Autologous Transplantation of RPE with Partial-Thickness Choroid after Mechanical Debridement of Bruch Membrane in the Rabbit

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PURPOSE. An improved translocation technique for autologous retinal pigment epithelium (RPE) transplantation is presented. The graft consists of a sheet of a partial-thickness choroid with RPE attached.

METHODS. Twenty-seven pigmented rabbits were used in this study. After mechanical debridement of Bruch membrane, partial-thickness RPE-choroid sheets were transplanted onto the subretinal space in 25 rabbits. The animals were examined by fundus photographs and fluorescein angiographs and were killed postoperatively at 1, 2, 4, 12, and 24 weeks. Eyecups containing the grafts were examined by light microscopy and immunohistochemistry. In addition, two partial-thickness RPE-choroid sheets were analyzed by transmission electron microscopy (TEM).

RESULTS. TEM revealed that the partial-thickness RPE-choroid graft consisted of retinal pigment epithelial cells, Bruch membrane, choriocapillaris, and ruptured middle vessels. The thickness of the graft was approximately 50 to 60 μm. Fluorescein angiography revealed neither fluorescein leakage nor staining in the graft at early or late phase. Light microscopy revealed that in 17 experiments in which the graft survived, the neural retina remained intact; however, in eight experiments with unsuccessful grafts, the neural retina degenerated. The surviving graft showed revascularization and monolayered retinal pigment epithelial cells. Furthermore, in sections in which the neural retina over the graft remained intact, all retinal pigment epithelial cells in the graft and rhodopsin in photoreceptor outer segments were positively labeled with antiretinaldehyde-binding protein antibodies and anti-opsin antibodies, respectively.

CONCLUSIONS. A partial-thickness RPE-choroid graft showed improved integration with the host choroid and photoreceptors. This technique has the potential to be a treatment for age-related macular degeneration. (Invest Ophthalmol Vis Sci. 2008;49:3185–3192) DOI:10.1167/iovs.07-1299

Although photocoagulation, photodynamic therapy, and antichoroidal neovascularization (CNV) therapy (pegaptanib sodium, bevacizumab, and ranibizumab) can be used to treat the exudative form of age-related macular degeneration (AMD),1–17 surgical excision of subfoveal choroidal neovascular membranes is necessary when AMD develops to a certain pathologic state. Submacular surgery has shown disappointing results because of the loss of the retinal pigment epithelium (RPE) concurrent with the removal of the neovascular membrane.18–23 Various attempts have been made to overcome this problem. Macular translocation appears to improve visual acuity.24–28 However, full or limited macular translocation bears considerable risk for proliferative vitreoretinopathy, retinal detachment, ocular torsion and diplopia, intraocular hemorrhage, and macular fold.29–34 The iris pigment epithelium (IPE) is easily accessible; however, it does not have all the functions of the RPE.35–38 Multilayers of IPE cells implanted onto the subretinal space may induce adverse focal effects on adjacent photoreceptors.39–41 Heterologous RPE transplantation to substitute for the loss of RPE is not feasible because of host-graft rejection.42–44 Therefore, autologous RPE transplantation was developed.

A method for autologous RPE transplantation is application of a suspension of cells.45–48 In this method, it is difficult to control the amount of retinal pigment epithelial cells and to induce the cells to form a monolayer on Bruch membrane. Another method is translocation of an autologous sheet of full-thickness RPE-choroid, belonging to the extramacular area, to the submacular space after the removal of CNV. The graft consists of RPE, Bruch membrane, choriocapillaris, and choroid. Some investigators have demonstrated the clinical feasibility of this technique49–56; however, MacLaren57 reported that the long-term results were not positive. Yepez et al.58 performed autologous RPE-choroid complex transplantation in five AMD patients by using a similar procedure. They found that subretinal fibrosis developed in all five patients and that graft pigmentation was lost between 30 and 60 days after the procedure. No significant improvement in visual acuity was observed in any of these patients. The authors believed that an autologous-free RPE-choroid graft is destined to fail.59 We speculate that the graft might be very thick, thereby preventing the transport of nutrients from the choroid to the RPE. If the thickness of the sheet of the RPE-choroid complex is reduced, the graft may survive more easily.

 Removing part of the choroidal tissue could make the graft thinner; this was termed partial-thickness RPE-choroid sheet. In the present study, we report the histologic outcomes of autologous transplantation of partial-thickness RPE-choroid sheet after abrasive debridement of Bruch membrane in rabbit models.

MATERIALS AND METHODS

Animals and Anesthesia

Twenty-seven pigmented rabbits, each weighing 2 to 2.5 kg, were used for this study. All surgical procedures were performed in one eye of...
each animal. The rabbits were anesthetized by intramuscular injection of a mixture of ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (15 mg/kg). Local anesthesia was applied to the experimental eye by a subconjunctival injection of 2% lidocaine hydrochloride (0.5 mL). The pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine hydrochloride. All animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Surgery

After anesthesia and pupil dilation, a sterile field was established around the experimental eye. A 360° conjunctival peritomy was performed. The lens was extracted by phacoemulsification through a 1.5-mm incision on the superior corneal limbus. The posterior capsular was preserved, and the incision of the limbus was closed. Next, an infusion cannula was inserted through the inferior nasal sclerotomy site, which was located 2 mm posterior to the limbus, and balanced salt solution (BSS) plus was used as an irrigating solution; standard three-port vitrectomy, including a posterior hyaloid detachment, was performed using a pneumatic cutter (Storz, St. Louis, MO) through the contact lens under a surgical microscope.

To prepare the graft, a donor area of approximately 2-optic disc (OD) diameters and located at 1.5-OD diameters inferior to the OD was treated by diathermy coagulation. The retina was removed, and the RPE in this area was exposed. One side of this RPE-choroid area was cut with a 20-gauge knife. Subsequently, a sharp spatula was inserted into the choroid to split the choroidal vascular tissues, and these tissues were removed by a subretinal forceps. The partial-thickness RPE-choroid complex appeared semitransparent. A sheet of 1- to 1.5-OD diameter of partial-thickness RPE-choroid graft was obtained after the other sides were cut using a vitreous scissor (Fig. 1).

In the same experimental eye, the area superior to the OD was selected as the recipient bed. A localized retinal detachment was created by subretinal injection of BSS through a 30-gauge metal cannula, and the retinotomy site was enlarged to enable the implantation of the graft. At the recipient bed, the RPE from a 1.5-OD diameter area of Bruch membrane was carefully debrided with the use of a silicon cannula, and the shed retinal pigment epithelial cells were carefully removed using a vitreous cutter. Mild Blanching of the choroidal vasculature was observed as the tip of the silicon cannula moved across the RPE-Bruch membrane surface; however, no choroidal bleeding occurred. Care was taken to avoid debridement directly under the retinotomy site.

After the recipient bed of Bruch membrane was prepared, the partial-thickness RPE-choroid graft was translocated to the area of denuded Bruch membrane by using a retinal forceps. The retina was repositioned and re附ixed by an air-fluid exchange, resulting in the enclosure of the graft within the retina. To complete the surgery, air was exchanged with silicone oil (5700 mPas viscosity; Bausch & Lomb, Waterford, Ireland). The area from which the partial-thickness RPE-choroid graft was obtained exposed the internal surface of sclera. Finally, the sclerotomy sites were closed, and the conjunctiva was sutured. Dexamethasone (2.5 mg) and gentamicin (20 mg) were injected subconjunctivally, and the eye was dressed with erythromycin ointment. After surgery, topical antibiotic and steroid were applied for 3 days.

Using the same surgical procedures, two partial-thickness RPE-choroid sheets were obtained from two rabbits (one sheet from one rabbit) without transplantation and subjected to morphologic examination by transmission electron microscopy (TEM).

Fundus Photography and Fluorescein Angiography

Color fundus photographs and fluorescein angiograms were obtained with a fundus camera (FF450 plus IR; Zeiss, Jena, Germany) at post-operative weeks 1, 2, 4, 12, and 24. Fluorescein angiography (FA) was performed by injecting 0.2 mL sodium fluorescein solution (10%) into the ear vein.

Histology

At postoperative weeks 1, 2, 4, 12, and 24, five experimental animals were sedated and killed, each by an intravascular overdose of 2% lidocaine. After death, each experimental eye was rapidly enucleated, punctured at the limbus, and immersed in 2.5% glutaraldehyde and 4% paraformaldehyde in a phosphate-buffer solution. After 2 hours, the anterior segment and intraocular silicone oil were removed. Subsequently, the posterior cup was fixed in the same solution for 48 hours.

To study each graft, the posterior cup was washed in 0.1 M phosphate-buffered saline (PBS) solution for 2 hours with three replacements. The tissue was then dehydrated in ascending ethanol concentrations. After dehydration, the tissue was cleared in xylene for 45 minutes and immersed in liquid paraffin for 1.5 hours at 60°C with three replacements. Subsequently, the area of the graft was cut as a small block of tissue, including the retina and sclera, embedded in paraffin wax, and processed for sectioning (5-μm sections). Groups of consecutive slides were stained with hematoxylin and eosin (HE) for light microscopy. If the neural retina over the graft was intact, its adjacent sections were treated for antigen retrieval and processed with immunolabeling of antibody against cellular retinaldehyde-binding protein (CRALBP; anti-CRALBP antibody, a gift from John C. Saari, University of Washington, Seattle, WA) and antibody against rhodopsin (monoclonal anti-opsin antibody O4886; Sigma-Aldrich, St. Louis, MO).

For the examination of the constituents of the graft, two sheets of partial-thickness RPE-choroid graft were immobilized on a nitrocellulose filter membrane (Hybond, Escondido, CA). The sheets were fixed in 2.5% glutaraldehyde at 4°C for 24 hours, postfixed with 1% osmium tetroxide at room temperature for 2 hours, and bloc stained and embedded in epoxy resin after dehydration in a graded acetone series. Semithin sections were stained with toluidine blue and observed under a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under an electron microscope (JEM1230; JEOL Inc., Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry was performed as follows. Sections were dewaxed and rehydrated in descending ethanol to water. The slides were incubated in 3% hydrogen peroxide for 15 minutes to quench endogenous peroxidase activity. Antigenicity was increased by incubating the sections in 0.125% trypsin (Zymed-Invitrogen, South San Francisco, CA) for 30 minutes at room temperature. To detect CRALBP, the pigment of the tissues was bleached by incubating the sections in 0.75% potassium permanganate for 5 minutes, followed by incubation in 2% oxalic acid for 30 seconds (this procedure was not used in the detection of rhodopsin). Nonspecific binding sites were blocked by incubating the sections in 5% bovine serum albumin (Roche, Basel, Switzerland), and the sections were then incubated with primary antibodies (rabbit anti-CRALBP, 1:4000 dilution; mouse anti-opsin antibody, 1:2500 dilution) overnight at 4°C in a humidity chamber. No primary antibodies were used in negative controls. The signal was amplified by incubating the sections with secondary antibodies for 45 minutes at 37°C, anti-rabbit IgG-polymer-horseradish peroxidase (HRP; Polink-1 HRP Bb Bulk Kit, D12-110; GBI, Mukilteo, WA) labeling for CRALBP and anti-mouse IgG-polymer-HRP (Polink-1 HRP Ms. Bulk Kit, D12-110; GBI) labeling for rhodopsin. For color development, the sections were incubated with diaminobenzidine substrate for 5 to 10 seconds and counterstained with hematoxylin.

RESULTS

Complications of Surgery

After excision of a sheet of partial-thickness RPE-choroid, the sheet shrank by one third because of tissue contraction. How-
ever, the graft did not fold. In all procedures, primarily the following complications occurred: choroidal hemorrhage, choroidal detachment, and retinal pigment epithelial damage. Choroidal hemorrhage can be avoided by careful diathermy coagulation. Choroidal detachment was induced by mechanical intervention when a spatula was used to split the choroidal tissues. However, the choroid was always reattached after air–fluid exchange and was retained in situ after surgery. RPE and Bruch membrane might be torn by the spatula when the choroidal tissues were separated.

After surgery, all rabbits showed exudation in the anterior chamber within 3 days. The exudation was subsequently absorbed without any special treatment. Proliferative vitreoretinopathy (or retina detachment) and hemorrhage were not observed. One rabbit had a corneal ulcer within a few days of surgery. The cornea of this rabbit became opaque after the ulcer was cured, which hindered observation of the fundus. Thus, fundus photography and FA could not be performed on this rabbit.
Fundus Photography and FA

Fundus photographs revealed that all grafts were localized at the site of retinal pigment epithelial debridement, except for one graft that was observed outside the area of retinal pigment epithelial debridement. Neither retinal detachment nor hemorrhage was observed. As healing occurred, the grafts became thinner, expanded slightly, and began to attach more firmly. Graft color was darker than that of the surrounding area. None of the grafts showed loss of pigment, significant shrinkage, or development of fibrosis.

In all grafts, from 1 week to 24 weeks after surgery, FA did not reveal fluorescein leakage or staining in the graft at early or late phase. Moreover, no window defect was observed in the grafts. However, FA revealed a window defect at the debridement site of Bruch membrane without graft covering (Fig. 2).

Histology and Immunohistochemistry

TEM showed that the 50- to 60-μm partial-thickness RPE-choroid graft consisted of retinal pigment epithelial cells, Bruch membrane, choriocapillaris, and ruptured middle vessels (Fig. 3). The microvilli of retinal pigment epithelial cells were fractured; however, retinal pigment epithelial cells, Bruch membrane, and capillaries were intact.

After implantation of the partial-thickness autologous RPE-choroid graft onto the subretinal space, light microscopy revealed two different outcomes for the neural retina over the graft. In successful grafts (17 eyes), the neural retina was almost intact. In these eyes, the retinal outer and inner nuclear layers, along with the ganglion cell layer, appeared normal, and, in some cases, photoreceptor outer segments were attenuated or shortened. The graft showed revascularization, and retinal pigment epithelial cells formed a monolayer. Inflammatory cells were not observed (Figs. 4A, 4B). In unsuccessful grafts (eight eyes), photoreceptor outer and inner segments disappeared, and the outer and inner nuclear layers became thinner or disappeared despite the graft vessels showing no obstructions and the graft retinal pigment epithelial cells appearing as a monolayer (Figs. 4C, 4D). These phenomena were observed postoperatively from 1 week to 24 weeks. Changes in the recipient Bruch membrane underneath the graft, which were examined by TEM, will be reported in another study.

In sections in which the neural retina over the graft was intact, all retinal pigment epithelial cells of the graft and rhodopsin in photoreceptor outer segments over the graft were positively labeled with anti-CRALBP antibodies and anti-opsin antibodies, respectively. No significant difference was observed in these sections at each time period after surgery. Furthermore, sections without the application of primary antibodies showed negative results for antibody labeling (Figs. 5A-D).

DISCUSSION

The RPE is a monolayer cuboidal epithelium located between the photoreceptors and the choroid. Many techniques have been developed for transplantation of a sheet of RPE. These techniques include culturing retinal pigment epithelial cells on an amniotic membrane, Descemet membrane, or anterior lens capsule.59–65 Using synthetic biodegradable substrate as a support material64–66 sandwiching a retinal pigment epithelial sheet between two sheets of gelatin for transplantation,67 and excising a peripheral patch of autologous full-thickness RPE-choroid and translocation to the submacular space.68–72

It is obvious that the technique of using an autologous retinal pigment epithelial sheet can minimize the risk for host-graft rejection and can reduce ethical or religious conflicts arising from transplants and the use of human fetal retinal pigment epithelial cells or cadaveric donors. Therefore, autologous retinal pigment epithelial sheet as a transplant is the optimal option for retinal pigment epithelial transplantation to the submacular space after CNV excision in AMD.50–58 However, in some cases, the results were disappointing, and the transplants developed subretinal fibrosis.51,57,58 In the study by Maaijwee et al.,68 although the full-thickness RPE-choroid graft showed revascularization in histologic examination, the neural retina did not remain intact after graft transplantation in pig models. We speculated that these grafts might not survive because of their thickness. Similarly, in skin transplantation, partial-thickness graft survives more easily than full-thickness graft.69 If the thickness of an RPE-choroid sheet is reduced by removing part of the choroidal tissues, the graft (termed partial-thickness RPE-choroid graft) would become thinner and survive more easily while allowing easy diffusion of nutrients from the choroid to the RPE and photoreceptors. Using the same assumption, Holz72 suggested intraocular microablution of the choroidal tissue by 308-nm laser energy (AIDA Excimer Laser System; TuiLaser AG, Germcring, Germany) for retinal pigment epithelial transplantation.

In our study, partial-thickness RPE-choroid was prepared with an intraocular sharp spatula by removing part of the choroidal tissues. All the surgical manipulations were thoroughly performed in the same eye. The grafts did not fold. After surgery, the grafts became flatter and appeared semitransparent; this showed that the grafts had grown together with the recipient bed by attaching more firmly to the bed. FA revealed that the grafts functioned as a barrier to both the passage of dye and the emission of fluorescence.

The finding that the RPE of the recipient bed was intentionally damaged by our technique was similar to that reported in the study by Maaijwee et al.68 Histologic examination revealed that the survived graft showed not only revascularization but also intact neural retina. This result was different from that reported in the study by Maaijwee et al.68 and demonstrated that the partial-thickness RPE-choroid graft performs a normal function of supporting the neural retina, even though the rabbit retina has no vascular supply except for a narrow horizontal band of medullated nerve fibers and is nourished by nutrients obtained from the choroid. If the graft shows only revascularization but a degenerated neural retina, we cannot affirm that the graft is successful because it does not perform its function of supporting the neural retina. Our experimental results revealed that the partial-thickness RPE-choroid sheet could support the neural retina.

Immunohistochemical examination revealed that in successful grafts, CRALBP in retinal pigment epithelial cells in the graft and rhodopsin in photoreceptors were positively labeled with the corresponding antibodies. CRALBP, a protein thought to participate in retinoid metabolism and visual pigment regeneration,71,72 is present in both the neural retina and the RPE.73 CRALBP was used previously as a marker for retinal pigment epithelial differentiation.65,77,78 Rhodopsin is present within internal membrane structures, known as disc membranes, found in the rod outer segments of photoreceptors in the retina. Rhodopsin is responsible for light trapping and optical signal transduction for photoreceptors.75–80 Retinal pigment epithelial cell damage affects the chromophore retinaldehyde-binding opsin, resulting in distortion of rhodopsin in the photoreceptors.81 Therefore, rhodopsin is an important cell marker for experimental studies on retinal transplantation.82–85 Because approximately 95% of the photoreceptors in the rabbit retina

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are rods, we selected rhodopsin as the cell marker to assess the results of retinal pigment epithelial transplantation.

In unsuccessful transplants, the neural retina over the graft was significantly damaged. It was concluded that the neural retina underwent necrosis, even if some grafts showed revascularization.

Why did some grafts fail to survive?

1. We did not use perfluorodecalin to reattach the retina during the surgery. After air–fluid exchange, a small amount of fluid sometimes remained in the subretinal space because the retinotomy site was located at a superior position. Therefore, the graft that was transplanted onto the subretinal space could not tightly adhere to Bruch membrane and obtain sufficient nutrients from the recipient bed.

2. The thickness of the graft might have influenced its survival rate given that some grafts were thicker because of technique. We speculated that the thicker the autologous-free RPE-choroid graft, the more difficult the reestablishment of blood circulation with local choroidal vessels and diffusion of nutrients from the choroid to the RPE and photoreceptors.

3. The blood supply of the choroidal recipient bed might have influenced the graft survival rate. It has been proven that choriocapillary atrophy and reduction in the flow through the choroidal vessels occur after RPE removal. In the present...
study, Bruch membrane was debrided at the recipient bed to mimic the damage after CNV extraction in AMD patients. If the local choroidal vessels had no perfusion, even if it was reversible, it would not be beneficial to the autologous-free graft survival. If the autologous-free graft does not receive nutrients rapidly from the recipient bed vessels, it will die or undergo necrosis within a short duration. On the other hand, a diffusion defect of the choriocapillaris and a decrease in the size of large choroidal vessels at the macular region are known to occur in AMD patients. Thus, this technique of autologous retinal pigment epithelial transplantation must be further evaluated in experimental and clinical research.

In conclusion, the present study demonstrates the feasibility and histologic outcomes of autologous transplantation of partial-thickness RPE/choroidal sheet. The graft not only survived but also performed its normal function, as demonstrated by graft revascularization and intact neural retina over the graft, respectively. This technique may be feasible for the surgical treatment of AMD.

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References


