Retinal Pigment Epithelium Wound Healing in Human Bruch’s Membrane Explants

Hao Wang,1 Yoshibiko Ninomiya,2 Ilene K. Sugino,1 and Marco A. Zarbin1

PURPOSE. To compare retinal pigment epithelium (RPE) resurfacing on the RPE basement membrane and inner collagenous layer (ICL) in human submacular Bruch’s membrane explants.

METHODS. Debridements were created in RPE-choroid-sclera explants (mean donor age 71.91 ± 7.76 years) to create defects exposing the RPE basement membrane (RPEbm(+)) defects), the ICL immediately below the RPE basement membrane (superficial ICL, [SICL]) or deeper layers of the ICL (DICL). Eleven pairs of eyes—four pairs with one eye having an RPEbm(+) defect and the fellow eye having an SICL defect and seven pairs with corresponding RPEbm(+) and DICL defects—were observed for 10 days by visualizing RPE ingrowth with 4’,6’-diamino-2-phenylindole (DAP) filters. At day 10, specimens were processed for scanning electron microscopy.

RESULTS. Resurfacing of localized RPE defects occurred to some degree in all 11 pairs of eyes. No significant difference in the percentage of resurfacing of RPEbm(+) defects (67.35% ± 18.82%) and SICL defects (64.26% ± 16.07%) was observed although healing of the SICL showed more variability in the morphology of RPE cells migrating into the defect. Significant differences in healing were observed between pairs with RPEbm(+) defects versus DICL defects (84.07% ± 15.35% and 54.00% ± 14.54% resurfacing, respectively). RPE ingrowth into DICL defects exhibited the greatest morphologic variability.

CONCLUSIONS. RPE basement membrane supports RPE resurfacing of localized RPE defects. The deeper portion of the ICL of aged submacular human Bruch’s membrane does not support RPE resurfacing to the same extent as does the RPE basement membrane. The finding that localized RPEbm(+) defects versus DICL defects mimics the histopathological findings in patients with age-related macular degeneration after excision of choroidal new vessels. (Invest Ophthalmol Vis Sci. 2003;44:2199–2210) DOI: 10.1167/iovs.02-04955

Age-related macular degeneration (AMD) is the major cause of irreversible loss of central vision among the elderly.1–12 Approximately 70% of cases of severe visual loss in AMD are due to growth of abnormal blood vessels, termed choroidal neovascularization (CNV), under the RPE and retina with secondary exudative retinal detachment, subretinal hemorrhage and lipid exudation, and outer retinal degeneration.13–14 Currently, there are approximately 100,000 new AMD-related cases of CNV per year, and the incidence will increase during the 21st century.15

Laser photocoagulation and photodynamic therapy (PDT) are the only CNV treatments with effectiveness that has been shown in randomized prospective multicenter clinical trials. Only a minority of patients with AMD involving CNV (~20%) are eligible for laser photocoagulation,16–17 which itself is associated with poor visual outcome and a high rate of recurrent CNV growth.18–22 Four years after treatment, the average visual acuity after laser photocoagulation of new subfoveal CNV is 20/320 versus 20/500 in untreated control subjects.23 CNV photocoagulation also can be associated with an immediate decrease in vision, although, on average, treated eyes have better vision than untreated eyes by 1 to 2 years after treatment. Eyes with occult CNV and poorly demarcated boundaries are not good candidates for laser photocoagulation.24 Also, in the case of classic CNV, if lesions are too large, or if patients have good visual acuity, laser photocoagulation is not a good option. In such patients, laser treatment can cause severe central visual loss that is worse than the natural course of the disease. An alternative treatment for CNV in AMD is PDT.25–27 A treatment benefit from PDT has been shown for eyes with predominantly (i.e., ≥50%) classic CNV and occult without classic CNV. PDT involves fluorescein angiography and an intravenous infusion of photosensitizing medication (verteporfin, Visudyne; Novartis, Summit, NJ) every 3 months for an indefinite period. The visual results with PDT are modest. Two years after the initiation of therapy, 59% of untreated patients with predominantly classic CNV experience moderate visual loss compared with 31% of treated patients. One year after PDT, the mean visual acuity is 20/160 ± 2 versus 20/200 in control subjects. Only approximately 16% of treated eyes experience an improvement of 1 line or more (versus 7% of control eyes). Many patients with AMD involving CNV are ineligible for PDT.28 In summary, the main benefit of laser photocoagulation and PDT is that they tend to reduce the rate of visual loss. These treatments restore lost vision in only a minority of cases. The visual benefit from these therapies is limited.

Submacular surgery with CNV excision offers the possibility of removing large or mixed classic-occult CNV while preserving the overlying retina, thus preventing or reversing photoreceptor damage and blindness associated with subretinal bleeding and scarring in AMD. Submacular surgery does not depend on a precise delineation of the CNV boundaries, in contrast to laser photocoagulation and PDT, and is therefore potentially applicable to a much larger proportion of all patients with AMD-associated CNV.29 In the only randomized prospective multicenter study comparing submacular surgery with laser photocoagulation, laser treatment and surgery were found to be equivalent. Two years after treatment 20 (65%) of 31 of laser-treated eyes and 14 (50%) of 28 of surgically treated eyes had visual acuity that was better than or no more than 1 line.
Table 1. Donor Information, Wound Type, and Resurfacing Outcome

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<th>Specimen</th>
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<th>D-Ex* (h)</th>
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<th>Wound Area (mm²)</th>
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* D-E, time between death of the donor and enucleation; D-Ex, time between death of the donor and processing for experiment.
† RPEbm(+), RPE defect with the RPE basement membrane intact. SICL, RPE defect after removal of RPE basement membrane, exposing the surface of the ICL that lies directly under the RPE basement membrane. DICL, RPE defect with basement membrane and part of the ICL removed, exposing more disrupted, less densely packed collagen fibrils composing the ICL.

worse than the baseline level. Visual recovery after CNV excision is usually poor in patients with AMD.

Clinical and histopathological studies indicate that in patients with AMD, CNV excision is usually associated with removal of adjacent native RPE and RPE basement membrane and incomplete or aberrant RPE growth into the dissection bed. Absence of RPE ingrowth into the dissection bed probably results in atrophy of the choriocapillaris and photoreceptors. These findings indicate two strategies for improving visual outcome after CNV excision in patients with AMD: RPE transplantation or stimulation of RPE resurfacing of the RPE basement membrane-deficient Bruch’s membrane by residual native RPE at the edge of the dissection bed.

Although RPE wound healing has been studied in vitro, it has not, to our knowledge, been studied on aged submacular human Bruch’s membrane that lacks native RPE basement membrane (the situation likely to be encountered clinically, as noted earlier). Previously described in vitro RPE wound-healing models are associated with complete wound resurfacing in contrast to the situation in patients with AMD who undergo CNV excision. The purpose of the current experiments is to determine whether the absence of the RPE basement membrane or superficial portion of the inner collagenous layer (ICL) of Bruch’s membrane affects the ability of native RPE to repopulate a localized RPE defect on aged submacular human Bruch’s membrane.

**Materials and Methods**

**Donor Eyes**

Eleven pairs of human donor eyes of age 65 to 85 years (71.9 ± 7.76, mean ± SD) were obtained through the National Disease Research Interchange (NDRI, Philadelphia, PA) and the North Carolina Eye Bank, a Vision Share (Apex, NC) member eye bank (Table 1). The donor inclusion criteria were as follows: (1) no history of chemotherapy, radiation to the head, or recent dependence on a ventilator; (2) up to 6 hours from death to enucleation; (3) up to 48 hours from death to experimentation; (4) intact RPE under the macula as visualized through a dissecting microscope; and (5) no contamination of either eye in each pair up to day 10. This research adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review board of the New Jersey Medical School.

**Preparation of Bruch’s Membrane Explants and RPE Defects**

Eyes were immersed briefly in 10% povidone iodine (Betadine solution; Purdue Frederick Co., Norwalk, CT) after remnants of conjunctiva, Tenon’s capsule, extraocular muscles, and orbital fat were removed carefully under a dissecting microscope. Eyes were then rinsed in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech, Herndon, VA) twice for 5 minutes each. A circumferential retinotomy was made posterior to the ora serrata so that by cutting the retina around the optic disc, the vitreous cavity and neural retina were removed with the anterior segment without touching the RPE surface. A 9 × 9-mm² explant of RPE-choroid-sclera with optic disc intact was isolated from the submacular area of each eyecup. Approximately 3-mm diameter RPE defects were made in the submacular area of each explant. RPE defects leaving intact the native RPE basement membrane (RPEbm(−) defects) were created by gently removing RPE with a loop of 9-0 nylon (Alcon Surgical, Fort Worth, TX). RPE defects with the native RPE removed (RPEbm(−) defects) were made by centripetally wiping the surface with a loop of plastic strip. To make defects with only RPEbm removed (superficial ICL defects, or SICL defects), approximately five wipes with the plastic strip were used. To expose the deeper portions of the ICL (deep ICL defects or DICL defects), approximately 20 wipes were used after removal of the native RPE.
Scanning Electron Microscopy and Transmission Electron Microscopy to Assess the Debridement Anatomy

Five pairs of eyes were used to assess the debridement anatomy. Four RPEbm(−) defects, three SICL defects, and three DICL defects were made in 10 donor eyes, as described. For each defect, after the debridements the tissue was fixed immediately in half-strength Karnovsky’s fixative containing 2.5% glutaraldehyde and 2.0% formaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C and bisected. Half of each defect was processed for scanning electron microscopy (SEM) and the other half for transmission electron microscopy (TEM). For SEM, after fixation, tissues were rinsed briefly with phosphate-buffered saline (PBS) and dehydrated in ascending ethanol concentrations. Tissues were then critical-point dried (Autosamdri-814; Tousimis Research Corp., Rockville, MD), mounted onto aluminum stubs, and sputter coated with 20 nm gold-palladium (Desk II; Denton, Moorestown, NJ). Explants were examined with a scanning electron microscope (JSM-35C; JEOL, Tokyo, Japan) equipped with an image-capture system (Digiscan; Gatan, Pleasanton, CA) at 25 kV accelerating voltage. For TEM, tissues were postfixed in 2% osmium tetroxide and stained en bloc in 2% uranyl acetate. After ethanol dehydration, tissues were embedded in epoxy resin. Ultrathin sections, 50 to 90 nm thick, were obtained with a microtome (Ultracut S; Leica, Heidelberg, Germany) and stained with uranyl acetate and lead citrate. Tissues were examined on a transmission electron microscope (JEM-100CX; JEOL). For both SEM and TEM, one observer was blinded to conditions of the samples. Two separate observers (one blinded to conditions of the samples) evaluated the debridement on each sample.

Monitoring Ongoing RPE Ingrowth with Fluorescence Microscopy

The remaining 11 pairs of eyes were used to study wound healing in the RPE on different surfaces. In 4 of the 11 pairs of eyes, for each pair, an RPEbm(−) defect was made in one eye, and an SICL defect was made in the fellow eye. In the other seven pairs, for each pair, an RPEbm(−) defect was created in one eye, and a DICL defect was created in the fellow eye (Table 1). The assignment of different defects to the eyes and the order in which the wounds were prepared was random.

RPE-choroid-sclera explants were cultured separately with the sclera side down in 35-mm diameter tissue culture dishes (BD Biosciences, Franklin Lakes, NJ) in a humidified atmosphere of 10% CO₂ and 90% air at 37°C for 10 days. Explants were maintained in DMEM supplemented with 15% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM l-glutamine (Life Technologies, Gaithersburg, MD), 2.5 μg/mL amphotericin B (Mediatech), 50 μg/mL gentamicin (Life Technologies), and 1 ng/mL bFGF (Life Technologies). Each dish contained 2.5 mL of medium that was changed every other day. After cell viability testing, explants were fixed immediately in half-strength Karnovsky’s fixative containing 2.5% glutaraldehyde and 2.0% formaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C and bisected. Half of each explant was postfixed, dehydrated, and critical-point dried before each change of medium. Tissues were then critical-point dried (Autosamdri-814; Tousimis Research Corp., Rockville, MD), mounted onto aluminum stubs, and sputter coated with 20 nm gold-palladium (Desk II; Denton, Moorestown, NJ). Explants were examined with a scanning electron microscope (JSM-35C; JEOL, Tokyo, Japan) equipped with an image-capture system (Digiscan; Gatan, Pleasanton, CA) at 25 kV accelerating voltage.

RPEbm(−) explants were cultured separately with the sclera side down in 35-mm diameter tissue culture dishes (BD Biosciences, Franklin Lakes, NJ) in a humidified atmosphere of 10% CO₂ and 90% air at 37°C for 10 days. Explants were maintained in DMEM supplemented with 15% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM l-glutamine (Life Technologies, Gaithersburg, MD), 2.5 μg/mL amphotericin B (Mediatech), 50 μg/mL gentamicin (Life Technologies), and 1 ng/mL bFGF (Life Technologies). Each dish contained 2.5 mL of medium that was changed every other day. After cell viability testing, explants were fixed and stained with uranyl acetate and lead citrate. Tissues were examined on a transmission electron microscope (JEM-100CX; JEOL). For both SEM and TEM, one observer was blinded to conditions of the samples. Two separate observers (one blinded to conditions of the samples) evaluated the debridement on each sample.

RESULTS

SEM and TEM Assessment of the Debridement Anatomy

In all four RPEbm(−) defects, both SEM and TEM showed intact RPE basement membrane with variable amounts of cell debris on the debrided surface (Figs. 3A, 3B). In all three SICL defects, SEM disclosed narrow rims of RPE basement membrane at the original wound edge and occasionally in the transition zone, whereas most of the extracellular showed tightly woven collagen fibers, characteristic of the ICL that lies directly under the RPE basement membrane (Fig. 3D). By TEM, RPE basement membrane was absent, and the ICL was fairly well preserved in most areas (Fig. 3C). In all three DICL defects, SEM disclosed narrow rims of SICL at the original wound edge and sometimes in the transition zone, whereas most areas showed more disrupted woven collagen fibers composing the deeper ICL (Fig. 3F). TEM showed the RPE basement membrane to be absent and that the superficial part of the ICL had also been removed in most areas. The thickness of the remaining ICL varied, with the thinnest ICL mostly in the center of the debridement (Fig. 3E).

Fluorescence Microscopy of RPE Ingrowth and Cell Viability

RPE cells were visualized with DAPI filters using the brownish autofluorescence of pigment granules, and RPE defects...
appeared blue with visible underlying choroidal vessels. The average wound area was $4.72 \pm 0.72 \text{mm}^2$ (mean $\pm$ SD, $n = 22$) with no significant difference between the different defects (i.e., RPEbm(+) defects versus SICL defects or RPEbm(+) defects versus DICL defects; Table 2, Fig. 4). At day 0, few RPE cells, if any, were seen in the RPE defect (Fig. 1A). RPE cells at the original wound edge started to enlarge and migrate into the defect from days 2 to 4. At day 10, all 22 wounds exhibited some degree of RPE resurfacing. RPE pigment at the wound edge appeared less dense than in intact RPE in DAPI images due to cells sliding out of their original location and into the debridement zone. Sliding cells lost their hexagonal shape, became enlarged, and often showed a small area with no autofluorescence within the cell, which appeared to correspond to the position of the nucleus (Fig. 1B). Most RPE cells at the original wound edge and in the defect were calcine-positive (Fig. 1C). Small patches of RPE cells that were unlabeled by calcine were distributed randomly at the original wound edge in four pairs of eyes.

**Figure 1.** (A) DAPI image of an RPEbm(+) defect at day 0. White outline: original wound edge. (B) DAPI image of the same defect at day 10 showing less dense RPE pigment at the original wound edge and many pigmented RPE cells in the defect. (C) Calcein image of the same defect showing darker areas in the wound covered by RPE cells, whereas lighter areas indicate uncovered basement membrane with cell debris. (D–B, arrows) Same RPE cell. Scale bar, 400 μm.

**Figure 2.** (A) Overlays of the calcine image and low-magnification SEM image of the defect shown in Figure 1. Because the images were overlaid in separate layers, switching between layers and turning off layer views enabled confirmation of cell locations. High-magnification images were examined with SEM to confirm the extent of cell spreading because cells that were extremely flattened could not be completely visualized in calcine images. (B) Low-magnification SEM image shows red lines defining unhealed edges of the defect. Insert: a higher magnification image showing edges of the epithelial defect (arrowheads). White outline: original wound edge. Scale bar, 400 μm.
SEM and Image Analysis of RPE Wound Healing

**RPEbm(+) Defects.** Eleven RPEbm(+) defects all had RPE basement membrane present, with some cell debris also present (Figs. 5, 6). The average resurfaced area and percentage of the area resurfaced were 3.48 ± 0.98 mm² and 78.00% ± 17.85% (mean ± SD, n = 11), respectively, at day 10. The resurfaced area and percentage of the area resurfaced did not correlate with donor age, time from death until enucleation, time from death until experiments, wound area, or wound perimeter. (Using multivariate correlation analysis, all correlations lie between 0.04 and 0.56.) Cells at the original wound edge showed ingrowth or migration along the entire edge with progressive elongation of the cells. Elongated cells at the original wound edge were oriented with the long axis perpendicular to the wound edge. Cells in front of them (Fig. 5, transition zone) were flattened and mostly formed monolayers (sometimes multilayers) resurfacing the defect. Cells at the front of migration (Fig. 5, leading edge) were also flattened with lamellipodia and filopodia extending toward the wound and neighboring cells. The flattened cells on the RPE basement membrane in the transition zone and leading edge were polymorphic. Some were polygonal and spread in a sheetlike manner, and some were elongated. Most cells located on the RPE basement membrane were in contact with other cells composing the migration front. Few flattened cells were also seen migrating singly. The presence of RPE basement membrane was confirmed in areas left uncovered by cells (Fig. 5C, asterisk; Fig. 6, bm).

**SICL-RPEbm(−) Defects.** The average wound size, resurfaced area, and percentage of the wound area that was resurfaced were not significantly different from RPEbm(+) defects (Tables 1, 2; Fig. 4). Similar to RPEbm(+) defects, cells located at the original wound edge were enlarged and elongated, pointing toward the wound center. In the transition zone, flattened, polymorphic RPE cells formed a monolayer with small spaces between cells revealing mostly the SICL and ocular cortex.

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<th>Wound Area (mm²)</th>
<th>RPEbm(+)</th>
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<td>Area Resurfaced (mm²)</td>
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<td>Debrided Area Resurfaced (%)</td>
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No significant difference was present between healing in the presence of native RPE basement membrane and SICL in terms of the wound size, area resurfaced, and percentage of the debrided area resurfaced (Student’s paired t-test, P > 0.05). The area resurfaced and the percentage of the debrided area resurfaced in RPEbm(+) defects and DICL defects were significantly different (t-test, P < 0.05), whereas the difference in the wound area between the two surfaces was not significant (t-test, P > 0.05). Data are reported as mean ± standard deviation.
casionally the RPE basement membrane. Cell coverage at the leading edge was different in appearance from that in RPEbm(+) defects. Flattened, sheet-like cells were present not only contiguous with the leading edge (Figs. 7C, 8A, asterisks) but also as multicellular patches and single cells. Some single spindle-shaped cells were also present near the leading edge (Fig. 7C, arrowhead; Fig. 8B, higher magnification of same cell) or in groups. Few cells near the leading edge of migrating cells were oriented parallel to the remaining rim of RPE basement membrane (Figs. 7C, arrow; 8A, higher magnification of same cells, arrows). The uncovered center of the wound showed tightly woven collagen fibrils of the SICL.

**DICL-RPEbm(−) Defects.** The average resurfaced area and percentage of the wound area that was resurfaced were significantly less than in RPEbm(+) defects, whereas the wound sizes of the two groups were comparable (Tables 1, 2; Fig. 4). Similar to healing of RPEbm(+) and SICL defects, cells at the original wound edge in DICL defects were enlarged and

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**FIGURE 4.** Comparison of percentage of resurfaced area in the different RPE defects. The percentage of resurfacing of RPEbm(+) defects (■) and SICL defects (□) is not significantly different (t-test, \( P > 0.05 \)), whereas the difference between RPEbm(+) defects (■) and DICL defects (□) is significant (t-test, \( P < 0.05 \)).

**FIGURE 5.** SEM of an RPEbm(+) defect. (A) White outline: original wound edge. RPE cells have covered 73.3% of the defect (darker areas). Square: area shown in (C). (B) Same image as in (A) with black lines showing the new edge of the healed defect. (C) At the original edge, cells are elongated with their long axis pointing toward the defect. Cells in front of them (transition zone) are flattened and form a monolayer or multilayer, covering the defect. At the leading edge of the resurfaced area, most flattened cells are in contact with one another composing the migration front. Uncovered areas show RPE basement membrane covered with debris (★). Scale bar: (A, B) 400 μm; (C) 100 μm.
and SICL defects was similar. Third, the extent of RPE wound healing in RPEbm(+) defects varied among donors.

Surgical excision of new vessels in patients with AMD is associated with removal of the adjacent native RPE.32–36 Repopulation of the dissection bed by native RPE is usually incomplete and aberrant,35–40 which probably results in chorio-capillaris and photoreceptor atrophy and poor visual recovery.35–39,41–44 Factors leading to the abnormal RPE repopulation of the dissection bed are unknown. New vessel excision often damages Bruch’s membrane, which is manifested by the presence of the RPE basement membrane, sometimes the ICL, and occasionally portions of the lamina elastica in the excised neovascular complex.35–36,39 We hypothesize that abnormalities in the dissection bed play a role in the poor RPE wound healing observed after CNV excision in patients with AMD. Studies in cat,44 pig,60 and monkey61 have demonstrated that native RPE can fully resurface intact Bruch’s membrane, mostly as a monolayer, whereas resurfacing of damaged aged human submacular Bruch’s membrane is incomplete.37–40 In this study, we compared RPE repopulation of the three different defects using human donor eyes in organ culture. RPE basement membrane was intact in RPEbm (+) defects. In SICL defects, RPE basement membrane was removed in most areas of the debridement with some residual RPE basement membrane left at the original wound edge and occasionally in the transitional zone. In DICL defects, more disrupted deeper ICL collagen appeared in most areas, with the SICL seen at the original edge and sometimes in the transition zone. RPE showed better resurfacing in RPEbm (+) defects than in DICL defects as evidenced by a significantly larger resurfaced area and higher percentage of the debrided area resurfaced in RPEbm (+) defects. Healing of SICL defects appeared similar to that in RPEbm (+) defects in terms of the area resurfaced and the percentage of the debrided area that was resurfaced. However, healing on these two types of surfaces is not identical as evidenced by different RPE cell morphology and by the fact that RPE cells tend to avoid the SICL in the presence of RPE basement membrane (Figs. 7C, 8A, arrows). These results suggest that RPE basement membrane supports RPE wound healing, and the deeper ICL does not support RPE resurfacing of localized defects in aged submacular human Bruch’s membrane to the same extent. The poor RPE ingrowth observed on the DICL defects mimics the histopathological findings in patients with AMD who have undergone CNV excision.37–40

It is not clear why RPE healing is better in RPEbm (+) and SICL defects than in DICL defects. In many cell types, the occurrence and speed of migration are influenced by integrin-extracellular matrix (ECM) ligand interactions, including integrin levels, integrin levels, and integrin-ligand binding affinities.62–69 The integrins or ligands involved in RPE migration on aged submacular Bruch’s membrane explants, with or without RPE basement membrane, have not been identified. However, Ho and Del Priore70 have shown that RPE attachment to Bruch’s membrane is mediated in part by fibronectin, laminin, vitronectin, and collagen IV, and that β1 integrin is an important mediator of adult human RPE attachment to Bruch’s membrane. Presumably, the presence of these ligands in the RPE basement membrane and SICL underlies the greater ability of RPE to resurface RPEbm (+) and SICL defects.1 The spindle-shaped RPE cells seen in SICL and DICL defects in this study are similar to the fibroblast-like RPE cells described by others.59 It has been shown that stationary nondividing RPE cells enter the cell cycle in pathologic conditions. These cells adopt the appearance of either macrophages or fibroblasts. In the presence of a substantial collagen matrix, RPE cells acquire a fibroblast-like morphology, whereas RPE cells acquire a macrophage-like morphology in the presence of an aqueous environment or photoreceptor debris in the subretinal space or loose collagen

**DISCUSSION**

The three major observations of this study are as follows. First, RPE wound healing, in terms of area resurfaced and percentage of the wounded area resurfaced, was similar in RPEbm (+) and SICL defects. Cell morphology on these two surfaces, however, was different. In RPEbm (+) defects, RPE cells were invariably flat, and most cells were in close contact with each other even at the resurfaced area’s leading edge. In SICL defects, at the resurfacing leading edge, flattened or spindle-shaped cells were present in groups or singly, and a few elongated cells were seen along the residual RPE basement membrane rim. Second, healing of the DICL defects was significantly less than that of the RPEbm (+) or SICL defects. In the transitional zone, only patchy flattened or spindle-shaped cells were observed, whereas in RPEbm (+) or SICL defects, monolayers of flattened cells were seen. Cell morphology at the leading edge in DICL defects was similar to the ^H9262/debris-covered RPE basement membrane. Scale bar, 10 μm.

**FIGURE 6.** Cells in the leading edge of the RPEbm (+) defect shown in Figure 5. A flattened RPE cell (levator) shows lamellipodia and filopodia extending onto an adjacent cell (levator) with other extensions (arrow) into the RPE basement membrane and cell debris (arrow bead). bm, debris-covered RPE basement membrane. Scale bar, 10 μm.
fibris in the vitreous. The difference in cell morphology of RPE cells that resurface RPEbm(+) defects and RPEbm(−) defects may reflect different cell-substratum interactions in the two conditions.

Healing of RPEbm(+) defects varied among the different specimens and did not correlate with age, time from death until enucleation, time from death until experiments, or wound size. Aside from damage to the peripheral RPE cells during the RPE debridement, which may compromise the viability of the cells, this variation may reflect an intrinsic disparity in the ability of RPE cells to regenerate and repopulate a wound in different donors. This result implies that the intrinsic properties of native RPE cells may also play a role in the abnormal repopulation of the dissection bed after CNV excision in patients with AMD. The poor repopulation of RPEbm(+) defects in some donors suggests that therapy to stimulate RPE regeneration and/or RPE transplantation may be necessary to promote repopulation of the dissection bed in some cases, even if the RPE basement membrane is intact.

Some RPE cells were able to repopulate the DICL defects. At the resurfacing leading edge of DICL defects, where the surface was most damaged, some cells appeared flattened and spread in a sheetlike formation, similar to the cells repopulating the RPEbm(+) defect, and some cells were elongated and spindle-shaped, exhibiting the morphology of migrating cells (Figs. 9C, 10A; arrowheads). These two types of RPE cells mostly were present as single cells or in small patches interspersed among elongated cells along the leading edge, seemingly avoiding the DICL (Fig. 10A, arrow). The polymorphic cell types at the leading edge of DICL defects may arise from subtle differences in the underlying surface and/or may reflect phenotypic heterogeneity of RPE cells in situ. Further investigation of the properties of the “hero” cells that are capable of repopulating DICL defects may provide information that is necessary for RPE cells to repopulate a damaged Bruch’s membrane in patients with AMD.

Potential mechanisms involved in resurfacing of the RPE defect include cell spreading, cell migration, and cell prolifer-
FIGURE 8. Cells in the leading edge of the SICL defect shown in Figure 7. Fused fibers of the superficial ICL in the area uncovered by cells (SICL) are shown. (A) One cell spreading out on the defect (★). Two elongated cells (arrows) parallel to the edge of remaining RPE basement membrane. (B) Spindle-shaped cell sending lamellipodia and filopodia into the SICL. Scale bars, 10 μm.

FIGURE 9. SEM of a DICL defect with (A) RPE cells covering 45.0% of the defect. White outline: original wound edge. Black square: area shown in (C); white square: area shown in Figure 10A. (B) The same image as in (A) with black lines at the new edge of the healed defect. (C) At the original wound edge, cells appeared similar to those in the RPEbm(+) and SICL defects. In the transition zone, patches of flattened cells were visible. At the leading edge, cells appeared thick (arrow), elongated, or spindle-shaped (arrowhead). Some flattened cells were also observed (★). Scale bar: (A, B) 400 μm; (C) 100 μm.
We believe cell spreading and migration are the principal initial mechanisms of RPE resurfacing in this aged human submacular Bruch's membrane organ culture preparation, as has been noted in other in vitro systems. This hypothesis is supported by the gradual, progressive enlargement and relatively uniform distribution of the cells from the wound edge to the transitional zone observed during follow-up with fluorescence microscopy and in SEM images. Small RPE defects are repaired by enlargement and sliding of the neighboring cells. Larger defects (>125 μm wide) are resurfaced by a combination of cell migration and proliferation. Because the wounds in the current system were fairly large (>2 mm diameter) and the cell density in the center of some defects was high, we suspect that cell proliferation also played a role in RPE resurfacing in this organ culture system.

This Bruch's membrane organ culture system allows the study of alterations in the cells (e.g., treating the cells in situ) as well as alterations in the RPE-denuded surface (e.g., removal of native RPE basement membrane, addition of extracellular matrix components to the surface). The methods established in this model system to estimate the degree of RPE repopulation and to monitor ongoing ingrowth of RPE allow quantitative assessment of RPE wound healing in response to potential therapeutic agents. Limitations of this in vitro system include (1) variations in specimen viability and inability to observe wound healing for more than 10 days because of contamination; (2) inability to study effects of overlying retina on cell attachment and/or migration; (3) inability to study the effect of the immune system on the retina-RPE-choriocapillaris; and (4) inability to study the effect of the inflammatory response on retina-RPE-choriocapillaris cell survival.

Nonetheless, that these in vitro resurfacing studies predict poor RPE resurfacing of iatrogenic RPE defects lacking RPE basement membrane, which appears to be the case in patients who have AMD and undergo CNV excision, suggests that the model has some utility. Improved understanding of the RPE wound healing response in this organ culture paradigm may enable stimulation of autologous RPE wound healing in denuded areas of Bruch's membrane, either in patients with AMD.
who undergo new vessel excision or in patients with incipient atrophy, and may avoid the immune rejection risks of alloge-
nic RPE transplantation.

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