Iris Pigment Epithelium Attachment to Aged Submacular Human Bruch's Membrane

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Purpose. To determine whether iris pigment epithelium (IPE) cells can attach to aged submacular human Bruch's membrane and to assess whether IPE cells express the integrin subunits that may be necessary to bind to the known extracellular matrix ligands present in Bruch's membrane.

Methods. IPE cells were seeded onto the RPE basement membrane (RPEbm) or inner collagenous layer (ICL) of aged submacular Bruch's membrane as microaggregates or were expanded in culture until enough cells could be obtained for seeding. Cell morphology and the percentage of cell coverage were determined 1 or 7 days after seeding. Messenger RNA was extracted from cultured and uncultured IPE cells and analyzed by RT-PCR. The expression of integrin subunits $\alpha1$ to $\alpha6$ and $\beta1$ mRNA was examined.

Results. Coverage by uncultured IPE was low on both surfaces at day-1 (RPEbm, 7.9% ± 4.8%; ICL, 5.0% ± 2.5%) with few intact cells present. Culturing IPE improved attachment with similar coverage on both surfaces and no significant difference between day-1 (RPEbm, 89.9% ± 9.1%; ICL, 63.4% ± 26.5%) and day-7 (RPEbm, 97.8% ± 2.3%; ICL, 94.7% ± 6.6%). By day-7, cell morphology and coverage on both surfaces was variable, ranging from few intact cells to a high degree of coverage by flattened cells. All integrin subunits studied were expressed in cultured cells, whereas $\alpha2$, $\alpha3$, and $\alpha4$ showed less or no expression in uncultured cells.

Conclusions. Upregulation of integrin mRNA expression may be one explanation for the difference in coverage by cultured versus uncultured IPE cells. The presence of dead, dying, or flattened cells at day 7 indicates that IPE may not survive or differentiate on aged submacular Bruch's membrane. (*Invest Ophthalmol Vis Sci.* 2004;45:4520–4528) DOI:10.1167/iovs.04-0684

Age-related macular degeneration (AMD) is the leading cause of severe visual loss in people older than 55 years in the United States. Development of subfoveal choroidal new vessels (CNVs) is the principal cause of visual loss among patients with AMD.[1] CNV extraction has been performed as a surgical treatment for AMD with the goal of eliminating subretinal hemorrhage, scarring, and photoreceptor damage.[2–4] CNV extraction produces a localized retinal pigment epithelium (RPE) defect in most cases.[3,5–7] In addition, portions of Bruch's membrane, mostly the native RPE basement membrane, are removed in many cases, exposing the underlying inner collagenous layer (ICL).[3,8] Loss of subjacent RPE cells can lead to photoreceptor degeneration.[9–11] In some animal models, transplanted RPE cells can rescue photoreceptors.[12–14] Thus, replacing RPE lost through disease (e.g., AMD) or by surgical excision of CNV may maintain photoreceptor function in patients.

RPE transplantation has been attempted after CNV extraction in AMD-affected eyes, but sustained visual improvement has not occurred.[15–18] Failure to recover vision may be due to RPE graft rejection or graft failure. To avoid graft rejection, autologous uncultured RPE cells have been transplanted.[16] Sustained significant visual improvement was not observed in most patients, although results in experimental animals indicate that this approach holds promise.[19] Systemic immune suppression combined with transplantation of allogeneic uncultured adult RPE cells has been performed in patients.[17] Significant visual improvement did not occur, and some patients had difficulty tolerating the combination therapy with prednisone-cyclosporine-azathioprine.[17] Histopathology of the transplant site in one patient indicated that the RPE cells were not organized as a monolayer and did not cover the entire dissection bed.[17] In addition, there was overlying photoreceptor atrophy. To avoid graft failure (i.e., RPE apoptosis), rapid RPE attachment to an appropriate surface is necessary (Ho TC, et al. *IOVS* 1996;37:ARVO Abstract 463).[20–24] Current evidence indicates that abnormalities both in the transplanted cells and in the recipient surface may underlie the poor results of clinical RPE cell transplants observed thus far.[24]

Because autologous iris pigment epithelium (IPE) cells can easily and safely be obtained by surgical iridectomy, some investigators have suggested IPE transplantation as an alternative to RPE transplantation.[25–29] IPE and RPE have the same embryonic origin[30] and share similar functional capabilities. In the iris, IPE cells are interconnected by tight junctions and form a blood–aqueous barrier,[30] whereas RPE cells form a blood–retinal barrier. In culture, rat, porcine, and human IPE can phagocytose photoreceptor outer segments.[31–33]

Thumann et al. [34] demonstrated that IPE cells transplanted into the subretinal space survived and did not adversely affect the photoreceptors in rabbits. Transplantation of IPE cells slowed down the degeneration of photoreceptors in the RCS rat.[35] Autologous IPE transplantation in patients with AMD undergoing CNV excision has been performed by several surgeons.[28,29,36,37] Overall, visual acuity improved in 10 patients, was unchanged in 25, and decreased in 2. Graft rejection was not recognized in any case, and, in one patient, the CNV recurred. However, the exact behavior of transplanted IPE in humans on aged submacular Bruch's membrane is not clear at this time, because no histopathology is available.

If autologous IPE transplants are to be successful in patients with AMD who undergo concomitant CNV excision, the IPE

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cells must attach, survive, and appropriately differentiate on aged submacular human Bruch's membrane. To determine whether cultured and/or uncultured aged human IPE cells can adhere and differentiate on aged submacular human Bruch's membrane, we conducted experiments in two areas. First, we assessed the ability of IPE cells to attach and survive in vitro on aged human submacular Bruch's membrane, which is the surface they are likely to encounter in situ after transplantation into humans with AMD. Previously, we have used this Bruch's membrane organ culture system to study the attachment, survival, and differentiation of transplanted RPE on aged submacular human Bruch's membrane.[21,23,24] This preparation allows one to study cell attachment in the presence and absence of native RPE basement membrane, which is important because transplanted cells encounter both surfaces in the CNV dissection bed.[3] Second, we studied integrin subunit mRNA expression in cultured and uncultured human IPE cells because the biology of integrin expression in IPE is unknown, and we believe that expression of particular integrins (e.g., those that mediate binding to Bruch's membrane components—namely, laminin, fibronectin, and collagen-I, -III, -IV, -V, and -VI)[38,39] is essential for IPE adhesion to Bruch's membrane. In previous work, we found that cultured and uncultured human RPE cells exhibit different integrin subunit mRNA expression, which may underlie their differing tendencies to adhere to Bruch's membrane (Gullapalli VK, et al. *IOVS* 2002;43:ARVO E-Abstract 3436).[24]

Materials and Methods

Human Donor Eyes

Human eyes were obtained from the National Disease Research Interchange (NDRI, Philadelphia) and the North Carolina Eye Bank, a Vision Share (Apex, NC) member eye bank, no more than 48 hours from the time of death. Thirty-seven pairs of eyes were used in the study. Donor ages ranged from 59 to 91 years (mean age ± SD, 76.0 ± 8.1). Death to enucleation times ranged from 1:05 to 7:07 hours (mean time ± SD, 3:39 ± 1:26 hours). This research followed the tenets of the Declaration of Helsinki and was approved by the institutional review board (IRB) of the New Jersey Medical School.

IPE Isolation and Culture

After removal of connective tissue, globes were immersed briefly in 10% povidone iodine (Betadine; Purdue Frederick Company, Norwalk, CT), rinsed with Balanced Salt Solution (Alcon Surgical, Fort Worth, TX), and rinsed twice in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA) containing 2.5 μg/mL amphotericin B for 10 minutes at 4°C.

The eyes were opened at the equator, and the anterior portions were used for IPE isolation. The posterior eyecups were used to prepare the Bruch's membrane explant or for another investigation.

The irides were incubated with 1 mg/mL type IV collagenase (Sigma-Aldrich, St. Louis, MO) in DMEM at 37°C for 20 to 25 minutes. After transfer to phosphate-buffered saline (PBS), IPE cells were peeled off as a sheet with a beveled flexible silicone tube, using a dissection microscope for visualization. Isolated sheets were cut into small pieces with scissors and seeded onto a culture dish precoated with bovine corneal endothelial extracellular matrix (BCE-ECM).[40] IPE cells were incubated in complete medium (15% fetal bovine serum, 2.5 μg/mL amphotericin B, 300 μg/mL L-glutamine, 0.05 mg/mL gentamicin, and 1 ng/mL basic fibroblast growth factor in DMEM) at 37°C in 10% CO_2 and refed every other day. The time in culture needed to grow enough IPE cells for seeding onto Bruch's membrane explants was 36.0 ± 12.1 days.

Immunocytochemistry

Immunocytochemical staining was performed to confirm the epithelial origin of the cells. After the culture dishes were rinsed with PBS three times, the cells were fixed with ice-cold 100% methanol for 5 minutes. After fixation, the dishes were rinsed with PBS containing 0.1% Triton X-100 three times. Nonspecific antigens were blocked with 2% normal goat serum, 2% bovine serum albumin, and 0.2% triton in PBS at room temperature for 20 minutes. Monoclonal mouse anti-cytokeratin (AE1/AE3) antibody (BioGenex, San Ramon, CA) was applied at 1:50 at 4°C overnight. After the dishes were rinsed with PBS containing 0.1% Triton X-100 three times, rhodamine-conjugated goat anti-mouse IgG-IgM antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied at 1:100 at room temperature for 1 hour. The dishes were rinsed with PBS three times, and the cells were observed with a microscope (Axiovert; Carl Zeiss Microimaging, Inc., Thornwood, NY) equipped with appropriate fluorescence filters.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Confluent cultures of IPE cells (n = 4, 79.5 ± 3.7 years) were washed with PBS. Single cell suspensions were prepared with brief trypsinization and then were washed and centrifuged. The pellet was frozen and stored at −80°C until further use. Uncultured IPE cells (n = 4, 73.8 ± 10.4 years) were frozen after isolation as sheets and stored at −80°C. Total RNA was extracted from the samples, and genomic DNA was lysed with a kit (BD-Clontech, Inc., Palo Alto, CA). The quality of the isolated RNA was examined by ascertaining 18S and 28S bands with agarose gel electrophoresis. The quantity of RNA was determined by spectrophotometry.

Reverse transcription–polymerase chain reaction (RT-PCR) was performed with gene-specific primers[24] for various integrin subunits using a kit (Superscript One-Step RT-PCR kit; Invitrogen, Carlsbad, CA). The concentration of RNA was adjusted to contain 0.1 μg of RNA in each solution. The thermocycler condition used for cDNA synthesis was 50°C for 30 minutes and for predenaturation was 94°C for 2 minutes; 35 cycles of PCR were performed as follows: denaturation, 94°C for 15 seconds; annealing, 55°C for 30 seconds; extend, 72°C for 30 seconds; and final extension at 72°C for 10 minutes. PCR products were examined with agarose gel electrophoresis. Cultured fetal human RPE RNA and GAPDH were run concurrently with each sample as positive controls. Reaction samples from which RNA was omitted served as the negative control.

Preparation of Bruch's Membrane Explants

After the anterior segment of the vitreous and the retina were removed, RPE cells were removed from the posterior eyecups. Two types of explants were prepared. The native RPE basement membrane was preserved in one eye (RPEbm), and the surface of Bruch's membrane was wiped to expose the inner collagenous layer (ICL) in the other eye, as described previously.[21,23,24] The complex of submacular Bruch's membrane, choroid, and sclera was punched out with a sterile 7.0-mm trephine. The bottom of the trephine, which contained the Bruch's membrane button, was then sealed by punching through a sheet of dental wax. Thus, the preparation consisted of a sterile trephine, with the bottom sealed with a button of dental wax, and a tissue button, which lay flat on the dental wax and flush against the trephine's wall. The dental wax was soaked in 70% alcohol and dried before use. Preliminary experiments did not reveal any adverse affect of the wax on cell growth. The trephine containing the button was transferred to a 24-well plate. IPE cells (cultured or uncultured) were added as described in the next section and incubated. Uncultured IPE cells were seeded onto Bruch's membrane explants prepared from the same donor eye.

IPE Transplantation

Single-cell suspensions of IPE in complete medium were prepared from cultures, and the concentration was adjusted to 6.05 × 10^5 cells/mL. Two hundred microliters of cell suspension was added to each trephine containing the Bruch's membrane explant (donor age, 72.8 ± 9.8 years). At this seeding density, fetal RPE cells can resurface the

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entire area by 24 hours.[21] The specimens were incubated at 37°C for 1 or 7 days.

Uncultured IPE cells were prepared as microaggregates from irides, as described earlier. Cells obtained from both irides of a pair of donor eyes were then equally divided between the two Bruch's membrane explants (~5 × 10^5 cells/explant) prepared from the same donor (77.6 ± 3.6 years). Because of poor cell survival at day 1, no specimens were prepared for analysis on day 7.

Scanning Electron Microscopic Analysis

Bruch's membrane specimens were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer at 4°C. After dehydration in increasing concentrations of ethanol, specimens were critical point dried (Autosamdri-814; Tousimis Research Corp., Rockville, MD), mounted on aluminum stubs, and left to dry overnight. Specimens were sputter coated with 20 nm gold-palladium (DESK II; Denton, Moorestown, NJ) and stored in a desiccator until they were analyzed.

Specimens were photographed by a scanning electron microscope at magnifications of 400× and 1500× (model JSM-35C; JEOL, Tokyo, Japan) equipped with an image-capture system (Digiscan; Gatan, Pleasanton, CA) at 25 kV accelerating voltage. For determination of the area covered by IPE cells, nonoverlapping images at a magnification of 400× were collected, and the percentage of total area covered by intact cells was measured with NIH image J v1.29 software (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Morphologic analysis was performed at 400× and 1500×.

Statistical Analysis

Data were analyzed by parametric Student's *t*-test. The paired *t*-test was used for comparison of paired data. All the data showed a normal distribution. Excel (Microsoft, Redmond, WA) and an add-on software (StatCel; OMS, Saitama, Japan) was used for analysis.

Results

IPE Growth in Cell Culture

IPE cells started growing on days 1 to 5 after seeding (Fig. 1A). Flattened, highly pigmented cells appeared along the edges of the IPE clumps seeded as microaggregates. As cells divided and migrated from a microaggregate, extremely elongate cells or large flattened cells commonly were observed at the original edge of the microaggregate. Elongate cells were oriented with the long axis pointed away from the microaggregate (Fig. 1B). The cytoplasm of the cells around the microaggregates was highly pigmented, whereas cells distal to the microaggregates were less pigmented (Fig. 1B). In some cultures, overlapping pseudopodia were observed (Fig. 1C; arrows). After approximately 2 weeks, single cells were observed migrating from the edge of the elongate cells. These cells were small and polymorphic (Fig. 1D). Proliferation of these cells (demonstrated by formation of cell patches in these areas that eventually became confluent) accounted for most of the new cells appearing in the culture dish. Once the cells came in contact with others, they became small and polygonal (Fig. 1E). These cells usually were distal to the original microaggregate and had various degrees of pigmentation, although most of the cells were lightly or not pigmented (Fig. 1F). Occasional, large pigmented cells were seen in this area (Fig. 1F, arrow). Pseudopodia overlapping adjacent cells were not observed. The pace of cell spreading varied among donors. The most active cultures became confluent in 3 weeks, whereas cells from some donors did not become confluent and ceased growing at 6 weeks. Confluent cultures exhibited polymorphic cells at the longest time observed (52 days). Hexagonally shaped cells were uncommon.

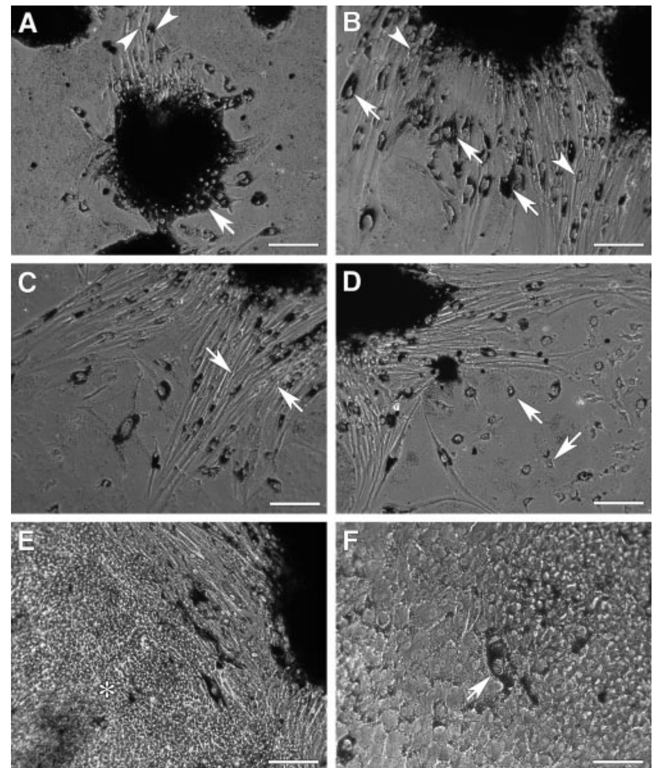

Figure 1. IPE cell culture images. (**A**) Day 4: donor age, 91 years. Highly pigmented flat cells were situated close to and at the edge of the microaggregate (*arrow*). Migrating elongate cells also extended from the edge of the microaggregate. Some of the elongate cells were less pigmented (*arrowheads*). (**B**) Day 11: same donor as in (**A**). Elongate cells were observed spreading out from the microaggregate. Highly pigmented cells were frequently visible (*arrows*). Occasional, less pigmented cells were close to microaggregates (*arrowheads*). (**C**) Day 11: same donor as in (**A**). Extremely elongate cells were more abundant. Some pseudopodia overlapped (*arrows*). Lightly pigmented cells were frequently visible. (**D**) Day 17, donor age, 71 years. Cells not as elongate as at day 11 commonly were seen migrating or spreading (*arrows*). Shapes and pigmentation of the small cells varied. (**E**) Day 46: donor age, 79 years. Small and nonelongate cells were confluent away from the microaggregate (*). Elongate cells around the microaggregate stayed the same shape as at earlier time points. (**F**) Day 46: same donor as in (**E**), higher magnification. Most of the cells were round or polygonal. Elongate cells were not visible. Highly pigmented large cells were seen occasionally (*arrow*). There were various degrees of pigmentation. Scale bar: (**A–E**) 200 μm; (**F**) 100 μm.

Cytokeratin Staining

All the cells stained positively for cytokeratin, regardless of cell shape.

IPE Growth in Organ Culture

Cultured IPE at Day 1. Cells attached onto native RPE basement membrane were highly variable in shape (elongate, flat, polygonal, or round) and size (Fig. 2). Cell extensions were common with lamellipodia (arrow, Fig. 2A) and filopodia (Fig. 2B, arrowheads,) extending over adjacent cells and onto the basement membrane. The surface of the attached cells exhibited varying amounts of short microvilli (Fig. 2B). In general, the enlarged, flattened cells had smooth surfaces. Degenerate cells or cell debris were visible on top of the attached cells or directly on the native RPE basement membrane. Degenerate or dying cells were smooth, partially disrupted and/or perforated (Fig. 2A; arrowheads). In no specimen did the layer of cells totally cover the surface. Even in specimens with a high degree

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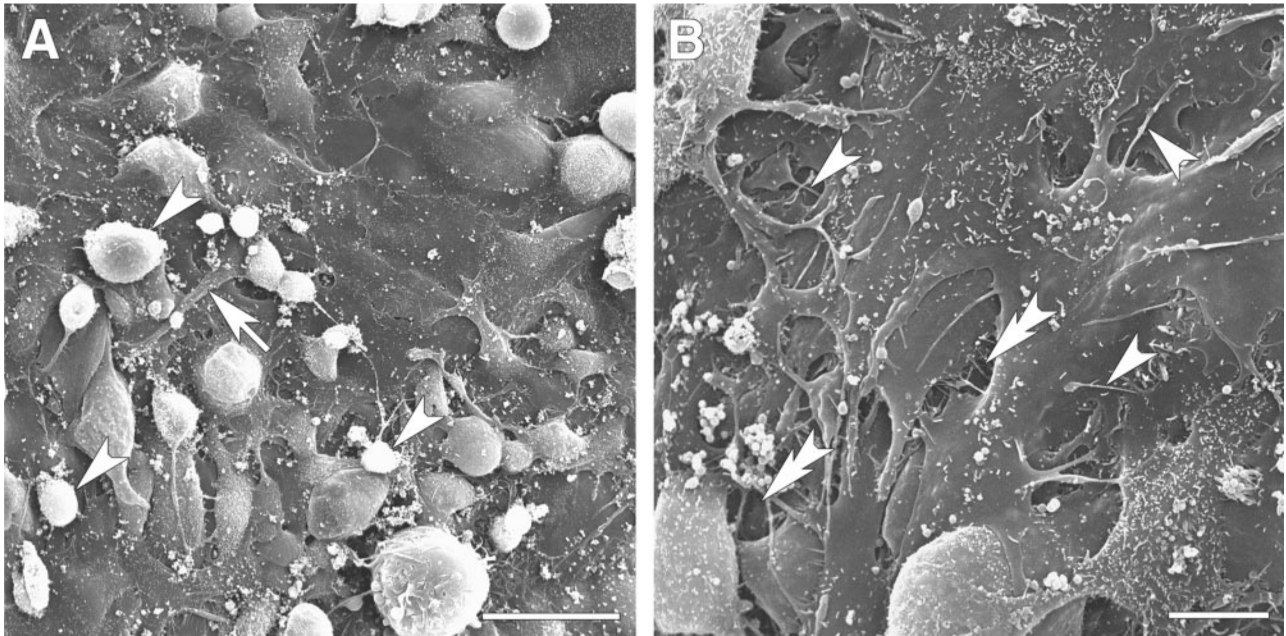

Figure 2. Cultured IPE cells from a 70-year-old donor 1 day after seeding on submacular RPE basement membrane (donor age, 76 years). (**A**) Cells present on the explant surface showed variable size and morphology. Dying cells on top of the attached layer were frequently visible (*arrowheads*). Cell extensions onto neighboring cells were common (*arrow*). (**B**) Higher magnification showing numerous filopodia extending onto neighboring cells (*arrowheads*). Cell surfaces exhibited varying amounts of short microvilli. Intercellular gaps were present (*double arrowheads*). Scale bar: (**A**) 30 μm; (**B**) 10 μm.

of resurfacing, the layer on native RPE basement membrane consisted of irregularly shaped cells with small intercellular gaps (Fig. 2B; double arrowheads) or many extensions over adjacent cells. Cells seeded onto the ICL were similar to those seeded onto native RPE basement membrane except cell coverage on the ICL appeared to be lower due to less cell spreading (Fig. 3), and there was more variability in coverage among donors (Table 1). However, the difference in coverage on RPEbm and ICL specimens was not statistically significant. Exposure of the native RPE basement membrane or ICL, depending on the goal of the debridement, was confirmed in uncovered areas.

Cultured IPE at Day 7. At this time point, there was no difference in the ability of native RPE basement membrane

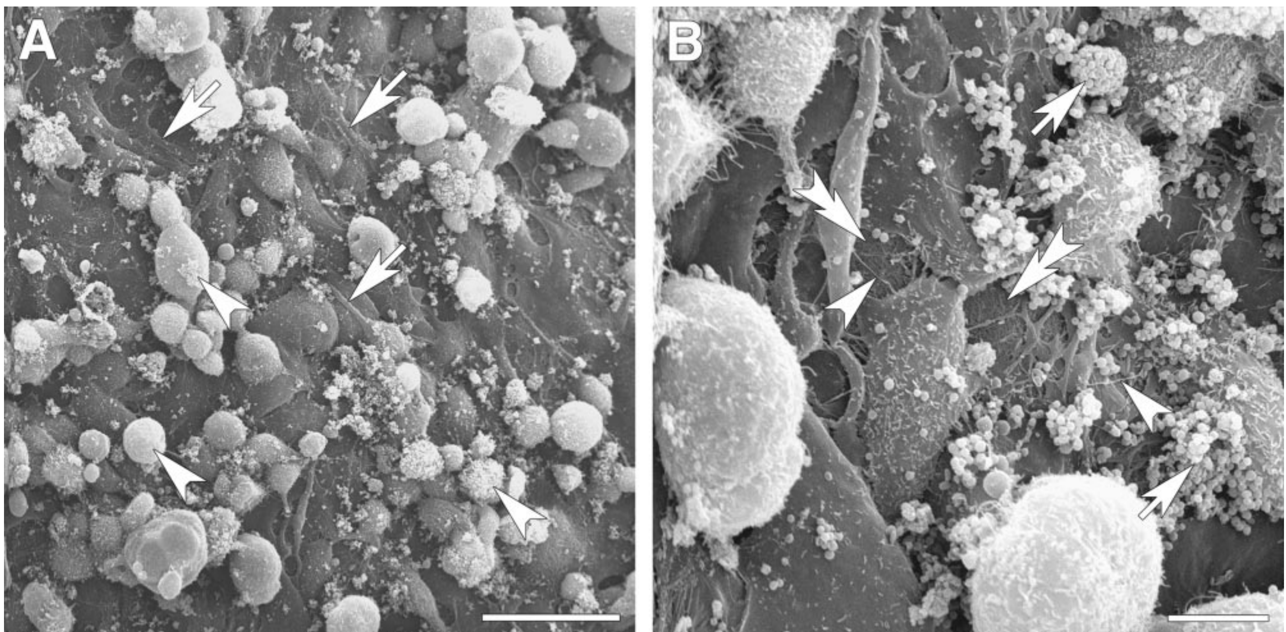

Figure 3. Cultured IPE cells 1 day after seeding on submacular ICL (same donor as in Fig. 2). (**A**) Similar to cells seeded on RPE basement membrane, IPE cells were highly variable in morphology but appeared less spread. *Arrows*: numerous lamellipodia extending over adjacent cells. *Arrowheads*: dead or dying cells. (**B**) Higher magnification showing filopodia (*arrowheads*) extending onto neighboring cells and onto the ICL (*double arrowheads*). Various amounts of microvilli were present on the cell surfaces. *Arrows*: cell debris. Scale bar: (**A**) 50 μm; (**B**) 10 μm.

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Table 1. Coverage of Day-1 Specimens with Cultured IPE

Donor Age (y)		% Coverage	
IPE	Explant	RPEbm	ICL
66	77	88.9	59.3
85	65	81.7	84.4
90	64	80.4	67.7
70	76	99.5	85.1
88	59	99.0	20.3
79.8 ± 11.0*	68.2 ± 7.9	89.9 ± 9.1*	63.4 ± 26.5†

* Mean ± SD.
† $P = 0.066$.

versus ICL to support IPE attachment (i.e., the coverage was highly variable, regardless of the surface and the donor). Nine of 10 explants (five on RPEbm, four on ICL) showed >90% of coverage by a monolayer of cells that were polygonal or elongate and very flat (Table 2). In general, cells were flatter than at day 1. Cell morphology was similar on most explants. Attached cells formed a monolayer of spread, polymorphic, and flattened cells interspersed with elongate cells (Fig. 4). In areas comprising predominantly polymorphic cells, individual cell borders were not evident. Some cells exhibited microvilli. Overlapping lamellipodia were present. Most cells on top of the flattened cell layer were dying or broken. In the remaining samples, large areas of the ICL were covered with flat, elongate cells oriented in the same direction (Fig. 5). The level of the debridement was confirmed in all explants in areas where there was no cell coverage.

Uncultured IPE at Day 1. Abundant cell debris was present on the surface of the explants. Few intact cells were present on both surfaces. When present, most intact cells were observed in microaggregates. Most of the cells in microaggregates had smooth surfaces (Fig. 6). No cell spreading was seen.

Area Analysis

The resurfaced area was calculated by measuring the area covered by intact cells and expressed as a percentage of total area (Tables 1, 2, 3). However, completely broken cells and cellular debris were not included as cell coverage.

One day after seeding, cultured IPE cells covered native RPE basement membrane or ICL significantly more than uncultured IPE (RPEbm: $P = 1.0 \times 10^{-7}$, ICL: $P = 0.0012$). Within the group of explants that were seeded with cultured IPE cells, no statistically significant difference was found between coverage on native RPE basement membrane and ICL or between coverage at days 1 and 7 after seeding (day-1 cultured IPE versus day-7 cultured IPE; RPEbm: $P = 0.18$, ICL: $P = 0.10$). Similarly, no differences were found among uncultured IPE with respect to coverage of RPE basement membrane versus ICL at day 1. There were no day-7 samples for uncultured IPE cells.

Table 2. Coverage of Day-7 Specimens with Cultured IPE

Donor Age (y)		% Coverage	
IPE	Explant	RPEbm	ICL
76	84	99.6	97.3
60	60	99.7	99.8
91	80	95.4	85.3
76	84	95.1	92.2
76	79	99.1	98.9
75.8 ± 11.0*	77.4 ± 10.0	97.8 ± 2.3*	94.7 ± 6.0†

* Mean ± SD.
† $P = 0.16$.

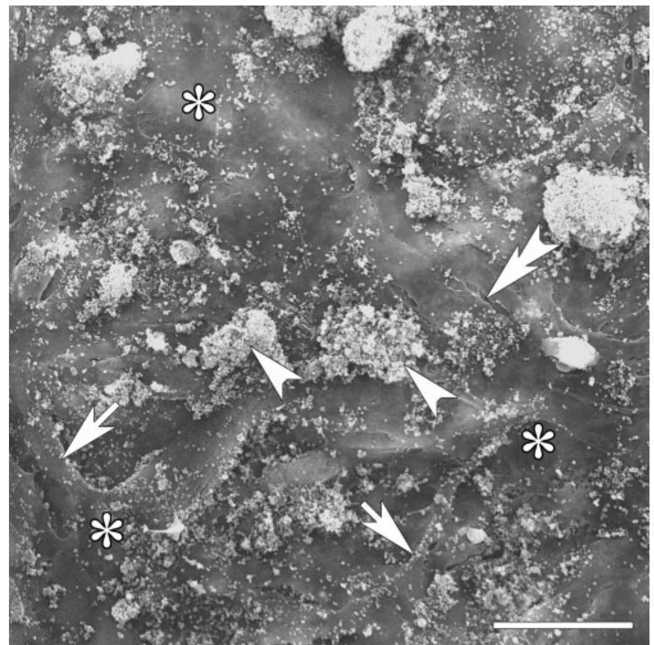

Figure 4. Cultured IPE cells from a 76-year-old donor 7 days after seeding on submacular RPE basement membrane (donor age, 84 years). Cells covering this explant formed a monolayer composed of enlarged, flattened cells (✻) and elongate cells (*arrows*). Small, intercellular gaps were present (*double arrowhead*). *Arrowheads*: cell debris. Scale bar: 50 μm.

Expression of Integrin Subunit mRNA

Subunit α1 mRNA expression was low in all four samples, both in uncultured and cultured IPE cells. Integrin α2, α3, and α4

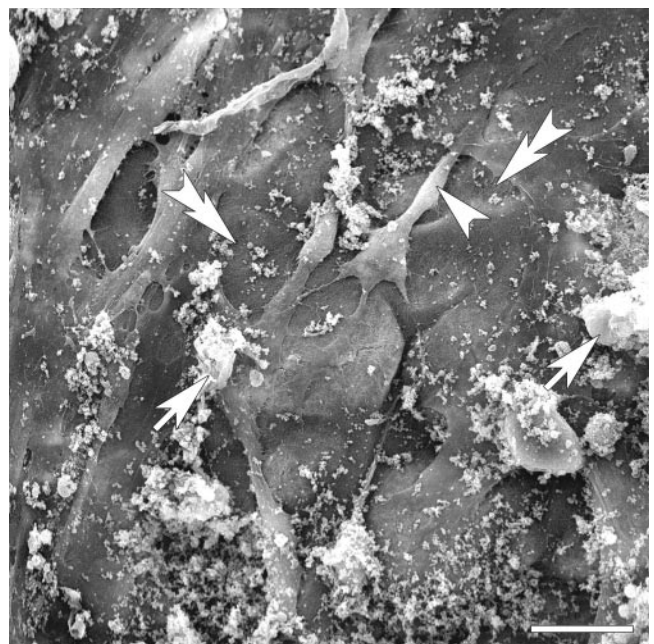

Figure 5. Cultured IPE cells from a 91-year-old donor 7 days after seeding on submacular ICL (donor age, 80 years). The coverage by cells on this donor was 85.3%. Some pseudopodia were rather thick (*arrowhead*). Dying or dead cells were also present (*arrows*). Uncovered areas revealed the ICL (*double arrowheads*). Scale bar: 50 μm.

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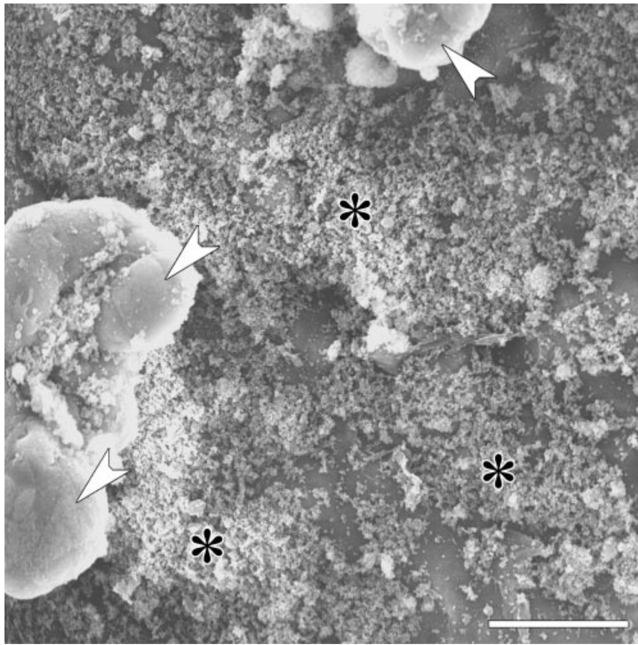

FIGURE 6. Uncultured IPE cells from a 74-year-old donor 1 day after seeding on submacular RPE basement membrane of the same donor. Most of the explant surface was covered with cell debris (✻). Intact cells in attached microaggregates were round and smooth (*arrowheads*). Scale bar: 50 μm.

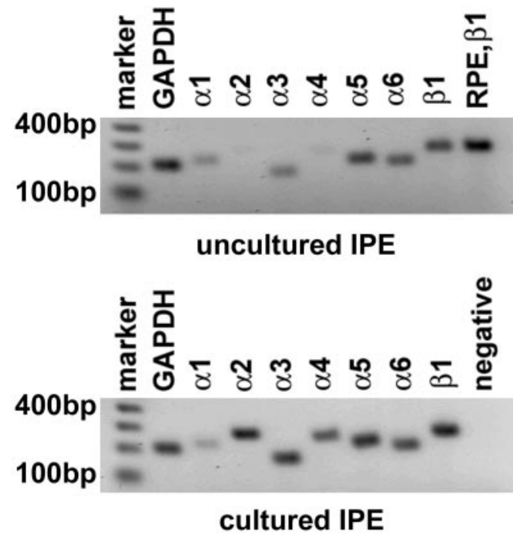

FIGURE 7. Agarose gel electrophoresis of RT-PCR products. *Top*: Bands of $\alpha2$ and $\alpha4$ integrin subunit mRNA were not clearly visible in uncultured IPE cells. Bands of $\alpha1$ and $\alpha3$ were weak. *Bottom*: Expression of $\alpha2$ and $\alpha4$ was much stronger in cultured IPE than in uncultured IPE cells. A band of $\alpha3$ was distinct in cultured IPE cells. Expression of the $\alpha1$-subunit was weak both in uncultured and cultured IPE cells. Alpha5, $\alpha6$, and $\beta1$ integrin subunit mRNA were expressed similarly in both cell types.

mRNA expression was less in uncultured IPE cells than in cultured IPE cells in all samples studied (Fig. 7). In one sample, the $\alpha2$ and $\alpha4$ subunits were not expressed at all. Cultured fetal RPE mRNA run concurrently and analyzed for β-1 mRNA showed levels of expression similar to those of IPE.

DISCUSSION

This study has three main findings. First, we established an efficient method for human IPE isolation, using type IV collagenase. This method allows easy removal of IPE cells from their underlying substratum. Second, cultured IPE resurfaced native RPE basement membrane or ICL of aged submacular human Bruch's membrane better than uncultured IPE. Third, uncultured IPE cells have lower expression of $\alpha2$ to 4 mRNA integrin subunits compared to cultured IPE cells.

Human IPE can be isolated as single cells with trypsin but require mechanical removal (e.g., with forceps or a Pasteur pipet) from the underlying stroma.[28,36,41] Harsh digestion with trypsin and mechanical removal could result in cell damage.[37] In the present study, IPE cells were isolated readily with type IV collagenase. The use of type IV collagenase, unlike trypsin, causes no disruption of cell–cell attachment among the IPE cells. However, type IV collagen is also a major component of the basement membranes of vascular cells and fibroblasts. Long incubation times release cells from the iris stroma into the media, resulting in contamination by other cells. Therefore, we used collagenase at a high concentration and for a short incubation time. Because collagenase does not disrupt cell–cell adhesion, IPE cells were isolated readily as a sheet. The use of 0.25% trypsin/1 mM EDTA solution after IPE isolation to separate the sheets into single cells did not yield better results than cutting the sheet with scissors. Attachment to bovine corneal endothelial cell extracellular matrix (BCE-ECM)–coated dishes, rate of cell growth, and cell proliferation were not as good if the cells were treated with trypsin (data not shown).

TABLE 3. Coverage of Day-1 Specimens with Uncultured IPE

Donor Age (y)	% Coverage	
IPE, Explant	RPEbm	ICL
80	3.1	6.0
82	3.7	0.9
74	7.9	4.4
74	10.4	7.3
78	14.6	6.5
77.6 ± 3.6*	7.9 ± 4.8*	5.0 ± 2.5†

* Mean ± SD.
† $P = 0.085$.

The behavior in cell culture of IPE isolated from aged donors with collagenase was similar to that of RPE from aged donors isolated using the same method.[42] The onset of growth showed a similar time course after the initial attachment. In both cell types, the main source of new cells was from small cells migrating from the microaggregate (Fig 1D and 1E). In addition, both IPE and RPE cells from aged donors showed variability in the rate and amount of cell growth. The main difference between cultured IPE and cultured RPE were the presence of extremely elongate cells in IPE cultures (Fig. 1B). These extremely elongate cells persisted in cultures grown to confluence.

Freshly harvested, uncultured IPE cells from aged donors did not attach or survive on aged human submacular Bruch's membrane 1 day after seeding. This behavior is similar to that of uncultured RPE from aged donors.[23] IPE cells, as do RPE cells, showed better attachment and coverage of this surface after culture. In addition, cultured IPE cells seeded onto aged submacular Bruch's membrane behaved similarly to cultured aged adult RPE seeded onto the same surface and at the same density at day 1. A comparison of cell coverage of IPE and RPE cells at the same time point, in similar organ explant culture conditions,[24] showed no difference in coverage between the cell types on native RPE basement membrane or on the ICL

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(Student's *t*-test, $P = 0.19$). Both cell types resurfaced the native RPE basement membrane and ICL by day 1 but showed a high degree of variability in coverage on the ICL. This variability may reflect differences in the ICL surface due to aging changes (e.g., lipid deposition, advanced glycation end product formation).[43–45] Morphologically, IPE resurfaces both the ICL and RPE basement membrane with cells that are flatter than RPE. However, compared with cultured IPE and RPE cells from older donors, fetal RPE cells appeared to attach more readily to aged human submacular Bruch's membrane. In a previously reported study, by 4 hours after seeding, cultured fetal human RPE effectively covered 92% of a surface comprising predominantly native RPE basement membrane in aged human submacular Bruch's membrane specimens; >99% of the denuded Bruch's membrane surface was covered by day 1 after seeding.[21] In contrast to IPE, fetal RPE cells covered the surface with a confluent layer of small, polygonal cells with tight junctions formed between cells.

There may be several reasons for differences in the behavior of uncultured versus cultured IPE. It is possible that by culturing, only a selected population of healthier and more responsive cells is expanded. Freshly harvested IPE cells are in a relatively quiescent state, and the cultured cells are in an active state before seeding. In culture, the cells are exposed to growth factors in the serum as well as fibroblastic growth factor while growing on an ECM ligand-rich surface. The integrins are a major group of cell–matrix attachment molecules, and the integrins expressed may be a reflection of the composition of the surface on which the cells are growing. Culturing per se and culturing on different substrates can affect the expression, not only of integrins[24,46] but also of other molecules.[47] Any or all the changes that occur during IPE cell culture may contribute to better survival and resurfacing on aged submacular human Bruch's membrane.

We examined the changes in the expression of integrin subunits because these are primary receptors involved in cell attachment to the ECM. In addition to acting as adhesion receptors, integrin binding to matrix ligands activates signal transduction pathways leading to cell proliferation,[48] cell growth, and prevention of apoptosis.[49,50] Messenger RNA of the $\alpha2$, $\alpha3$, and $\alpha4$ subunits was expressed more in cultured than in uncultured IPE cells when the cultured cells were grown on BCE-ECM–coated dishes. The $\alpha2$ subunit is involved in cell adhesion to collagen (types I, III, IV), fibronectin, and laminin.[51–53] The $\alpha3$ subunit is involved in cell adhesion to collagen type I, fibronectin, and laminin.[51] The $\alpha4$ subunit is involved in cell adhesion to fibronectin.[54] BCE-ECM contains type IV collagen, fibronectin, and laminin.[55] A change in environment, such as removal of cells from their original basement membrane, growth on BCE-ECM, incubation in culture medium containing various cytokines, and the proportion of anterior and posterior IPE cells,[30] may underlie the differences in mRNA expression in cultured versus uncultured IPE cells. Different integrin subunit combinations can bind to the same extracellular matrix ligand, but the receptors recognize different sites. For example, $\alpha4\beta1$ and $\alpha5\beta1$ recognize the CS1 and RGDS sites, respectively, of fibronectin.[54,56,57] The ECM molecules found in Bruch's membrane include laminin, collagen IV, fibronectin and heparan sulfate in the basal lamina, and collagens I, II, and III in the ICL.[38,39,58] Although it is tempting to speculate that increased expression of the $\alpha2$, $\alpha3$, and $\alpha4$ integrin subunit mRNA may have improved the coverage of cultured IPE cells on Bruch's membrane, at this time we have no direct evidence that these subunits bind to molecules on Bruch's membrane and no direct evidence that lack of these particular integrin subunits is the critical reason for the poor survival of uncultured IPE cells on Bruch's membrane.

In explants incubated with cultured IPE cells for 7 days, most of the attached cells were remarkably flat or elongate. This morphology indicates that IPE cells grown on aged human submacular Bruch's membrane in organ culture had not differentiated, even 7 days after seeding. We have no evidence from the cultures we studied that the IPE cells elaborated a basement membrane during the first 7 days in organ culture. By 7 days in cell culture, human RPE cells deposit ECM ligands,[59] but as far as we know, they have not been shown to deposit a basal lamina per se at this time point. Factors that promote differentiation of IPE cells to acquire RPE functions have not been elucidated. There is some evidence that cultured IPE cells show signs of differentiation. For example, they have been shown to express mRNA of RPE65 and 11-*cis*-dehydrogenase (although the protein expression has not been demonstrated), and cultured IPE can phagocytose photoreceptor outer segments.[31–33,60,61] Morphologic data from this study suggest that cultured IPE cells may not differentiate adequately to support photoreceptor survival if grown on aged submacular human Bruch's membrane. Determining whether the cells are capable of taking on RPE-like functions despite an undifferentiated morphology in the explant system requires further study. We note that the organ culture system used in these experiments lacks an overlying neural retina, an underlying functional choriocapillaris circulation, and vision-related cellular activity. The latter features may influence IPE cell differentiation on Bruch's membrane. In view of the absence of bona fide animal models of the aging of human Bruch's membrane, however, this organ culture paradigm provides an opportunity to begin to study cell-aged submacular human Bruch's membrane interactions.

Although autologous IPE transplants in patients do not appear to be subject to rejection, the results do not indicate improved visual outcome after IPE transplantation, even if the cells are cultured before transplantation. Abe et al.[28] reported the results of seven patients who underwent CNV removal and autologous cultured IPE transplantation. Six of seven patients showed improved visual acuity. However, no significant difference was seen in comparison with CNV removal without IPE transplantation. No rejection was recognized during the follow-up period (6–14 months). Results using uncultured IPE cells were not significantly different, which is consistent with the results we report in this study.[29,37]

In conclusion, cultured IPE cells were found to be more suitable than uncultured cells for transplantation onto aged submacular human Bruch's membrane, regardless of whether the attachment surface was native RPE basement membrane or the ICL. Cultured IPE cells adhered equally well to Bruch's membrane native RPE basement membrane and ICL in these experiments. Use of autologous IPE cells for transplantation is attractive because the cells can be obtained relatively easily and safely, and graft rejection should not occur. However, because in these experiments the IPE cells did not appear morphologically differentiated on aged submacular human Bruch's membrane, their suitability for transplantation in patients with AMD is uncertain. Further experiments are needed to determine whether IPE cells can assume RPE functions on the attachment surface encountered in situ after transplantation in AMD-affected eyes that have undergone CNV excision.

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