Repopulation of Different Layers of Host Human Bruch’s Membrane by Retinal Pigment Epithelial Cell Grafts

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PURPOSE. To determine the morphology of human retinal pigment epithelium (RPE) after reattachment to different ultrastructural layers of human Bruch’s membrane (BM).

METHODS. Bruch’s membrane explants were prepared from eyes of 23 human donors (age range, 11–89 years). The basal lamina of the RPE, inner collagenous layer, and elastin layer were removed sequentially by mechanical and enzymatic techniques. First-passage cells of human RPE (15,000 cells/6 mm explant) from three donors (ages, 52, 64, and 80 years) were plated onto different layers of human BM, and the explants were examined by scanning and transmission electron microscopy up to 21 days later.

RESULTS. RPE flattened and extended footplates 6 hours after plating onto basal lamina. Cells remained round 6 and 24 hours after plating onto the inner collagenous, elastin, or outer collagenous layer. The RPE cells became confluent 14 days after plating onto basal lamina but did not become confluent up to 21 days after plating onto the inner collagenous or elastin layer. Sparse round cells were observed 21 days after plating onto deeper layers, suggesting extensive loss of RPE.

CONCLUSIONS. The morphology and subsequent behavior of the RPE reattached to BM depends on the anatomic layer of BM available for cell reattachment. The results suggest that the ability of transplanted RPE to repopulate BM in age-related macular degeneration and other disorders may depend on the layer of BM available to serve as a substrate for cell reattachment. (Invest Ophthalmol Vis Sci. 1999;40:767–774)

Transplantation of the RPE has been attempted in humans after surgical excision of a subfoveal choroidal neovascular membrane in exudative AMD. Some macular dystrophies including AMD may be caused by a mutation in a photoreceptor-specific protein. However, a rationale exists for transplantation of the RPE in wet AMD because removal of the RPE during subfoveal surgery leads to secondary atrophy of the choriocapillaris. Algvere et al. have shown that patients fixate over the grafts of RPE immediately after surgery but lose their ability to fixate over the grafts 3 months later. This result suggests that the transplanted RPE did not survive in the subretinal space, perhaps because of immune rejection or the failure of the transplant to reattach to host BM. Rapid reattachment of transplanted cells to BM is important because harvested dissociated RPE undergo apoptosis unless they reattach to a substrate.

The RPE can reattach to its native basal lamina on the inner surface of BM after harvesting by trypsinization. This reattachment is mediated through a specific interaction between a β₁-integrin subunit on the cell surface and ligands present in the basal lamina that include laminin, fibronectin, collagen IV, and vitronectin. However, potential candidates for transplantation of the RPE include patients with tapetoretinal degenerations, advanced geographic atrophy in nonexudative AMD, and subfoveal choroidal neovascularization in exudative AMD. It is unlikely that the basal lamina will be intact in these patients, because components of the basal lamina are synthesized and degraded by the RPE. In addition, numerous structural alterations occur throughout BM in patients with severe exudative and nonexudative AMD and surgically...
excised choroidal neovascular membranes contain fragments of the basal lamina of the RPE, inner collagenous layer, and occasionally, elastin. Thus, it is unlikely that the host basal lamina remains intact throughout the dissection bed after surgical removal of subfoveal neovascularization. In view of these considerations, it is important to investigate the effects of the substrate on human RPE plated onto different anatomic layers of BM, including native basal lamina, the inner collagenous layer, the elastin layer, and the outer collagenous layer.

METHODS

Culturing Human RPE

Harvesting RPE for Tissue Culture. Cultures of RPE were prepared from eyes of three human donors (age, 52, 64, and 80 years) obtained from the Mid-America Tissue Bank (St. Louis, MO) within 6 to 24 hours of death. Briefly, eyes were cleaned of extraocular tissue. The suprachoroidal space was sealed with cyanoacrylate glue, and a small scleral incision was made 3 mm posterior to the limbus until the choroidal vessels were exposed. Tenotomy scissors were introduced through this incision into the suprachoroidal space, and the incision was extended circumferentially. Four radial relaxing incisions were made, and the sclera was peeled away from the periphery to the optic nerve. The eyecup was then incubated with 25 U/ml dispase (Gibco, Grand Island, NY) for 30 minutes. The eye was rinsed with carbon dioxide-independent medium (Gibco), and a circumferential incision was made into the subretinal space along the ora serrata. The RPE-choroid complex was removed from the remainder of the ocular tissue and transferred to another dish. Loosened sheets of human RPE were collected with a pipette and pooled in Dulbecco’s modified Eagle’s medium (DMEM H16, Gibco) containing 10% fetal bovine serum. The cells were centrifuged at 1000 rpm for 5 minutes, the supernatant discarded, and the pellet resuspended in approximately 10 days, and cultures were passaged by trypsinization of the cells. First-passage RPE was used in all experiments.

Cytokeratin Staining. Cells that were grown to confluence were stained with a mixture of monoclonal anti-pancytokeratin antibodies to cytokeratins 1, 4, 5, 6, 8, 10, 15, 18, and 19 (Sigma, St. Louis, MO), as previously described. All the cells were positive for pancytokeratin, indicating the cells were of epithelial origin.

Harvesting of Human Bruch’s Membrane

Explants of human BM were prepared from the periphery of 23 donors (age range, 11-89 years; mean, 54 ± 25 years). A full-thickness circumferential incision was made posterior to the ora serrata, and the anterior segment and vitreous were removed carefully. The posterior pole of each eyecup was inspected visually with direct and retroillumination under a dissecting microscope. None of the study eyes had evidence of subretinal blood, extensive drusen, or irregular pigmentation of the macular RPE. The eyecups were put in carbon dioxide-independent medium and a scleral incision was made 3 mm from the limbus and extended circumferentially. Four radial incisions were made, and the sclera was peeled away. A circumferential incision was made into the subretinal space 1 mm posterior to the ora serrata. The choroid-BM-RPE complex was then carefully peeled toward the optic disc and removed after trimming its attachment to the optic nerve. Native RPE was removed by bathing the explant with 0.02 N ammonium hydroxide in a 50-mm petri dish (Falcon; Becton Dickinson, Lincoln Park, NJ) for 20 minutes at room temperature, followed by washing three times in PBS. The explant was then floated in carbon dioxide-independent medium over an un laminated, hydrophobic 125- to 175-μm-thick polytetrafluoroethylene membrane (Millipore, Bedford, MA) with 0.45-μm pores, with the basal lamina of the RPE facing the membrane. The curved edges were flattened from the choroidal side with fine forceps, and care was taken to avoid touching BM. Agarose (4%; Sigma) was poured on the BM-choroid complex from the choroidal side, and the tissue was kept at 4°C for 2 to 3 minutes to solidify the agarose. The polytetrafluoroethylene membrane was peeled off, and 6-mm circular buttons were then trephined on a teflon sheet from peripheral BM and placed on 4% agarose at 37°C in nontreated polystyrene wells of a 96-well plate (Corning Costar, Cambridge, MA). The high viscosity of the agarose solution prevented it from flowing over the apical explant surface. The agarose solidified within 2 to 3 minutes at room temperature, thus stabilizing the BM explant. Typically, 6 to 8 explants could be harvested from each eye.

Preparation of Different Layers of Bruch’s Membrane

Explants containing different layers of BM on the apical surface were prepared as follows.

Heparinase or Chondroitinase-Treated Explants. Triplicate buttons from each donor were treated at 37°C with 2.4 U/ml heparinase (Sigma) in PBS (pH 7.5) for 2 hours to remove the heparan sulfate proteoglycans from the basal lamina of BM or with 0.1 U/ml chondroitinase ABC (Sigma) in Tris buffer (pH 7.5) for 2 hours to digest chondroitin A, B, and C and dermatan sulfate proteoglycan groups of the basal lamina. Some buttons were treated with heparinase and then with chondroitinase to determine the effects of removing both proteoglycan groups on the morphology of the reattached RPE. After enzymatic treatment, the exposed surface was washed three times with PBS for 5 minutes. Control explants were washed three times with PBS.

Sequential Removal of Bruch’s Membrane Layers. Removing the basal lamina of the RPE from the fellow eye exposed the inner collagenous layer. This was done by placing the explant on a 12- to 18-μm-thick hydrophilic polycarbonate-polyvinylpyrrolidone membrane with 0.4-μm pores (Millipore) with the basal lamina facing toward the membrane. We then poured liquid 4% agarose on the explant while applying suction to the membrane, cooled the specimen to 4°C to solidify the overlying agarose, and gently peeled off the basal lamina. The elastin layer was exposed by treating the explant surface with 1 mg/ml collagenase (Sigma) in PBS (pH 7.5) for 1 hour at 37°C, and the outer collagenous layer was exposed by treating
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Reattachment Studies

All experiments were performed using first-passage RPE as soon as the cells reached confluence. Confluent cell cultures were synchronized by placing them in serum-free minimum essential medium (MEM) for 24 hours before harvesting with 0.25% trypsin-0.25% EDTA in Hanks’ balanced salt solution for 10 minutes. Two milliliters of 0.1 mg/ml aprotinin (Sigma) in HEPES buffer (pH 7.5) was added to quench the trypsin reaction, and the cell suspension was centrifuged for 5 minutes at 800 rpm. The cell pellet was washed three times and resuspended in phenol red-free MEM (Gibco) without serum. Cell viability was assessed using a kit (LIVE/DEAD Viability Kit; Molecular Probes, Portland, OR); the viability of harvested cells was always more than 97%. For short-term studies (24 hours or
RESULTS

The morphology of retinal pigment epithelium (RPE) 6 hours after plating onto basal lamina on the inner aspect of BM is shown in Figure 1. Flattened footplates (FP, Figs. 1A, 1D) were present within 6 hours after plating trypsinized RPE onto basal lamina. Numerous filamentous pseudopodia (f, Fig. 1C) extended from the RPE to the basal lamina. No footplates extended from the cell to an area of exposed inner collagenous layer (ICL, Fig. 1B). Prominent buds were present in the previously trypsinized RPE (b, Fig. 1B). Debris that may represent basal laminar deposit was visible after ammonium hydroxide treatment of explants harvested from a 69-year-old donor (arrow, Fig. 1C). Transmission EM confirmed the presence of flattening at the cell borders (Fig. 1D). Twenty-four hours after plating onto basal lamina, RPE was present on the surface (Fig. 1E), and focal contacts (open arrows, Fig. 1F) separated by cleftlike spaces (solid arrow, Fig. 1F) were present between the flattened, elongated RPE and the basal lamina of the explant.

The morphology of the RPE 6 hours after plating onto the inner collagenous layer harvested from a 56-year-old donor is shown in Figure 2A. Transmission EM revealed focal contacts (black arrows, Fig. 2B) separated by cleftlike spaces (open arrow, Fig. 2B) between the plated cell and the inner collagenous layer. Cells remained round 24 hours after plating onto the inner collagenous layer (Figs. 2C, 2D) although occasional focal contacts (open arrows, Fig. 2D) were present between the RPE and this layer.

The morphology of RPE plated onto the elastin layer of BM harvested from a 69-year-old donor is shown in Figure 3A. Six hours after plating, the reattached cells remained round, but occasional filamentous cellular extensions (f, Fig. 3A) were observed in the elastin layer. Transmission EM confirmed that the plated cells reattached but remained round 24 hours after plating onto the elastin layer harvested from a 45-year-old donor (Fig. 3B). Flat footplates (FP, Fig. 3C) were visible on cells reattached to the heparinase-treated basal lamina. Fine, filamentous pseudopodia (f, Fig. 3D) extended from the cell to the heparinase-treated basal lamina. The morphology of the RPE reattached to its basal lamina after treatment with chondroitinase is shown in Figure 3E. Removal of the elastin layer exposed the outer collagenous layer (Fig. 3F). Cells remained round after they reattached to this substrate, with rare extensions from the cell to the exposed collagen (open arrow, Fig. 3F).

The RPE became nearly confluent within 14 days after plating onto basal lamina derived from a 37-year-old donor (Fig. 4A) or a 74-year-old donor (Fig. 4B), with occasional gaps in the cell layer (arrow, Fig. 4B). However, the RPE did not become confluent 21 days after plating onto the inner collagenous layer (Fig. 4C) or the elastin layer (Fig. 4D) harvested from the same 74-year-old donor. In fact, only occasional round and shrunken cells were present on either layer 21 days after plating (Figs. 4C, 4D).

DISCUSSION

We have previously shown that the initial reattachment rate of human RPE seeded onto human BM depends on the layer of BM available for cell reattachment.17 The reattachment rate of the RPE is highest to the inner aspects of BM and decreases as deeper layers of BM are exposed. The reattachment rate to the basal lamina is highest, followed by the inner collagenous layer, elastin layer, and the outer collagenous layer. The fate of human RPE also depends on the layer of BM available for cell attachment.33 The apoptosis rate of attached cells increases as deeper layers of BM are exposed, whereas the proliferation rate and mitotic index are higher on basal lamina than on deeper layers.31

The present study shows that the layer of BM available for cell reattachment also affects the morphology of adult human RPE that reattach to BM. The RPE flattened initially after plating onto basal lamina but remained round after reattachment to the inner collagenous layer, elastin layer, or outer collagenous layer. Dissociated RPE became confluent within 14 days after plating onto basal lamina at a density of 15,000 cells per 6-mm explant; this plating density corresponded to the plated cells’ covering 15% of the explant surface. The RPE did not become confluent after plating onto the inner collagenous layer or the elastin layer. In fact, only occasional round, shrunken cells could be found on the inner collagenous or elastin layer 21 days after plating. This is not surprising, because cell attach-
FIGURE 2. Retinal pigment epithelium (RPE) reattached to inner collagenous layer (ICL) of BM. (A) Six hours after plating onto ICL from a 56-year-old donor, cells remained round without pseudopodia extending from cells to the ICL (compare with Fig. 1). Filamentous pseudopodia extending between cells were occasionally seen (arrow). (B) Six hours after plating onto ICL from a 56-year-old donor, areas of focal contact (filled arrowheads) and large cleftlike spaces (open arrowhead) were present between cells and the ICL. Curling of BM was an artifact from tissue processing. (C) Twenty-four hours after plating onto ICL from a 15-year-old donor, cells remained round. (D) Twenty-four hours after plating onto ICL from a 15-year-old donor, focal contacts (open arrows) were present between the RPE and the ICL. The elastin layer (EL) was also visible. Bar, (C) 10 μm; (A, B, D) 5 μm.

Cell shape is correlated with subsequent cell survival and proliferation in other epithelial cells; cells forced to extend themselves over a large surface area survive longer and proliferate faster than cells with a more rounded shape.32 Our results are consistent with those in previous studies showing that the extracellular matrix affects the morphology and subsequent behavior of cells.33–42 RPE removed from BM and plated onto tissue culture plastic flatten, develop a fibroblastic morphology, lose their apical-basal polarity, and become depigmented.37–38 The RPE differentiates to neurons when plated onto a laminin substrate,34 and cell morphology can be altered by soluble cytokines and cytokines within the extracellular matrix.30 Extracellular matrix derived from the RPE is superior to tissue culture plastic, gelatin, laminin, fibronectin, and bovine corneal endothelial cell extracellular matrix in promoting proliferation of the RPE and maintaining cell polarity in vitro.30

The adhesion of a cell to its substrate is a complex, multistage molecular process that involves initial cell attachment, spreading, and the formation of focal adhesions that requires a specific interaction between receptors on the cell surface and ligands in the extracellular matrix.43–44 The RPE contains a β1-subunit of integrin that mediates the attachment of RPE to extracellular matrix derived from the RPE and extracellular matrix components on BM that include fibronectin, collagen IV, vitronectin, and laminin.16 Integrins are transmembranous glycoproteins that attach to the cell substrate externally and the cell cytoskeleton internally.52 The binding of integrin receptors on the surface of the RPE to extracellular matrix ligands causes numerous structural and morphologic changes in the RPE and controls cell survival, migration, proliferation, growth, and differentiation. Integrin binding regulates an intracellular focal adhesion kinase that provides a link between the extracellular matrix and internal cell biochemistry and provides a molecular mechanism by which the substrate can determine the fate of the transplanted RPE.

Our results have important implications for studies involving transplantation of the RPE in AMD and other disorders. If the basal lamina of BM is intact, transplanted RPE may be able to reattach to this surface and rapidly flatten and proliferate to repopulate bare areas of BM. However, it is unlikely that the basal lamina is intact in many patients who are candidates for transplantation of the RPE. In nonexudative AMD, areas of geographic atrophy are devoid of RPE and may not contain normal basal lamina. In exudative AMD, transplantation of the RPE is performed after surgical removal of subfoveal choroidal neovascularization.3–4,46 Native RPE is often removed with the subfoveal neovascular membrane in exudative AMD and may be removed with the choroidal neovascular membrane in other disorders as well.47–51 Although we do not know the exact location of the cleavage plane that develops when the subfoveal neo-
vascularization is removed, it is unlikely that the basal lamina is intact after surgery because surgically excised choroidal neovascular membranes contain fragments of basal lamina, inner collagenous layer, and occasionally, elastin.27 Thus, in exudative and nonexudative AMD, RPE harvested for transplantation probably has to reattach to the inner collagenous or deeper layers of BM. Our results suggest that transplanted adult RPE may not be able to repopulate this abnormal surface.

For transplantation of the RPE to be successful, it is important to know the substrate that is available for cell reattachment in each disease state and the effects of this substrate on the reattachment, survival, and proliferation of the transplanted cells. Many other factors may also affect the ability of donor RPE to repopulate BM, including the age of the donor RPE (i.e., fetal versus adult),28 prior passaging of the RPE in tissue culture, the technique used to harvest the RPE for transplantation, the presence of cytokines and/or cell attachment factors in the subretinal space,29 and alterations in the molecular composition of the reattachment surface caused by AMD-related changes within BM.30 Successful transplantation of the RPE in AMD and other disorders may require a complete...
understanding of these variables to ensure rapid repopulation of human BM and to avoid secondary atrophy of the choriocapillaris. 

References