Diverse Applications of Gene Therapy

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Preface

This book has been a concerted effort by a group of academicians, researchers and scientists, who have contributed their research works for the realization of the book. This book has materialized in the wake of emerging advancements and innovations in this field. Therefore, the need of the hour was to compile all the required researches and disseminate the knowledge to a broad spectrum of people comprising of students, researchers and specialists of the field.

Gene therapy has been extensively researched in this all-inclusive book. Gene therapy as a field has gained its due regard after all these years of poor outcomes. Issues in the past which were troubling scientists and practitioners are now being easily resolved. The growth of secure and effective gene transfer and development in the field of cell therapy has now brought new ways to deal with varied diseases. The book aims at compiling information from different resources about various gene therapy tools, practical achievements of gene therapy and its future usage. Some of the important chapters discuss non-viral delivery systems in gene therapy, transgene expression, plasmid transgene expression in vivo - promoter and tissue variables, DNA electrotransfer as an effective tool for gene therapy, siRNA and gene formulation.

At the end of the preface, I would like to thank the authors for their brilliant chapters and the publisher for guiding us all-through the making of the book till its final stage. Also, I would like to thank my family for providing the support and encouragement throughout my academic career and research projects.

Editor

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1. Introduction

The retina comprises diverse differentiated neurons that have specific functions. Photoreceptor cells, the first-order neurons in the retina, have photopigments (rhodopsin and opsin) that absorb photons. Signals produced by the photoreceptor cells are transmitted to second-order neurons. Finally, visual signals are transmitted to the brain from the third-order neurons, the retinal ganglion cells (RGCs). Major diseases that cause blindness in advanced countries include glaucoma, diabetic retinopathy, retinitis pigmentosa (RP), and age-related retinopathy. Loss of vision due to these diseases is irreversible. However, with regard to glaucoma, eye drops that have the effect of reducing intraocular pressure have been developed. In diabetic retinopathy, effective surgical treatments such as vitrectomy and photocoagulation have been established. Blindness due to glaucoma and diabetic retinopathy can be prevented by administering these treatments in the early phase. On the other hand, in diseases caused by gene mutations, such as RP, effective treatments for delaying photoreceptor degeneration have not yet been established. Degeneration of photoreceptor cells results in loss of vision, even if other retinal neurons are intact [1-3].

RP is a disease that causes blindness due to photoreceptor degeneration. Symptoms include night blindness and loss of peripheral and central vision. Approximately 1 in 4,000 people are affected by this disease [4]. In 1990, Dryja et al. [5] first identified a point mutation in the rhodopsin gene from RP patients. A number of gene mutations responsible for RP has subsequently been identified. Most of these genes are associated with the phototransduction pathway in the retina. In some cases, the mutated gene exists not only in photoreceptor cells but also in retinal pigment epithelial cells. To date, 53 causative genes and 7 loci of RP have been identified (http://www.sph.uth.tmc.edu/Retnet/). Leber's congenital amaurosis (LCA) is another retinal degenerative disease predicted to affect approximately 1/81000 individuals [6]. Most LCA patients have

severe visual defects in childhood. Histological analysis of the retinas of LCA patients shows marked retinal atrophy in the outer retinal layer, vascular thickening and sclerosis, and atrophy of the retinal pigment epithelium (RPE) [7]. Leber classified the disease as a type of RP on the basis of these characteristics. Later, Franceschetti and Dieterle differentiated it from retinal dystrophy based on the features of electroretinograms (ERGs) in these patients. Many gene mutations involved in LCA have been identified and the disease has been classified into 15 subtypes based on the affected gene [8-13]. Among these, LCA2, accounting for 10% of LCA cases [14], is due to a mutation in the RPE65 gene, which encodes all-*trans* retinyl ester isomerase. Deficiency in RPE65, leads to severe loss of visual function. Thus, in the case of LCA2, the cause of the disease is clearly identified as the biochemical blockade of the visual cycle caused by RPE65 deficiency [11,12]. Replacement therapy using the RPE65 gene is a candidate therapeutic strategy for LCA2. Indeed, successful results have been reported in RPE65 replacement therapy with the LCA2 animal model, Briard dogs [15]. After proof-of-principle studies [16], phase I trials using adeno-associated virus vector type 2 were conducted in 3 independent groups [17]. The results showed no adverse effects such as systemic dissemination of vector or immunological responses to the vector or transgene. Importantly, improvement of visual function as evaluated by microperimetry was observed in 1 subject [18,19]. Two other groups also reported improvement in visual function [20,21]. Continuous follow-ups for 1.5 years [22] have confirmed the safety and tolerability of replacement gene therapy [23]. The various hereditary forms of RP are as follows: autosomal dominant, recessive, and X-linked recessive. The Pro23 -> His gene mutation in the rhodopsin gene [24,25] occurs in 20–30% of all RP patients in Europe and the U.S. In contrast, the occurrence in Japan is only a few percent. Thus, in addition to the diversity of the gene mutations, their frequencies vary characteristically among different races. Differences in the progression, clinical findings, and development of the disease are also observed among different patients, even in those with the same mutation. A common feature of photoreceptor cell death caused by various gene mutations is eventual apoptosis via a common pathway [26]. Based on this rationale, various kinds of methods to prevent apoptosis, such as chemical treatment [27,28] and gene therapy, including gene replacement and neurotrophic factor supplementation [29-31], have been investigated. However, these strategies have not been successful in the complete prevention of cell death, although they have been shown to delay degeneration. The diversity of clinical features and gene mutations makes it difficult to develop effective treatments for RP.

A retinal prosthesis, comprising electrodes, an image processor, and a camera, is the only method to restore vision that has been studied [32-36]. Recently, a new strategy involving gene therapy for restoring vision has been developed using bacteriorhodopsin family genes [37,38]. The channelrhodopsin-2 (ChR2) gene derived from the green alga *Chlamydomonas* functions as a photoreceptor and cation-selective channel [39]. After the absorption of photons by photopigments, photon acquisition is completed by a chain reaction involving certain photoreceptor-specific proteins. Thus, the phototransduction pathway in photoreceptor cells requires not only photopigments but also certain photoreceptor-specific proteins, which complicates the reaction. Due to the inherent characteristics of ChR2, photosensitive neurons can be produced by the transfer of the ChR2 gene into neurons [40-42]. Here, we introduce new strategies for restoring vision by using channelrhodopsins.

2. Materials and methods

All the experiments performed for this report were approved by the Tohoku University Animal Care Committee, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the number and suffering of animals used in the following experiments.

Animals

We used 2 types of photoreceptor degeneration models: a genetically blind rat model and a light-induced photoreceptor degeneration model. The experimental design for each of these models is shown in Fig. 1.

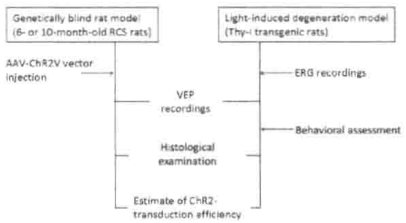

Figure 1. Experimental design. Two types of photoreceptor degeneration models were used in this study. The photoreceptor cells of RCS rats degenerate by 3 months after birth due to the Mertk gene mutation. On the other hand, Thy-TG rats have native photoreceptors. Therefore, we subjected TG rats to continuous light exposure to induce photoreceptor degeneration. To confirm photoreceptor degeneration, ERGs were recorded before performing behavioral assessments. Finally, the eyes from all animals were subjected to histological examination.

Genetically blind rats

Royal College of Surgeons (RCS; rdy/rdy) rats [43,44] were used as model animals for photoreceptor degeneration in our experiments. The RCS rat, an animal model of recessively inherited retinal degeneration, is widely used in the study of photoreceptor degeneration. The gene responsible is the receptor tyrosine kinase gene Mertk [45], and mutations in MERTK, the human ortholog of the RCS rat retinal dystrophy gene, cause RP [46]. Photoreceptor degeneration is almost complete by 3 months after birth. We intravitreously injected the AAV-ChR2V vector into 6-month- or 10-month-old RCS rats. The rats were obtained from CLEA Japan, Inc. (Tokyo, Japan).

Thy-I ChR2 transgenic rats

We established transgenic (TG) rats harboring the ChR2 gene regulated by the Thy-1.2 promoter to investigate contrast sensitivity at each spatial frequency [47]. The rat Thy-1.2 antigen has been found to be abundant in the brain and thymus [48,49]. In the retina, the Thy-1.2 antigen is recognized as a marker specific to RGCs [50,51]. It is necessary to induce the degeneration of native photoreceptor cells in order to investigate the visual function conferred by ChR2-expressing RGCs, because the Thy-I TG rat has native photoreceptor cells. For this purpose, Thy-I TG rats were subjected to light-induced photoreceptor degeneration. Briefly, Thy-I TG rats were kept in cyclic light (12 hours ON/OFF: 5–10 lux/dark) for at least 2 weeks

prior to light exposure. The rats were then exposed to a 3000-lux intensity of fluorescent light for 7 days [28]. We used a light exposure box (NK Systems, Tokyo, Japan) to control the timing and light intensity for the induction of photoreceptor degeneration. After induction, we recorded ERGs to confirm photoreceptor degeneration.

Preparation of the adeno-associated virus vector

The adeno-associated virus (AAV) vector with the ChR2 gene was constructed as described previously [38]. Following this, the AAV Helper-Free System (Stratagene, La Jolla, CA) was used to produce infectious AAV-Venus (control) and AAV-ChR2V virions, which were purified by a single-step column purification method as previously described [52].

Recording of ERGs and visual electrophysiology (VEP)

ERGs and VEP readings were recorded using a Neuropack (MEB-9102; Nihon Kohden, Tokyo, Japan) according to methods previously described [38,53]. Briefly, rats were dark-adapted overnight, and the pupils were dilated with 1% atropine and 2.5% phenylephrine hydrochloride. Small contact lenses with gold wire loops were placed on both corneas, and a silver wire reference electrode was inserted subcutaneously between the eyes. Eyes were stimulated with flash light stimuli of 10-ms duration using a blue LED. Full-field scotopic ERGs were recorded, band-pass filtered at 0.3–500 Hz, and averaged for 5 responses at each light intensity. For VEP recordings, recording electrodes (silver-silver chloride) were placed epidurally on each side, 7 mm behind the bregma and 3 mm lateral of the midline, and a reference electrode was placed epidurally on the midline 12 mm behind the bregma, at least 7 days before the experiments [54,55]. Under ketamine-xylazine anesthesia, the pupils were dilated with 1% atropine and 2.5% phenylephrine hydrochloride. The ground electrode clip was placed on the tail. Photic stimuli of 20-ms duration were generated under various intensities by pulse activation of a blue LED. The high- and low-pass filters were set to 50 kHz and 0.05 kHz, respectively. One hundred consecutive response waveforms were averaged for each VEP measurement.

Determination of transduction efficiency

At the end of the experiment, RCS and Thy-TG rats were sacrificed, and their eyes were resected and fixed in 4% paraformaldehyde and 0.1 M phosphate buffer, pH 7.4 [56]. The eye of each rat was flat-mounted on a slide and covered with Vectashield medium (Vector Laboratories, Burlingame, CA) to prevent the degradation of fluorescence. Then, the number of positive cells was counted.

3. Behavioral assessment

The spatial vision of each animal was quantified by its optomotor response. We used a virtual optomotor system to evaluate the contrast sensitivities of each spatial frequency. The original virtual optomotor system described by Prusky et al. [57] was modified for rats [47]. When a drum is rotated around an animal with printed visual stimuli on the inside wall, the animal tracks the stimulus by turning its head. A light-dark grating pattern was displayed on

computer monitors (ProLite E1902WS; Iiyama, Tokyo, Japan) arranged in a square around a platform. The software controlled the speed of virtual optomotor rotation, which was set at 12 degrees per second (2 rpm) in all experiments. The spatial frequency and the contrast of the grating pattern were varied but the average brightness was kept constant.

The animal was allowed to move freely on the platform in the virtual optomotor system. The grating session was started at a low spatial frequency (0.06 cycles/degree) with maximal contrast. An experimenter assessed whether the animals tracked the rotation, by monitoring the head movement and the presented rotating stimulus simultaneously on another display connected to the video camera. If head movement simultaneous with the rotation was evident, the experimenter judged that the animal could discriminate the grating, and proceeded to the next grating session. If the movement was ambiguous, the same grating session was presented again. All behavioral tests were double-blinded and performed during the first few hours of the animals' light cycle (light on at 8 AM).

4. Results

4.1. Recording of VEP measurements in RCS rats

VEP measurements in 6- or 10-month-old RCS rats are expected to be abolished due to loss of photoreceptor cells. Generally, in RCS rats, photoreceptor degeneration is almost complete by 3 months after birth. Indeed, VEP measurements were not evoked even by the maximal LED flash in any of the aged RCS (rdy/rdy) rats (Fig. 2A). On the other hand, robust VEPs were evoked by the blue LED flash in RCS rats injected with the AAV-ChR2V vector (Fig. 2A). Initially, small VEP responses were observed at 2 weeks after AAV injection (data not shown), and the maximum amplitudes of VEP were observed 8 weeks later [58]. There were notable differences in sample waveforms from 6- and 10-month-old rats injected with AAV-ChR2V. Amplitudes and latencies of VEPs from 6-month-old rats were larger and shorter, respectively, than those from 10-month-old rats (Fig. 2B).

4.2. Transduction efficiencies of ChR2 in retinas of RCS rats

The expression of the ChR2 gene was evaluated by measuring Venus fluorescence in RCS rat retinas (Fig. 3A). The number of positive cells in rats injected at 10 months of age was significantly less than that injected at 6 months of age (Fig. 3B). The number of RGCs decreased linearly with age, following photoreceptor degeneration in the RCS rats (Fig. 3C). We have previously shown [56] that the ChR2 gene is mainly expressed in RGCs upon intravitreous injection of the AAV-ChR2V vector. Therefore, the observed decrease in the number of RGCs with age suggests that the transduction efficiencies at both ages are very similar.

4.3. Photoreceptor degeneration in Thy-I TG rats

There were 11–12 rows of photoreceptor nuclei in the outer nuclear layer (ONL) of the Thy-1 TG rats; this is a number usually observed in rodents without retinal degeneration [59].

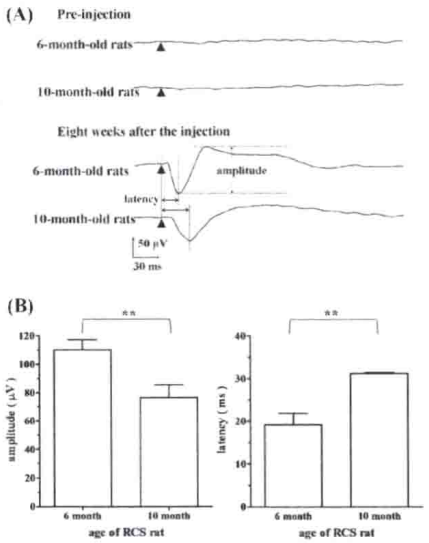

Figure 2. VEP recordings before and after the injection of AAV-ChR2V. (A) VEP recordings from both 6-month- and 10-month-old RCS rats showed no responses. However, VEPs responses were clearly elicited 8 weeks after injection. (B) The amplitudes and latencies from rats injected with AAV-ChR2 at 6 months of age (n = 8) were significantly larger and shorter than those injected at 10 months of age (n = 4).

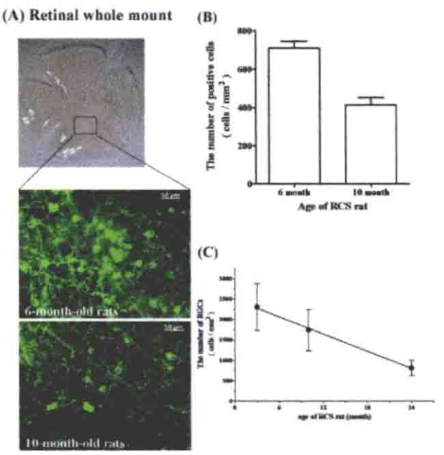

Figure 3. Transduction efficiencies of ChR2 in retinas of RCS rats. (A) Retinal whole-mount specimens obtained from rats injected with AAV-ChR2 at 6 and 10 months of age. (B) Venus-positive cells expressing the ChR2 gene were observed in whole-mount specimens. (C) The number of RGCs decreased with age.

Following continuous light exposure, photoreceptor cells disappeared (Fig. 4A). ERGs showed no response, indicating that the photoreceptor cells degenerated in the whole retina (Fig. 4B).