Transplanted and repopulated retinal pigment epithelial cells on damaged Bruch's membrane in rabbits

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Abstract

Aims—The authors studied how artificially damaged Bruch's membrane influenced growth and differentiation of transplanted embryonic retinal pigment epithelial (RPE) cells and of host RPE cells in rabbits.

Methods—Embryonic RPE cells obtained from pigmented rabbits were transplanted into the subretinal space of adult albino rabbits. The host RPE was removed with a silicone cannula, and Bruch's membrane was damaged by scratching with a micro-hooked 27 gauge needle under the detached retina in closed vitrectomy. The transplantation sites were examined 3, 7, and 14 days after surgery by light and electron microscopy.

Results—Varying degrees of damage in Bruch's membrane were observed. Pigmented and hypopigmented RPE cells showed a normal polarity and tight junctions were seen at the sites of mild to moderate damage 3–7 days after the surgery. In contrast, fibroblast-like cells with no such features of RPE cells formed multiple layers at the sites of severe damage involving the full thickness of Bruch's membrane and the choriocapillaris even 14 days after the surgery. Without transplantation, host RPE cells repopulated the damaged areas in the same way as transplanted RPE cells.

Conclusions—Transplanted embryonic RPE cells as well as host RPE cells grew and differentiated on the moderately damaged Bruch's membrane, while the severely damaged Bruch's membrane did not allow differentiation of RPE cells although these cells could grow and cover the damaged areas.

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cells, leading to poor visual outcome. Histopathological studies of excised CNVs from the eyes in AMD or presumed ocular histoplasmosis syndrome, indeed, revealed that they contained fragments of Bruch's membrane such as the RPE basement membrane and lamina elastica, indicative of surgical damage to Bruch's membrane.^{7–9} In addition, RPE transplantation tried in patients with AMD after excision of CNVs by some surgeons has proved unsuccessful after a long period of observation.^{10–12} Recently, the relation between RPE transplantation and Bruch's membrane has been pointed out.^{12–14}

In animal models, experimental removal of RPE cells is followed by their repopulation,^{15–17} and cultured RPE cells have been also successfully transplanted onto denuded intact Bruch's membrane in rabbits.^{18–21} Valentino and co-workers, however, reported that RPE did not repopulate as a monolayer where Bruch's membrane had been experimentally damaged in the monkey, instead fibroblast-like cells covered the defective area.¹⁶

The damage to Bruch's membrane is considered to be one of the reasons for preventing transplanted RPE cells from reattachment to Bruch's membrane and their normal growth. In this study, we developed an animal model for eyes with damaged Bruch's membrane, and investigated the growth of transplanted RPE cells from embryos as well as that of host RPE cells by histological examination.

Material and methods

ISOLATION OF EMBRYONIC RPE

A total of 108 pigmented embryonic rabbits were used as donors while 30 albino adult rabbits as hosts. Three adult rabbits were used at 3, 7, and 14 days after surgery, either with damaged or non-damaged Bruch's membrane. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

After anaesthesia by intravenous injection of sodium pentobarbitone (50 mg/kg), the abdomens of pregnant pigmented rabbits at 25–30 days of gestation were incised. The embryos were taken out, and their eye balls were enucleated. The whole eye balls were then rinsed with 0.1% chlorhexidine, 70% ethanol, and calcium/

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Bruch's membrane is damaged and retinal pigment epithelial (RPE) cells are removed during surgical excision of choroidal neovascular membranes (CNVs) in patients with age related macular degeneration (AMD).^{1–6} The surrounding RPE cells do not repopulate the defective area as the normally functioning

Figure 1 Phase contrast micrograph of retinal pigment epithelial cells from rabbit embryos in primary culture used for transplantation. Bar = 100 μ m.

magnesium-free phosphate buffered saline (PBS). After the anterior segment with the lens and vitreous was removed, the neural retina with adherent vitreal remnants was cut at the optic disc and discarded. The posterior segment was incubated with 0.25% trypsin and 1 mM EDTA in Hanks' balanced salt solution for 10 minutes, and the RPE cells were detached by gentle aspiration with a glass pasteur pipette. The cells were then pelleted by centrifugation and washed with calcium/magnesium-free PBS. The RPE cells were incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal calf serum, streptomycin (100 mg/l), and ampicillin (100 mg/l) under a humidified

atmosphere of 95% air and 5% carbon dioxide at 37°C. The cells reached confluency in 1–2 weeks, and no contamination with other types of cells was confirmed (Fig 1). Before transplantation, these primary culture cells were trypsinised and suspended in PBS at a concentration of approximately 5000–10 000 cells per 100 μ l.

SURGICAL TECHNIQUES

Each adult albino rabbit at the age of 5–10 months, weighing 2–3 kg, was anaesthetised with intravenous sodium pentobarbitone (50 mg/kg). The pupil was dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride. A conjunctival flap was formed and two ports at the pars plana were made with a stylet blade for controlling the intraocular pressure by infusion at one port and introducing instruments at another. A 30 gauge blunt tipped micro needle attached to a 1 ml disposable syringe was introduced through the pars plana incision and visualised in the vitreous cavity under a surgical microscope (Takagi, Nakano, Japan) with a corneal contact lens. The needle was guided to the neural retina about two disc diameters from the optic disc. A jet stream of balanced salt solution (BSS) was ejected from the needle to produce a small retinal hole and a bleb retinal detachment.

The RPE cells were then removed by brushing with a 20 gauge soft tipped silicone cannula (subretinal cannula, Eagle Laboratories,

Figure 2 Non-damaged versus damaged Bruch's membrane (b) immediately after the surgery. The denuded Bruch's membrane with the intact choriocapillaris (A), in contrast with haemorrhage (arrow in B) from a rupture of Bruch's membrane with plugged choriocapillaris (arrowhead in C) at one site, and the RPE basement membrane (arrowhead in D) removed from the elastic layer (arrow in D) at another site. (A), (B) PAS stain, bar = 100 μ m. (C), (D) Uranyl acetate and lead citrate stain, bar = 2 μ m.

Figure 3 Seven days after the surgery without transplantation. The host RPE cells without polarity layer on the damaged area (between arrows in A) of Bruch's membrane (b), while monolayered RPE cells with the normal polarity repopulate the non-damaged Bruch's membrane (b) in close contact with retinal photoreceptor outer segments (B). Uranyl acetate and lead citrate stain, bar = 2 μ m.

Rancho Cucamonga, CA, USA) inserted under the detached retina. Bruch's membrane was also damaged mechanically by scratching with a 27 gauge microhooked needle.

When choroidal haemorrhage was induced, the bleeding was stopped with high intraocular pressure induced by raising an infusion bottle of BSS for 10–20 minutes. Subretinal haemorrhage and dissociated RPE cells were washed out from the subretinal space with a jet injection of BSS, and removed from the vitreous cavity with a vitreous cutter (Ocutome: Alcon Laboratories, Fort Worth, TX, USA). A 100 μ l suspension of embryonic RPE cells in primary culture was injected through the retinal hole into the subretinal space using a 30 gauge needle attached to a 1 ml disposable syringe. Reflux of the injected RPE cells into the vitreous cavity was then aspirated by a vitreous cutter. The sclerotomy sites and conjunctival flap were closed with 10–0 nylon sutures.

HISTOLOGICAL EXAMINATION

The animals were killed with an overdose of pentobarbitone sodium 3, 7, or 14 days after

the surgery. The eyes were enucleated and fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight. The eyes were processed through a graded series of ethanol and xylene, and embedded in paraffin. Sections were cut at 5 μ m thickness and stained with periodic acid Schiff (PAS) for light microscopy. For transmission electron microscopy, tissue fragments containing the transplantation sites were cut out, post fixed in 1% osmium tetroxide, dehydrated with a graded ethanol series, and embedded in epoxy resin. The sections were cut with a diamond knife, placed on copper grids, stained with uranyl acetate and lead citrate, and photographed with an electron microscope (HS-9: Hitachi, Tokyo, Japan). Thick resin sections were also stained with azure for light microscopy.

Results

DENUDED VERSUS DAMAGED BRUCH'S MEMBRANE
Brushing of the RPE with a soft tipped silicone cannula left intact the full thickness Bruch's membrane, including the RPE basement

Figure 4 RPE cells on damaged Bruch's membrane (b) 3 days after transplantation. Pigmented (arrows in A) and non-pigmented RPE cell layer over the damaged area of Bruch's membrane. RPE cells with melanin granules cover a break (between arrows in B) in Bruch's membrane (b). Basal infoldings and microvilli are absent. (A) Azure stain, bar = 100 μ m. (B) Uranyl acetate and lead citrate stain, bar = 2 μ m.

Figure 5 RPE cells on moderately damaged versus severely damaged Bruch's membrane (b) 7 days after transplantation. Viable pigmented RPE cells (small arrow in A) showed apical microvilli and basal infoldings spreading on the mildly damaged Bruch's membrane. Some RPE cells lose viability as indicated by a small number of intracellular organelles (asterisks in A, B, C). Bruch's membrane (b) has such aberrant sites as a fibroblast invading its inner collagenous layer (large arrow in A), protrusion of a disrupted end of the elastic layer (arrow in B), or convoluted RPE basement membrane (arrowheads in C) with basal infoldings (arrow in C). Fibroblast-like cells form multiple layers on the severely damaged Bruch's membrane (D). Uranyl acetate and lead citrate stain, bar = 2 μ m.

membrane (Fig 2A), and the retina became reattached within 7 days after the surgery in the eyes with non-damaged Bruch's membrane. In contrast, further mechanical scratching of the denuded Bruch's membrane with a microhooked needle sometimes induced choroidal haemorrhage (Fig 2B). A retinotomy site remained open, and the retina was not reattached completely for 2 weeks after the surgery in the eyes with damaged Bruch's membrane.

Ultrastructurally, the damage varied from a rupture of Bruch's membrane (Fig 2C) to simple loss of the RPE basement membrane with the elastic layer preserved (Fig 2D). Three degrees of damage were mechanically caused to Bruch's membrane. The first degree of damage (mild damage) was damage only to the RPE basement membrane and the inner collagenous layer (Fig 2D). The second degree of damage (moderate damage) involved the elastic layer, resulting in its waving and partial defects (see Fig 5B). The third degree of damage (severe damage) involved the choriocapillaris by perforation of the elastic layer, sometimes leading to choroidal haemorrhage (Fig 2B).

Host RPE cells repopulated the damaged areas of Bruch's membrane 1 week after the surgery. These repopulated cells did not show a normal polarity such as basal infoldings and apical microvilli. Cells were irregularly layered over each other, while a few tight junctions were detected (Fig 3A). In contrast, monolayered or bilayered RPE cells with a normal polarity repopulated the non-damaged Bruch's membrane, and the retina was attached (Fig 3B).

THREE DAYS AFTER TRANSPLANTATION ON DAMAGED BRUCH'S MEMBRANE

The damaged areas of Bruch's membrane were covered by pigmented and non-pigmented presumed RPE cells in a few layers (Fig 4A). Ultrastructurally, pigmented RPE cells covered small breaks of Bruch's membrane with neighbouring RPE cells. These pigmented immature RPE cells at this stage did not show basal infoldings or apical microvilli, and fibroblast-like cells formed multiple layers (three to five layers) (Fig 4B).

ONE WEEK AFTER TRANSPLANTATION ON DAMAGED BRUCH'S MEMBRANE

Viable pigmented RPE cells, with high electron density cytoplasm due to rich rough surfaced

Figure 6 RPE cells on damaged Bruch's membrane (b) 14 days after transplantation. Subretinal fibrovascular tissue with capillary lumens (arrows in A) above PAS positive Bruch's membrane (arrowheads in A), containing pigmented RPE cells (arrows in B) with tubuloacinar formation. A pigmented RPE cell with normal apical microvilli (arrowheads in C) and basal infoldings (arrows in C) on mildly damaged Bruch's membrane, in contrast with a pigmented cell (asterisk in D) which covers a break of full thickness of Bruch's membrane in the severely damaged area and reproduces its basement membrane (arrowheads in D). The inner nuclear layer of the retina (arrows in E) is attached to fibrous scar, and the outer nuclear layer (arrowhead in E) is in close proximity to pigmented RPE cells at some sites. (A), (E) PAS stain, bar = 100 μ m. (B), (C), (D) Uranyl acetate and lead citrate stain, bar = 2 μ m.

endoplasmic reticuli, showed apical microvilli and basal infoldings spreading on the mild to moderately damaged Bruch's membrane (Fig 5A). However, some RPE cells lost viability as indicated by a small number of intracellular organelles (Fig 5A, B, C). Bruch's membrane under the RPE cells had numerous aberrant sites caused by the mechanical damage itself as well as by the regenerative processes as follows. Fibroblastic cells invaded the inner collagenous zone (Fig 5A), and disrupted ends of the elastic layer protruding into the choroid under the reproduced RPE basement membrane (Fig 5B). The RPE basement membrane was convoluted

together with its basal infoldings, dropping down into the choriocapillaris (Fig 5C).

In contrast with mild to moderately damaged sites of Bruch's membrane described above, severely damaged areas were not attached by RPE cells, but were overlaid by fibroblast-like cells with collagen bundles deposited among them (Fig 5D).

TWO WEEKS AFTER TRANSPLANTATION ON DAMAGED BRUCH'S MEMBRANE

A fibrovascular tissue with fibroblasts and capillary lumens was located over the PAS positive Bruch's membrane (Fig 6A). Ultrastructurally,

Figure 7 RPE cells on non-damaged Bruch's membrane (b) 7 days after transplantation. The retina is attached to pigmented RPE cells (arrows in A). These cells on the preserved basement membrane of the host RPE form monolayers or bilayers and show basal infoldings, apical microvilli, and tight junctions (arrow in B). (A) Azure stain, bar = 100 μ m. (B) Uranyl acetate and lead citrate stain, bar = 2 μ m.

the tissue contained not only fibroblasts but also many pigmented RPE cells (Fig 6B). Pigmented RPE cells with normal apical microvilli and basal infoldings were attached to the mildly damaged Bruch's membrane (Fig 6C). Even at the sites of a severely damaged Bruch's membrane, RPE-like cells with melanin granules covered breaks of the elastic layer, and reproduced a basement membrane (Fig 6D). These cells showed tubuloacinar formation as their proliferating process, but did not develop basal infoldings or apical microvilli. The retina remained detached in most areas, but partial adhesions between Bruch's membrane and the degenerated retina were observed at some sites (Fig 6E). The outer nuclear layer was shortened, and inner and outer segments of the photoreceptor cells were almost lost.

TRANSPLANTATION ON NON-DAMAGED BRUCH'S MEMBRANE

Pigmented RPE cells existed as a monolayer or bilayer on the non-damaged Bruch's membrane under the attached retina (Fig 7A), in contrast with multiple layers of fibroblast-like cells on the damaged membrane as described above (Fig 5D). They were attached to the preserved host RPE basement membrane and showed basal infoldings, apical microvilli, and tight junctions at 7 days after the transplantation (Fig 7B).

Discussion

Most of the mechanical damage caused to Bruch's membrane in this study belonged to mild to moderate degrees of the damage without a rupture of the choriocapillaris since the elastic layer was so resilient²² and less prone to mechanical agitation. Fibroblast-like cells formed several layers over the severely damaged areas of Bruch's membrane where the elastic layer was damaged, and the choriocapillaris was substituted for fibrosis. Since RPE cells are known to become like fibroblasts when their proper interaction with extracellular matrix is disturbed,^{23, 24} fibroblast-like cells, piling up and covering the severely damaged areas of Bruch's membrane with fibrotic choriocapillaris, were considered to originate both from choroidal fibroblasts and from RPE cells.

Transplanted RPE and regrown host RPE cells, firstly, have to attach to the extracellular matrix and then initiate their proliferation and differentiation. RPE cells which lose contact with their basement membrane have been known to become apoptotic.²⁵⁻²⁷ In addition, an in vitro study by Ho *et al*²⁷ showed that RPE cells, cultured on a sheet of extracellular matrix, including type IV collagen, fibronectin, and laminin, were less prone to become apoptotic. RPE cells produce type IV collagen which is a main component of their basal lamina.^{28, 29} RPE cells could be attached to the mildly or moderately damaged Bruch's membrane and reproduce their basement membrane, which would, in turn, influence their survival. The severely damaged Bruch's membrane in this study, however, hampered transplanted RPE cells from attachment and differentiation.

Pigmented embryonic RPE cells were used as transplants since they had a higher potential for growth and differentiation than adult ones. Pigments in RPE cells were used to distinguish transplanted cells from host cells. The presence of pigments has been recognised as a less reliable marker since RPE cells can phagocytose pigments, and such markers as Barr bodies³⁰ and sex chromatin³¹ are recommended for use. The present study was also limited to as short a period as 2 weeks of observation to see the early phase of the transplantation, since long term experiments have to overcome immunological rejection of transplanted allogeneic RPE cells, which is known to occur at around 3 months.¹⁸

The retina remained basically detached in the eyes with damaged Bruch's membrane during a 2 week period, in contrast with the eyes with non-damaged Bruch's membrane in which the retina was reattached. Choroidal haemorrhage and breakdown of the outer blood-retinal barrier, caused by mechanical damage of Bruch's membrane, would result in persistent retinal detachment. Experimental retinal detachment in itself has been shown to induce stratification of RPE cells and their undifferentiated features.³²⁻³⁴ The part of the features, as observed in the present study,

could be attributable to persistent retinal detachment. However, the differences in the differentiated extent of RPE cells between the mild to moderate damage and the severe damage existed under the same condition of persistent retinal detachment.

Two to 3 month old young rabbits were used as hosts in this study, and their RPE cells showed a high potential for repopulation after the removal from Bruch's membrane. In eyes with AMD, in contrast, RPE cells hardly repopulate the defective area after their surgical removal together with excision of CNV, and later, the underlying choriocapillaris becomes atrophic.³⁵ To stop this vicious cycle leading to the choriocapillaris atrophy, RPE cells would be transplanted immediately after CNV surgery. However, severe full thickness damage of Bruch's membrane is induced in a large area surrounding the stalk of CNV after its surgical excision, and results in the formation of fibrous tissue. Under the circumstances, stratification of transplanted RPE cells with poor differentiation, as observed in the previous and present studies, hampers their normal interaction with retinal photoreceptor outer segments. This problem might be overcome by modulating transplanted RPE cells by gene transfer or by the addition of growth factors in the future.

In conclusion, the extent of the damage in Bruch's membrane influenced attachment, growth, and differentiation of the transplanted RPE cells and host RPE cells. The severe full thickness damage of Bruch's membrane involving the elastic layer as well as the choriocapillaris did not allow RPE cells to grow as a monolayer with the normal polarity. If the damage to Bruch's membrane was not severe, transplantation of RPE cells would be effective in covering the area which lost host RPE cells. RPE cells in a sheet or together with full thickness Bruch's membrane, in contrast with suspension of dissociated cells used in this study, would be transplanted as a better alternative in cases of severe damage to Bruch's membrane.

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