Reengineering of Aged Bruch’s Membrane to Enhance Retinal Pigment Epithelium Repopulation

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PURPOSE. An earlier study showed that age-related changes in the inner collagen layer (ICL) inhibit RPE cell repopulation of human Bruch’s membrane. The present study was undertaken to determine the effect of cleaning and/or an extracellular matrix (ECM) protein coating on the reattachment, apoptosis, proliferation, and final surface coverage of the transplanted RPE cells.

METHODS. Explants of aged Bruch’s membrane with ICL, exposed were prepared from five human cadaveric eyes (donor ages, 69–84 years) and treated with Triton X-100 and/or coated with a mixture of laminin (350 μg/mL), fibronectin (250 μg/mL), and vitronectin (35 μg/mL). Viable human fetal and ARPE-19 cells (n = 15,000) were plated onto the surface and the RPE reattachment, apoptosis, and proliferation ratios were determined on the modified surfaces. Cells were cultured up to 17 days to determine the surface coverage. Ultrastructure of the modified Bruch’s membrane and RPE morphology were studied with transmission and scanning electron microscopy.

RESULTS. Reattachment ratios of fetal human RPE and ARPE-19 cells were similar on aged ICL (41.5% ± 1.7% and 42.9% ± 2.7%, P > 0.05). The reattachment ratio increased with ECM protein coating and decreased with detergent treatment. Combined cleaning and coating restored the reattachment ratio of the fetal RPE cells, but failed to increase the reattachment ratio of ARPE-19 cells. The highest apoptosis was observed on untreated ICL. Cleaning and the combined procedure of cleaning and ECM protein coating decreased fetal RPE cell apoptosis. Only RPE cells plated on cleaned or cleaned and ECM-coated ICL demonstrated proliferation that led to substantial surface coverage at day 17.

CONCLUSIONS. Age-related changes that impair RPE repopulation of Bruch’s membrane can be significantly reversed by combined cleaning and ECM protein coating of the ICL. Development of biologically tolerant techniques for modifying the ICL in vivo may enhance reattachment of the RPE and its repopulation of aged ICL. (Invest Ophthalmol Vis Sci. 2004;45:3337–3348) DOI:10.1167/iovs.04-0193

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Preparation of RPE Cultures

All study protocols adhered to the provisions of the Declaration of Helsinki for research involving human tissue. Human fetal RPE cells
were harvested from 14- and 17-week-old human fetuses processed within 6 hours of elective abortion. The techniques for harvesting and culturing the RPE cells have been published. Briefly, on receipt, eyes were cleaned of extraneous tissue. A circumferential scleral incision was made 1.5 mm posterior to the limbus, and the sclera was peeled away. The eyecup was then incubated with 25 U/ml dispase (Invitrogen-Gibco, Grand Island, NY) for 30 minutes and rinsed with CO2-free medium (Gibco). Loosened RPE sheets were collected with a Pasteur pipette and plated onto bovine corneal endothelium-ECM-coated, 60-mm treated plastic dishes (Falcon; BD Biosciences UK, Plymouth, UK). The cells were incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C and maintained in Dulbecco’s modified Eagle’s medium (DMEM H16; Invitrogen-Gibco) supplemented with 15% FBS, 100 IU/ml penicillin G, 100 μg/ml streptomycin, 5 μg/ml gentamicin, 2.5 μg/ml amphotericin B, and 1 ng/ml recombinant human basic fibroblast growth factor (bFGF; Invitrogen-Gibco), to promote RPE cell growth. The medium was changed every other day and the cells observed daily. Cells became confluent in approximately 10 days, and confluent cultures were passaged by trypsinization. First-passage RPE cell lines were used in these experiments.

The ARPE-19 cell line was obtained from the American Type Culture Collection (Manassas, VA). This is a line of spontaneously immortalized RPE cells that have morphologic and functional characteristics similar to those of adult human RPE cells. Cells were maintained in a 1:1 mixture of DMEM and Ham’s F-12 with HEPEs buffer containing 20% FBS (Invitrogen-Gibco), 56 mM final concentration sodium bicarbonate, and 2 mM l-glutamine (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C in 10% CO2.

Cytokeratin Labeling

Cells were stained with a pancytokeratin antibody to verify that all cells were of epithelial origin. For this purpose, harvested RPE sheets were rinsed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 30 minutes, and washed again with PBS. The cells were treated for 1 hour at room temperature with 3% bovine serum albumin (Sigma-Aldrich) in PBS to block nonspecific binding sites. The cells were then incubated at 37°C for 1 hour with an FITC-conjugated monoclonal anti-pan cytokeratin antibody to cytokeratin-5, -6, and -8 (Sigma-Aldrich). The cells were washed three times with PBS and then incubated under a fluorescence microscope. An irrelevant isotypic IgG primary antibody (anti-human von Willebrand antibody; Sigma-Aldrich), coupled with an FITC-conjugated secondary antibody was also tested and showed no background staining. All the harvested cells were positive for pancytokeratin, indicating that the cells were of epithelial origin.

Harvesting of Human Bruch’s Membrane Explants

Explants of the inner collagen layer (ICL) of human Bruch’s membrane were prepared from the peripheral retinas of eyes of four elderly donors (average age, 77 ± 6 years [SD]; range, 69–84 years old) obtained within 24 hours of death. The harvesting technique has been described. Briefly, a full-thickness circumferential incision was made posterior to the ora serrata, and the anterior segment and vitreous were carefully removed. The posterior pole of each eyecup was inspected visually with direct and retroillumination under a dissecting microscope, and globes were discarded if there was any evidence of subretinal blood, previous surgery, or any extensive structural or vascular alteration of the posterior segment due to a disease process, such as proliferative diabetic retinopathy or proliferative vitreoretinopathy. The eyecups were put in CO2-free medium (Invitrogen-Gibco), and a scleral incision was made 3 mm from the limbus and extended 360°. Four radial incisions were then 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-Bruch’s membrane-RPE complex was then carefully peeled toward the optic disc and removed after its attachment to the optic nerve was trimmed. Native RPE cells were removed by bathing the explant with 0.02 N ammonium hydroxide in a 50-mm polystyrene Petri dish (Falcon; BD Biosciences) for 20 minutes at room temperature, followed by washing three times in phosphate-buffered saline (PBS). The Bruch’s membrane explant from the fellow eye was prepared by removing the RPE with 0.02 N ammonium hydroxide as just described. The Bruch’s membrane explant was then floated in carbon dioxide-free medium over a 12- to 18-μm-thick hydrophilic polycarbonate-polynylpyrrolidione membrane with 0.4-μm pores (Millipore, Bedford, MA) with the basal lamina facing the membrane. The curled edges were flattened from the choroidal side with fine forceps without touching Bruch’s membrane. Four percent agarose (Sigma-Aldrich) was poured on the Bruch’s membrane-choroid complex from the choroidal side, and the tissue was kept at 4°C for 2 to 3 minutes to solidify the agarose. The hydrophilic membrane was peeled off along with the basal lamina of the RPE, thus exposing the bare ICL. Circular buttons (6-mm diameter) were then trephined from the peripheral Bruch’s membrane on a Teflon sheet and placed on 4% agarose at 37°C in nontreated polystyrene wells of a 96-well plate (Corning Costar Corp., Cambridge, MA). The agarose solidified within 2 to 3 minutes at room temperature, thus stabilizing the Bruch’s membrane explant. The wells were gently rinsed with PBS three times for 5 minutes, gamma sterilized (20,000 rad), and then stored at 4°C.

Different Treatments of ICL of Bruch’s Membrane

Explants containing ICL of aged Bruch’s membrane on the apical surface were prepared as described earlier and processed further to create four experimental plating surfaces: (1) cleaned ICL: For this purpose, triplicate explants were treated with 0.1% Triton X-100/0.1% sodium citrate solution for 20 minutes at 4°C; (2) ECM-protein-coated ICL: To coat ICL with ECM protein, another set of triplicate buttons were incubated with an ECM protein mixture containing laminin (330 μg/mL), fibronectin (250 μg/mL), and vitronectin (33 μg/mL) at 37°C for 30 minutes; (3) cleaned and ECM-protein-coated ICL: Some buttons were first cleaned and then coated with ECM protein; and (4) untreated buttons: These were used to determine the fate of the fetal and ARPE-19 RPE cell lines on aged ICL. After the cleaning and/or coating process, the exposed surfaces were washed three times with PBS for 5 minutes, and explants were stored at 4°C.

RPE Reattachment Studies

Confluent cell cultures were synchronized by placing them in phenol-free MEM (Invitrogen-Gibco) without serum for 24 hours before harvesting with 0.25% trypsin/0.25% EDTA in Hanks’ balanced salt solution (HBSS) for 10 minutes. Two milliliters of 0.1 mg/mL aprotinin (Sigma-Aldrich) 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 then resuspended in phenol red-free MEM without serum. The number of cells was determined by cell counter (model Z-1; Coulter Scientific, Hialeah, FL), and cell viability was assessed with a kit (Live/Dead Viability Kit; Molecular Probes, Eugene, OR). At least 250 cells were examined under 100× magnification, and the viability was expressed as the average ratio of live cells to the total number of cells in three different areas.

Fifteen thousand viable RPE cells were plated on different layers of Bruch’s membrane explants and serum- and phenol-free MEM containing 100 IU/ml penicillin G, 100 μg/ml streptomycin, 5 μg/ml gentamicin, and 2.5 μg/ml amphotericin B was added to reach a final volume of 200 μL in each well. At this plating density, the RPE cells covered approximately 15% of the plating area, assuming a cell diameter of 20 μm. RPE cells were allowed to attach to the surface for 24 hours in a humidified atmosphere of 95% air/5% CO2 at 37°C in phenol red-free MEM (Invitrogen-Gibco) without serum. Unattached cells were removed from the tissue culture plates by gently washing the wells three times with MEM.
**Assay for RPE Adhesion**

The number of attached live RPE cells in each well was determined with a colorimetric assay that indirectly estimates the number of live cells by measuring intracellular dehydrogenase activity (CellTiter 96 Aqueous nonradioactive cell proliferation assay; Promega, Madison, WI). Dehydrogenase enzymes found in live cells reduce MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) into the aqueous-soluble formazan in the presence of an electron-coupling agent (phenazine methosulfate; PMS). The quantity of the formazan product can be determined from the absorbance at 490 nm and is directly proportional to the number of living cells in culture.

The assay was performed in dark conditions, because of the light sensitivity of MTS and PMS. MEM (100 μL) without phenol red was added to each well. The added solution contained 1.0 g/mL glucose in a bicarbonate-based buffer that maintains the pH at 7.3 to 7.4 in 5% CO₂ and 95% air, thus minimizing the effects of changes in glucose and pH on the colorimetric assay. Twenty microliters of freshly prepared MTS/PMS solution (20:1) was added to each well, resulting in a final concentration of 333 μg/mL MTS and 25 μM PMS. Plates were incubated for 4 hours at 37°C and 100 μL of medium from each well was transferred to a corresponding well of another 96-well plate and read at 490 nm with an ELISA plate reader. The corrected absorbance was obtained by subtracting the average optical density reading from triplicate sets of controls containing the Brücher's membrane explant on 4% agarose without plated cells. The number of viable cells was estimated from standardized curves obtained by plating 100 to 13,000 viable, synchronized fetal RPE and ARPE-19 cells separately in triplicates on Brücher's membrane explants stabilized on 4% agarose. A linear relationship between the number of viable cells and the absorbance at 490 nm (data not shown). The RPE reattachment ratio for a substrate was the ratio of attached cells to the entire plated cell population for that substrate: ratio = [attached/(attached + unattached)].

**Assay for RPE Apoptosis**

Twenty-four hours after cells were plated in triplicate wells, the wells were washed gently three times with MEM and fixed with 4% paraformaldehyde for 4 hours. Apoptotic cells were identified using the TUNEL method. For this purpose, cells were permeabilized with 0.2% Triton X-100 in 0.2 M sodium citrate solution at 4°C for 4 minutes. Explants were washed three times with PBS and incubated with a mixture of fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase (TdT) from calf thymus for 60 minutes. TdT catalyzes the polymerization of labeled nucleotides to free 3'-OH terminals of DNA fragments. DNA breaks were then observed under a fluorescence microscope. For this purpose, explants were carefully removed from the wells and flipped over on a coverslip. The total number of apoptotic cells was counted under a fluorescence microscope. The apoptosis ratio on each plating surface was the ratio of apoptotic cells to the total number of attached cells on that surface.

**Assay for RPE Proliferation**

Twenty-four hours after plating, RPE cell proliferation was stimulated by replacing the medium with MEM supplemented with 15% fetal bovine serum (FBS) and 1 ng/mL recombinant bFGF (Invitrogen-Gibco). The number of cells on each explant was determined with the MTS assay 24 hours after growth stimulation, as described earlier. The proliferation ratio was the ratio of the number of viable and attached cells 24 hours after growth stimulation to the initial number of viable and attached cells on a certain surface.

**Assay for RPE Repopulation**

In triplicate wells, RPE cells were maintained in 200 μL of MEM containing inert fluorescent beads (Lumafour, Stony Point, NY) and supplemented with 15% FBS, 100 IU/mL penicillin G, 100 μg/mL streptomycin, 5 μg/mL gentamicin, 2.5 μg/mL amphotericin B, and 1 ng/mL recombinant human bFGF (Invitrogen-Gibco). The culture medium was changed every other day, and cell growth was monitored daily for up to 17 days with an inverted fluorescence microscope (Olympus, Tokyo, Japan) equipped with a 20 X long-working-distance objective (numeric aperture [NA]: 0.4, ULWD CDPlano 20PL; Olympus). At the end of the observation period, explants were removed from the wells and mounted upside down on coverslips. Fluorescence microscopy was used to obtain images from 10 representative areas. Total surface coverage, expressed as the percentage of the total surface area, was calculated from the collected images, using image-analysis software (MetaMorph 4.5; Universal Imaging Corporation, Downingtown, PA). Results were confirmed with scanning electron microscopy (SEM).

**Scanning Electron Microscopy**

Explants with RPE cells were fixed in modified Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer [pH 7.4]) at 4°C overnight. They were then postfixed in 1% osmium tetroxide in 0.16 M cacodylate buffer (pH 7.4) for 1 hour, stained in 1% uranyl acetate in 0.1 M sodium acetate buffer, and dehydrated in a graded series of ethyl alcohol (30%-100%). The samples were then critical point-dried (E5000; Polaron, Watford Hertfordshire, UK), mounted on aluminum specimen stubs with carbon-conductive tabs grounded with colloidal silver liquid paint, and sputter coated with 15.0 nm of gold (E5000; Polaron). Samples were examined by SEM (model S-4500 FEG; Hitachi, Tokyo, Japan) at 15 kV accelerating voltage and the images recorded (55 P/N film; Polaroid Corp., Cambridge, MA).

**Statistical Analysis**

Triplicate wells were used to calculate the average reattachment, apoptosis, and proliferation ratios and the final fate of RPE cells seeded onto each substrate. Because of the limited number of explants that could be harvested from an eye, typically, SEM studies were performed in duplicate only. Data from all experiments were pooled and expressed as the mean ± SD. The reattachment, apoptosis, and proliferation ratios and surface coverage on different substrates and RPE cell populations were analyzed in pairs by the Dunn’s multiple comparison test. A confidence level of P < 0.05 was considered to be statistically significant.

**RESULTS**

**Reattachment Ratio**

Reattachment ratios of fetal human RPE and ARPE-19 cells on treated and untreated aged (≥60 years) human ICL are shown in Figure 1. Twenty-four hours after plating, the overall reattachment ratios to untreated ICL were comparable for both cell lines (41.5% ± 1.7% for fetal human RPE and 42.9% ± 2.7% for ARPE-19, P > 0.05). Coating of the ICL with a mixture of ECM protein increased the reattachment ratios of both cell lines compared with untreated ICL (48.9% ± 2.3%, P < 0.01 for fetal human RPE and 53.0% ± 4.9%, P < 0.01 for ARPE-19). The cleaning procedure significantly decreased the reattachment ratio in both cell lines (35.4% ± 2.3%, P < 0.01 for fetal human RPE and 21.1% ± 2.3%, P < 0.01 for ARPE-19), although the reattachment of ARPE-19 cells was decreased more (P < 0.01). Combined cleaning and ECM protein coating restored the reattachment ratio of fetal human RPE to levels comparable to untreated ICL (42.3% ± 2.3%, P > 0.05), but still remained significantly lower than ECM-protein-coated ICL (P < 0.01). In
contrast, ECM protein coating after cleaning failed to increase the reattachment ratio of ARPE-19 cells to the level of untreated ICL (22.6% ± 1.4%, P > 0.05).

**Apoptosis Ratio**

Both cell types demonstrated the highest ratio of apoptosis on untreated ICL (192.7 ± 32.1 per 100,000 cells for fetal human RPE versus 41.5 ± 18.0 per 100,000 cells for ARPE-19 cells, P < 0.05; Fig. 2). ARPE-19 cells were significantly more resistant to apoptosis on untreated ICL than were fetal human RPE cells (P < 0.01). ECM protein coating lowered the apoptosis ratio significantly and eliminated the difference in apoptosis rates between these two cell populations (22.7 ± 7.9 per 100,000 fetal human RPE cells and 16.8 ± 7.3 per 100,000 ARPE-19 cells, P > 0.05). Cleaning the ICL alone lowered the apoptosis ratios for both cell types, but this was significant only for fetal RPE cells (13.3 ± 11.5 per 100,000 fetal human RPE cells, P < 0.05, and 21.1 ± 18.3 per 100,000 ARPE-19 cells, P > 0.05). Combined cleaning and ECM protein coating resulted in the lowest apoptosis ratios for fetal human RPE cells (5.3 ± 9.1 per 100,000 fetal human RPE cells, P < 0.05). The observed decrease in the apoptosis ratio of ARPE-19 cells on
cleaned and ECM-protein–coated ICL was not statistically significant (19.7 ± 17.0 per 100,000 ARPE-19 cells, \( P > 0.05 \)).

**Proliferation Ratio**

The proliferation ratios of RPE cells 24 hours after growth stimulation on different treatment groups of ICL are shown in Figure 3. The number of fetal human RPE cells attached to untreated ICL decreased within 24 hours (proliferation ratio = 0.87 ± 0.04, \( P < 0.05 \)), whereas the number of ARPE-19 cells remained the same (proliferation ratio = 1.05 ± 0.08, \( P > 0.05 \)). The difference between the behavior of these two cell lines was statistically significant (\( P < 0.05 \)). ECM coating prevented loss of fetal RPE (1.05 ± 0.05 for fetal human RPE cells, \( P < 0.01 \)) but had no effect on ARPE-19 (0.91 ± 0.07 for ARPE-19 cells, \( P > 0.05 \)). Cells plated on cleaned ICL increased significantly in number, as evidenced by higher proliferation ratios (1.52 ± 0.07 for fetal human RPE cells and 1.74 ± 0.17 for ARPE-19 cells, \( P < 0.01 \) when compared with untreated ICL). Combined cleaning and ECM coating yielded the highest proliferation ratios in both cell lines (1.62 ± 0.05 for fetal human RPE cells and 1.89 ± 0.22 for ARPE-19 cells, \( P < 0.01 \) when compared with untreated ICL). These ratios correspond to doubling times of 34.7 ± 2.3 hours for fetal human RPE cells and 28.5 ± 5.1 hours for ARPE-19 cells on cleaned and ECM-protein–coated ICL.

**Ability of RPE Cells to Repopulate ICL**

The surface coverage 17 days after initial plating is shown in Figure 4. RPE cells plated on untreated (0.28% ± 0.01% for fetal human RPE cells and 0.55% ± 0.05% for ARPE-19 cells) and ECM-protein–coated ICL (0.55% ± 0.10% for fetal human RPE cells and 0.30% ± 0.04% for ARPE-19 cells) failed to survive. RPE cells plated on either cleaned (6.64% ± 1.51% for fetal human RPE cells and 19.9% ± 3.2% for ARPE-19 cells) or cleaned and ECM-protein–coated ICL (20.8% ± 3.3% for fetal human RPE cells and 32.1% ± 7.2% for ARPE-19 cells) showed significant surface coverage 17 days after plating. The highest surface coverage was obtained with combined cleaning and ECM protein coating of the ICL.

**Cellular and Ultrastructural Morphology**

Figure 5 shows the effect of different treatments on the ultrastructural features of the ICL. Untreated aged ICL mainly consisted of cross-linked collagen fibers arranged in fused bundles that run unidirectionally (Fig. 5A). Delicate interdigitations between collagen fibers were lost, and large pockets of interfibrillar spaces were filled with extracellular debris. Globular ECM proteins, which formed aggregates in some locations, were attached along the course of the fibers. ECM protein coating without cleaning increased the number of globular ECM proteins attached to the fibers (Fig. 5B) with additional smaller clumps of ECM debris on and between cross-linked collagen fibers. Treating the matrix with Triton X-100 and sodium citrate removed the microaggregates and most of the globular ECM proteins, and created empty spaces between bare bundles of collagen fibers (Fig. 5C). Cross-linked collagen bundles partially broke up and a morphologic appearance developed that more closely resembled the native architecture of collagen fibers within Bruch’s membrane. Cleaning and subsequent coating led to breaking of cross-links, regular spacing of collagen fibers, removal of debris, and homogenous coating with globular structures that may represent clusters of ECM proteins. These clusters were diffusely distributed over the regenerated collagen framework and did not form aggregates as seen on uncleared ICL (Fig. 5D). Removal of macroaggregates left empty spaces between collagen fibers.

TEM of the untreated aged ICL revealed cross-linked collagen bundles and electron-lucent material that resembled basal linear deposits (Fig. 6A). Occasional electron-dense ECM protein aggregates were noted on the collagen fibers. The addition of ECM proteins without cleaning yielded an increase in the number of electron-dense particles among cross-linked collagen fibers (Fig. 6B). Cleaning the matrix broke the cross-links between collagen fibers, creating gaps in the collagen framework (Fig. 6C). After cleaning, an electron dense ring appeared around the electron-lucent vesicular debris noted in untreated samples. This probably represents the reconstitution of the proteins within the lipoproteinaceous debris with their hydrophilic surface exposed to the outside, because of the effect of Triton X-100. Electron-dense particles increased considerably among dissociated collagen fibers after the cleaning and coat-
ing process. An increase in these anionic binding sites may indicate a shift toward hydrophilicity.

There was no clear difference in the morphology of both RPE cell populations seeded onto similar surfaces. RPE cells that attached to untreated and ECM-protein–coated ICL failed to flatten at 24 hours after plating (Fig. 7A). Although ECM protein coating increased the number of attached cells, these cells failed to flatten (Fig. 7B). Cleaning the ICL resulted in the attachment of fewer RPE cells but the attached RPE cells flattened (Fig. 7C). Combined cleaning and ECM protein coating of the ICL not only resulted in flattening of the attached RPE cells but also in the expression of differentiated RPE cell features, such as apical microvilli (Fig. 7D). On day 17, only rare cellular debris was noted on untreated and ECM-protein–coated ICL (Fig. 8A, 8B). A few RPE cells plated onto the cleaned surface flattened and developed long extensions (Fig. 8C), but cleaned and ECM-protein–coated ICL was markedly different, with many patches of the ICL covered by a RPE monolayer (Fig. 8D).

### DISCUSSION

**Effects on RPE Behavior of Cleaning and Recoating Bruch’s Membrane**

Figure 9 summarizes the effects of cleaning and resurfacing the ICL on RPE cell attachment, apoptosis, and proliferation. Young Bruch’s membrane contains regularly spaced collagen fibers with no significant cross-linking or accumulation of extracellular deposits. ECM ligands, such as laminin fibronectin and vitronectin, are present (Fig. 9, column 1, row 1). Changes develop within the ICL as a function of age, including collagen cross-linking and the accumulation of ECM deposits (Fig. 9, column 1, row 2). Coating the ICL without cleaning increases the number of receptors available on the inner aspects of the ICL, but collagen cross-links and lipoproteinaceous debris are still present (Fig. 9, column 1, row 3). Cleaning old ICL reverses the collagen cross-linking and removes the ECM deposits, but also removes most of the ECM ligands necessary for cell attachment (Fig. 9, column 1, row 4). Coating of cleaned ICL refurbishes Bruch’s membrane by removing the deposits, removing the cross-links, and then providing again a normal

![Figure 4](image-url) **Surface coverage of RPE on modified aged ICL 17 days after plating.** Few cells survived on untreated and ECM-coated (ECM-P) ICL. Although the initial attachment ratios were low on cleaned ICL, RPE cells proliferated on this layer, resulting in higher surface coverage. The highest surface coverage was obtained with ARPE-19 cells on cleaned and ECM-protein–coated ICL.

![Figure 5](image-url) **SEM of ICL from an 84-year-old donor.** (A) Untreated ICL revealed cross-linked bundles of collagen (arrows). Small globular structures on the collagen fibers probably represent aggregates of abnormal ECM proteins (arrowbeads). Macrodeposits filled interfibrillar spaces (✱). (B) ECM protein coating without cleaning increased the amount of ECM protein aggregates on the collagen matrix (arrowbead). Cross-linking of collagen fibers was not affected by the coating (arrows). (C) Cleaning with Triton X-100 and sodium citrate removed the aggregates of ECM proteins and debris and resulted in gaps between collagen fibers (✱). Note that cross-links between collagen fibers were broken, yielding individual fibers (arrowbeads). In some areas there was incomplete separation of the macrofibers (arrows). (D) Cleaning and subsequent ECM protein coating broke the cross-links between collagen fibers and resulted in deposition of ECM proteins onto the regenerated collagen framework (arrowbeads). The ECM proteins coating the surface were smaller in size and did not form multimeric aggregates on the native matrix. The gaps (arrow) remained between the collagen fibers, probably corresponding to areas where macrodebris was removed (compare to A). Bars, 0.5 μm.
complement of ECM ligands for cell attachment (Fig. 9, column 1, row 5).

In Figure 9, columns 2 to 4 show the effects of these ultrastructural changes on RPE attachment, apoptosis, and proliferation. The RPE attachment and proliferation rates are high, and apoptosis is low on young ICL (Fig. 9, row 1). Age-related changes in the ICL of Bruch’s membrane adversely affect RPE attachment and proliferation rates and increase the RPE apoptosis rate (Fig. 9, row 2). Simple ECM protein coating alone is not sufficient to restore the integrity of Bruch’s membrane for RPE behavior. Coating alone significantly increases RPE attachment to the abnormal surface, probably by simply supplying the necessary ECM ligands. Despite the decreased apoptosis rate, the proliferation rate remains low when only the surface is coated (Fig. 9, row 3), suggesting that different cell-surface interactions control each of these functions. Cleaning alone lowers the attachment rate further, probably by removing some normal ECM ligands present within the ICL, but the attached cells flatten and undergo proliferation, unlike RPE cells on ECM-protein–coated ICL (Fig. 9, row 4).

Cleaning followed by ICL resurfacing is necessary to reverse the age-related changes partially and maximize the surface repopulation of the ICL (Fig. 9, row 5). Cleaning hard ECM protein coating restores RPE cell attachment, subsequent cell spreading and proliferation and decreases the apoptosis rate. Electron microscopy reveals that surface cleaning eliminates the collagen cross-links and removes ECM deposits. At the same time, removing the debris and regenerating the collagen framework probably vacates binding sites of ECM proteins on collagen fibers, onto which newly added ECM proteins can polymerize to create a structure that is closer to the native ultrastructural architecture.

**Implications for Cellular Replacement in AMD**

The neurosensory retina overlying choroidal neovascularization has the potential to recover, as evidenced by the preservation of foveal photoreceptors in eyes with exudative AMD. Even at later stages of AMD, 25% to 30% of the photoreceptors remained structurally intact. Foveal translocation surgery has also supplied clues that remaining photoreceptors are sufficient to restore central vision, as some patients achieve a final visual acuity of 20/40 or better once the photoreceptor-RPE interface is restored.

Adult human RPE cells can attach and repopulate the innermost basal laminar layer of both young and aged (<60) Bruch’s membrane, whereas they fail to survive on the deeper layers of aged human Bruch’s membrane. Thus, the fate of an RPE graft at the time of surgical excision of a neovascular complex is dependent on the layer of Bruch’s membrane exposed. However, Bruch’s membrane may be abnormal in patients with AMD. Thickening of Bruch’s membrane and the formation of basal laminar deposits, basal linear deposits, and drusen occur early in the pathogenesis of AMD. Furthermore, surgical removal of subfoveal choroidal neovascularization in AMD may disrupt the inner layers of Bruch’s membrane, so that the lamellae of Bruch’s membrane available for RPE reattachment may not be uniform throughout the transplantation bed. RPE cells plated onto deeper layers of aged human Bruch’s membrane fail to attach and eventually die by apoptosis. Failure of RPE to survive and repopulate diseased and damaged areas of Bruch’s membrane may be one of several factors accounting for the fact that uncontrolled series of human transplantation studies with allogeneic and autologous fetal and adult human RPE failed to show any biological benefit. This is in contrast to the anatomic and functional success of RPE transplantation in animal models that lack age-related ultrastructural alterations in human Bruch’s membrane. Therefore, restoration of foveal vision in exudative AMD may require modification of aged Bruch’s membrane to improve RPE repopulation of this structure.

We have previously shown that age-related alterations in the molecular composition and ultrastructure of human Bruch’s membrane make it an unfavorable substrate for the attachment and survival of grafted RPE cells. Apoptotic mechanisms are activated within the harvested RPE graft as soon as cells are detached from their native substrate during the harvesting procedure. RPE cell death can be suppressed by RPE reattachment and subsequent spreading on a substrate through the interaction between integrin receptors on the basal surface of RPE and their specific ligands within the ECM. Failure to reestablish this interaction after RPE harvesting inevitably results in rapid RPE death by apoptosis. The current report shows that age-related structural alterations in human Bruch’s...
membrane can be at least partially reversed by cleaning and coating with ECM proteins. Such treatment can reestablish the native ECM framework to an extent adequate enough to alter the dismal fate of human RPE grafts seeded onto the ICL of aged Bruch’s membrane.

Previous studies have demonstrated that several structural and molecular alterations occur within human Bruch’s membrane as a function of age. These changes, which disrupt the delicate molecular architecture of Bruch’s membrane, include (1) structural changes in the main collagen framework, including cross-linking and deposition of long-spaced collagen \(^3^8\); (2) qualitative and quantitative changes in the native ECM molecules \(^3^9\); (3) deposition of abnormal extrinsic molecules \(^4^0\), and (4) macromolecular changes in the structure of Bruch’s membrane, such as drusen formation, calcifications, and cracks or loss of inner layers due to inadequate basal membrane regeneration, as in geographic atrophy \(^3^1,1^5\). Additional structural

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**FIGURE 7.** SEM of RPE on ICL. (A) RPE cells plated on untreated ICL failed to flatten within 24 hours. (B) ECM protein coating increased the number of attached cells; however, these cells remain rounded. (C) Cleaning of the ICL resulted in fewer attached RPE cells but they were flattened. (D) Combined cleaning and ECM protein coating yielded flattened RPE cells with differentiated cell features such as microvilli. RPE cells extended foot processes and formed focal adhesion plaques (arrows). Bars: (A) 7.5 μm; (B, C) 5.0 μm; (D) 3.0 μm.

**FIGURE 8.** SEM of RPE 17 days after plating. (A) Occasional cellular debris was noted on untreated aged ICL. (B) ECM protein coating did not increase RPE cell survival. Seventeen days after plating, surface showed numerous areas of cellular debris. (C) Surviving RPE on cleaned ICL showed long cellular extensions (arrows). (D) RPE cells plated on cleaned and ECM-protein-coated ICL proliferated and formed patches of an epithelial monolayer (arrows). Bars: (A) 5 μm; (B) 30 μm; (C) 25 μm; (D) 50 μm.
alterations can be induced by submacular surgery, because excised neovascular membranes in AMD eyes contain fragments of the basal lamina and deeper layers of Bruch’s membrane, thus exposing the ICL and perhaps other layers. The chemical treatments we used to reengineer the aged human Bruch’s membrane act by (1) liquefying and extracting membranous lipoprotein debris from the ICL to expose ECM protein receptors on native collagen fibers, 40,41; (2) reestablishing the native collagen framework by dissolving long-spacing collagen and breaking collagen cross-links 42; and (3) polymerizing a layer of ECM proteins onto the rejuvenated core collagen matrix of Bruch’s membrane. 44 A nonionic detergent ( Triton X-100) was used to extract membranous debris from the aged Bruch’s membrane while preserving the anionic glycosaminoglycan bridges between the collagen fibrils and the native structure of collagen. 43 At the concentrations we used, Triton X-100 dissolved the membranous debris of age-related photoreceptor outer segments 44 without disrupting the ultrastructure of the matrix. 45 It also did not interfere with the subsequent adhesion of ECM proteins to the collagen fibers 46 and allowed them to polymerize in their native form on the collagen matrix. 47 Detergent treatment before ECM protein coating avoided the binding of ECM molecules to lipoprotein debris with a consequent abnormal configuration. 48 The reducing agent sodium citrate was added to solubilize the lipid debris and to facilitate the breakdown of age-related pentosidine cross-links between collagen fibers. 49 In theory, the removal of the lipoprotein debris and the secondary increase in anionic binding sites may induce a shift toward hydrophilicity and increased hydraulic conductivity of the ICL. 50 Taking all results together, we believe that the chemical treatments we used to reengineer the aged human Bruch’s membrane acted by liquefying and extracting membranous lipoprotein debris from the ICL to expose ECM protein receptors on native collagen fibers, 40,41 thus reestablishing the native collagen framework by dissolving long-spacing collagen and breaking collagen cross-links. 42 This allowed proteins subsequently placed on this surface to polymerize onto the rejuvenated core collagen matrix of Bruch’s membrane. 44

Our data suggest that simple cleaning of the aged ICL lowers the reattachment of both RPE cell types, possibly by removal of ECM proteins serving as adhesion molecules. This hypothesis is supported by the disappearance of globular proteins from and between collagen fibers detected by on SEM. This conclusion is further supported by our observation that replenishing the ECM proteins—namely, laminin, vitronectin, and fibronectin—significantly increased the attachment rate of fetal RPE on cleaned ICL. Failure to restore the reattachment of ARPE-19 cells by ECM protein coating of cleaned ICL suggests these cells may depend on different ECM receptors or an unique three-dimensional architecture of binding sites for attachment. This is not surprising, because different cell lines and even different passages of the same cell line may express different integrin heterodimers to attach to a substrate. 53

Although cleaning alone decreased the reattachment of both RPE cell lines, the cells flattened and had significantly lower apoptosis rates on cleaned ICL. Integrin-dependent reorganization of the cytoskeleton is thought to be responsible for cell flattening after attachment. 54 Flattening of RPE on cleaned ICL, where ECM proteins were removed by deter-
gents, may be due to lower surface tension due to concurrent removal of lipid debris. The fact that cleaning of the ICL lowered RPE apoptosis, despite also lowering cell attachment, implies that age-related changes in Bruch’s membrane may trigger RPE apoptosis by a mechanism more complex than simply interfering with cell attachment. This may involve interfering with the ability of a cell to spread along the surface and adopt a distinct morphology.

Supplying the ICL with high concentrations of ECM proteins alone increased RPE reattachment but did not result in RPE flattening. A random polymerization pattern of supplemental ECM proteins on the ICL may prevent them from acquiring their native three-dimensional organization and expose their binding epitopes at regular intervals. The resultant inadequately spaced multiple focal adhesion plaques may not generate cytoskeletal modifications to trigger RPE flattening. Although the addition of ECM proteins on the ICL increased the RPE attachment rate, it did not decrease the apoptosis rate. This suggests that increased RPE attachment alone is not sufficient to increase survival on aged ICL. The decreased survival of RPE cells on ECM-protein–coated ICL was associated with a failure of RPE flattening, implying, as stated earlier, that cytoskeletal alterations are essential for suppression of detachment-induced apoptosis.

On the appropriate substrate, RPE cell adhesion to a surface is followed by cell spreading, formation of focal adhesions, and development of stress fibers with subsequent cell proliferation and migration. Cell proliferation is controlled by many of the same signaling proteins that play a role in adhesion, and also requires a proper interaction of integrin receptors with their ECM ligands. Inadequate binding sites on aged ICL may prevent attached RPE from undergoing proliferation. We observed that only RPE cells attached to cleaned ICL flattened and proliferated. However, even on cleaned and ECM-protein–coated ICL, where RPE obtain the highest proliferation rate, we were able to populate only approximately one third of the bare ICL during the observation period. Further modification of the ICL or an increase in the number of RPE cells may allow complete resurfacing of the epithelial defect in a more timely fashion.

The fate of the human fetal and ARPE-19 cell lines seeded onto untreated aged human ICL is similar to that of adult human RPE cells, although ARPE-19 cells are more resistant to detachment-induced apoptosis. The resistance of ARPE-19 cells to apoptosis on untreated ICL may be due to a deficiency in two major apoptosis execution pathways within these cells: induction of nuclear calcium-dependent endonucleases and activation of the interleukin-1β-converting enzyme family of proteases. Despite their increased resistance, alterations in the chemical composition of the aged ICL can still induce apoptosis in ARPE-19 cells and ultimately lead to the same fate as that of adult and fetal human RPE cells.

Age-related changes in the Bruch’s membrane, such as collagen cross-linking, elastin fragmentation, and deposits of abnormal material may precede cellular changes by decades. Similar age-related changes occur in the ECMs of various other tissues and organs. For example, skin wrinkling is characterized by collagen cross-linking, fragmentation of elastin, and alteration of matrix metalloproteinase activity. In Alzheimer’s disease, there is an age-related aggregation of β-amyloid within the ECM of the brain that induces secondary changes in neural cells. Aging of the ECM is also responsible for a pro-oncogenic milieu that manifests itself as an exponentially increasing incidence of epithelial cancers with aging. In the eye, age-related changes in the glycosaminoglycan in the trabecular meshwork contribute to the development of open-angle glaucoma. To our knowledge, the present study is the first to demonstrate that it is possible to reverse the age-related changes in Bruch’s membrane composition and structure in AMD. We demonstrated that correction of this change leads to changes in RPE behavior. This view offers a different perspective of the role of the age-related changes in the ECM in altering cell behavior and presents a unique opportunity to intervene by reversing this process.

In summary, we have shown that age-related changes within the ICL inhibit RPE cell repopulation after subfoveal membrane resection in AMD. We now demonstrate that we can partially reverse this process in vitro by reengineering Bruch’s membrane—namely, by cleaning the ICL with a nonionic detergent and reforming it with ECM proteins (laminin, vitronectin, fibronecgin). This study has several limitations that should be addressed before in vivo application: (1) The use of detergents in vivo may be limited by cell membrane damage, although this may be avoided by using biodetergents, such as fluorinated surfactants or lipid-degrading enzymes such as lipoprotein lipase (Curcio CA, personal communication, 2005); (2) the effect of the overlying sensory retina on RPE cell attachment and proliferation must be considered, although in vitro studies remain the only way to study RPE-human ICL interaction; and (3) the effects of other ECM proteins on RPE survival should be studied to determine the most effective combinations and concentrations. Despite these limitations, our study serves as an important proof of principle and demonstrates that reengineering Bruch’s membrane may result in enhanced resurfacing of iatrogenic or age-related defects in AMD.

References


