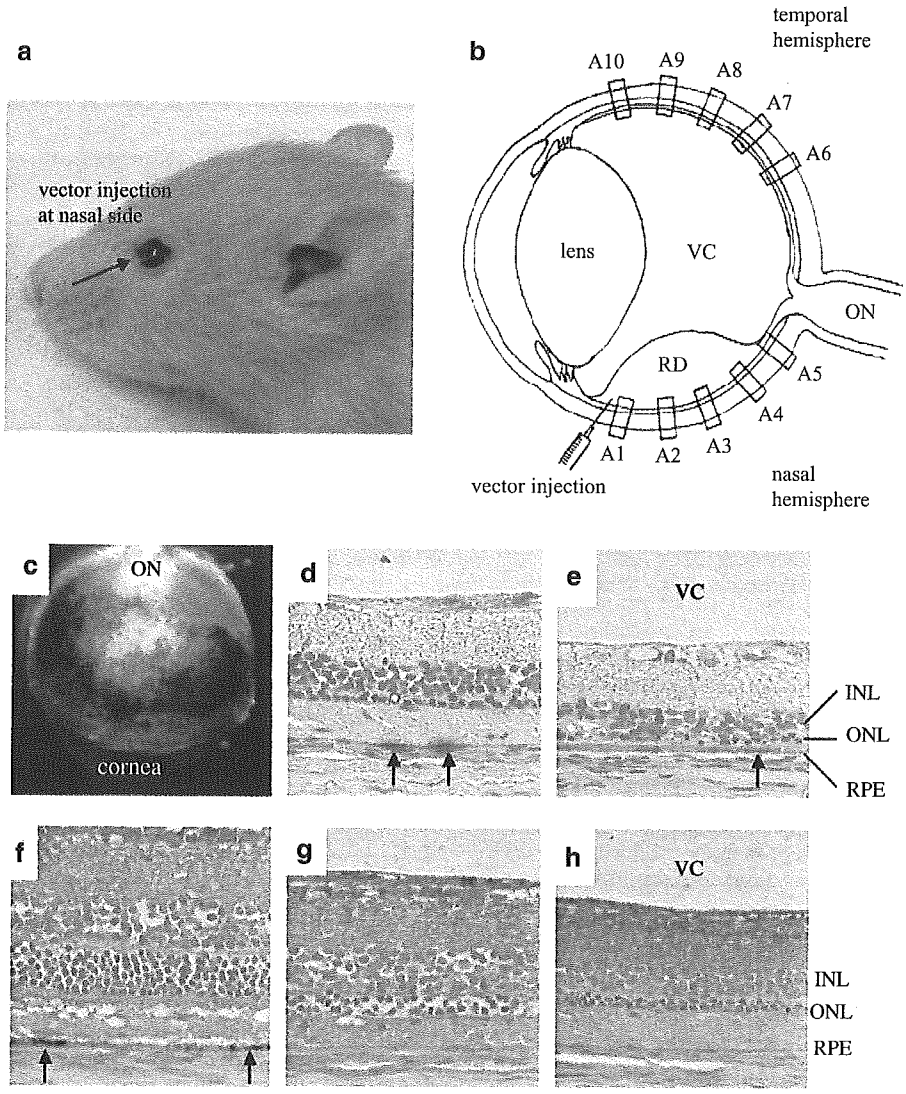

Figure 1 *The gene transfer technique (a, b). A 30-gauge needle was inserted into the subretinal space of the peripheral retina in the nasal hemisphere via an external trans-scleral transchoroidal approach. We counted the number of PCs per 100 μm at the 10 points shown in this figure. Panels (c, d, e) demonstrate the location and duration of SIV-mediated transgene expression* in vivo. *Panels (c, d) show the results of X-gal histological studies at 4 weeks after the subretinal injection of SIV-lacZ. Gene expression of lacZ persisted up to at least 24 weeks (e). The results of immunohistochemical studies against human PEDF at 4 weeks after subretinal injection of SIV-PEDF in the nasal hemisphere are shown in (f), those in the temporal hemisphere in (g), and SIV-lacZ in (h). PEDF expression was observed in RPE only in the case of SIV-PEDF subretinal injection. Original magnifications: c, × 8; d–h, × 200.*

Possible gene therapy approaches to prevent retinal degeneration have been suggested, including (1) supplementation of normal gene to PCs directly,[6,9,10,15–17] (2) prevention of the accumulation of abnormal proteins by enhanced degradation using ribozyme in PCs,[8,18] (3) inhibition of apoptotic loss of PCs via cellular antiapoptotic genes,[19] and (4) suppression of apoptotic loss of PCs via the overexpression of secreting neurotrophic factors, including FGF-2[20,39] and CNTF.[7,11,40,41] Clearly, the former three approaches require highly efficient gene transfer to PCs, while the latter does not, indicating that the tropism of the vector and the choice of promoter may be critical factors in the therapeutic outcome. We therefore first characterized the gene transfer of SIV with CMV promoter in the retina of RCS rats using nls-lacZ and PEDF genes.

As shown in Figure 1, SIV-mediated retinal gene transfer driven by CMV promoter was relatively limited to the RPE, although it was rarely seen in some PCs. This result was equivalent to that reported previously in Wistar rats.[22] This gene transfer and expression profile suggests that, in the use of the SIV-CMV promoter construct, gene transfer of a secreting neurotrophic factor might be a reasonable approach. This approach may be advantageous compared to gene supplement approaches, because normal gene supplementation requires a number of different therapeutic genes and vectors for individuals. The use of neurotrophic factor may not always depend on the genetic status, which suggests a practical advantage in clinical settings.

PC protection gene therapy using neurotrophic factors has been evaluated and reported by some laboratories,[7,11,41] and these reports demonstrated a significant level of prevention of PC degeneration in RP model animals. Some of these studies, however, assessed the effects only at specific time points. Here, we examined

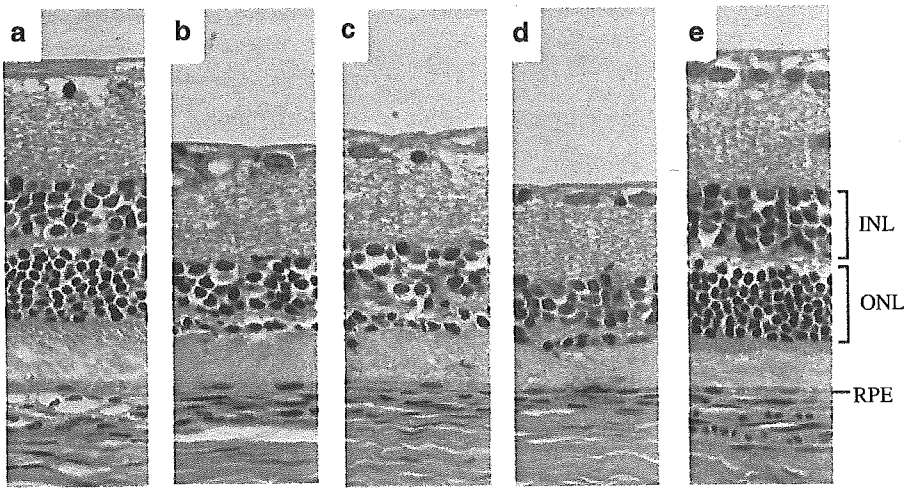

Figure 2 *Histological findings of the nasal retina of congenic RCS-rdy rats (a), untreated RCS rats (b), and RCS rats injected with: BSS (c), SIV-lacZ (d) and SIV-PEDF (e) at 4 weeks after injection. The SIV-PEDF-injected retina displayed five to six rows of PCs in the nasal hemisphere, compared with two to three rows in the other control retinas. On the other hand, in the temporal hemisphere, the SIV-PEDF-injected retina was similar to that of the other controls. Staining was made with hematoxylin eosin. Original magnifications: a–e, × 400.*

the time course of the effect of neurotrophic gene transfer, and clearly observed a significant delay in histological and electrophysiological PC degeneration, but not their complete prevention in comparison with the control congenic RCS-rdy rats (Table 1), even though we confirmed immunohistochemically sustained expression of PEDF throughout the experimental course *in vivo* (data not shown). This limited biological effect of PEDF demonstrated in this study may be explained by (1) our limitation of the gene-transfer area to approximately 1/4–1/6 of the whole retinal square, or (2) the limited therapeutic potential of PEDF for retinal degeneration. The former limitation was an intentional part of our experimental design; we injected vectors to a limited area in order to obtain evidence of a therapeutic effect of PEDF that was specific to the transfer area, as well as to gain an internal control in the noninjected area of the same eye. Also, extensive detachment during gene transfer may cause traumatic loss of vision, which of course was not ethically acceptable. We are currently assessing the use of multiple small injections over a wider retinal region to determine whether extension of the gene-transfer area may yield more functional benefits.

Regarding the second point – the possibility that PEDF had a limited therapeutic effect on RP – this should be considered in more detail, since it is related to the choice of therapeutic gene. As demonstrated in Table 1, PEDF treatment reproducibly resulted in a-wave>b-wave amplitude ratio. This result was fairly unusual, because the amplitude of b-wave, which corresponds to the mass response amplifying the signals from PCs, should be greater than that of a-wave. To explain this, we are now assessing the therapeutic effect of FGF-2, an alternative neurotrophic factor, on RCS rats via SIV-mediated gene transfer. Interestingly, although FGF-2 treatment resulted in a less pronounced effect in terms of both histology and a-wave amplitude than did PEDF treatment, b-waves in eyes treated with FGF-2 inversely demonstrated approximately two-fold higher amplitudes than those seen with PEDF (unpublished observation). Although it is still premature to reach a conclusion regarding these findings at present, a possible explanation might be that PEDF dominantly rescues PCs, but not secondary neurons. This hypothesis would be supported by the following findings: (1) neurotrophic factor-mediated activation, including FGF-2 but not PEDF, of intracellular signaling pathways has been observed in Muller cells and other cells of the inner retina, but not in PCs,[43] and (2) a possible PEDF receptor is located in the inner segment of PCs and the ganglion cell layer, but not in the inner nuclear layer.[44] In addition, the occurrence of an ERG pattern in which the a-waves have a greater amplitude than the b-waves is not unheard of, since such an unusual ERG ratio has been observed both in BALB/cByJ nob/nob mice and in patients with complete X-linked congenital stationary night blindness type 1.[45] According to our current data, PEDF holds the potential to provide retinal protection; however, we do not know whether this therapeutic gene will be sufficient to treat RP in human subjects. Our current study is, to the best of our knowledge, the first demonstration of a significant therapeutic effect of gene transfer in the use of PEDF, and we believe that PEDF is likely to be one of the better genes among the various neurotrophic factors because of its antiangiogenic ability through the induction of endothelial apoptosis.[34–36] Since the unexpected proliferation of neovessels is likely to worsen the patient's vision, PEDF would seem to be a good candidate for retinal gene therapy. Therefore, further studies will be needed to assist in the choice of appropriate therapeutic gene(s), and theoretically, the combination of PEDF and FGF-2 may be an option for more effective gene therapy.

Finally, while discussions regarding the relevancy of the delay of disease progression sufficient to matter to patients should be ongoing, we consider that it may be reasonable, because majority of RP diseases in human subjects progress over several decades. This clinical feature of RP suggests that a doubling of the disease time course by gene therapy may, at least in theory, be sufficient to allow patients with RP to retain their vision. Further evaluation of the utility of delaying the disease progression is needed, especially through clinical studies.

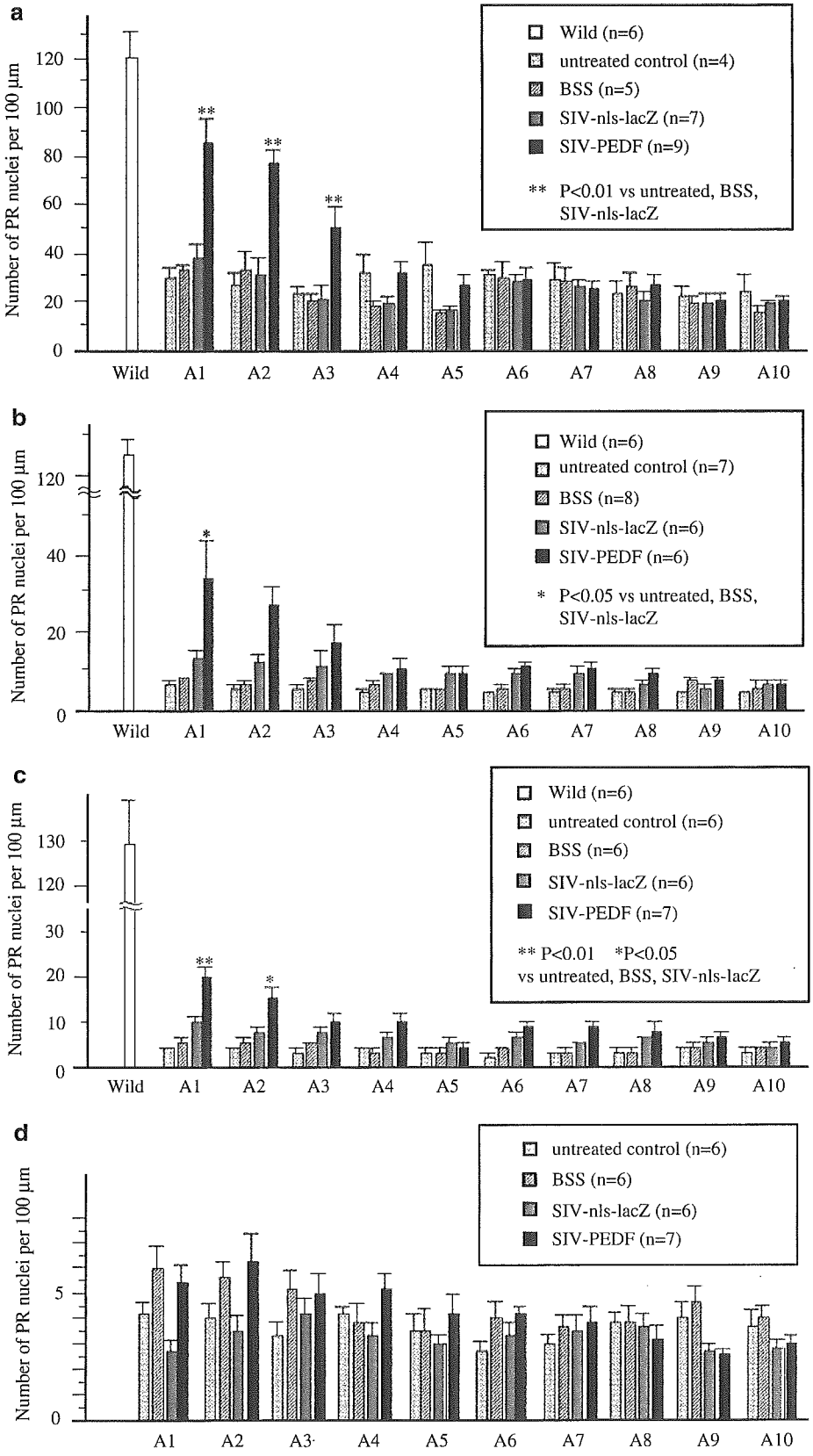

Figure 3 The mean number of photoreceptor nuclei per 100 µm retina along the horizontal meridian of the eye from the optic nerve head to the ora serrata of RCS rats at 4 weeks (a), 8 weeks (b), 12 weeks (c), and 24 weeks (d) after injection. The nearer the region was to the SIV-PEDF injection site, the better preserved were the photoreceptor nuclei.

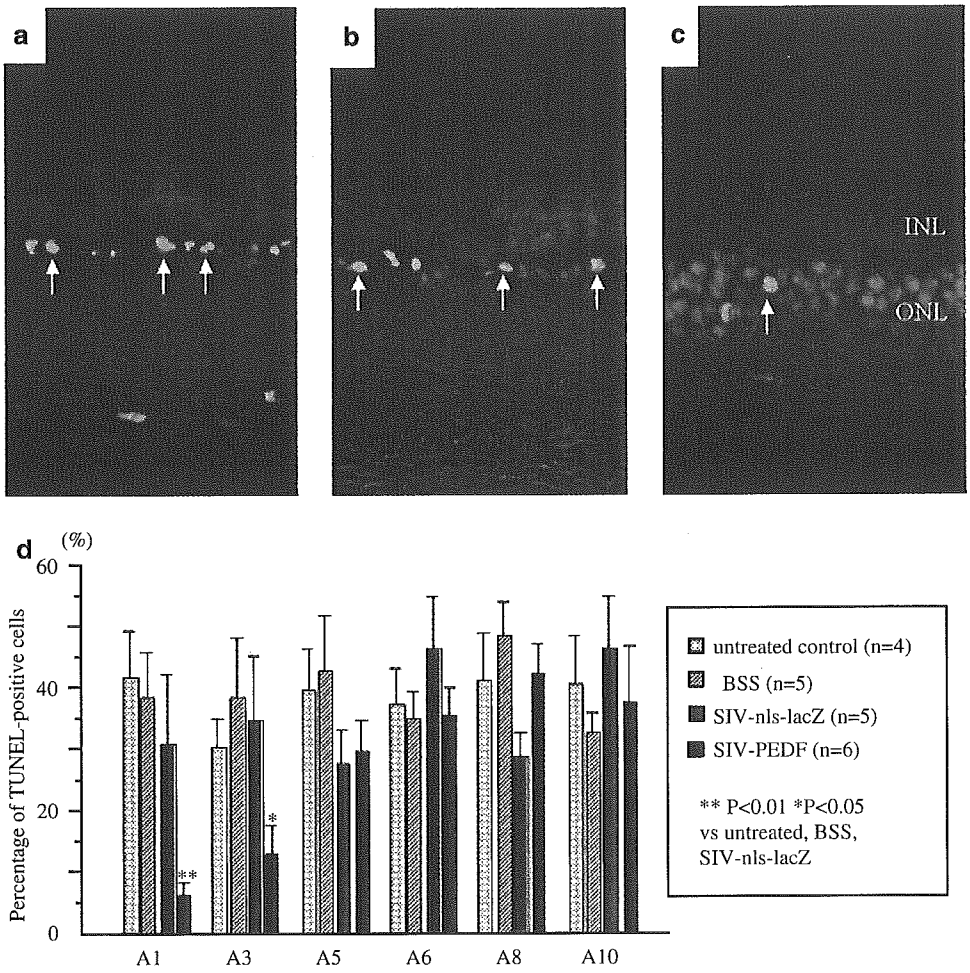

Figure 4 *Fluorescence photomicrographs of TUNEL-processed retinal sections at 4 weeks after injection ((a) untreated control eye; (b): BSS-injected eye; (c): SIV-PEDF-injected eye). The density of TUNEL-positive photoreceptor nuclei is smaller in the SIV-PEDF-treated retina than in the other control eyes (d). Original magnifications: a–c, × 200.*

Table 1 Average of a- and b-wave amplitude from dark-adapted ERG analysis at 4 and 8 weeks after injection

Time (after injection)	*No. of eyes*	*Treatment*	*a-Wave. (μV)*	*b-Wave (μV)*
4w	2	Wild type	344.1±14.5	991.4±11.2
	10	SIV-PEDF	72.5±23.2*	47.0±15.3
	6	SIV-nls-lacZ	14.7±7.9	5.5±3.9
	6	BSS	0.0	0.20±0.20
	8	Untreated	0.09±0.09	0.15±0.15
8w	8	SIV-PEDF	25.9±8.0*	10.5±5.3
	6	SIV-nls-lacZ	0.8±0.5	3.3±2.2
	1	BSS	0	0
	3	Untreated	0	0

*$P<0.05$ vs untreated control, BSS, and SIV-nls-lacZ.

In summary, we demonstrated that SIV-based lentivirus vector-mediated PEDF gene transfer to the retina of RCS rats could significantly protect against retinal degeneration, morphologically and functionally, and that this effect occurred, at least in part, via the inhibition of photoreceptor cell apoptosis. These findings suggest that SIV-based lentivirus vector-mediated PEDF gene transfer to the retina is a useful approach for RP.

Materials and methods

SIV vectors

Recombinant SIVs were constructed as previously described[21]. The SIVs encoding the *E.coli* lacZ gene, with simian virus 40 large T antigen nuclear localizing signal (SIV-nls-lacZ) and pigment epithelium-derived factor (SIV-PEDF), were propagated. A U3 region in the 3′ and 5′ long terminal repeat (3′ and 5′ LTR) of the SIV was deleted for self-inactivation, and the SIV vectors were pseudotyped with vesicular stomatitis virus envelope glycoprotein G (VSV-G). The virus titer was determined by transduction of the human embryonic kidney 293T cell line, and these viruses were kept at −80°C until just before use.

Animals

The 3-week-old inbred RCS rats, which are pink-eyed, tan-hooded rats (gift of Professor Tamai, Tohoku University, Sendai, Japan), and congenic RCS-*rdy* rats of the wildtype at the *rdy* locus were maintained humanely, with proper institutional approval, and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were carried out 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 Pathogens Experiments at Kyushu University and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government. In the sections below, *n* is used to indicate the number of treated eyes.

Gene transfer procedures

The subretinal injection of each solution was performed as previously described with minor modifications.[21] Briefly, rats were anesthetized by inhalation ehtel. The following procedures were then performed using an operating microscope. A 30-gauge needle was inserted into the anterior chamber at the peripheral cornea, and 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. The vector solution (10μl)(SIV-PEDF or SIV-nls-lacZ) (2.5×10^7 transducing units (TU)/ml) or BSS: (137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, and 13 mM Tris, pH 7.6) was injected, and excess solution from the injection site was washed out using phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM KH_2PO_4, pH 7.2). Approximately 2–3 μl of solution remained in the subretinal space (data not shown). 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.

X-gal histochemistry

The rats were killed, and the eyes were enucleated and fixed with ice-cooled 2% paraformaldehyde with 0.25% glutaraldehyde in 0.1 M PBS for 10 min. Then the eyes were washed with 0.1 M PBS containing 0.1% Triton-X followed by X-gal staining (solution: 5 mM potassium ferrous cyanide, 5 mM ferric cyanide, 2 mM magnesium chloride, 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) for 3 h at room temperature. The X-gal-stained tissue was refixed and mounted in paraffin, and 5 μm-thick sections counterstained with nuclear fast red were examined under a light microscope.

Immunohistochemistry

The eyes of animals injected with SIV-PEDF, SIV-nls-lacZ, and BSS were enucleated at 4, 8, 12, and 24 weeks after injection. Eyecups were fixed with ice-cooled 4% paraformaldehyde in PBS for 1 day at room temperature. The eyes were mounted in paraffin, and 5 μm-thick sections were made and allowed to dry overnight. The sections were incubated for 1 h at room temperature using a monoclonal antibody to PEDF (CHEMICON International, Temecula, CA, USA) diluted in 1% bovine serum albumin (1/250). DACO catalyzed signal amplification (CSA) System peroxydase (DAKO, Carpinteria, CA, USA) was then used to amplify the signal of PEDF. Bound antibodies were detected by color modification of DAB by $CoCl_2$ to distinguish the signal from the melanin pigments.[46] After being washed with water, slides were counterstained with methyl green.

TUNEL staining

The rats were killed, and their eyes were enucleated and fixed with 4% paraformaldehyde in PBS. The eyes were mounted in paraffin in 5 mm-thick sections. To detect apoptotic cells, retinal sections were processed for TUNEL, using the ApopTag Fluorescein Direct *In Situ* Apoptosis Detection Kit (Intergen, Purchase, NY, USA) according to the recommendations of the manufacturer. After labeling, propidium iodide was used to counterstain the nuclei.

Electroretinograms

To exclude interanimal variation in the ERG recordings, in the case of SIV-PEDF injection to one eye, the opposite eye was usually treated with SIV-nls-lacZ or BSS, or was not treated, as an internal control (4 weeks after injection: PEDF *versus* lacZ=four animals, PEDF *versus* BSS=three animals, PEDF *versus* untreated=three animals, lacZ *versus* untreated=two animals, BSS *versus* untreated=three animals; 8 weeks after injection: PEDF *versus* lacZ=five animals, PEDF *versus* untreated=three animals, lacZ *versus* BSS=one animal). The data from some control animals are also included in Table 1 (where '*n*' indicates the number of eyes). ERGs were recorded by a masked examiner (YG) without any information on whether the eyes were treated or untreated. Each animal was subjected to ERG study at several time points. The rats were kept in a dark room at least overnight before the experiment. The electroretinographic evaluations were performed using previously described methods.[47,48] The rats were anesthetized with an intraperitoneal injection of 15 μl/g body weight of saline solution containing ketamine (1 mg/ml), myoblock (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. ERGs were recorded using a coiled stainless-steel wire containing the anesthetized (1% proparacaine HCl) corneal surface through a layer of 1% methylcellulose, and a similar wire was also placed in each of the leads. The responses were differentially amplified (band pass: 0.8–1200 Hz), averaged, and stored by a minicomputer (Signal Processor 7T17, NEC Sau-ei). Xenon strobe flash stimuli ($t<1$ ms) were presented by a Ganzfeld stimulator (VPA-10; Cadwell, Kennewick, WA, USA) in the dark.

The dark-adapted ERGs were obtained using a flash intensity of 1.30 log cd-s/m^2. The responses to five successive flashes in each rat were averaged. In addition, a 1-min interflash interval was used for this session. The ERG experiment was done in an identical manner 4 and 8 weeks after injection. PEDF-treated eyes always showed better ERG amplitudes than those seen in internal control eyes.

Statistical analyses

All values were expressed as the means ± s.e.m. The data were analyzed by using a nonparametric test (Mann–Whitney *U*-test). A *P*-value of less than 0.05 was considered to be statistically significant.

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