Reattachment of Cultured Human Retinal Pigment Epithelium to Extracellular Matrix and Human Bruch’s Membrane

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Purpose. To determine the mechanism of reattachment of harvested human retinal pigment epithelium (RPE) to RPE-derived extracellular matrix and Bruch’s membrane.

Methods. Confluent first- to third-passage human RPE were harvested from tissue culture and plated onto RPE-derived extracellular matrix or human Bruch’s membrane exoplants denuded of cells by treatment with 0.02 N ammonium hydroxide. The authors measured RPE reattachment to uncoated surfaces or surfaces precoated with extracellular matrix proteins (fibronectin, laminin, vitronectin, or type IV collagen), antibodies to extracellular matrix proteins, or the synthetic peptide RGDS (arginine-glycine-aspartate-serine). Some RPE were pretreated with anti-β1 integrin antibodies before plating onto either substrate.

Results. Coating the surface of either RPE-derived extracellular matrix or Bruch’s membrane with fibronectin, laminin, vitronectin, or type IV collagen increased the RPE attachment rate. Exposing RPE to anti-β1 integrin antibodies or RGDS or precoating the surface with antibodies to fibronectin, laminin, vitronectin, or type IV collagen decreased the RPE attachment rate to both surfaces. The RPE attachment rate to Bruch’s membrane was lower when the exoplants were harvested from the macula of older (age, 70 to 90 years) versus younger (age, 30 to 40 years) persons (52.4 ± 3.6% versus 64.3 ± 3.5%, respectively; P < 0.05).

Conclusions. The attachment of cultured human RPE cells to human Bruch’s membrane or to RPE-derived extracellular matrix is mediated by an interaction between the β-subunit of integrin on the RPE surface and ligands in the extracellular matrix that include laminin, fibronectin, vitronectin, and type IV collagen. The lower rate of RPE reattachment to the macula from older human cadaveric eyes may have implications for studies aimed at RPE transplantation in elderly persons. Invest Ophthalmol Vis Sci. 1997;38:1110-1118.

During the past decade, investigators in several laboratories have been attempting to transplant retinal pigment epithelial (RPE) cells onto Bruch’s membrane. These efforts were fueled initially by the hope that patients with tapetoretinal degenerations might benefit from RPE transplantation, but a more immediate need for RPE transplantation exists in the evolving field of surgical management of subfoveal neovascularization. It is technically feasible to excise subfoveal choroidal new vessel membranes in age-related macular degeneration (AMD), presumed ocular histoplasmosis, and numerous other conditions. In presumed ocular histoplasmosis, many patients undergoing surgical excision of a subfoveal choroidal neovascular membrane experience significant improvement in central visual function, whereas improvement in central visual acuity to better than 20/200 is unusual in patients with AMD. This may be because the RPE is usually removed with the neovascular membrane during surgery in AMD, and areas of Bruch’s membrane that are debried of native RPE at the time of submacular surgery are not repopulated completely by migration and proliferation of adjacent host RPE. Absence of the RPE has been shown to cause secondary atrophy of the choriocapillaris in experimental studies, and choriocapillaris atrophy also...
To date, RPE transplantation has been reported in five patients with subfoveal neovascularization in exudative AMD. Scanning laser ophthalmoscopic microscopy showed that all five patients were able to fixate over the area of the RPE graft immediately after surgery, but an absolute scotoma developed in this region within 3 months after surgery. There are at least two possible explanations for this observation: The transplanted tissue may have been rejected immunologically or the transplant may have not survived because of the status of the underlying Bruch’s membrane. We have shown previously that separation of the RPE from its substrate can induce apoptosis, and reattachment of harvested RPE to a substrate is necessary to prevent programmed cell death. Thus, successful transplantation of the RPE requires that the transplanted cells reattach rapidly to the host Bruch’s membrane. However, thickening of Bruch’s membrane and the formation of basal laminar deposits, basal linear deposits, and drusen occurs early in the pathogenesis of AMD, so that the inner lamellae of Bruch’s membrane will be abnormal in the patients with AMD who are candidates for RPE transplantation. Successful reattachment of transplanted RPE to this substrate will require a complete understanding of the interaction between the transplanted RPE and healthy and diseased host Bruch’s membrane.

Several investigators previously have characterized the molecular constituents that may play a role in the reattachment of human RPE to human Bruch’s membrane. The RPE basement membrane and the inner collagenous zone contain laminin, fibronectin, vitronectin, and collagen type IV, and RPE cells contain a β₃-subunit of integrin. These molecules are known to play a role in the attachment of various epithelial cells to their substrate and have been shown to play a role in the reattachment of harvested RPE to Petri dishes coated with laminin or fibronectin. Retinal pigment epithelium can reattach and spread on untreated Petri dishes, but RPE reattachment is greater to laminin-coated or fibronectin-coated Petri dishes. The synthetic tetrapeptide RGDS (arginine-glycine-aspartate-serine), which is derived from the cell-binding domain of fibronectin, will decrease RPE binding to laminin-coated or fibronectin-coated dishes. When added simultaneously with the cells, RPE binding to fibronectin-coated dishes and laminin-coated dishes can be blocked by an antibody to the β₃-subunit of integrin, but RPE cell binding to charged tissue culture plastic is not affected by the antibody. When RPE are allowed initially to attach and spread on fibronectin or laminin-coated Petri dishes, addition of antibodies to fibronectin or laminin causes the attached cells to detach from the surface and round up.

Although interactions between the β₃-integrin subunit and extracellular matrix ligands have been shown to be important for RPE binding onto coated Petri dishes, the role of integrin subunits and extracellular matrix molecules in RPE reattachment to RPE-derived extracellular matrix and Bruch’s membrane has not been shown unequivocally. The purpose of the current experiments is to determine the role of these molecules in attachment of human RPE cells to extracellular matrix produced by human RPE in tissue culture and to Bruch’s membrane harvested from cadaveric human eyes.

**METHODS**

**Harvesting of Human Retinal Pigment Epithelium**

Human eyes from a single 32-year-old donor were obtained from Mid-American Eye and Tissue Bank (St. Louis, MO) and transported to our laboratory in sterile vials. Remnants of conjunctiva, Tenon’s capsule, extraocular muscle, and orbital fat were removed carefully under a dissecting microscope. The globe then was sterilized by rapid dipping in 70% ethanol, air dried, and transferred to Eagle’s minimum essential medium (Gibco, Grand Island, NY) with 100 U/ml penicillin G sodium, 0.1 mg/ml streptomycin sulfate, and 2.5 μg/ml fungizone (Sigma, St. Louis, MO) (MEM PSF) and remained in this medium for the surgical dissection.

A full-thickness circumferential incision was made posterior to the ora serrata and the anterior segment, vitreous, and neural retina were removed carefully. With careful attention to ensure that the meniscus of fluid did not extend above the cut edge of the RPE, MEM PSF with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) (MEM/10 PSF) was pipetted gently into the eyecup. Retinal pigment epithelial cells then were harvested by gently brushing Bruch’s membrane with a fire-polished pipette or the blunt end of a laboratory spatula. The RPE suspension was aspirated from the eyecup, placed in a centrifuge tube, and pelleted by centrifugation at 800 revolutions per minute for 5 minutes. The cell pellet was resuspended in MEM/10 PSF. The cells harvested from each eye were plated in 60-mm dishes and grown to confluence in MEM PSF with 20% fetal bovine serum for primary culture in an atmosphere of 95% air/5% carbon dioxide at 37°C. Cells were fed with fresh MEM/10 PSF supplemented with 50 μg/ml of gentamicin (Sigma) and 1 ng/ml of basic fibroblast growth factor (Gibco).
twice per week. Typically, it took 3 to 4 weeks for the primary cultures to reach confluence. Cells were split 1:5 at confluence and serially passaged in six well plates or 60-mm dishes.

**Preparation of Human Retinal Pigment Epithelial Extracellular Matrix**

The RPE cells were plated into 96-well plates (8000 viable cells/well) and grown to confluence. Three to four weeks after the cells reached confluence, the wells were washed three times with Hanks balanced salt solution. Extracellular matrix was prepared by exposing the confluent cells to three freeze-thaw cycles, with the temperature cycling between −70°C and room temperature, followed by treatment with 0.02 N ammonium hydroxide for 5 minutes, and washing five times in phosphate-buffered saline (PBS).

**Preparation of Human Bruch’s Membrane Exoplants**

Exoplants of human Bruch’s membrane were prepared by taking cadaveric human eyes harvested as above through three freeze-thaw cycles, with temperature cycling between −70°C and room temperature. A full-thickness circumferential incision was made posterior to the ora serrata and the anterior segment and vitreous were removed carefully. The posterior pole was placed in wells coated with extracellular matrix or cells to alter cell adhesion components. Bovine serum albumin (BSA, 2 mg/ml in PBS) was added to the wells coated with extracellular matrix or placed on the surface of harvested Bruch’s membrane. After 1 hour, the wells and the exoplants were washed with PBS three times before the reattachment studies were performed.

**Pre-treatment of Extracellular Matrix, Bruch’s Membrane, or Cells to Alter Cell Adhesion**

Coating Surface With Extracellular Matrix Components. One hundred microliters of fibronectin, laminin, or type IV collagen dissolved in PBS (5 mg/l) was placed in wells coated with extracellular matrix or placed on the surface of harvested Bruch’s membrane. After 4 hours, the wells and the exoplants were washed with PBS three times before the reattachment studies were performed.

Coating Surface With Antibodies Against Extracellular Matrix Components. Bovine serum albumin (BSA, 2 mg/ml in PBS) was added to the wells coated with extracellular matrix or placed on the surface of harvested Bruch’s membrane exoplants for 1 hour. Excess BSA then was removed by washing with PBS. Stock solutions of anti-fibronectin (6.6 mg/ml), anti-laminin (7.8 mg/ml), anti-collagen IV (4.6 mg/ml), and anti-vitronectin (1.4 mg/ml) antibodies (Sigma) were diluted 1:10, 1:100, and 1:200 in PBS. One hundred microliters of the appropriate diluted solution then was added to wells coated with extracellular matrix or placed on the surface of harvested Bruch’s membrane. The RPE reattachment studies then were performed by adding the cells to the wells, followed by incubation at 37°C for 6 hours as described below.

Coating Cells With RGDS. The synthetic peptide RGDS (Sigma) was dissolved in MEM (final concentration = 1 mg/ml) containing 2 mg/ml bovine serum albumin immediately before use and added to wells coated with extracellular matrix or wells containing exoplants of harvested Bruch’s membrane. After 1 hour, the wells and the exoplants were washed with PBS three times before the reattachment studies were performed.

Coating Cells With Antibodies Against β1 Integrin. The RPE cells harvested from tissue culture as described below were resuspended in MEM containing an anti-β1 integrin antibody (Sigma) diluted to a concentration of 20 μg/ml in MEM. The harvested RPE were maintained in this suspension for 1 hour before the reattachment studies were performed.

**Retinal Pigment Epithelial Reattachment Studies**

Confluent first- to third-passage human RPE cells were harvested by incubation with 0.25% trypsin/0.25% ethylenediaminetetraacetic acid in Hanks balanced salt solution for 20 minutes. Ten milliliters of MEM/15 was added to quench the reaction, and the cell suspension was centrifuged for 5 minutes at 800 revolutions per minute. The cell pellet was washed three times and then resuspended in MEM without serum, and 100-μl aliquots containing 8000 viable cells were added to the wells containing extracellular matrix or Bruch’s membrane exoplants.

The cells were allowed to attach to the surface for 6 hours. Unattached cells were removed from the tissue culture plates by washing the wells three times with Hanks balanced salt solution to remove the unattached cells. Unattached cells were removed from the Bruch’s membrane exoplants by picking up the tissue with fine forceps, dipping it three times in Hanks balanced salt solution to remove the unattached cells, and placing the tissue in a new well of a 96-well plate. Data were analyzed using an analysis of variance followed by a multiple comparison test (Figs. 3 and 4) or a two-tailed t-test (Fig. 5); P < 0.05 was considered statistically significant.

**Assay for Retinal Pigment Epithelial Adhesion**

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide] (Sigma) is a dye whose absorption
There was excellent correlation ($r = 0.99$) between log (optical density) and log (number of viable cells) using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide] assay for unattached cells, cells reattached to tissue culture plastic, and cells attached to Bruch's membrane exoplants (mean ± standard deviation, $n = 9$).

Characteristics change when it is dehydrogenated by cellular mitochondrial dehydrogenase; the activity of this latter enzyme is proportional to the number of live cells exposed to the dye. Thus, the use of MTT allows us to determine the number of live cells present. The MTT-based cell assay was performed as follows: 15 µl MTT (5 mg/ml) in PBS was added to wells containing 100 µl of media and an unknown number of cells, and the cells were incubated at 37°C for 4 hours. Dehydrogenation was terminated by adding 100 µl of lysing buffer [(20% sodium dodecyl sulfate in 50% of dimethylformamide in distilled water (Sigma); pH adjusted to 4.7 by adding 2.5 ml of 80% acetic acid and 0.8 ml of 1 N hydrochloric acid) to 100 ml solution] and incubating overnight at room temperature. The amount of yellow-reduced tetrazolium was quantified with an enzyme-linked immunosorbent assay reader with a 570-nm filter. The solid tissue was removed from the wells containing exoplants, and the 96-well plates then were read with an enzyme-linked immunosorbent assay reader. The number of cells attached to the surface then was calculated by comparing the enzyme-linked immunosorbent assay readings obtained on the wells with an unknown number of cells to a standardized curved ob-
The percent attachment rate, defined as the percent of cells that had attached to the surface in 6 hours, was always higher than the attachment rate to Bruch's membrane (35.6 ± 4.1%; P < 0.05 for all comparisons). Group 2 (diagonal stripes): The addition of extracellular matrix components increased the attachment rate over baseline. The attachment rate after the addition of fibronectin (72.4 ± 6.8%), laminin (68.2 ± 4.6%), collagen type IV (74.6 ± 5.7%), or vitronectin (75.6 ± 4.9%) was always higher than the attachment rate to Bruch's membrane alone (64 ± 9.5%; P < 0.05 for all comparisons). Group 3 (horizontal stripes): The addition of antibodies against Bruch's membrane components decreased the attachment rate over baseline. The attachment rate after the addition of anti-fibronectin (47 ± 10%), anti-laminin (52.6 ± 9.6%), anti-collagen type IV (51.5 ± 3%), or anti-vitronectin (42.2 ± 3.3%) was always lower than the attachment rate to Bruch's membrane alone (64 ± 9.5%; P < 0.05 for all comparisons).

The studies of cell attachment to Bruch's membrane were performed as above, except that nine exoplants from each of the two groups were randomly assigned to one of the following three treatments: coating the exoplant surface with fibronectin, laminin, type IV collagen, and vitronectin simultaneously to maximize the attachment of cells to the exoplant surface; coating the exoplant surface with anti-fibronectin, anti-laminin, anti-collagen IV, and anti-vitronectin simultaneously in an attempt to maximally inhibit the attachment of cells to Bruch's membrane; or no pretreatment with extracellular matrix proteins or antibodies to these proteins (control specimens).

### Scanning and Transmission Electron Microscopy of Bruch's Membrane Exoplants

The morphology of the exoplants after ammonium hydroxide treatment was determined by electron microscopy. For transmission electron microscopy, the specimens were washed in 0.2-mol sodium cacodylate buffer and placed in 1% osmium tetroxide at room temperature for 1 hour. Samples then were dehydrated in graded alcohol and embedded in Araldite resin (Polysciences, Warrington, PA). Thin sections were cut for transmission electron microscopy, placed on copper grids, and stained with saturated uranyl acetate and 0.3% lead citrate. Sections then were viewed with a JEOL-100B electron microscope (JEOL, Peabody, MA). For scanning electron microscopy, tissue was fixed and dehydrated as above. The specimens then were critical point dried using liquid carbon dioxide as the transitional fluid, sputter coated with gold, and viewed with a scanning electron microscope.

### RESULTS

Figure 1 shows that there is a linear relation between the optical density measurements and the number of viable cells for three populations of cells: unattached cells, immediately after placing RPE into wells; RPE attached to extracellular matrix-coated plastic, and cells attached to Bruch's membrane exoplants. There was an excellent correlation (r = 0.99) between log

![Graph showing attachment rate](image-url)
FIGURE 5. Human retinal pigment epithelial cell adhesion to Bruch’s membrane harvested from the macula of young (white bars) and older (shaded gray bars) persons. Data are divided into three groups. Group 1 (open bars): The attachment rate on Bruch’s membrane alone is higher for younger versus older Bruch’s membrane (64.3 ± 2.5% versus 52.4 ± 3.6%). Group 2 (diagonal stripes): The simultaneous addition of fibronectin, laminin, collagen type IV, and vitronectin to Bruch’s membrane exoplants increased the attachment rate over baseline for younger (75.5 ± 4.1%) and older (76.5 ± 4.8%) persons and eliminated the age-dependent difference in cell reattachment rate. Group 3 (horizontal stripes): The simultaneous addition of anti-fibronectin, anti-laminin, anti-collagen type IV, and anti-vitronectin markedly decreased the reattachment rate to younger (32.6 ± 2.5%) and older (34.6 ± 2.5%) Bruch’s membrane and eliminated the age-dependent difference in cell reattachment rate. Data presented as mean ± standard deviation, n = 9. BM = Bruch’s membrane; anti-IgG = an irrelevant IgG antibody; FN = fibronectin; LM = laminin; C IV = type IV collagen; VN = vitronectin; RGDS = (arginine-glycine-aspartate-serine); anti-β1 = antibody to β1-subunit of integrin.
each compared to Bruch’s membrane alone). The addition of anti-fibronectin, anti-laminin, anti-collagen IV, and anti-vitronectin (1:100 dilution) decreased the attachment rates to 47 ± 10%, 52.6 ± 9.6%, 51.5 ± 3%, and 42.4 ± 3.3%, respectively (P < 0.05 for each compared to Bruch’s membrane alone). The simultaneous addition of anti-fibronectin, anti-laminin, anti-collagen IV, and anti-vitronectin antibodies decreased the attachment rates further to 35.6 ± 4.1% (P < 0.05 compared to Bruch’s membrane alone). Treatment with RGDS markedly decreased the RPE reattachment rate to 33.3 ± 3% (P < 0.05 compared to Bruch’s membrane alone). Treatment of RPE cells with anti-β1 integrin antibodies before plating the cells decreased the attachment rate to 37.4 ± 5.9%. The reattachment rate of human RPE to uncoated tissue culture plastic was 25.8 ± 4.5%.

There is an age-dependent difference in RPE adhesion to Bruch’s membrane derived from the macular region of human eye bank eyes (Fig. 5). The reattachment rate of RPE to Bruch’s membrane harvested from the macula of younger persons (Fig. 5, white bars) was significantly higher than the reattachment rate of RPE to Bruch’s membrane harvested from older (Fig. 5, shaded gray bars) persons (64.3 ± 2.5% versus 52.4 ± 3.6%; P < 0.05). Interestingly, the simultaneous addition of fibronectin, laminin, vitronectin, and collagen IV increased the attachment rates to 75.5 ± 4.1% and 76.5 ± 4.8% (P < 0.05 for each compared to uncoated Bruch’s membrane) for younger and older Bruch’s membrane, respectively, and eliminated the difference in the attachment rates between young and old Bruch’s membranes (P > 0.05). The simultaneous addition of anti-extracellular matrix proteins antibodies decreased the attachment rate of RPE cells to young and old Bruch’s membrane to 32.6 ± 6.5% and 34.6 ± 2.5%, respectively (P < 0.05 for each compared to uncoated Bruch’s membrane), and again eliminated the difference in attachment rates to young and older Bruch’s membrane (P > 0.05).

Figure 6 shows the ultrastructure of Bruch’s membrane after ammonium hydroxide treatment. All of the native RPE cells have been removed. On scanning electron microscopy, the RPE basement membrane is visible (thin black arrow, Fig. 6 left) as are gaps between patches of basement membrane (white arrow, Fig. 6 left). Transmission electron microscopy shows that the RPE basal lamina, inner collagenous zone, elastin layer, outer collagenous zone, and basement membrane of the choriocapillaris are preserved after ammonium hydroxide treatment (Fig. 6 right).

**DISCUSSION**

We have shown that the binding of RPE cells to RPE-derived extracellular matrix and to human Bruch’s membrane depends on laminin, fibronectin, vitronectin, and collagen IV in the extracellular matrix, because antibodies to these extracellular matrix proteins decreased RPE binding to both substrates. The β1 subunit of integrin is important in RPE reattachment to each substrate, because addition of a β1-integrin antibody markedly decreased RPE binding to each substrate (Figs. 3 and 4).

In our studies, 8000 viable cells were added to each well containing extracellular matrix or Bruch’s membrane exoplants. Because the wells were 6.5 mm in diameter, the plating density was 240 cells/mm². If we assume a cell diameter of 20 to 25 μm, the 8000 viable cells would be expected to cover approximately 8% to 12% of the reattachment surface. We intentionally chose this low-plating density for these studies to ensure that we counted...
only RPE that were reattached to the surface rather than to one another. We recognize that RPE will spread out and assume a flattened morphology after they reattach to the surface at this plating density, and we are currently investigating the effects of plating density and cell morphology on the expression of β₁-integrin and other integrin subunits on the cell surface. We also are using this system to investigate the reattachment of RPE harvested in sheets to Bruch's membrane.

Our data suggest that there is a small but significant difference between RPE reattachment rates to Bruch's membrane exoplants harvested from the macula of younger versus older persons (64.3 ± 2.5% versus 52.4 ± 3.6%, respectively). The age of the donor RPE did not vary during this experiment, because all RPE were harvested from a single 32-year-old patient. The addition of extracellular matrix components (such as laminin, fibronectin, collagen IV, or vitronectin) increased the RPE reattachment rate for both groups and eliminated the difference in RPE reattachment to the macula of younger and older eyes. Although the reasons for the age-related difference in RPE binding to macular Bruch's membrane cannot be discerned from this study, our results suggest that there may be an age-related decline in the number of binding sites for RPE on extracellular matrix proteins within Bruch's membrane. We are attempting to address this issue by quantifying the number of receptors in the inner lamellae of Bruch's membrane as a function of age.

An age-related difference in RPE binding to macular Bruch's membrane is present despite the fact that we excluded eyes that exhibited submacular pathology visible at the dissecting microscope, such as submacular blood, submacular scars, or visible choroidal neovascularization. Because these disorders may well degrade the integrity of the inner aspects of Bruch's membrane, it is likely that RPE reattachment to Bruch's membrane may be reduced further in these disease states. We are in the process of using our system to determine the RPE reattachment rate as a function of the disease status of the host macula. We are hopeful that a complete understanding of the factors involved in RPE reattachment to normal and diseased human Bruch's membrane will maximize the chances of successful transplantation of RPE into the subretinal space and ultimately will improve the visual prognosis after surgical excision of subfoveal choroidal neovascularization.

Key Words
Bruch's membrane, cell adhesion, extracellular matrix, retinal pigment epithelium, retinal pigment epithelial transplantation

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References


