# ANALYSIS OF RETINAL PIGMENT EPITHELIUM INTEGRIN EXPRESSION AND ADHESION TO AGED SUBMACULAR HUMAN BRUCH'S MEMBRANE

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**ABSTRACT**

*Purpose:* Uncultured aged retinal pigment epithelium (RPE) does not resurface aged Bruch's membrane after 24 hours in organ culture. These experiments assess whether culturing alters RPE integrin expression and resurfacing of Bruch's membrane.

*Methods:* RNA was isolated from uncultured and cultured RPE of aged adult donor and fetal eyes. Integrin subunit messenger RNA (mRNA) expression was studied by reverse transcriptase–polymerase chain reaction (RT-PCR) and semiquantitative analysis of the amplified products. Cell surface integrin expression was assessed using flow cytometry. Passaged cultured fetal RPE and primary cultured aged RPE were seeded onto Bruch's membrane, and resurfacing was assessed with scanning electron microscopy.

*Results:* Uncultured fetal RPE had low levels of α3 and β5 mRNA compared to passaged cultured fetal RPE. Uncultured aged RPE had decreased α1-5 mRNA compared to primary cultured aged RPE. Cultured aged RPE had decreased β4 and β5 mRNA compared to passaged cultured fetal RPE. Flow cytometry confirmed the expression of α1-5, αv, and β1 protein on cultured fetal RPE and α1-3 and β1 protein on cultured aged RPE. Twenty-four hours after seeding, cultured fetal and aged RPE resurfaced 99% ± 1.3% and 76% ± 22%, respectively, of aged submacular Bruch's membrane specimens from which native RPE had been debrided, exposing the native RPE basement membrane. Cultured fetal and aged RPE resurfaced 97% ± 3.1% and 39% ± 35%, respectively, of specimens in which the inner collagenous layer was exposed.

*Conclusions:* Uncultured aged RPE has low amounts of integrin subunits that form receptors for laminin, fibronectin, and collagens. Culturing up-regulates integrins and promotes more efficient aged RPE attachment to and survival on aged Bruch's membrane.

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## INTRODUCTION

The main goal of this research is to develop a treatment that will restore lost vision and stabilize good visual acuity in patients with subfoveal choroidal neovascularization due to age-related macular degeneration (AMD). The central hypothesis of this research is that choroidal new vessel (CNV) excision coupled with effective retinal pigment epithelium (RPE) replacement can restore lost vision and preserve good visual acuity in patients with AMD-associated CNVs, provided that surgery is undertaken before significant photoreceptor atrophy has occurred.

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### BACKGROUND AND SIGNIFICANCE

AMD is the major cause of irreversible loss of central vision among the elderly.[1-7] Approximately 70% of cases of severe visual loss in AMD are due to growth of CNVs under the RPE and retina with secondary exudative retinal detachment, subretinal hemorrhage and lipid exudation, and outer retinal degeneration.[8-11] CNVs can be classified according to their appearance on fluorescein angiography.[12] CNVs that hyperfluoresce and leak dye relatively early during fluorescein angiography and are well circumscribed are termed *classic* CNVs. CNVs that leak dye later in the angiogram are termed *occult* CNVs. CNVs that exhibit features of both classic and occult CNVs are termed *mixed* CNVs. Most CNVs are occult or mixed.[13-15]

Current treatments for CNVs in AMD patients generally do not yield good visual acuity. Laser photocoagulation and photodynamic therapy (PDT) are the only CNV treatments whose effectiveness is proved in randomized prospective multicenter clinical trials. Only a

minority of AMD patients with CNVs (~20%) is eligible for laser photocoagulation,[13,14] which itself is associated with poor visual outcome and a high rate (~50%) of recurrent CNV growth.[16-21] Four years after treatment, the average visual acuity with laser photocoagulation of new subfoveal CNVs is 20/320 versus 20/500 in untreated controls.[19] CNV photocoagulation also can be associated with an immediate decrease in vision, although, on average, treated eyes have better visual acuity than untreated eyes by 1 to 2 years after treatment. Eyes with occult CNVs and poorly demarcated boundaries are not good candidates for laser photocoagulation.[22] Also, in the case of classic CNVs, if lesions are too large or if patients have good visual acuity, laser photocoagulation is not an attractive option. In such patients, laser photocoagulation can cause severe central visual loss. A treatment benefit from PDT has been shown for eyes with predominantly (ie, ≥50%) classic CNVs and occult without classic CNVs in AMD.[23,24] The visual results with PDT are modest. Two years after the initiation of therapy, 59% of untreated patients with predominantly classic CNVs experienced moderate visual loss compared to 31% of treated patients. One year after PDT treatment, the mean visual acuity is 20/160 + 2 versus 20/200 in controls. Only about 16% of treated eyes experienced an improvement of ≥1 line (versus 7% of control eyes). Many AMD patients with CNVs are not eligible for PDT.[13-15] 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 only in a minority of cases. Restoration/preservation of reading vision after these therapies is uncommon.

Submacular surgery with CNV excision offers the possibility of removing large CNVs while preserving the overlying retina, thus preventing/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 CNVs.[25] In the only published 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 laser-treated eyes and 14 (50%) of 28 surgery-treated eyes had visual acuity that was better than or no more than 1 line worse than the baseline level.[25] Visual recovery after CNV excision is usually poor in AMD patients.[26,27] Clinical and histopathological studies indicate that in AMD patients, CNV excision is usually associated with (1) removal of adjacent native RPE and RPE basement membrane[28-31] and (2) incomplete/aberrant RPE growth into the dissection bed.[31-36] Lack of RPE ingrowth into the dissection bed probably results in choriocapillaris and photoreceptor atrophy.[31,35-41] These findings indicate that RPE transplantation might improve visual outcome after CNV excision in AMD patients.

Our research goal is to develop an improved treatment for the exudative complications of AMD that results in preservation or restoration of precision vision (ie, visual acuity that would sustain activities of independent living, such as reading and driving). Because of the limitations in laser photocoagulation and PDT, many alternative approaches to treatment are being explored, including gene therapy, anti-angiogenic therapy, radiation therapy, feeder vessel photocoagulation, thermotherapy, surgery to relocate the affected retina, intravitreal injection or oral administration of steroids, and cellular transplantation combined with CNV excision.[23,24,42-59] It is not clear at this time that any single treatment will both be applicable to a substantial majority of AMD patients with CNVs and provide or maintain excellent visual acuity.

## RATIONALE FOR RPE TRANSPLANTATION

Combined CNV excision–RPE replacement might be a treatment that restores good visual acuity and is applicable to many people, including those not treatable by any currently available modality. This reasoning is based on (1) the ability to excise all CNVs, (2) good visual results in some patients undergoing macular translocation, and (3) the ability of RPE to rescue photoreceptors and reconstitute the choriocapillaris in experimental models. As already noted, the rationale for surgical excision of CNVs is that removal of the CNV eliminates the source of bleeding, serous retinal detachment, and subretinal fibrosis, all of which can cause retinal atrophy. Thus, before excessive photoreceptor atrophy occurs, patients with subfoveal CNVs have potentially reversible visual loss.[60] Surgical techniques exist for complete CNV excision (including classic, occult, and mixed CNVs), but visual outcome after such surgery is usually poor in AMD patients.[26-28,31,35,61] Why is vision poor after CNV excision in AMD patients? Potential explanations include preoperative, intraoperative, and postoperative factors, as detailed in the following paragraphs.

### *Preoperative Factors*

*Photoreceptor Degeneration.* Most surgeons do not recommend CNV excision unless visual acuity is 20/200 or less, which may mean some degree of photoreceptor degeneration has occurred.[26-28,31,35,61] However, patients with CNVs due to the presumed ocular histoplasmosis syndrome (POHS) with vision of 20/200 or worse often can improve to 20/40 or better after CNV excision,[28,62-64] so preoperative visual acuity per se may not be a decisive limiting factor provided that surgery is undertaken in a

timely manner.

*Choriocapillaris Degeneration.* Ramrattan and coworkers[65] showed that there is a progressive decrease in the thickness of the choroid from 200 µm at birth to 80 µm by age 90 years. Choriocapillaris density and lumen diameter decrease, and the width of the intercapillary pillars increases with age.[37,60,65-69]

*Intraoperative Factors*

*Retinal Damage.* The existence of retinal damage is proven by intraoperative clinical observation of retina-CNV adhesions and the presence of photoreceptor fragments in excised CNV complexes.[29,31] Similar damage can occur, however, with CNV excision in patients with POHS, yet one can still obtain significant visual recovery after surgery.[29,30]

*Removal of CNV-Associated RPE.* Histopathology of excised CNV complexes demonstrates the presence of native RPE and fragments of subjacent RPE basement membrane with CNVs in 70% to 90% of AMD cases, owing to the intimate association of RPE with CNVs.[29-31] In POHS, on the other hand, CNVs sometimes grow internal to the RPE (rather than external to it, as is more often the case in AMD), which permits CNV excision with preservation of subjacent RPE.[70] The preservation of subfoveal RPE may contribute to the better visual outcome after CNV excision in POHS versus AMD patients.[71,72]

*Intraoperative Choriocapillaris Damage.* Histopathology of excised CNVs rarely demonstrates the presence of choriocapillaris.[29-31]

*Postoperative Factors*

*Subfoveal Choriocapillaris Nonperfusion.* Choriocapillaris nonperfusion is seen in about 75% to 90% of AMD patients after CNV excision and very likely is an important factor limiting visual recovery.[31,35,36,40] Choriocapillaris nonperfusion is probably related to RPE removal at surgery, although in some cases preoperative and intraoperative choriocapillaris damage may play a role. Choriocapillaris nonperfusion is indirect evidence for inadequate RPE resurfacing of the dissection bed.[41,73-77] Human histopathological studies have provided direct evidence for inadequate RPE resurfacing of the dissection bed.[33,34] Long-term human studies suggest that choriocapillaris nonperfusion can progress after CNV excision, which also indicates inadequate RPE survival in the dissection bed.[78]

*Postoperative Photoreceptor Degeneration.* Human clinical studies indicate that periods of macular detachment up to 2 weeks are compatible with recovery of visual acuity of 20/50 in a substantial number of patients.[79] Monkey and cat experiments indicate that many photoreceptors survive during retinal detachment periods of several weeks duration, although some photoreceptors definitely die.[80,81] Approximately 80% of the cat outer nuclear layer survives during 3 days of retinal detachment.[82] The number of photoreceptor nuclei in detached cat retina does not begin to decline significantly (ie, >20% decline in density) until detachment periods of over 13 days.[83] The holangiotic cat retina is rod dominated, however. Data from cat retinal detachment studies indicate that 14-day detachment followed by 30-day reattachment is associated with rod and cone outer segment length similar to that observed after 5-day detachments.[81] In contrast, preliminary data regarding cone survival indicate that cones may be more prone to apoptosis with detachment (versus rods) and that 44% of cones die during a 3-day detachment in cats.[84] (It is not clear that all cones in detached retina were identified in the latter study owing to down-regulation of cone-identifying molecules [eg, calbindin D] after detachment.) Additional experiments in the cone-dominated ground squirrel confirm these impressions.[85] While published experimental data do not indicate clearly what the exact survival of cones is after 2-week periods of retinal detachment, a reasonable estimate is that 40% to 60% survive in otherwise healthy retina. In addition to duration of detachment, the height of detachment influences photoreceptor survival. The macular detachments that would arise from CNV excision are shallow (<2 to 3 mm height), which also favors photoreceptor survival during a 2-week RPE resurfacing period. Since clinical data indicate that relatively small numbers of preserved cones are needed to support visual acuity of 20/30,[86] it appears that enough photoreceptors could survive combined CNV excision and RPE transplantation to support reading vision, provided that a properly functioning RPE monolayer can be re-established within 2 weeks of surgery. Thus, RPE transplantation, which is demonstrably effective in laboratory animals (please see below), probably is feasible in human patients despite its possible association with photoreceptor death during the process of resurfacing Bruch's membrane.

Visual results with macular translocation surgery might mean that combined CNV excision–RPE transplantation will result in good visual acuity in patients with AMD. Results of an uncontrolled retrospective consecutive series indicate that by 6 months after surgery, 15 (48%) of 31 patients gained ≥2 lines of vision on a Snellen chart.[45] (Complications and physical constraints on the degree of macular relocation achieved routinely may limit the number of patients who are eligible for treatment with this approach.[44-48,87,88])

## RESULTS OF RPE TRANSPLANTS IN HUMANS WITH AMD

Thus far, allogeneic RPE transplants in AMD patients

that have undergone CNV excision have failed, with resulting poor vision and, in patients who are not immune suppressed, subretinal fibrosis and chronic fluid leakage in the dissection bed.[50,89-93] Why?

*Immune Rejection*

RPE transplants are rejected in mice, despite the presence of some degree of immune privilege in the subretinal space.[94-96] Possible immune rejection of RPE allografts has been described in other experimental animals.[97-100] Transplants in some AMD patients undergoing CNV excision have used allogeneic cultured, fetal human RPE, which renders them susceptible to rejection; culturing also can induce major histocompatibility antigen expression.[101] In a pilot study, AMD patients receiving uncultured adult human RPE underwent immune suppression with prednisone, cyclosporine, and azathioprine, and the grafts did not appear to be rejected.[91] These elderly patients, however, could not tolerate systemic triple therapy for an extended period of time. Local immune suppression is somewhat effective in laboratory experiments,[102] but it has not been reported in human RPE transplants. Methods for harvesting RPE for autologous transplants exist,[103,104] but in vitro data indicate that harvested aged human RPE does not adhere well to aged submacular Bruch's membrane, even in the presence of native RPE basement membrane.[105] If effective immune suppression can be achieved, then use of allogeneic cultured cells could be considered.[106,107]

*Graft Failure*

Binder and coworkers[53] have transplanted autologous RPE in AMD patients undergoing CNV excision. Most patients achieved postoperative visual acuity close to 20/200. Three (21%) of the 14 patients had postoperative visual acuity of 20/60 or better. Of these three, 1 patient (7%) had preoperative visual acuity of 20/50. Thus, it is not clear that autologous RPE transplantation had a significant effect on visual outcome in the majority of patients described in this report. In this series and in the previously cited reports of RPE transplants in AMD eyes, the RPE transplants may not have survived in the subretinal space independent of immune rejection. Tezel and Del Priore[108] have shown that if RPE cells cannot adhere to their basement membrane (or a comparable surface) within 24 hours, they undergo apoptosis. All previous demonstrations of successful RPE transplants in laboratory animals have involved transplantation onto normal Bruch's membrane or onto native RPE (eg, references [96-100,102,103,106,109-123]). In AMD, Bruch's membrane is itself abnormal as a result of lipidization, protein cross-linking, protein deposition, and changes in hydraulic conductivity.[124-130] Transplanted fresh adult human RPE does not adhere or show limited adherence to aged submacular human Bruch's membrane in vitro.[105] Consistent with these in vitro observations, histopathology of an immune suppressed patient that underwent CNV excision plus RPE transplantation indicates that the cells were not organized as a monolayer, and there was photoreceptor atrophy over the transplant.[52] The poor visual results associated with autologous iris pigment epithelium transplants may result from graft failure and, perhaps less likely, from limitations in the ability of iris pigment epithelium to replace RPE.[131-140] Currently, techniques for routine harvest and transplantation of stem cell RPE precursors are not available.

## BIOLOGY OF CELL ATTACHMENT

Analysis of human CNV excision–RPE transplant failure leads one to focus on improving human RPE adhesion to and survival on aged submacular human Bruch's membrane in the presence and absence of native RPE basement membrane.

*Biology of Cell Attachment in Model Systems*

Cells adhere to their extracellular matrix by binding to ligands in the extracellular matrix. In metazoa, this binding is mediated primarily by a class of proteins called *integrins*.[141] Integrins are a family of transmembrane heterodimers (ie, comprising an $\alpha$ and a $\beta$ subunit). There are 18 $\alpha$ and eight $\beta$ subunits that are known to assemble into 24 distinct integrins.[141] Integrin heterodimers utilizing the $\beta 1$ subunit tend to mediate epithelial cell–extracellular matrix interactions.[142] Following binding to the extracellular matrix, integrins aggregate at focal sites of contact in which many proteins are assembled and activated on the integrins' cytoplasmic ends. This protein assembly is termed a focal adhesion.[141,143] The reorganization of cytoskeletal and other adhesion-related proteins leads to cell adhesion. Upon adhesion to a suitable substrate, anchorage-dependent cells respond efficiently to growth factors through activation of protein kinase C and mitogen-activated protein kinase.

Among the proteins comprising a focal adhesion, focal adhesion kinase (FAK) plays an important role as a positive regulator of cell migration and proliferation and in the prevention of apoptosis. FAK is a tyrosine kinase that binds to the $\beta$ subunit of integrins either directly[144] or via talin and undergoes autophosphorylation at tyrosine residue 397.[145] Recruitment and phosphorylation of FAK initiate a number of signal pathways that result in cell growth.[146-149] Tyrosine phosphorylation of FAK followed by binding of Src kinases eventually leads to activation of Rho, CdC42, Rac, Ras, and phosphoinositide 3-kinase (PI-3K) that ultimately results in cell adhesion,

proliferation, and inhibition of apoptosis.[146,150-152]

Focal adhesions confer structural integrity by influencing reorganization of cytoskeletal elements in the cell. Stable focal adhesions result in cell adhesion, while dynamically regulated formation of focal adhesions is a key element in cell migration. Assembly of focal adhesions is under the control of the small GTPase, Rho, whose activity is under the control of FAK.[153-155]

*Factors Affecting Cell Adhesion*

As already noted, cell adhesion to the extracellular matrix is mediated primarily by integrins. Cell-permeable inhibitors of the $Ca^{2+}$-dependent protease, calpain, stabilize peripheral focal adhesions, increase adhesiveness, and decrease the rate of cell detachment.[156] When increases in cytosolic $Ca^{2+}$ concentration are inhibited, neutrophils adhere to fibronectin or vitronectin.[157] Inhibitors of nitric oxide synethetase, NG-monomethyl-L-arginine or NG-nitro-L-arginine-methyl ester HCL, increase the number of adherent and immigrated leukocytes in cat mesenteric preparations.[158] This effect is partially reversed by L-arginine.

*Anoikis*

Adhesion to a substrate is essential for the survival and proliferation of anchorage-dependent cells.[159-161] Anchorage-dependent cells dissociated from their extracellular matrix undergo apoptosis. Cell death induced due to loss of anchorage is termed *anoikis*.[162-164] Cell shape (mediated by the cytoskeleton) and signal pathways activated by cell binding to the extracellular matrix regulate apoptosis.[165,166] Rescue from cell death involves cooperation of cytoskeletal proteins and certain integrin molecules. In some cells, for example, the integrin subunit, α5, can rescue cells from anoikis, but αv cannot. In the presence of constitutively active FAK, apoptosis can be prevented in unattached cells,[163] while inactivation of FAK causes apoptosis.[167] As already noted, FAK activates PI-3K. PI-3K is a lipid kinase that is involved in integrin-initiated signal transduction. It is a heterodimeric enzyme comprising a catalytic 110 kd subunit (p110) and a 85 kd regulatory subunit (p85). PI-3K associates with FAK following integrin activation,[168,169] which in turn activates protein kinase B and Akt. Pro-apoptotic molecules Bad, caspase 9, and repressing fork head transcription factor are all inactivated by protein kinase B/Akt, thus preventing apoptosis.[170] (PI-3K also plays a regulatory role in cell migration.[150,171-175]) Also, integrin subunit α5-mediated attachment results in detectable levels of bcl-2 that are not seen with other integrins.[176] Bcl-2 inhibits caspases and, ultimately, apoptosis. In addition, bcl-2 expression leads to decreased p21 and p27 (in suspended cells), which arrests the cell cycle. Such an arrest is a requirement for apoptosis to occur.[177] Rapid, complete attachment to the dissection bed by transplanted RPE cells is highly desirable to avoid apoptosis,[108] to avoid egress of RPE into the vitreous cavity, which can lead to complications such as epiretinal membrane formation,[178] and to promote photoreceptor survival.[39,179]

*Relevance to RPE Transplantation in AMD Eyes*

Histopathology of excised CNVs indicates that the surgical cleavage plane is through Bruch's membrane. In most (~70% to 90%) specimens, extensive fragments of native RPE basement membrane are removed with the CNV.[29-31] In vitro RPE adhesion experiments using cultured fetal and aged human RPE indicate that RPE adhesion to Bruch's membrane lacking native RPE basement membrane is deficient (please see discussion that follows).[105,180] Thus, the abnormal surface of the dissection bed may underlie inadequate resurfacing of the iatrogenic RPE defect, at least in part.

*RPE Attachment to Model Surfaces.* In vitro experiments have shown that normal, untransformed epithelial cells can attach rapidly and spread on attachment factors such as fibronectin, laminin, and epibolin.[181-186] In general, epithelial cells do not appear to have an absolute requirement for only one attachment protein.[184-187] Studies of RPE cells have shown that attachment and proliferation of these cells can be facilitated by combinations of laminin, fibronectin, collagen type IV, and other components of the extracellular matrix as well as by heparin and growth factors.[181,188,189] This lack of absolute specificity for attachment factors suggests that RPE cells are capable of interacting with multiple factors, and therefore a combination of the various attachment factors in appropriate proportions may facilitate the attachment of transplanted RPE cells onto denuded Bruch's membrane. This combination is likely present in the areas of residual RPE basement membrane (Table I). RPE basement membrane and the inner collagenous layer of Bruch's membrane contain laminin, fibronectin, vitronectin, and collagen type IV, and RPE cells contain a β1 integrin subunit.[182,183,190-192] Studies by Campochiaro and Hackett[188] show that RPE cells not only attach and proliferate in culture but also differentiate and show density-dependent inhibition when grown on laminin supplemented with basic fibroblast growth factor and heparin. Ho and Del Priore[193] have shown that RPE attachment to Bruch's membrane is mediated in part by fibronectin, laminin, vitronectin, and collagen IV. Presumably, the presence of these ligands in the RPE basement membrane underlies the preferential adherence of RPE to RPE basement membrane versus other layers of Bruch's membrane.[105,108,180,194-196]

Several studies have demonstrated integrins in human RPE. Anderson and coworkers[197] demonstrated that cultured human RPE exhibit α5β1 immunoreactivity,

TABLE I: DISTRIBUTION OF VARIOUS EXTRACELLULAR MATRIX LIGANDS IN HUMAN BRUCH'S MEMBRANE[130,191]

| BRUCH'S MEMBRANE LAMINA | EXTRACELLULAR MATRIX LIGAND |
|---|---|
| RPE basement membrane | CIV, CV, laminin, HS |
| Inner collagenous layer | CI, CIII, CV, fibronectin, CS, DS |
| Elastic lamina | Elastin, CVI, fibronectin |
| Outer collagenous layer | CI, CIII, CV, fibronectin, CS, DS |
| Choriocapillaris basement membrane | CIV, CV, CVI, laminin, HS |

CI through CVI, collagen types I through VI; HS, heparin sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; RPE, retinal pigment epithelium.

and Meitinger and coworkers[198] showed that α2, α3, and α5 subunits are present in these cells. In an immunohistochemical study of two adult donor eyes (one had squamous cell carcinoma, and the other had choroidal melanoma) by Brem and coworkers,[199] RPE in situ expressed α4 and β2 subunits. Blocking β1 integrin subunit binding in human RPE decreases adhesion to RPE-derived extracellular matrix as well as to Bruch's membrane.[193] Studies performed using chick, monkey, and cat RPE have demonstrated the presence of α3, α6, and β1 integrin subunits.[181,197,200] Mousa and coworkers[201] showed that αvβ5 mediates the attachment of cultured human RPE, including the ARPE-19 cell line, to vitronectin, and α5β1 mediates binding to fibronectin.

RPE adhesion to extracellular matrices can be affected by drugs that modulate intracellular $Ca^{2+}$ (eg, ionomycin) and calmodulin signaling systems. Wagner and coworkers[202] showed that tamoxifen and the experimental calmodulin antagonist, J8, produced significant inhibition of RPE attachment that was partially inhibited by serum. Up-regulation of the protein kinase C pathway using the phorbol ester, phorbol myristate acetate, also inhibited RPE attachment to fibronectin. Down-regulation of cyclic AMP using 2′,5′-dideoxyadenosine caused a slight reduction in attachment.

*Human RPE Attachment to Human Bruch's Membrane.* Human RPE cells attach rapidly to RPE basement membrane compared to the other layers of Bruch's membrane.[105,180,194,195] The degree of attachment to and resurfacing of Bruch's membrane varies, however, with the source of RPE cells. Trypsin-harvested, cultured fetal human RPE attaches to aged human submacular Bruch's membrane explants with intact native RPE basement membrane and, by 1 hour after seeding, resurfaces 92% ± 9% of a 7-mm-diameter surface.[180] The percentage of resurfacing at 1 hour decreases to 37% ± 33% if the inner collagenous layer is exposed. In contrast, uncultured, collagenase IV-harvested aged RPE attaches to and resurfaces only 8.7% ± 2.6% of aged submacular Bruch's membrane explants with intact native RPE basement membrane and 0.63% ± 0.42% of aged submacular Bruch's membrane inner collagenous layer by 4 hours after seeding.[105] Twenty-four hours after seeding uncultured aged RPE, most of the aged human submacular Bruch's membrane surface is covered by debris, presumably from dying cells. Using cultured adult human RPE and peripheral human Bruch's membrane, Tezel and coworkers[194] showed that the attachment rate was highest on RPE basement membrane and was lower on the outer layers of Bruch's membrane. Similarly, the proliferation rate was highest and apoptosis rate was lowest on native RPE basement membrane compared to the other layers of Bruch's membrane. Cultured adult RPE resurfaced peripheral Bruch's membrane almost completely by 14 days when native RPE basement membrane was present but failed to do so on the inner collagenous layer even after 3 weeks.[195] Ho and Del Priore[193] reported that 6 hours after seeding, approximately 52% of cultured, passaged RPE cells from a 32-year-old donor attached onto submacular Bruch's membrane of 70- to 90-year-old donors. Other studies from the same laboratory also showed higher attachment rates with cultured RPE from older donors when seeded onto peripheral Bruch's membrane of older persons.[193,194,196] The higher attachment rate might reflect a difference in integrin expression of primary isolated RPE cells versus cultured cells. It also might reflect differences in the extracellular matrix composition of peripheral versus submacular Bruch's membrane.[126,203] Histology of excised CNVs,[29-31] histopathology of eyes after CNV excision,[33,34] and postoperative clinical findings[31,35,36,40] all suggest that the CNV dissection bed exposes both the superficial and deeper portions of the inner collagenous layer, which will constitute much of the surface to which transplanted cells must adhere and on which they must survive. In contrast to uncultured aged human RPE, cultured fetal human RPE can adhere and can spread to some degree on the inner collagenous layer.[180] Scanning electron microscopy studies show that a tightly woven "basket weave" of collagen fibers is present on the superficial inner collagenous layer surface, as described by Goldbaum and Madden.[204] In addition, the collagen fibers are fused in some areas, leaving a smooth surface that may be more suitable for cell attachment owing to greater extracellular matrix ligand availability.[165,205]

Our research group hypothesized that differences in integrin expression might be responsible for the variation in attachment and resurfacing on different layers of Bruch's membrane by uncultured aged and cultured fetal human RPE. This hypothesis could not be confirmed or refuted by previous studies of integrin expression in RPE

cells. Therefore, in this study, the expression of cell-extracellular matrix adhesion-mediating α and β integrin subunits was compared in uncultured and cultured aged and fetal human RPE using the reverse transcriptase–polymerase chain reaction (RT-PCR). The presence of these receptors on the cell surface was assessed with flow cytometry. The effect of cell culture–induced changes in integrin expression on attachment was examined by seeding cultured RPE on aged submacular human Bruch's membrane.

## METHODS

### DONOR TISSUE

Adult human donor eyes were obtained from various eye banks in the United States through the National Disease Research Interchange, Philadelphia, PA, and the North Carolina Eye Bank, a Vision Share (Apex, NC) member eye bank. Eyes from donors ≥55 years were used. Mean donor age was 72 ± 8.2 years (n = 22). Eyes from fetuses of 14 to 20 weeks gestation period were obtained through Advanced Bioscience Resources, Inc (ABR, Alameda Calif) or the Central Laboratory for Human Embryology (University of Washington, Seattle, Wash). The mean fetal age was 18 ± 3.8 (n = 8) gestational weeks. This research followed the tenets of the Declaration of Helsinki and was approved by the institutional review board of the New Jersey Medical School.

### RPE CULTURE

The external surface of donor eyes was trimmed, and globes were immersed in 10% povidone iodine briefly. This was followed by two 10-minute incubations in Dulbecco's modified Eagle's Medium (DMEM) (Mediatech, Herndon, VA) containing 2.5 μg/mL amphotericin B. The anterior segment, vitreous, and retina were dissected out. Posterior segments of fetal eyes, consisting of RPE-choroid and sclera, were incubated in 0.8 mg/mL collagenase type IV (Sigma, St Louis, MO) at 37ºC in 10% $CO_2$ for approximately 90 minutes. In the case of aged human eyes, the RPE-choroid layers were detached from the sclera and incubated in 0.4 mg/mL collagenase type IV for 30 to 45 minutes. RPE sheets were dissected out carefully at the end of the incubation using 25-gauge needles, rinsed several times in DMEM, triturated with a 200-μL pipette, and plated on bovine corneal endo thelial cell–extra cellular matrix (BCE-ECM)-coated dishes.[104,105,180] Cells were cultured in complete medium, which is DMEM supplemented with 15% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 2 μM L-glutamine (Gibco Life Technologies, Carlsbad, Calif), 2.5 μg/mL amphotericin B (Gibco-BRL, Grand Island, NY), 50 μg/mL gentamicin (Gibco Life Technologies), and 1 ng/mL basic fibroblast growth factor (Gibco Life Technologies) at 37ºC in 10% $CO_2$[180] Upon reaching confluency, fetal cells were passaged at 1:4 ratios while adult RPE, not passaged, was used for RNA, flow cytometry, or attachment studies. Second-passage fetal cells were suspended in 90% fetal bovine serum/10% dimethyl sulfoxide and frozen at –80ºC until further use. Cultured fetal RPE of second to fourth passage were used for these experiments. Purity of the cultures was determined by morphology and cytokeratin staining.[104] For cytokeratin staining, cells were fixed with ice-cold methanol for 5 minutes, then blocked for 20 minutes with 2% bovine serum albumin, 0.2% Triton, and 2% normal goat serum. Monoclonal anticytokeratin antibody (AE1/AE2 cocktail; Biogenex, San Ramon, Calif) was applied at a concentration of 1:50 overnight at 4°C. The keratin antibody was diluted with blocking solution. For negative controls, cells were incubated with blocking solution instead of primary antibody. After washing with cold phosphate buffered saline (PBS), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma) was applied at a concentration of 1:100 for 1 hour at room temperature. Cells were washed with cold PBS and mounted with Vectashield fluorescent mounting medium (Vector Laboratories, Burlingame, Calif). Slides were examined with fluorescent microscopy.

### REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

To obtain RNA from uncultured RPE, the posterior eyecup was prepared from adult donor and fetal eyes as already described, and the RPE was gently brushed off into Trizol (Gibco, Rockville, MD) solution. Total RNA was extracted from uncultured (adult, n = 5; fetal, n = 4) or cultured (adult, n = 4; fetal, n = 4) RPE using Trizol according to the manufacturer's instructions. Genomic DNA was digested by incubating RNA samples in excess of RNase-free DNAse I (Gibco) prior to amplification. Quality of the RNA was determined by electrophoresis of a small sample. Previously published gene-specific primer sequences were used with the exceptions of α1 and actin, which were designed using Primer 3 software (Table II).[206] RT-PCR was done with 0.1 mg of RNA using Superscript One-Step RT-PCR kit (Gibco) under the following conditions in a GeneAmp Perkin Elmer thermocycler: RT:cDNA synthesis 50ºC (54ºC for β4), 30 minutes; predenaturation 94ºC, 2 minutes; 35 cycles of PCR: denature 94ºC, 15 seconds; anneal 55ºC, 30 seconds; extend 72ºC, 30 seconds; final extension: 72ºC for 10 minutes. Since melanin inhibits PCR,[207] 0.1 mg of RNA was diluted with 2.5 mg of RNAse- and DNase-free bovine serum albumin (Sigma) for 30 minutes at 4ºC prior to use in the reaction mixture.[208] For negative controls, primers or total RNA were excluded from the reaction mixture, and

TABLE II: GENE-SPECIFIC PRIMERS TO HUMAN INTEGRIN SUBUNITS AND EXPECTED PCR PRODUCT SIZE

| PRIMER | 5′–3′ SEQUENCE | PRODUCT SIZE |
|---|---|---|
| α1 sense | AAGTGCAACAAGTGACAGCG | 237 |
| antisense | TCTGGCATTGGAAAAGATCC | |
| α2 sense | CACTCGATTTGGTTCAGCAA | 283 |
| antisense | GAACCACTTGTCCAAAGGCA | |
| α3 sense | GCCAGCATTGGTGACATCAA | 179 |
| antisense | GAATAGCCGAAGGTGGCCAA | |
| α4 sense | ATGCTGCAAGATTTGGGGAA | 265 |
| antisense | GCACCAACTGCTACATCTAC | |
| α5 sense | CCAGGATGGCTACAATGATG | 222 |
| antisense | CCCACAATCAGATCAGGATA | |
| α6 sense | CAAGATGGCTACCCAGATAT | 210 |
| antisense | CTGAATCTGAGAGGGAACCA | |
| αv sense | AGATCTGGACCAGGATGGTT | 197 |
| antisense | ATCTGTGGCTCCTTTCATTG | |
| β1 sense | GTTACACGGCTGCTGGTGTT | 264 |
| antisense | CTACTGCTGACTTAGGGATC | |
| β4 sense | AACGATGAACGGTGCCACCT | 222 |
| antisense | CTCCACGATGTTGGACGAGT | |
| β5 sense | AGGATGCACTGCATTTGCTG | 273 |
| antisense | TCCACCGTTGTTCCAGGTAT | |
| β6 sense | GGAATGGACAGCAAACTAGC | 243 |
| antisense | GGAGTCCTTCTGAAGTAGAC | |
| actin sense | AAGTACTCCGTGTGGATCGG | 286 |
| antisense | CACCTTCACCGTTCCAGTTT | |

RNAse-free water was used instead. PCR products were separated on 1.5% agarose gel, stained with 0.5 mg ethidium bromide for 30 minutes, and scanned using a fluorimager (Molecular Dynamics, Sunnyvale, Calif). The fluorescence intensities were normalized to that of actin. PCR products were sequenced using BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Inc, Foster City, Calif) on the model 3100 genetic analyzer (Applied Biosystems) at the New Jersey Medical School Molecular Resource Facility. Sequences were analyzed using Sequence Analysis software obtained from the same vendor and compared with the respective gene sequences available in the public domain using the National Center for Biotechnology Information's BLAST program.

### FLOW CYTOMETRY

Single cell suspensions of cultured adult and fetal cells were prepared by detaching cells from culture dishes with trypsin 0.5%/EDTA 0.5 mM solution. Cells were incubated with Dulbecco's phosphate buffered saline (DPBS; without $Ca^{2+}$, $Mg^{2+}$) containing 1% fetal calf serum and 1% pooled human serum (blocking solution) for 10 minutes to block nonspecific binding. This step was followed by incubation for 1 hour at 4ºC with one of the following mouse monoclonal antibodies (all from Chemicon, Temecula, Calif) at a dilution of 1:100: α1, α2, α3, α4, α5, αv, β1, α2β1, α5β1. Cells were washed and incubated in FITC-conjugated goat anti-mouse antibody (Sigma) at a dilution of 1:75 for 45 minutes. All washes and incubations were done in DPBS containing 1% bovine serum albumin and 0.1% $NaN_3$ and at 4°C to reduce metabolic activity of the cells. Dead cells were labeled with 10 μg/mL propidium iodide solution prior to analysis of the cells. Negative control samples included samples incubated with secondary antibody alone, stained with propidium iodide alone, and incubated with blocking solution alone. At least 10,000 cells were analyzed with a Becton Dickinson FACStar flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data from the cells were collected using a four-decade log amplifier.

### RPE ATTACHMENT TO BRUCH'S MEMBRANE IN ORGAN CULTURE

Submacular Bruch's membrane explants were prepared using previously published methods.[105,180] After dissecting out the anterior segment, vitreous, and retina from donor eyes, submacular RPE was debrided using a microsurgical sponge (Alcon, Fort Worth, Tex). One eye of each pair was debrided to create a surface with intact native RPE basement membrane by wiping the RPE gently two to four times with a sponge moistened with balanced salt solution (BSS; sodium chloride 0.64%, potassium chloride 0.075%, calcium chloride dehydrate 0.048%, magnesium chloride hexahydrate 0.03%, sodium acetate trihydrate 0.39%, sodium citrate dehydrate 0.17%, sodium hydroxide, hydrochloric acid, and water) under a dissecting microscope. The number of wipes was determined by the color change to a lighter appearance that occurs upon RPE removal. In the fellow eye, the inner collagenous layer was exposed by repeated (up to 50 times) firm wiping of the submacular Bruch's membrane with a relatively dry sponge. The technique has been validated using scanning

electron and transmission electron microscopy.[105] Using a 7-mm-diameter corneal trephine, a full-thickness button that included the underlying sclera was punched from the debrided macular area. With the button still in the trephine, the floor of the trephine was sealed with wax to support the tissue. Cultured fetal or aged human RPE was seeded onto the submacular Bruch's membrane explant preparation at a density of $1.21 \times 10^5$ cells/button in 200 μL of complete medium (please see discussion that follows). RPE-choroid-sclera explants were cultured with the sclera side down in 35-mm-diameter tissue culture dishes (Becton Dickinson) in a humidified atmosphere of 10% $CO_2$ and 90% air at 37°C. Explants were maintained in complete medium. Following a 24-hour incubation, the tissue was fixed in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The tissue was dehydrated using graded concentrations of ethanol, critical point dried (Tousimis Autosamdri-814, Rockville, MD), mounted onto aluminum stubs, and sputter-coated with 20 nm gold-palladium (Denton DESK II, Moorestown, NJ). Explants were examined with a scanning electron microscope (JEOL JSM-35C, Tokyo, Japan) equipped with a Digiscan image capture system (Gatan, Inc, Pleasanton, Calif) at 25 kV accelerating voltage. For measuring the area of the explant resurfaced by the cells, digital images were grabbed at 200× magnification from 8 to 10 nonoverlapping areas using Digital Micrograph software (Gatan). The area covered by the cells, as well as the total area of the 200× field, was measured using NIH Image J software, and the ratio was expressed as percent coverage.

#### STATISTICAL ANALYSIS

Fluorescence intensities of agarose gels of RT-PCR products were analyzed statistically by analysis of variance (ANOVA), and comparisons were made with the Tukey-Kramer test. If the data did not satisfy the assumptions of normal distribution (as determined using the Shapiro-Wilk W test), the Kruskal-Wallis rank test, a nonparametric test, was used. Resurfacing of Bruch's membrane by cultured fetal or aged human RPE was compared using Student's *t* test.

## RESULTS

#### REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

We hypothesized that differences in attachment of passaged cultured fetal RPE and uncultured aged human RPE could be due to differences in integrin expression. We extracted RNA directly from the eyecup rather than enzymatically isolate RPE first in order to control for the possibility that the amount of mRNA in cells would change during the short incubation with enzyme. This procedure was done under microscopic visualization, and there was no choroidal contamination as judged by the clinical appearance of the intact Bruch's membrane surface.

RT-PCR of integrin subunit mRNA showed low to absent expression of α1-5 and β6 in uncultured aged human RPE. Primary cultured aged RPE grown on BCE-ECM-coated culture dishes contained higher levels of these mRNAs (Figures 1 and 2). Expression of β4 and β5 subunit mRNA was similar in uncultured and cultured aged RPE. Uncultured fetal RPE had low levels of α3 and β4 mRNA, and expression of both of these mRNAs was higher in passaged cultured fetal cells. These RT-PCR experiments provide semiquantitative data and do not indicate the absolute mRNA transcript levels, but only indicate relative amounts for the four different cell types analyzed.

The Shapiro-Wilk W test indicated that the α2, α3, β4, and β6 mRNA fluorescence intensities were not distributed normally, reflecting the skewed distribution of their fluorescence intensities among the four different cell types, especially in uncultured aged human RPE. In these cases, the Kruskal-Wallis rank test was used to assess statistical significance of differences in fluorescence intensities among the different cell types. In all other cases, ANOVA was used for this assessment. The four cell types exhibited statistically significant differences in integrin mRNA subunit expression in the following cases: α1 ($P = .047$), α2 ($P = .0093$), α3 ($P = .0049$), α4 ($P = .016$), α5 ($P = .00040$), β4 ($P = .0091$), β5 ($P = .00010$), and β6 ($P = .016$). For a given integrin subunit mRNA exhibiting statistically significant differences among the four cell types, individual comparisons of mRNA expression among the four cell types were made using the Tukey-Kramer test. A $q$ of 2.94 (based on $\alpha \leq 0.05$ and 16 degrees of freedom) was used in the calculations of comparisons, and the following statistically significant differences were noted. Expression of α1, α2, α3, α4, and α5 mRNA in uncultured aged human RPE was significantly less than that in primary cultured aged RPE. Expression of α3 and β5 subunit mRNA was significantly less in uncultured than in passaged cultured fetal RPE. Expression of α2, α3, and α5 mRNA in uncultured aged RPE was significantly less than in passaged cultured fetal RPE. Expression of β5 mRNA was significantly higher in fetal RPE than in aged RPE regardless of whether the cells were uncultured or cultured. Cultured fetal RPE had significantly higher levels of β4 mRNA compared to the other three cell types. With the exceptions of the β4 and β5 subunits, there were no statistically significant differences in integrin mRNA expression between passaged cultured fetal and primary cultured aged RPE. Despite the finding that β6 integrin subunit mRNA expression exhibited significant variation

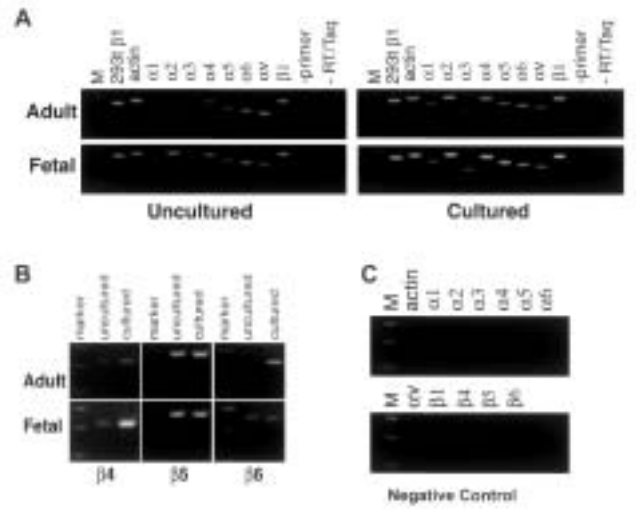


FIGURE 1
Expression of integrin subunit mRNA in cultured and uncultured fetal and aged human RPE. Integrin mRNA was amplified by RT-PCR using gene-specific primers. PCR products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. A, Expression of α1-6, αv, and β1 subunit mRNA. Uncultured aged human RPE shows low levels of α1-4 mRNA compared to cultured aged or fetal RPE. α3 mRNA is low in uncultured fetal RPE. M = 100bp marker; 293t cells with β1 primers used as positive control. Primers were eliminated in the –primers lane. Reverse transcriptase and Taq polymerase enzymes were eliminated in the –RT/Taq lane. B, Expression of β4, β5, and β6 subunit mRNA. β6 is absent in uncultured aged human RPE. Marker = 100bp. C, Negative control. No bands were seen when RNA was eliminated from the samples.

with the Kruskal-Wallis rank test, Tukey-Kramer testing did not reveal significant differences in β6 subunit mRNA expression among the four cell types.

### FLOW CYTOMETRY

Since mRNA expression does not demonstrate the presence of integrin proteins on the cell surface and since integrins can undergo posttranslational modifications, flow cytometry studies were done to explore further the results of RT-PCR experiments. Cultured fetal RPE expressed α1, α2, α3, α4, α5, αv, and β1 subunits on the surface (Figure 3). In addition, the heterodimers α2β1 and α5β1 were expressed. Consistent with the RT-PCR data, primary cultures of aged human RPE expressed α1 and α3 subunits and the α2β1 heterodimer on the cell surface. At least three samples were examined for each cell type and for each integrin subunit or heterodimer tested. Experiments (n = 2) using nonenzymatic isolation of cells did not show any difference in integrin expression compared to trypsinized cells (data not shown). Similarly, incubation of isolated cells for 20 minutes at 37°C before proceeding with the flow cytometry experiment did not reveal any significant differences in expression of integrins on the surface compared to cells that were not incubated (n = 2; data not shown).

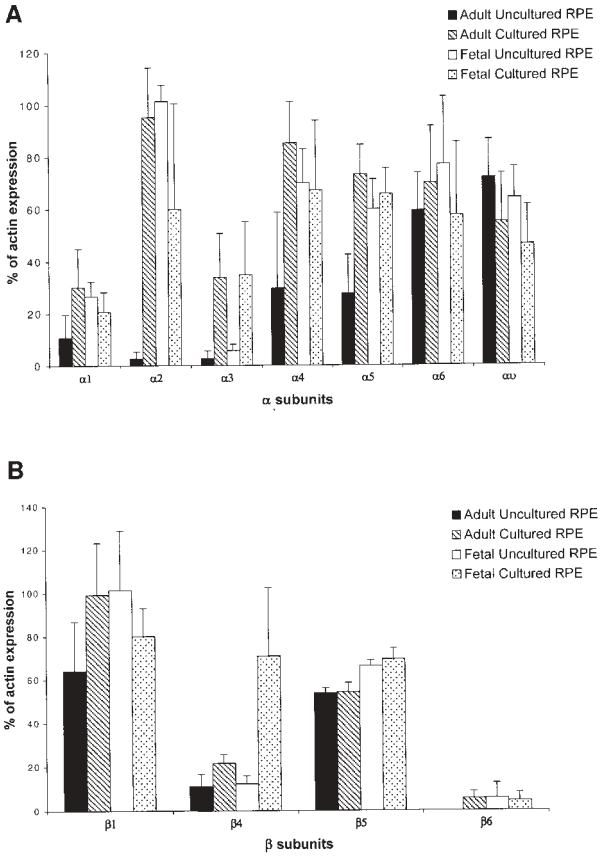


FIGURE 2
Semiquantitative analysis of integrin subunit mRNA expression (A, α subunits; B, β subunits) in cultured and uncultured fetal and aged human RPE relative to the expression of actin. RT-PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and the intensity of fluorescence was measured with a fluorimager. Uncultured fetal RPE had low levels of α3 and β5 mRNA compared to passaged cultured fetal RPE. Uncultured aged human RPE had decreased α1-5 mRNA compared to primary cultured aged RPE. Cultured aged human RPE cells had decreased β4 and β5 mRNA compared to passaged cultured fetal RPE.

### CULTURED RPE ATTACHMENT TO AGED HUMAN BRUCH'S MEMBRANE

The results reported here indicated that, in contrast to uncultured RPE, cultured aged human RPE expresses integrins needed for attachment and survival on Bruch's membrane. To test this hypothesis, primary cultures of aged human RPE cells were seeded on aged submacular human Bruch's membrane debrided to expose the native RPE basement membrane or the inner collagenous layer. We compared the attachment and survival of the same number of passaged, cultured fetal human RPE on similar surfaces. Twenty-four hours after seeding, RPE morphology was examined by scanning electron microscopy.

Passaged cultured fetal RPE and primary cultures of aged human RPE attached and spread on native RPE basement membrane as well as on the inner collagenous

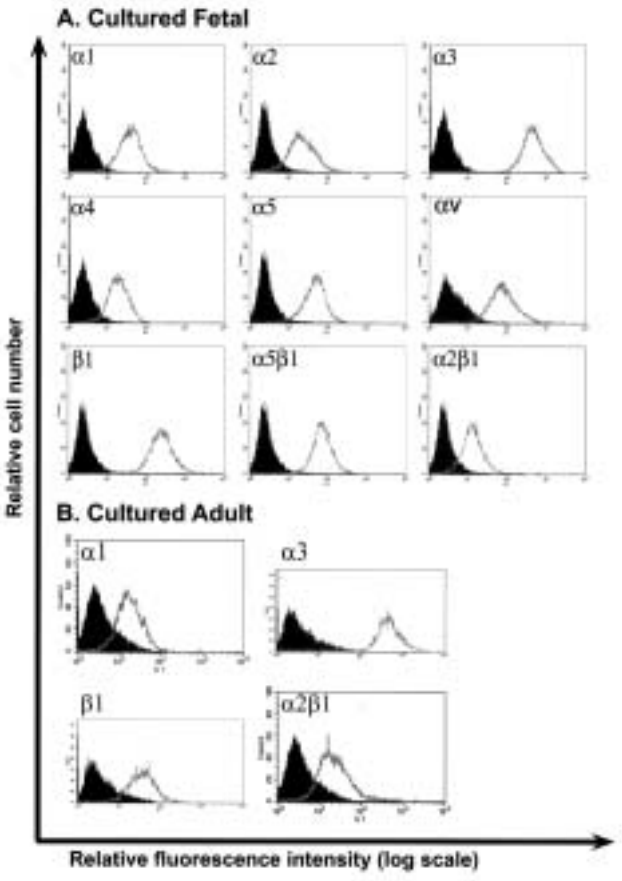


FIGURE 3

Flow cytometry analysis of cell-surface expressed integrin subunits in second to fourth passage cultures of fetal RPE (A) and primary cultures of aged human RPE (B) shows the expression of various subunits on the cell surface (green curve). Negative control is shown closer to the y-axis as a black shaded curve. Cells were detached from cultures with trypsin/EDTA, and a single cell suspension was incubated with antibody against one of the integrin subunits or a heterodimer. Bound antibody was labeled with fluorescein-tagged secondary antibody. Primary antibody was omitted in negative control samples (shaded curve). X-axis represents relative log fluorescence intensity, and y-axis represents cell count.

layer of aged submacular human Bruch's membrane (Figures 4 and 5, Table III). There were, however, significant differences in the behavior of the two cell types. Cultured fetal RPE consistently attached to and resurfaced almost the entire explant area by 24 hours after seeding. These cells formed a monolayer that was not completely confluent, as demonstrated by the presence of intercellular gaps (Figure 4). In some areas, cellular processes extended over neighboring cells (Figure 4). The degree of cultured fetal RPE resurfacing was similar on native RPE basement membrane (99% ± 1.3% surface coverage at 24 hours after seeding, n = 4) and on the inner collagenous layer (97% ± 3.1% surface coverage at 24 hours after seeding, n = 6). No statistically significant difference was noted in resurfacing of the native RPE

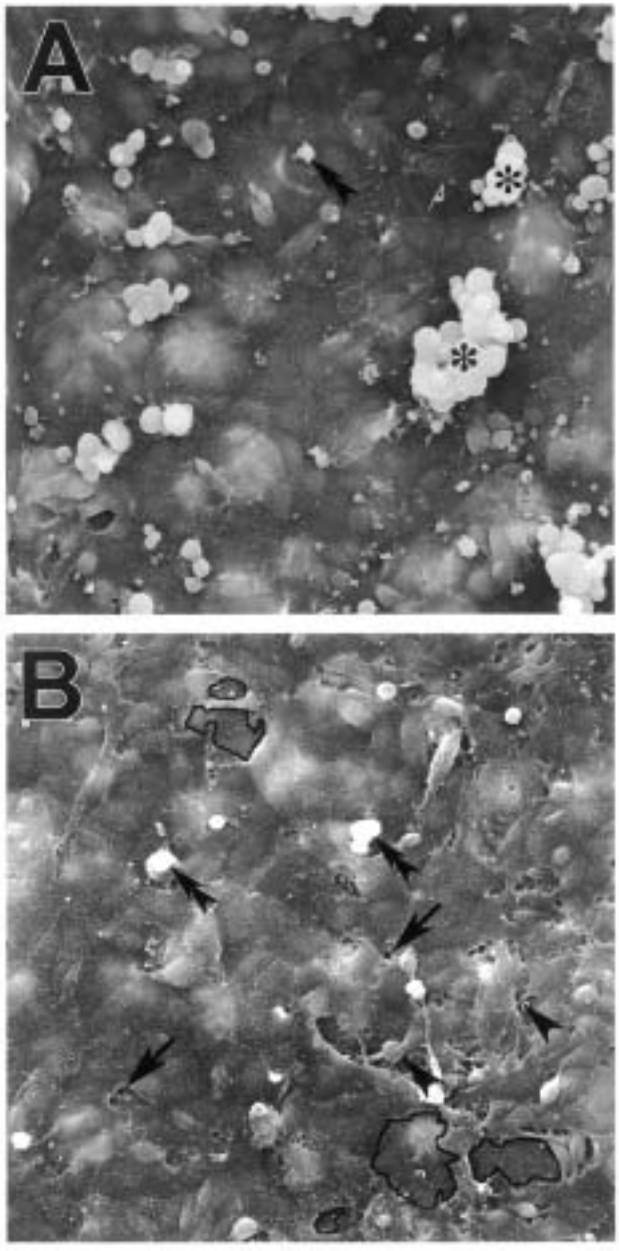


FIGURE 4

Attachment of cultured fetal human RPE to aged submacular human Bruch's membrane. Second passage cultured fetal human RPE (121,000 cells) was seeded onto 7-mm-diameter Bruch's membrane explants from which the native RPE was debrided to the level of the RPE basement membrane or the inner collagenous layer. Scanning electron microscopy shows that by 24 hours most of the surface is covered by RPE cells in each case, although some areas of bare Bruch's membrane are present. A, Cells on RPE basement membrane show complete coverage in this area. Supernumerary cells are present as clumps (asterisks) or single cells (double arrowheads). B, Cells on the inner collagenous layer show incomplete resurfacing. Small gaps are present between cells (arrows), and larger uncovered areas are evident (outlined by polygons). Supernumerary cells are present (double arrowheads). In some areas, numerous cell extensions are present (arrowheads). A and B show explants from the same donor (aged 81 years). Cells are from a 17-week fetal eye. Original magnification ×400.

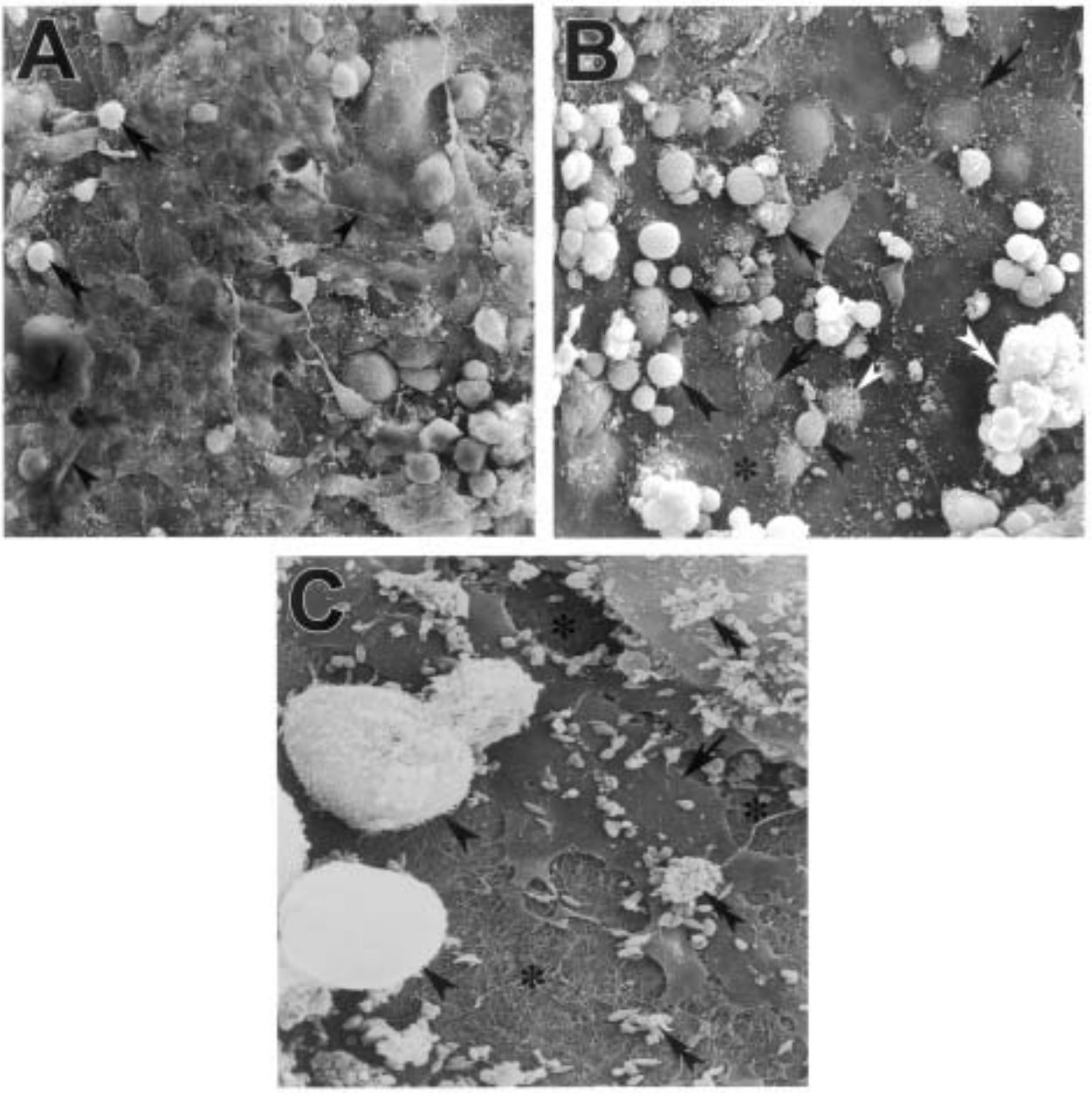


**FIGURE 5**

Attachment of cultured aged human RPE to aged submacular human Bruch's membrane. Primary cultured aged human RPE (121,000 cells) was seeded onto 7-mm-diameter Bruch's membrane explants from which the native RPE was debrided to the level of the RPE basement membrane or the inner collagenous layer. Scanning electron microscopy shows RPE attachment and spreading on both surfaces at 24 hours after seeding, but resurfacing is not complete. Cell morphology is highly variable on both surfaces. A, Cells on RPE basement membrane show complete coverage in this area, but the cells show more morphological variability than fetal RPE cells on similar surfaces. Numerous elongated cell processes are present extending over neighboring cells (arrowheads). Supernumerary cells are present (double arrowheads). B, Cells on the inner collagenous layer show incomplete coverage of the surface. Supernumerary cells are present, despite incomplete coverage, as single cells (black double arrowheads) or cell clumps (white double arrowheads). Cells attached to the collagen surface (asterisk) are either rounded (black arrowheads) or flattened (arrow). A cell showing apoptotic blebbing is present on the collagen surface (white arrowhead). C, High magnification of cells on the inner collagenous layer. Collagen fibers are evident at this magnification confirming the identity of the attachment surface (asterisks). A flattened cell process (arrow) and rounded cells (arrowheads) are shown on the collagen surface. Double arrowheads point to cell debris adherent to the cell surface. A, B, and C are from the same donor (RPE cells, patient aged 81 years; Bruch's membrane explant, patient aged 66 years). A and B, original magnification ×400. C, original magnification ×2,000.

TABLE III: RESURFACING OF AGED HUMAN SUBMACULAR BRUCH'S MEMBRANE BY PASSAGED CULTURED HUMAN FETAL RPE AND PRIMARY CULTURED AGED RPE*

| | CELL TYPE | |
|---|---|---|
| ATTACHMENT SURFACE | PASSAGED CULTURED FETAL HUMAN RPE | PRIMARY CULTURED AGED HUMAN RPE |
| Native RPE basement membrane | 99% ± 1.3% (n = 4)† | 76% ± 22% (n = 5)‡ |
| Inner collagenous layer | 97% ± 3.1% (n = 6) | 39% ± 35% (n = 11)§¶ |

*Cells were seeded onto Bruch's membrane, and resurfacing was assessed 24 hours later by scanning electron microscopy.
†Data refer to the percent of a 7-mm-diameter area of Bruch's membrane that is resurfaced 24 hours after native RPE ± subjacent native RPE basement membrane has been debrided and seeded with cultured fetal or aged human RPE cells.
‡Not statistically different from fetal RPE resurfacing on basement membrane ($P$ = .080).
§Significantly different from fetal RPE resurfacing on the inner collagenous layer ($P$ = .0011).
¶Significantly different from aged RPE resurfacing on basement membrane ($P$ = .046).

basement membrane versus the inner collagenous layer ($P$ = .33) at 24 hours. A previous study has shown that 1 hour after seeding, RPE attachment to aged Bruch's membrane is significantly greater compared to attachment to the inner collagenous layer.[180]

Bruch's membrane resurfacing by primary cultures of aged human RPE, on the other hand, was variable (Table III). In many specimens, RPE cells attached and spread on the native RPE basement membrane as well as on the inner collagenous layer, as was noted with passaged fetal RPE (Figure 5). In some specimens, numerous rounded (versus flattened) and shrunken cells were present on the inner collagenous layer, indicative of inability to attach (Figure 5).[105] Many of the rounded cells exhibited smooth membranes sometimes with perforations, indicative of late-stage apoptosis,[105] while others were partially fragmented, indicative of necrotic-phase apoptosis. In such specimens, a substantial amount of debris was present on Bruch's membrane, possibly the residuum of cells that had already undergone apoptotic death. Blebbing and surface debris were not present to any significant degree in passaged cultured fetal RPE seeded onto Bruch's membrane.

By 24 hours after seeding, primary cultures of aged human RPE resurfaced the inner collagenous layer to a lesser degree (39% ± 35% surface coverage, n = 11) than they did the native RPE basement membrane (76% ± 22% surface coverage, n = 5), and the difference was statistically significant ($P$ = .046). (In contrast, uncultured aged human RPE exhibited less than 15% surface coverage of Bruch's membrane 24 hours after seeding onto native RPE basement membrane of aged submacular human Bruch's membrane explants; the amount of coverage was even less on the inner collagenous layer.[105]) At 24 hours after seeding, passaged cultured fetal RPE resurfaced the inner collagenous layer of Bruch's membrane to a significantly greater degree than primary cultured aged human RPE ($P$ = .0011). Differences in cultured fetal and aged human RPE resurfacing 24 hours after seeding onto native RPE basement membrane were not significantly different (Table III).

The behavior of aged human RPE cells tended to be similar within a given pair of donor Bruch's membrane explants. If few RPE cells attached to one explant, the explant from the fellow eye also exhibited poor RPE cell attachment. In contrast, if RPE cells attached to explants with intact native RPE basement membrane, they also attached to the fellow eye specimen from which the native basement membrane had been debrided, exposing the inner collagenous layer. These data indicate that primary cultures of aged human RPE from different donors behave differently and/or that submacular Bruch's membrane from different donors supports primary cultured aged human RPE attachment to different degrees.

## DISCUSSION

Although several different groups of cell-substrate receptors exist, integrins constitute the dominant group and are the main receptor types used by cells for adhesion to the extracellular matrix. Combinations of the β1 subunit with various a subunits form most of the receptors for extracellular matrix molecules: α1β1, α2β1, α3β1, and α6β1 are receptors for laminin and/or collagens while α5β1, α4β1, and αvβ6 are the major receptors for fibronectin.[209-211] Several studies using species ranging from *Xenopus* to human have demonstrated the expression of one or more integrins in RPE cells.[182,183,197,200,212-215] In the present study, we assessed the differences in expression of integrin subunits mediating cell-extracellular matrix adhesion in fetal and aged human RPE. In aged RPE, α1, α2, α3, α4, and α5 integrin subunit mRNA was significantly lower in uncultured compared to primary cultured cells. In fetal RPE, α2, α3, α5, β4, and β5 subunit mRNA was significantly lower in uncultured compared to passaged cultured cells. Primary cultured aged RPE cells and passaged cultured fetal human RPE cells did not have identical

integrin subunit mRNA expression, as β4 and β5 mRNA levels were higher in fetal cells. Usually, mRNA expression reflects protein expression unless there is a regulation at the level of translation, which can occur in cultured RPE. Liu and Redmond,[216] for example, have shown translational inhibition of RPE-65 transcripts. Since studies of integrins in aged human RPE using confocal microscopy are difficult to interpret because of the presence of large amounts of pigment and lipofuscin granules, and since immunohistochemistry and immunoblots depend on the availability of specific antibodies capable of detecting the protein, RT-PCR provides an efficient and highly specific assay for integrins. Flow cytometry data indicate that at least some integrin subunits detected by RT-PCR also are expressed on the cell surface. RT-PCR and flow cytometry results reported here indicate that uncultured aged human RPE is unlikely to attach to aged submacular human Bruch's membrane upon being transplanted into the subretinal space and that culturing RPE might promote proper attachment to this surface.

In patients with AMD undergoing CNV excision, the surfaces most likely to be encountered by transplanted RPE in the dissection bed are residual native RPE basement membrane and/or the inner collagenous layer of Bruch's membrane. The extracellular matrix components of Bruch's membrane vary with the lamina. RPE basement membrane contains collagens IV and V and laminin. Collagens I, III, and V and fibronectin are present in the inner and outer collagenous layers. The elastic lamina contains elastin, fibronectin, and collagen VI, and choriocapillaris basement membrane contains collagens IV, V, and VI, and laminin.[130,191,217] Receptors for collagen include α1β1 and α2β1.[218-220] Other integrins may be weak receptors for collagens.[221] The relative deficiency of these integrins in aged uncultured human RPE would lead one to predict poor RPE attachment to these extracellular matrix molecules. Several different α and β subunit combinations, however, can form a receptor for a particular extracellular matrix ligand, although with varying affinity.[222] Therefore, mere absence or low expression of α1β1 and α2β1 may not completely explain the poor adhesion of uncultured aged human RPE. Because a number of integrin subunits do not appear to be expressed in these RPE cells, the likelihood of multiple combinations of integrin subunits promoting RPE attachment to the RPE basement membrane or the inner collagenous layer is low. In addition, lower amounts of integrins result in lower magnitude and slower kinetics of activation intracellular molecules such as FAK and mitogen-activated protein kinase.[223] As a result, uncultured aged human RPE has a lower chance of attachment and survival on aged submacular human Bruch's membrane than primary cultured aged RPE or passaged cultured fetal human RPE cells.

In a previous study,[180] RPE basement membrane was shown to improve attachment of passaged cultured fetal human RPE to aged Bruch's membrane 1 hour after seeding. In the current study, attachment to Bruch's membrane was assessed 24 hours after seeding for two reasons. First, the suspended cells have more time to attach to Bruch's membrane, leading to a more accurate assessment of attachment capacity. Second, anatomic evidence of attachment is more obvious than at 1 hour because if the cells do not attach by 24 hours, they undergo apoptosis.[108] If the cells are attached effectively at this time, they show no signs of apoptosis (eg, membrane blebbing).[108,224] Twenty-four hours after seeding, primary cultures of aged human RPE seeded on Bruch's membrane were able to attach and spread, although numerous cells were round, most frequently on the inner collagenous layer. In contrast, few if any surviving cells are identified on aged submacular human Bruch's membrane at this time point if uncultured aged human RPE is seeded.[105] Compared to primary cultured aged RPE, passaged cultured fetal RPE had more consistently uniform attachment to aged submacular human Bruch's membrane, and the cells had somewhat healthier-appearing morphology 24 hours after seeding (eg, fewer rounded cells were present on Bruch's membrane), independent of whether the cells were seeded onto native RPE basement membrane or the inner collagenous layer. While resurfacing of Bruch's membrane explants by primary cultures of aged human RPE was not equal to that of passaged fetal RPE, it was clearly higher than uncultured aged human RPE.[105]

The difference in the behavior of cultured, passaged fetal human RPE and primary cultures of aged human RPE might reflect qualitative and/or quantitative differences in integrin expression (eg, β4 and β5 subunits). In addition to differences in integrin expression, reasons for the discrepancy between the degree of resurfacing by primary cultures of aged human RPE and passaged fetal RPE might include the following. There may be variability in the health or phenotype of aged human RPE cells in primary culture.[225,226] Cells derived from primary cultures tend to exhibit more phenotypic variability than passaged cells. In these studies, we used primary cultures of aged human RPE cells because passaged aged human RPE does not grow well in culture.[104] In contrast, fetal RPE was used after having been passaged two to four times. Variable age-related Bruch's membrane changes might alter extracellular matrix ligand availability, which in turn might affect the ability of aged RPE to resurface Bruch's membrane. Other AMD-associated changes in Bruch's membrane might be inhibitory to RPE cell attachment.[227]

The different integrin subunits that are expressed upon culturing and the similar expression among cultured

fetal and cultured aged human RPE are likely to be a result of culturing on a BCE-ECM-coated surface. BCE-ECM contains fibronectin, laminin, and collagens III, IV, and VI,[228] and studies indicate that human RPE cells grow well on this surface.[181,188] The up-regulation of integrins seen in cultured aged human RPE does not appear to occur when uncultured aged human RPE is seeded onto aged submacular human Bruch's membrane in organ culture.[105] It is possible, however, that up-regulation of integrins needed to bind to Bruch's membrane may not occur quickly enough to avoid the onset of cell death. For example, freshly isolated adult RPE cells, despite low levels or absent integrins, are still capable of attaching to and surviving on BCE-ECM, yet they cannot seem to carry out these functions on aged submacular human Bruch's membrane. Thus, as already noted, factors in addition to a paucity of appropriate integrins on the cell surface may be responsible for poor attachment of aged human RPE cells. In contrast to aged submacular human Bruch's membrane, extracellular matrix molecules in BCE-ECM may be arranged in a way that favors attachment.

In vivo and in vitro RPE transplantation models exist, but they do not appear to be directly relevant to AMD patients undergoing CNV excision. Previously reported successful in vivo RPE transplants in laboratory animals involve attachment to normal Bruch's membrane or native RPE. One can model the situation encountered in situ after CNV excision by studying human RPE attachment to submacular Bruch's membrane in vitro.[105,180] The system uses aged human submacular Bruch's membrane in organ culture from which native RPE has been debrided surgically (ie, mechanically), exposing native RPE basement membrane or the inner collagenous layer, depending on the debridement depth. (Peripheral and submacular Bruch's membrane may differ in their ability to support RPE attachment;[193] RPE resurfacing in patients with AMD must occur under the macula, the site of most CNV ingrowth.) This in vitro system permits one to study alterations in the cells (eg, treating the cells in situ; study attachment of fetal versus adult versus transfected cells) as well as alterations in the RPE-denuded surface (eg, removal of native RPE basement membrane, addition of extracellular matrix components to the surface, treatment of the surface to expose relevant "masked" extracellular matrix ligands). Limitations of this organ culture system include (1) inability to study the effects the overlying retina might have on RPE cell attachment and/or migration and proliferation;[229-234] (2) inability to study the effects of various substances added to stimulate RPE attachment and/or migration and proliferation on the retina or choriocapillaris; (3) inability to study the effect of the immune system on retina-RPE-choriocapillaris cell survival; and (4) inability to study the effect of the inflammatory response on retina-RPE-choriocapillaris cell survival. Despite its limitations, the paradigm appears to be relevant to RPE replacement in humans with AMD. In vitro attachment studies using this system predict poor uncultured adult RPE survival in the CNV dissection bed,[105,180] which is what has been observed in immune suppressed patients undergoing RPE transplantation after CNV excision.[52]

One way to transplant RPE cells in AMD patients and avoid immune rejection is to use autologous grafts.[53,103-105,235] RPE isolated from a biopsy could be transplanted under the macula. On the basis of the results of this study, as well as those of Tsukahara and coworkers,[105] adult RPE enzymatically isolated from the biopsy for transplantation may not lead to a successful graft. Culturing RPE cells may improve the probability of attachment and survival in the eye. Culturing also provides an opportunity to manipulate the cells prior to transplantation and to establish cell lines for transplantation.

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