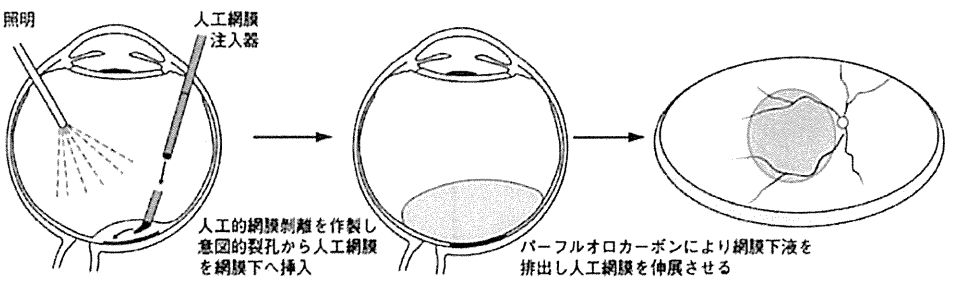

図3　色素結合薄膜型の人工網膜(OUReP™)の埋め込み手術

医療機器製造販売承認を最終的に申請することは，企業のみが可能であり，大学はできないという厳然たる事実がある。だからこそ企業を早く見つけて技術を移転する必要がある。治験の途中で品質が変わると，治験をやり直さなければならなくなる。治験開始時の工学部製造品と技術移転後の企業製造品とは同等の品質であることを目指して，最初から製造工程管理と品質管理が完成した工学部製造品で治験を始められるように準備している。2013年4月には，岡山大学病院が臨床研究中核病院として厚生労働省に選定され，その中で人工網膜の医師主導治験を進めることも決まった。

10. 生物学的安全性評価

「医療機器の製造販売承認申請等に必要な生物学的安全性評価の基本的考え方について」が，厚生労働省医薬食品局によって薬食機発0301第20号として2012年3月1日に発せられた。これは，ISO10993 "Biological evaluation of medical devices" に基づいている。岡山大学方式の人工網膜は，細胞毒性試験，感作性試験，遺伝毒性試験(復帰突然変異試験，染色体異常試験)，埋植試験，刺激性試験，全身毒性試験において毒性はなかった。また，人工網膜の部材である光電変換色素自体にも，すべての試験で毒性はみられなかった。

11. 治験の枠組み

First-in-humanの体内植込み型の医療機器の治験をどのような枠組みで行うかについては，植込み型補助人工心臓の治験が参考になる。2008年4月4日に厚生労働省医薬食品局より発せられた薬食機発第0404002号「次世代医療機器評価指標の公表について」の中に，(別添1)「次世代型高機能人工心臓の臨床評価のための評価指標」の(参考4)「治験の症例数と期間」の項目がある。そこに，「症例数は当面安全性を考慮したFeasibility studyの性格を持つものは5例前後，Pivotal studyは15例前後が適切だと考えられる」とある。

12. おわりに

先行事例がない中で次から次へと迫りくる問題を解決しながら[21),22)]，倫理的で科学的な医師主導治験を準備している。2013年には薬事法が改正され，法律名も「医薬品，医療機器等の品質，有効性及び安全性の確保等に関する法律」と変更されて2014年に施行される。製造工程管理と品質管理を進めて，治験実施計画書を作成し，治験計画の届出をPMDAに提出することを目指して薬事戦略相談を重ねている。治験申請が岡山大学病院の治験審査委員会に承認された後，松尾俊彦が専門とする網膜硝子体手術を活かして治験を行う予定である(図3)[23)～26)]。患者の気持ちと安全をまずは第一に考え，患者会とも連携して進めていきたいと思う。多くの人達に支えられて，やっと治験の入り口まで辿り着いた。

本稿のすべての著者には規定されたCOIはない。

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Vision maintenance and retinal apoptosis reduction in RCS rats with Okayama University-type retinal prosthesis (OUReP^{TM}) implantation

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Abstract Photoelectric dye-coupled polyethylene film, designated Okayama University-type retinal prosthesis or OUReP^{TM}, generates light-evoked surface electric potentials and stimulates neurons. In this study, the vision was assessed by behavior tests in aged hereditary retinal dystrophic RCS rats with OUReP^{TM}, retinal apoptosis and electroretinographic responses were measured in dystrophic eyes with OUReP^{TM}. The dye-coupled films, or plain films as a control, were implanted in subretinal space of RCS rats. On behavior tests, RCS rats with dye-coupled films, implanted at the old age of 14 weeks, showed the larger number of head-turning, consistent with clockwise and anticlockwise rotation of a surrounding black-and-white-striped drum, compared with rats with plain films, under the dim (50 lux) and bright (150 lux) conditions in the observation period until the age of 22 weeks ($n = 5$, $P < 0.05$, repeated-measure ANOVA). The number of apoptotic cells in retinal sections at the site of dye-coupled film implantation was significantly smaller, compared with the other retinal sites, neighboring the film, or opposite to the film, 5 months after film implantation at the age of 6 weeks ($P = 0.0021$, Friedman test). The dystrophic eyes of RCS rats with dye-coupled films showed positive responses to maximal light stimulus at a significantly higher rate, compared with the eyes with no treatment ($P < 0.05$, Chi-square test). Electroretinograms in normal eyes of Wistar rats with dye-coupled or plain films showed significantly decreased amplitudes ($n = 14$, $P < 0.05$, repeated-measure ANOVA). In conclusions, vision was maintained in RCS rats with dye-coupled films implanted at the old age. The dystrophic eyes with dye-coupled films showed electroretinographic responses. Five-month film implantation caused no additional retinal changes.

Keywords Retinal prosthesis · Photoelectric dye · RCS rat · Apoptosis · Electroretinogram

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Introduction

Retinitis pigmentosa is a hereditary disease, caused by mutations in many different kinds of genes which are expressed in retinal photoreceptor cells or retinal pigment epithelial cells [1]. Slowly progressive death of photoreceptor cells begins usually in the peripheral retina and approaches to the posterior pole of the eye, leading to visual field narrowing and finally to visual acuity reduction. Strategies for the treatment of retinitis pigmentosa will be in two directions: the first is to stop or at least make slower the progression of the disease with drugs in patients who still maintain poor but useful vision, and the second is to implant a substitute for photoreceptor cells in patients who have lost the vision. Retinal prosthesis, or artificial retina, is the most promising therapeutic modality to replace the lost photoreceptor cells [2, 3].

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The prevalent system of retinal prosthesis transfers the image, captured by a digital videocamera, to electric signals, and outputs electric current from an electrode array, implanted around the degenerated retina to stimulate the remaining neurons which send axons to the brain. In 2013, a first type of retinal prosthesis, Argus II^{TM} retinal prosthesis system [4], which uses this system, was approved by the United States Food and Drug Administration as a medical device.

The wider visual field plays an important role in ambulatory vision. The electrode arrays of retinal prostheses have a technical limitation in stimulating the wider retinal area to obtain the wider visual field. To accomplish a wider visual field with high resolution, we have developed a photoelectric dye-based retinal prosthesis, called Okayama University-type retinal prosthesis, or OUReP^{TM} (Fig. 1a, b) [5–12] and are currently preparing a clinical trial (Fig. 1c) [12–14]. In contrast with electrodes [2] or photodiodes [3] which output electric current to stimulate the remaining retinal neurons, the photoelectric dye, coupled to polyethylene film surface, outputs electric potential in response to light. The photoelectric dye-coupled polyethylene film (OUReP^{TM}) generates light-evoked surface electric potentials and does not output electric currents, but stimulates retinal neurons [8, 9, 12]. A rat strain with hereditary retinal dystrophy, called Royal College of Surgeons (RCS) rats, restored the vision in behavior tests after subretinal implantation of the dye-coupled films [15]. Indeed, the use of electric potential overcomes a major problem of photodiodes which generate too low electric currents to stimulate retinal neurons [3].

In this study, we assessed the vision by behavior tests in RCS rats with the dye-coupled films (OUReP^{TM}), implanted at the old age of 14 weeks, and measured electroretinograms in normal eyes of Wistar rats and in dystrophic eyes of RCS rats with OUReP^{TM} implantation. In addition, we analyzed retinal apoptosis 5 months after OUReP^{TM} implantation.

Materials and methods

Preparation of dye-coupled polyethylene film

Thin films were made from polyethylene powder and exposed to fuming nitric acid to introduce carboxyl moieties on the film surface. Photoelectric dye molecules, 2-[2-[4-(dibutylamino)phenyl]ethenyl]-3-carboxymethylbenzothiazolium bromide (NK-5962, Hayashibara, Inc., Okayama, Japan), were coupled to carboxyl moieties of the polyethylene film surface via ethylenediamine (Fig. 1b), as described previously [6, 8, 15]. The fuming nitric acid-treated only polyethylene film and the photoelectric dye-coupled polyethylene film were designated as the plain film and the dye-coupled film (Fig. 1a), respectively.

Animals and experimental design

Behavior tests were done in 10 male RCS rats with no treatment at the age of 6, 8, 10, 12, and 14 weeks. At the age of 14 weeks, 5 of these 10 RCS rats underwent subretinal implantation with dye-coupled films of 1 × 5 mm

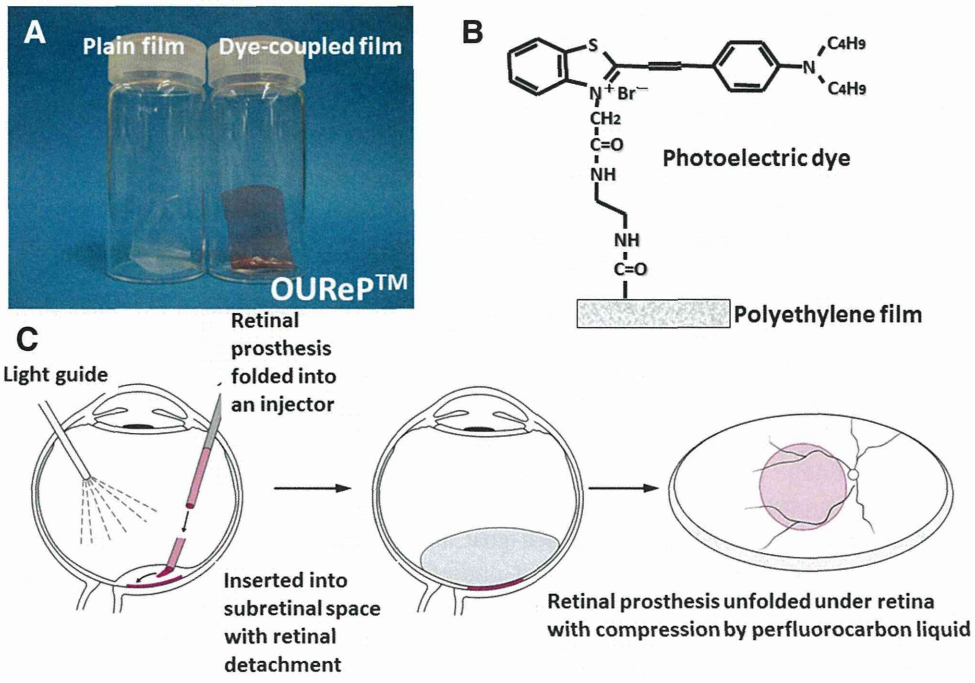

Fig. 1 **a** Plain polyethylene film (plain film, *whitish*) and photoelectric dye-coupled polyethylene film (dye-coupled film, *reddish*, OUReP^{TM}). **b** Photoelectric dye molecule, coupled to polyethylene film surface. **c** Schematic drawing to show subretinal implantation of dye-coupled film (OUReP^{TM}) at vitreous surgery

size in both eyes while the remaining 5 rats underwent plain film implantation in both eyes, via scleral incision as described previously [10, 15].

The behavior tests were repeated in the 10 rats with film implantation at the age of 16, 18, 20, and 22 weeks. At the age of 22 weeks, rats were sacrificed to confirm the subretinal film implantation. As another series of experiments, dye-coupled films were implanted in 7 RCS rats and plain films implanted in 7 RCS rats at the age of 6 weeks. These rats were sacrificed for hematoxylin and eosin-stained histology, immunohistochemistry, and apoptosis detection 5 months after the implantation. Each rat was housed in a standard rat cage in the 12-hour-each light and dark cycle at the Animal Center of Okayama University. This study was approved by the Animal Care and Use Committee in Okayama University, based on the Animal Welfare and Management Act in Japan.

Behavior test

A drum (diameter = 40 cm), with the inside painted with black-and-white stripes, was rotated around a rat, housed in a transparent-walled cage (diameter = 30 cm), at the speed of 2 or 4 rounds per minute (rpm) [15]. Rats' behavior was recorded by a videocamera from above. The drum was rotated clockwise for 3 min and anticlockwise for 3 min after the 3-min interval. The testing was done in the bright condition at 150 lux under the usual fluorescence ceiling light (Fig. 2a), and was repeated in the dim condition at 50 lux, illuminated with a fluorescence light source, placed on the floor (Fig. 2c), as described previously [15]. The combined number and the total time of head-turning or body-turning, consistent with clockwise and anticlockwise drum rotation, were used for statistical analysis (repeated-measure analysis of variance, ANOVA).

Immunohistochemistry and apoptosis detection

The eyes with film implantation were immersed in 4 % paraformaldehyde for 2–3 h, and cut into halves. Frozen sections were stained immunohistochemically, as described previously [15]. The primary antibodies were: anti-calbindin D-28 K (1:500 dilution, rabbit polyclonal, Millipore, Temecula, CA, USA), anti-protein kinase C (PKC)-α (1:250 dilution, mouse monoclonal, Sigma-Aldrich, St. Louis, MO, USA), anti-synaptophysin (1:500 dilution, mouse monoclonal, Sigma-Aldrich), and anti-glial fibrillary acidic protein (GFAP) (1:200 dilution, mouse monoclonal, Chemicon, Temecula, CA, USA). Second fluorescence-labeled antibodies were Alexa-350-labeled goat anti-mouse IgG antibody or Alexa-350-labeled goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA).

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated fluorescein-conjugated-dUTP nick-end-labeling (TUNEL) assay (In Situ Cell Death Detection Kit, Fluorescein, Roche Diagnostics, Mannheim, Germany). The number of apoptotic cells was counted in retinal sections with 1 mm width, perpendicular to the vitreous-retinal pigment epithelial axis. Three areas were chosen for comparison of the number of apoptotic cells: the area apposed to the film, the area neighboring the film, and the area opposite to the film across the posterior pole of the eye [15].

Electroretinographic recording

Seven male Wistar rats at 6 weeks of the age had dye-coupled films implanted in the right eye and plain films in the left eye while 7 male Wistar rats at 6 weeks had plain films implanted in the right eye and dye-coupled films in the left eye. Electroretinograms in both eyes were recorded at 6 weeks of the age just before the implantation, at 8 and 10 weeks of the age, 2 and 4 weeks, respectively, after the implantation.

Dye-coupled films were also implanted in both eyes of 5 male RCS rats at 6 weeks of the age while plain films were implanted in both eyes of 3 male RCS rats. Four male RCS rats served as non-treated controls. Electroretinograms in both eyes were recorded at 8, 10, 12, and 14 weeks of the age, corresponding to 2, 4, 6, and 8 weeks, respectively, after the implantation.

Rats were placed overnight in a dark room for dark adaptation. Rats were anesthetized and placed on a heating pad, set at 37 °C. After mydriasis, a contact lens electrode with white light-emitting diode (LED) was placed on the corneal surface, with no air bubble trapped between the cornea and the contact lens, a reference electrode was put into the mouth, and an earth clip was placed along the tail. Rod response (dark-adapted 0.01 ERG with 1000 cd/m^2 × 10 μs), and maximal responses (dark-adapted 3.0 ERG with 10,000 cd/m^2 × 0.3 ms and dark-adapted 10.0 ERG with 10,000 cd/m^2 × 1 ms) with standard flash, were sequentially recorded at the interval of 90 s, based on the International Society for Clinical Electrophysiology of Vision (ISCEV) standards (PuREC and LED Visual Stimulator LS-100/200, Mayo Corporation, Aichi, Japan).

Results

Behavior test

The combined number of head-turning, consistent with the direction of clockwise and anticlockwise drum rotation at 2 rpm, was significantly larger in RCS rats with dye-coupled films in the time course of 22 weeks of the observation, compared with rats with plain films, both under the

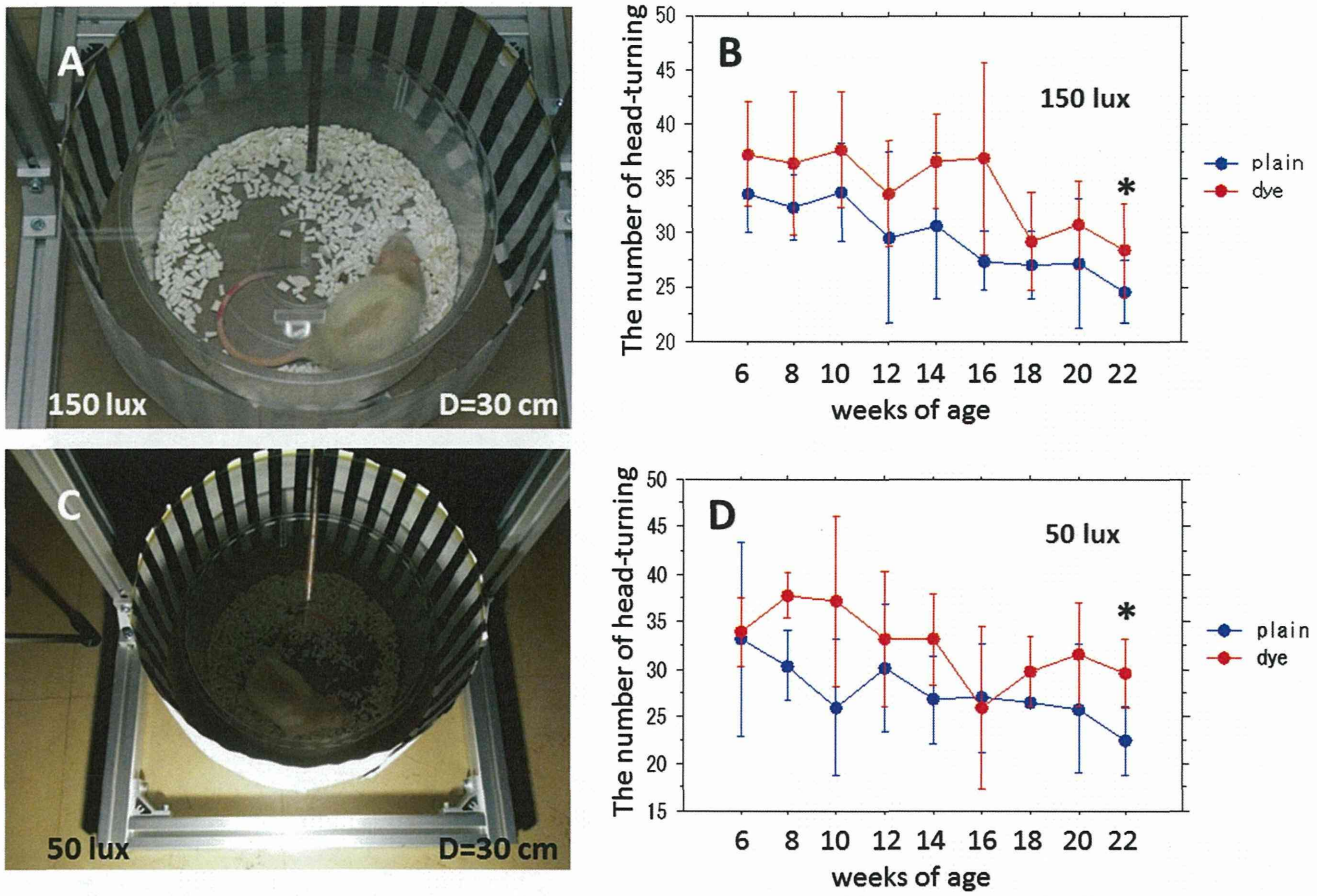

Fig. 2 Photographs (**a**, **c**) and results (**b**, **d**) of behavior tests in RCS rats. A *black-and-white-striped* drum is rotated around a rat in a transparent-walled cage (30 cm in diameter, $D = 30$ cm) at the speed of 2 rounds per minutes (rpm) in the bright (150 lux, **a**, **b**) or the dim (50 lux, **c**, **d**) condition. Behavior tests were done at 6–14 weeks of the age without treatment. A half of rats ($n = 5$), designated as "dye", had dye-coupled film implantation and the other half ($n = 5$), as "plain", had plain film implantation at 14 weeks of the age. Behavior tests were repeated at 16–22 weeks of the age. The combined number of head-turning, consistent with the direction of clockwise and anticlockwise drum rotation, are significantly larger in RCS rats with dye-coupled films, compared with rats with plain films, under the bright light ($P = 0.0147$), and under the dim light ($P = 0.0201$, repeated-measure analysis of variance, $n = 5$). T *bars* indicate standard deviation

bright light ($P = 0.0147$ for class and $P = 0.0002$ for time course, Fig. 2b) and under the dim light ($P = 0.0201$ for class and $P = 0.0123$ for time course, repeated-measure ANOVA, $n = 5$ for each group, Fig. 2d). The other behavioral indicators did not reach statistical significance.

Immunohistochemistry and apoptosis

No inflammatory cell, necrotic cell, or apparent gliosis or fibrosis was noted in the retina of the eyes with dye-coupled films or plain films at two time points, at 22 weeks of the age after the implantation at 14 weeks of the age (data not shown) and 5 months after the implantation at 6 weeks of the age (Fig. 3). PKC-α-stained rod bipolar cells, calbindin-stained horizontal cells and amacrine cells, and GFAP-stained Muller cells were observed in three areas, apposed to the film, neighboring the film, and opposite to the film, at the same level between the eyes with dye-coupled films and plain films. Synaptophysin staining was fully preserved in the inner plexiform layer at the same level between dye-coupled film and plain film implantation.

The number of apoptotic cells was smaller, although not significantly, at the site of dye-coupled film implantation at 22 weeks of the age, compared with the other sites, neighboring the film or opposite to the film ($P = 0.0970$, $n = 3$, Friedman test, Fig. 4, bottom left panel). Furthermore, the number of apoptotic cells was significantly smaller at the sites of dye-coupled film and plain film implantation, 5 months after film implantation, compared with the other sites ($P = 0.0021$ and $P = 0.0038$, respectively, $n = 7$, Friedman test, Fig. 4, bottom right panel).

Electroretinograms

In Wistar rats with the normal retina, rod response (Fig. 5a) and maximal responses (Fig. 5b) were recorded in the eyes,

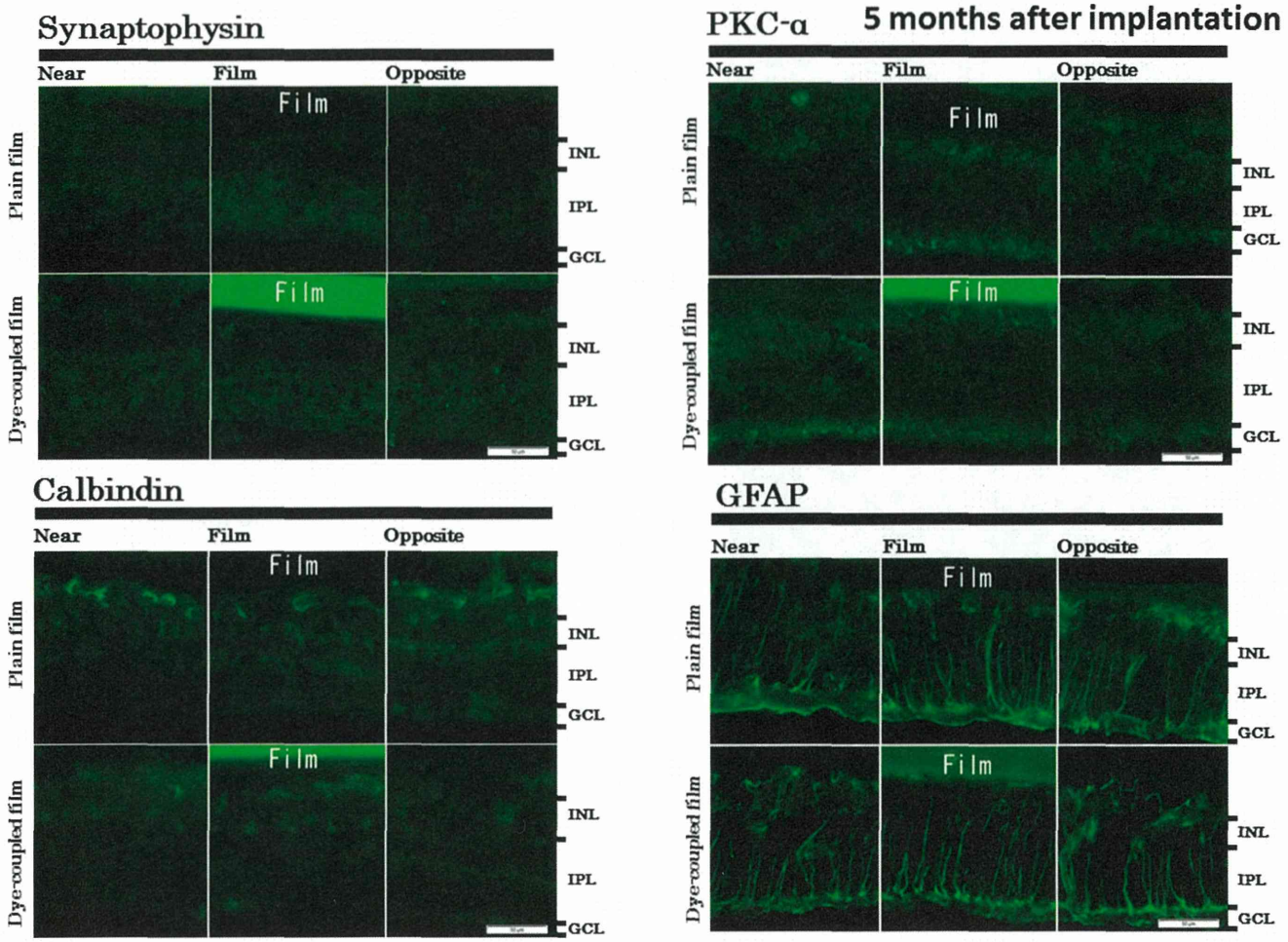

Fig. 3 Immunohistochemical staining in three areas of retinal sections of RCS rats' eyes with subretinal plain films or dye-coupled films: at the site of film implantation (*Film*), neighboring the film (*Near*), and opposite to the film across the posterior pole of the eye (*Opposite*). 5 months after the implantation at 6 weeks of the age. Synaptophysin to stain inner plexiform and outer plexiform layer, protein kinase C-α (PKC-α) to stain rod bipolar cells, calbindin to stain horizontal cells and amacrine cells, and glial fibrillary acidic protein (GFAP) to stain glial cells (Muller cells). Note autofluorescence of dye-coupled films but no fluorescence of plain films (*Film*). INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer, *bar* = 50 μm

just before the dye-coupled or plain film implantation at 6 weeks of the age, 2 and 4 weeks after the film implantation. The amplitudes of the rod response, the a-wave and b-wave of maximal responses did decrease in the time course, both in the eyes with dye-coupled films and in the eyes with plain films ($P < 0.05$), but did not show significant difference between the eyes with dye-coupled and plain film implantation (repeated-measure ANOVA, $n = 14$ for each group of eyes, Fig. 5c).

In RCS rats, a peak-like electroretinographic response to standard flash, in the maximal response (dark-adapted 10.0 ERG with 10,000 cd/m^2 × 1 ms), was recorded in eyes of RCS rats with dye-coupled film or plain film implantation or with no treatment (Fig. 5d). The positive response, defined as the b-wave amplitude with voltage, 50 μV or larger, was noted at a significantly higher rate in eyes with dye-coupled films, only at 4 weeks after the implantation, compared with the eyes of no treatment ($P = 0.0425$, Chi-square test, Table 1).

Discussion

In our preceding study, the dye-coupled polyethylene films were implanted in the subretinal space of RCS rats' eyes at the age of 6 weeks. This age was chosen because the retina of RCS rats at the age has lost almost all photoreceptor cells. Even after the age of 6 weeks, the retina in RCS rats continues to lose neurons in the outer and inner nuclear layer, and in the ganglion cell layer.

In a coming clinical trial, candidates for retinal prostheses will be patients with retinitis pigmentosa who have no light perception for a certain period of time. Under the circumstances, neuronal death will continue to occur in the

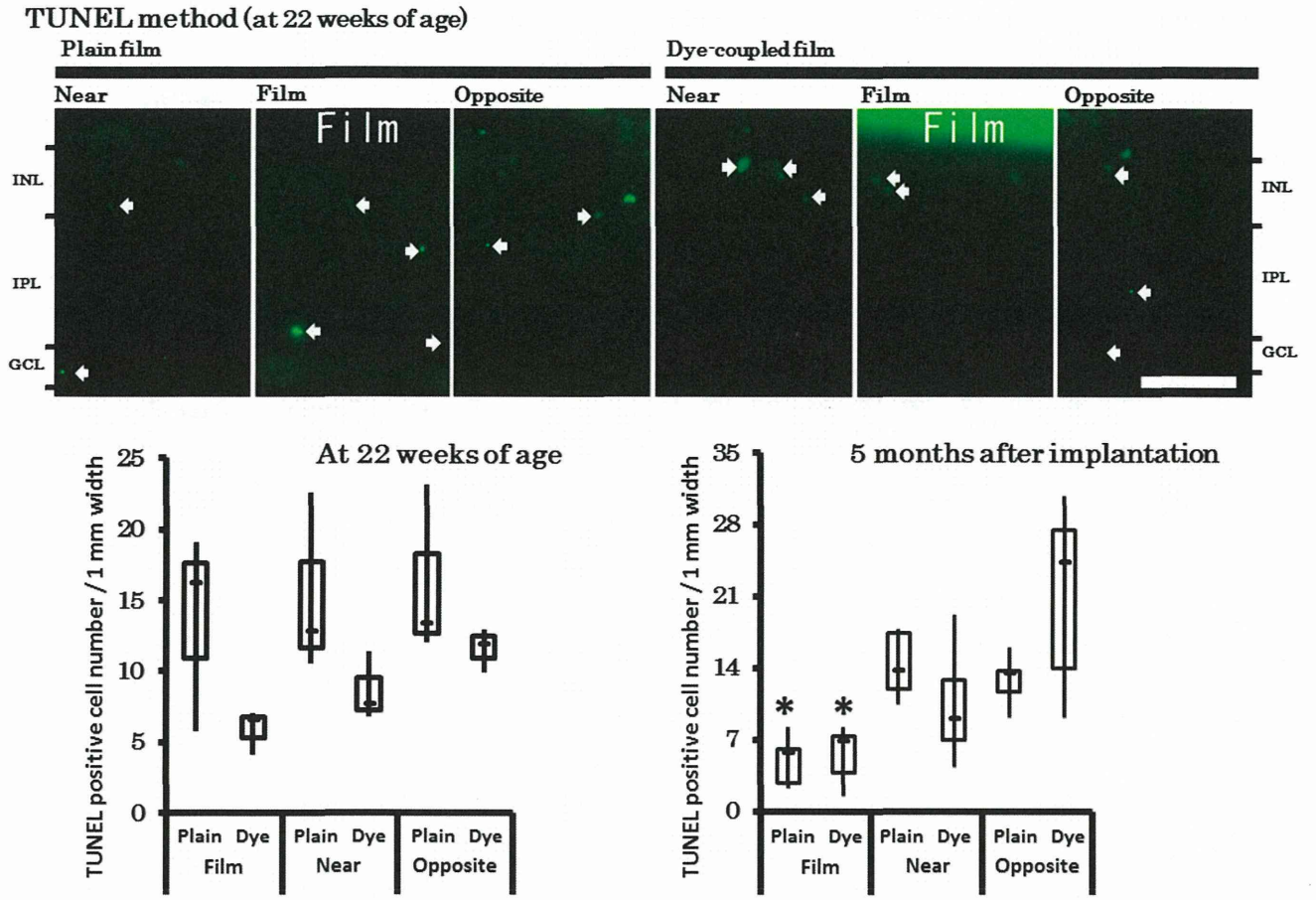

Fig. 4 Top panel. Apoptotic cells (*arrows*) in three areas of retinal sections of RCS rats' eyes with subretinal plain films (*Plain*) or dye-coupled films (*Dye*): at the site of film implantation (*Film*), neighboring the film (*Near*), and opposite to the film across the posterior pole of the eye (Opposite). 8 weeks after the film implantation at 14 weeks of the age. INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer, *bar* = 50 μm. Bottom panels. The number of apoptotic cells is smaller, although not significantly, at the site of dye-coupled film implantation at 22 weeks of the age, compared with the other sites, neighboring the film or opposite to the film ($P = 0.0970$, $n = 3$, Friedman test, *left panel*). The number of apoptotic cells is significantly smaller at the sites of dye-coupled film and plain film implantation, 5 months after film implantation, compared with the other sites ($P = 0.0021$ and $P = 0.0038$, respectively, $n = 7$, Friedman test, right panel). A box with a *bar* indicates the upper 75 percentile and lower 25 percentile, with a median, maximum, and minimum

inner nuclear layer and ganglion cell layer. This study was, therefore, planned to test the effect of dye-coupled film implantation on the vision in aged RCS rats. Vision was maintained in aged RCS rats, as in younger rats [15]. This study also confirmed that head-turning, in response to rotations of the back-and-white-striped drum at 2 rpm, is a good indicator for assessing the vision in rats under the dim or bright condition. The size of dye-coupled films, inserted in rats' eyes, was roughly estimated to occupy one-clock hour meridian of the retina, and the visual acuity was estimated as 0.005, based on the visual angle of the black-and-white stripes.

The dye-coupled and plain films were implanted for 5 months in the subretinal space of RCS rats' eyes to examine their biological effect on the degenerating retina. The remaining neuronal layers were maintained at retinal sites with dye-coupled films 5 months after the implantation, comparable to the other retinal sites, neighboring the film implantation and opposite to the film implantation [16, 17]. It should be noted that the dye-coupled films still kept autofluorescence, indicating stable photoelectric dye molecules on the film surface, even 5 months after the film implantation.

Neuronal apoptosis was reduced at retinal sites with dye-coupled films, at two time points, at the age of 22 weeks with 8-week implantation, and also after 5 months with the implantation, compared with the other retinal sites, neighboring the film implantation and opposite to the film implantation. Furthermore, apoptosis was reduced in retinal sites of plain film implantation, similar to the sites with dye-coupled films, 5 months after the film implantation. The results showed that the dye-coupled and plain films were not

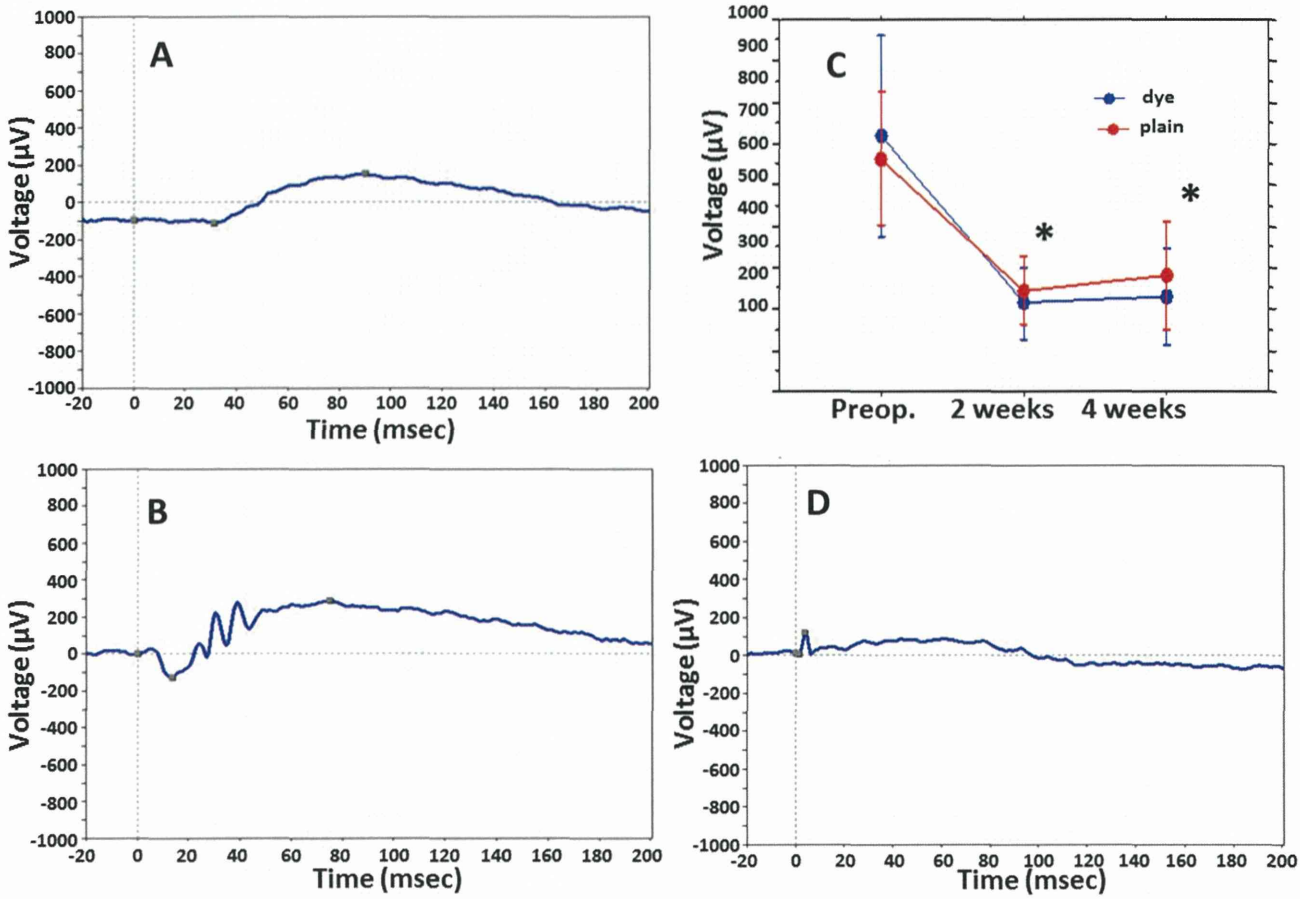

Fig. 5 Electroretinograms in Wistar rats (*right panels*, **a** and **b**) and RCS rat (**d**). Rod response (dark-adapted 0.01 ERG, **a**) and maximal response (dark-adapted 10.0 ERG, **b**) in the right eye of a Wistar rat, 4 weeks after dye-coupled film implantation at 6 weeks of the age. The amplitude (voltage) of the b-wave significantly decreases ($P < 0.0001$) in the time course but is not significantly different between the eyes with dye-coupled films (*dye*) and plain films (*plain*) ($P = 0.7877$, repeated-measure ANOVA, **c**). A peak-like response as maximal response (dark-adapted 10.0 ERG, **d**) in the left eye of a RCS rat, 2 weeks after dye-coupled film implantation at 6 weeks of the age

Table 1 Electroretinographic (ERG) response in dystrophic eyes of RCS rats with no treatment, plain polyethylene film implantation, or photoelectric dye-coupled polyethylene film implantation at the age of 6 weeks

	ERG response	Age of rats			
		8 weeks	10 weeks	12 weeks	14 weeks
No treatment ($n = 8$)	Yes	3	0	0	0
	No	5	8	8	8
Plain film ($n = 6$)	Yes	0	1	0	1
	No	6	5	6	5
Dye-coupled film ($n = 10$)	Yes	2	4	0	0
	No	8	6	10	10

The positive response (Fig. 5d), defined as the b-wave amplitude with voltage, 50 μV or larger, is noted at a significantly higher rate in eyes with dye-coupled films, at the age of 10 weeks, namely, 4 weeks after the implantation, compared with the eyes of no treatment ($P = 0.0425$, Chi-square test)

harmful to the remaining retinal neurons. The protective effect of the plain films on the retina would be supported by a previous study which demonstrated the neuroprotective effect of inactive implants in RCS rats' eyes [18].

Electroretinograms were recorded first in the normal eyes of Wistar rats with dye-coupled or plain film implantation. The rod response, and the a-wave, b-wave, and oscillatory potentials of maximal responses to standard

flash could be recorded 2 and 4 weeks after the implantation. The decrease in the amplitude of the waves would be attributed, not only to surgical intervention of film implantation, but also to film influence on metabolic interaction between retinal photoreceptor cells and retinal pigment epithelial cells. The electroretinographic responses to standard flash were recorded more frequently in dystrophic eyes of RCS rats with dye-coupled film implantation at the timing of 4 weeks after the surgery, compared to the eyes with no treatment or with plain film implantation. This timing of recording would suggest the recovery from surgical intervention, and also progressive retinal degeneration afterward. The electroretinographic results gave a line of objective evidence to show the function of the dye-coupled film as retinal prosthesis.

In conclusions, the photoelectric dye-coupled polyethylene film, OUReP^{TM}, could maintain the vision of RCS rats when the film was implanted in the dystrophic eyes at the old age as 14 weeks. The retina showed no additional abnormalities after 5-month OUReP^{TM} implantation in the dystrophic eyes of RCS rats. A positive response was detected by electroretinographic recording in the dystrophic eyes of RCS rats with OUReP^{TM} implantation. Toward the preparation for a clinical trial, no toxicity has been found for OUReP^{TM} or for the photoelectric dye in any tests for biological evaluation of medical devices, based on the International Organization for Standardization (ISO) 10993.

Conflict of interest The authors declare that they have no conflict of interest.

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