Comparison of External and Internal Approaches for Transplantation of Autologous Retinal Pigment Epithelium

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The feasibility of autologous transplantation of retinal pigment epithelial (RPE) cells from just posterior to the ora serrata to the posterior pole was demonstrated in the rabbit model. Two techniques for introducing the transplanted cells were compared: an internal (anterior transvitreal) and an external (posterior transscleral) penetration to the subretinal space. In both approaches, RPE cells were obtained by biopsy from the peripheral retina of a rabbit eye, cultured, labeled with a fluorescent dye and 3H-thymidine, and transplanted to the posterior pole of the same or contralateral eye. The external approach consistently resulted in a greater number of transplanted cells on Bruch’s membrane. The internal technique was more precise because it permitted direct visualization of the placement of the transplanted RPE. Transplantation of autologous RPE is a possibility that should be further pursued.

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The retinal pigment epithelium (RPE) is essential for the vitality and functioning of the photoreceptors. This cell layer, a major component of the blood-retina barrier, phagocytoses the growing tips of photoreceptors and transports nutrients and wastes between the photoreceptors and the choriocapillaris. Several inherited forms of retinal degeneration, which cause blindness, have been attributed to genetic mutations that alter RPE function.

Age-related macular degeneration (AMD) also is considered a consequence of impaired RPE function, possibly the result of age-related accumulation of lipofuscin in the RPE lysosomes. The spatial distribution of lipofuscin coincides with the preferential expression of AMD in the macula, sparing the fovea, and with the low probability of degeneration in the peripheral regions of the retina. The amount of lipofuscin in the RPE cells has been directly correlated with the amount of debris in Bruch’s membrane (ie, drusen) and inversely correlated with the number of photoreceptors present in the aging fovea. These data suggest that the loss of RPE cells or accumulation of lipofuscin in the macular RPE may contribute to the atrophic changes of AMD.

Hypothetically, retinal degenerations from either genetic or age-related compromise of RPE function could be clinically treated by transplantation of healthy RPE cells. In fact, photoreceptor loss in Royal College of Surgeons (RCS) rats has been prevented by transplantation of RPE cells. However, bringing an experimental model to clinical practice will require resolution of several issues, including selection of the optimal surgical approach and avoiding the rejection of the transplanted tissue. Allograft transplantation has resulted in a focal granulomatous reaction within 1–2 wk; consequent use of immunosuppression often was associated with infection and toxicity. Autologous transplantation should eliminate these problems. If preferential accumulation of lipofuscin causes or exacerbates progression of AMD, transplantation of peripheral RPE cells (with lower levels of lipofuscin) may stabilize the disease process in AMD and other degenerative diseases while circumventing the problem of tissue rejection and immunosuppression.

Previous researchers reported on their experiences with the internal\textsuperscript{8-11} or external\textsuperscript{7,12,13} approach only. The current study compares two different transplantation techniques: the internal (anterior transvitreal) and external (posterior transscleral) transplantation approaches. We performed each technique in a different eye of the same rabbit, the same technique in both eyes of the same rabbit, or a single technique in just one eye of a rabbit. We have attempted to answer the
following questions. Is autologous RPE transplantation a viable process? How can each approach be improved using precision injection equipment? Which approach results in fewer complications?

Materials and Methods

Animals and Anesthesia

Twenty-nine pigmented rabbits (weight range, 2.5–3.0 kg) were maintained in a 12-hr light–dark cycle. All animals were handled according to the Public Health Service and National Institutes of Health Policy for Humane Care and Use of Laboratory Animals and the ARVO Resolution on the Use of Animals in Research.

Local anesthesia (topical 0.5% proparacaine) was applied to the experimental eye, and the pupil was dilated with 1% tropicamide and 10% phenylephrine. General anesthesia was induced with intramuscular 60 mg/kg ketamine hydrochloride (IM) injections of ketamine hydrochloride 60 mg/kg and acepromazine maleate 3 mg/kg. To maintain anesthesia, approximately 1 mg/kg (0.1 ml of a 50% dilution of an original concentration of 65 mg/ml) of sodium pentobarbital was given intravenously (IV) as needed every 10–30 min. Topical polymyxin B ointment was applied to the operated eye, and 1 ml of procaine penicillin (100,000 U/kg) was given IM prophylactically at the end of each operation. The animals were killed by IV administration of sodium pentobarbital 100 mg/kg and perfused through the heart with fixative as described later in this article.

Transplant Procedure

Autologous RPE transplantation involved three steps. First, a peripheral biopsy of RPE cells was removed from the right eye anterior to the equator 4–6 mm behind the corneal limbus. Then, the RPE cells were cultured from the biopsy and allowed to grow until sufficient in number for transplantation. Finally, the RPE cells were labeled with fluorescent dye and ³H-thymidine and transplanted into (1) the posterior pole of the fellow eye, (2) the same right eye by either the internal or external approach, or (3) both eyes of the same rabbit with the internal approach performed on the left eye and the external on the right.

Biopsy of RPE Cells

RPE cells were obtained from the choriocapillaris–Bruch's membrane–RPE biopsy either including (full thickness) or excluding (partial thickness) the sensory retina. When the sensory retina was excluded, a Fleeringa ring was sutured to the sclera, then a 4 × 6 mm scleral flap, hinged posteriorly, was created 4–6 mm behind the corneal limbus. This flap, nearly the full thickness of the sclera, was dissected by grasping an edge with a 0.12 mm corneal forceps and cutting close to the base of the sclera with a microsurgery knife (681.01; Grieshaber). The thin floor of the flap formed a translucent membrane through which the choroid could be seen. Occasionally, when necessary, diathermy was applied to the thin sclera over the exposed choroid at the flap's periphery to help maintain hemostasis. A 30 G irrigation cannula was placed through the exposed choroid into the subretinal space, and 100 μl of sterile phosphate-buffered saline (PBS) was injected to create a small retinal detachment. A drop of sterile cyanoacrylate glue was then placed on the exposed choroid to prevent the specimen from rolling up and later to identify the choroidal surface for cell culture trypanization. The tissue was then resected and placed in a well of a 96-well culture dish (Costar, Cambridge, MA) containing PBS. The scleral flap was closed by a 7–0 or 8–0 silk suture.

When the sensory retina was included, the flap size was decreased to 4 × 4 mm because no subretinal bleb was to be formed. The full-thickness biopsy was performed immediately after preparation of the flap and without making a bleb or using glue. The scleral flap was closed as before.

Culture and Preparation of RPE Cells for Transplantation

Trypsin (200 μl of a 0.25% solution) was placed into the well with the tissue after the PBS was removed. After a 5 min incubation at room temperature, the RPE cells were dislodged by gentle trituration, and the cell suspension was transferred to a 35-mm culture dish (Falcon Primaria; Becton Dickinson, Lincoln Park, NJ) containing 2 ml of Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (HyClone, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml; all from Gibco, Grand Island, NY). The cultures were labeled with 3H-thymidine (NET-355; New England Nuclear, Boston, MA) in the medium for 7 days, and a fluorescent stain was added. either 4',6-diamidino-2-phenylindole (DAPI) hydrochloride (Molecular Probes, Eugene, OR) or fast blue (Sigma, St. Louis, MO), for 1 hr. The fast...
blue was solubilized by sonification in PBS, and both stains were filter sterilized. For transplantation, the cells were trypsinized, and 60,000–140,000 cells were resuspended in 100 μl of PBS. RPE cell viability was determined by the trypan blue exclusion test (GIBCO). Periodically, cell viability after passage through the injector needles and pipettes was also determined by the trypan blue exclusion test. It was consistently found to be greater than 80%.

Transplantation

**Internal approach:** Micropipettes and a microsyringe manipulator were used for more accurate placement of the transplanted cells. The micropipettes were drawn from glass (outer diameter, 1.2 mm; inner diameter, 60 μm; Sutter, San Rafael, CA) on an Ealing vertical microelectrode puller at a tension of 7 arbitrary units. The tip was smoothed after ascertaining that it was not obstructed, and the shaft was glued into the hub of the plastic connector cut off from a 23-gauge butterfly needle.

The internal approach was performed on 14 eyes from 14 rabbits. (Six rabbits underwent both approaches; the internal technique was performed on the left eye and the external, on the right. In the eight remaining rabbits, one eye only was used.) A peritomy was made in the superior quadrant of the eye, and the superior rectus muscle was hooked and tagged with a 4–0 suture to rotate the eye. After exposing the sclera, a sclerotomy was made on an area 3–6 mm behind the corneal limbus. The micropipette, connected to a 250-μl Hamilton syringe (VWR, Boston, MA) positioned on an electronic injection microsyringe manipulator, was inserted through the sclerotomy, anterior to the equator, at an angle that prevented it from touching the large lens of the rotated eye of the rabbit. The pipette was advanced vertically by turning the knob of the micromanipulator until it penetrated the retina to no more than 200 μm. An aliquot (30–40 μl) of the labeled cell suspension was then slowly injected with an electronic micromotor injector attached to the syringe plunger, producing a localized bleb detachment of the sensory retina and dislodging the host RPE cells at the tip of the micropipette. No reflux could be seen during the procedure as long as the pipette remained in the retinal hole. As the tip was removed, the bleb formed. At that point, reflux was possible, especially if the pipette had entered the retina tangentially, where the tapering shaft was likely to tear the retina. Direct visualization of the procedure, using either the microscope and contact lens or the indirect ophthalmoscope, allowed the surgeon to control the size and placement of the bleb.

**External approach:** A guide pipette of the same glass type as used for the internal approach was cut to a length which allowed a Hamilton 33-gauge needle that was inserted through the pipette to protrude ex-
The external procedure was performed in 31 eyes from 21 rabbits. (Of the total, six rabbits underwent both approaches with the internal one on the left eye and the external one on the right. The external approach only was used in the 15 remaining animals, both eyes in 10 animals and one eye in 5.) A peritomy was made, the eye was rotated, and one extraocular muscle was disinserted. Both the origin and end of the disinserted rectus muscle were tagged with 5-0 sutures to expose the posterior sclera. A nearly full-thickness scleral flap was dissected by grasping an edge of the flap with a 0.12 mm corneal forceps and cutting a flap of the desired size with a microsurgery knife to provide a window at the transplantation site; the thin floor of the flap formed a translucent membrane through which the needle tip that was penetrating the choroid could be seen (Fig. 1). The shaft and the tapered tip of the guide pipette were secured so that the tip was angled at 15° to the sclera (to allow the 33 G needle inserted into it to be held at the same angle) by two 6-0 silk sutures 1-cm apart. Then the 33 G needle was advanced through the guide pipette, protruding 4 mm to penetrate the choroid tangentially into the subretinal space. A 250 \( \mu l \) Hamilton syringe was used to inject 100 \( \mu l \) of 0.02% ethylenediaminetetraacetic acid (EDTA) into the subretinal space. All injections and withdrawals were manually controlled in the external approach. No reflux was visible unless the needle was not correctly placed in the subretinal space or more than 100 \( \mu l \) of fluid was injected. The EDTA was then aspirated, bringing with it a number of RPE cells released from Bruch’s membrane. (To confirm this removal, RPE cultures have been successfully established from the aspirated cells. Choroidal melanocytes have a distinctive morphology that permits positive identification of the cells grown here as RPE cells.) The syringe containing the aspirated EDTA and RPE cells was then removed from the hub of the injecting needle and replaced with a second 250 \( \mu l \) syringe containing 50 \( \mu l \) of the labeled autologous cultured RPE cell suspension. These cells were then slowly injected with minimal force, the needle was removed, and the scleral flap was closed. After the injection of the transplanted RPE cell suspension, no visible reflux was noted. Bleb formation was examined with an indirect ophthalmoscope.

**Histopathologic Evaluation**

The rabbits were killed at various times after transplantation. Some of the experimental rabbits were perfused through the heart with 300 ml of warm (37°C) saline with 0.5% NaNO3 for 5 min and then with 1 l of 4% paraformaldehyde with 0.1% glutaraldehyde, pH 7, for 1 hr. Each experimental eye was enucleated, immersed for 24 hr in 4% paraformaldehyde, pH 10, after the perfusion or, alternatively, without perfusion. The low concentration of glutaraldehyde served to minimize artifactual fluorescence. The higher pH in the immersion buffer improved fixation (and adhesion) of the retina to the globe. The transplantation area was excised under a dissecting microscope and embedded in paraffin. Serial 6-\( \mu m \) sections, after deparaffinization and mounting in Aqua-Mount (Lerner, New Haven, CT), were examined by transmitted visible illumination and ultraviolet induced fluorescence on a Zeiss III photomicroscope.

Fig. 2. Primary cultures of rabbit RPE cells in phase contrast. (A) A 1-month-old culture established from cells trypsinized from an explant. This sparse non-confluent culture exhibits elongated RPE cells that lost their melanin during extensive proliferation. (B) Confluent culture initiated with cells from an eye cup contains both elongate cells and smaller polyhedral cells organizing into a cobblestone pattern in areas of confluence. Because they were plated at a high concentration, most cells in culture retain melanin pigment. (X350.)
scope. Fluorescence microscopy of transplanted cells stained with either fast blue or DAPI was performed using a 365-nm excitation filter and a 460-nm emission barrier.

Identification of Transplanted RPE Cells

Because RPE cells lose melanin pigmentation as they undergo cell division in vitro, amelanotic cells found on Bruch’s membrane were closely examined. A lack of melanin is not sufficient proof of a transplanted cell, however, because disrupted host RPE cells can also lose pigment and transplanted cells can phagocytose unassociated host pigment. The presence of fluorescent dye was therefore required for positive identification of transplanted RPE cells.

Radioactive Label

The cultured RPE cells were labeled with \(^{3}H\)-thymidine for approximately 1 wk at a mean label of 68.5 ± 37 cpm/1000 cells (mean ± standard deviation). Vitreous samples from 18 eyes were counted at different times from 2 days to 4 wk after transplantation. Vitreous samples (0.1–0.5 ml of vitreous in 5 ml of scintillation fluid from 15 experimental eyes were counted on a Beckman LS1701 scintillation counter (Beckman Instruments, Fullerton, CA) for 1 min to measure the amount of \(^{3}H\)-labeled cells or debris leaking into the vitreous. Three eyes that had undergone biopsy with no later surgical intervention were used as controls. Only one of the 14 eyes from the external approach which were counted had a retinal hole; the only eye counted from the internal technique also had a hole. The scintillation counting efficiency was 40%.

Results

Specimens that included the sensory retina with the RPE (full-thickness biopsy) were more successfully cultured (32 of 37, 86%) than those without the sensory retina (partial-thickness biopsy) (1 of 6, 17%). Rabbit RPE cells in culture form monolayers that are more irregular than the cobblestone pattern typical of cultured bovine, porcine, monkey, and human RPE cells. In the first few days of culture, elongated pigmented RPE cells appeared. With extensive proliferation the RPE cells from the biopsy specimen produced patches of disorganized RPE cells (Fig. 2A). With time, these patches would coalesce to form irregular mosaic monolayers, but the cultures were usually trypsinized for transplantation before confluence. Rabbit RPE cells prepared from an eyecup also exhibited elongated RPE cells (Fig. 2B). Reaching confluence with substantially fewer generations, these cultures exhibited irregular monolayers of predominantly pigmented cells (Fig. 3B).

Surgical Techniques

A fluid bleb formed under the retina upon injection of the transplanted cells. The bleb was completely flat at the end of 1 wk after the internal approach but took more than 2 wk to flatten after the external approach. In neither case was further retinal detachment observed.

In the internal approach, access to the posterior pole transplantation site was easier, and the placement of the pipette tip into the subretinal space could be monitored more accurately. The retinal hole created by the micropipette was at least equivalent in size to the pipette tip, approximately the size of a 27–30 G needle.

Using the external approach, we gained access to the transplantation site at the posterior pole by detaching one of the extraocular muscles at the desired quadrant. The 33-gauge needle created a small hole (approximately 40 μm in diameter) at the scleral entrance site. Occasional subretinal hemorrhages found in early experiments were avoided by using a smaller-gauge needle, carefully choosing the entry site, and using diathermy. Iatrogenic retinal holes (the result of overpenetration of the needle) occurred in only 20% of our cases. Some of the differences between the two surgical procedures are presented in Table 1.

Histologic Evaluation

Fluorescent dye-labeled transplanted RPE cells were found on denuded Bruch’s membrane up to 18 days after transplantation in the external approach and 14 days in the internal. No evidence of a granulomatous immune reaction was ever observed throughout this study.

Fifteen days after transplantation by the external approach procedure, nonpigmented cells were observed on Bruch’s membrane by light microscopy (Fig. 3). Fluorescence microscopy revealed that these were transplanted RPE cells stained with the fast blue fluorescent dye. An eye enucleated 18 days after an external procedure had a prominent multilayered focus of transplanted RPE in the center of the bleb region (Fig. 4). More peripherally in the same bleb region, Bruch’s membrane was less denuded, but a cluster of transplanted cells was inserted into the interrupted layer of host RPE cells. At the outermost edge of the transplantation site, the interspersed transplanted and host RPE cells were in contact with surviving photoreceptors.

The presence of transplanted RPE was also confirmed by histologic examination in eyes that had un-
dergone the internal-approach technique. However, removal of host RPE was spotty, and the transplanted cells were intercalated among and over the host cells, forming a bumpy mixed mosaic. In any one high dry field (125X or 200X), we never found more than a few transplanted RPE cells.

Table 1. Comparison of the two surgical approaches

<table>
<thead>
<tr>
<th>Procedures</th>
<th>External approach</th>
<th>Internal approach</th>
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<tbody>
<tr>
<td>Entrance</td>
<td>Access to posterior pole difficult, requires disinsertion of rectus muscle</td>
<td>No disinsertion required</td>
</tr>
<tr>
<td>Entrance site</td>
<td>Usually no retinal hole</td>
<td>Entrance produces tiny retinal hole</td>
</tr>
<tr>
<td></td>
<td>May be used in macula and at multiple sites</td>
<td>May be used in macula and at multiple sites</td>
</tr>
<tr>
<td>Equipment</td>
<td>500-μl syringe</td>
<td>Microsyringe-manipulator</td>
</tr>
<tr>
<td>Injection</td>
<td>Manual injection</td>
<td>Electronic microinjection at constant rate</td>
</tr>
<tr>
<td>RPE washout</td>
<td>Yes, with EDTA</td>
<td>No</td>
</tr>
</tbody>
</table>

Radioactive Label

In those eyes in which a retinal hole was not present, the range of $^3$H-thymidine counted was 10–28 cpm/0.5 ml of vitreous, slightly above the background level of the control eyes (10–12 cpm/0.5 ml of vitreous). The count increased in the two eyes with retinal holes. Three weeks after an external-approach transplantation, one had a count of 60 cpm/0.5 ml of vitreous, and the other (removed 1 wk after the internal-approach procedure) contained 67 cpm/0.5 ml of vitreous. Because each vitreous was sampled far from the transplanted site, these results suggest that radioactively labeled cells can percolate through the retinal hole into the vitreous; the counts suggest as many as 2000 cells had penetrated into the vitreous. This possibility was affirmed when we found a layer of transplanted cells organized on the inner limiting membrane in one eye after an internal-approach transplantation.

Complications

At each biopsy site, chorioretinal scarring was noted. No postbiopsy retinal detachment was found.
Fig. 4. External approach: day 18. Unstained 8-μm sections examined by phase contrast (A, C) and fluorescence (B, D) microscopy. A multilayered clump of RPE transplant cells was found at the center of a bleb. The transplanted RPE cells (arrowheads) contact the denuded Bruch's membrane (open arrow). Just below the open arrow a number of autofluorescent erythrocytes can be seen in the choriocapillaris. Although the outer nuclear retina appear atrophic in the detached center of the bleb (A, B, small arrows), the periphery of the same bleb retained all the retinal layers (C, D). In the area of reattachment several cells in the RPE monolayer exhibited both markers for transplant cells: they are amelanotic (C) and they have the fluorescent marker (D). The three dark structures in (C) and (D) are deeply pigmented RPE cells; the four arrows identify loci of four transplant cells. Fluorescence below the Bruch's membrane may be RPE cells that percolated into the choroid along the trail of the injection needle. Labels in (C) indicate the photoreceptors (PR), outer and inner nuclear layers (ONL and INL, respectively), the outer and inner plexiform layers (OPL and IPL) and the ganglion cell layer (GC). Slight movement of the microscope stage between (C) and (D) caused a shift in the images. Bar = 10 μm.
The detached bleb did not expand after either approach in our study. After 1 wk, the bleb was flat in eyes injected internally (which always resulted in a retinal hole). The blebs formed using the external approach persisted for nearly 3 wk. However, when hemorrhage was visible, the blebs flattened more slowly. Six eyes from the internal approach and three from the external approach were examined after 1 mo; no proliferative vitreoretinopathy (PVR) was observed by indirect ophthalmoscopy in these 9 eyes or in the 36 eyes observed for shorter periods.

Endophthalmitis, probably contracted from a contaminated needle, developed in one eye 24 hr after the external approach, which also had produced an iatrogenic retinal hole. Dense cataracts developed in two animals: one after a repeat RPE biopsy of the peripheral retina near the lens the other after transplantation with the internal approach. All others were free from cataracts.

Table 2 summarizes the major complications observed with each technique. With the internal approach, the creation of a retinal hole was unavoidable. The external approach at times produced iatrogenic retinal holes; however, this complication decreased to less than 20% with adequate precautions, i.e. careful monitoring under an operating microscope, maintaining the angle of the injection needle precisely at 15°, and limiting penetration into the subretinal space under the 4 X 3 mm scleral flap window. Complications encountered with both approaches were retinal schisis, subretinal hemorrhage, and retinal atrophy, especially loss of the outer nuclear layer, which was often observed in the central bleb area.

**Discussion**

We introduced a procedure for autologous transplantation and compared results using the internal and external approaches each in a different eye from the same animal or the same technique in both eyes of the same animal. In our preliminary data, a comparison between two different autologous transplantation techniques in paired eyes from one animal and from different animals presented no significant differences. The biopsy sites at the periphery of the retina resulted in chorioretinal scars but no retinal detachment. Placement of a needle into the subretinal space was routinely achieved with both techniques. Injection of the transplanted cells caused a retinal bleb that was resorbed without problems. In all operations that produced a retinal hole (all internal procedures and some external), the blebs were flat within 1 wk. This was consistent with previous studies of retinal detachment and iatrogenic holes in rabbits. The blebs formed during the external approach without complications flattened more slowly and were still seen 2 wk after surgery. A retinal hole did not progress to retinal detachment. PVR was not observed in eyes followed for more than 1 mo, indicating that only some cells had reached the vitreous.

Modifications introduced here on the rabbit model that facilitated successful results in one or both techniques included the following: (1) autologous transplanted cells to prevent granulomatous immune reactions, (2) full-thickness biopsies in the peripheral retina to obtain a high yield of cultured RPE cells, (3) the use of a micromanipulator to place the micropipette into the subretinal space and electronic micromotor control of the injection under direct visualization of the indirect ophthalmoscope or surgical microscope and corneal contact lens in the internal approach, (4) a guide pipette to place correctly and limit penetration of the needle into the subretinal space during the external transplantation procedure, and (5) a brief EDTA treatment in the external approach that allowed more RPE cells to be removed from Bruch's membrane.

**Problems Encountered**

Partial-thickness specimens did not produce cultures as reliably as did full-thickness specimens. Forming the bleb in the subretinal space necessary for a partial-thickness specimen was difficult because of strong adhesive forces between the retina and RPE in vivo. In freshly enucleated rabbit eyes we found most of the RPE cells separated from Bruch's membrane and came off with the retina when it was peeled (data not shown), even with an injection of fluid into the subretinal space—a finding consistent with other studies. The RPE cells that remained with Bruch's membrane and were removed with the biopsy grew
less successfully in culture than did full-thickness specimens, perhaps indicating an injury. Although we cannot exclude the possibility that cyanoacrylic glue was toxic to these cells, our observations strongly concur with previous reports that rabbit RPE cells adhere more strongly to the sensory retina than to Bruch's membrane.

A biopsy using partial-thickness specimens may also not be advantageous because the surviving photoreceptor cells appear to be atrophic. Atrophy of photoreceptor cells over the transplanted area was also observed in both techniques, probably in response to the detachment, because photoreceptor cell death and necrosis in the outer nuclear layer has also not been advantageous because the surviving photoreceptor cells appear to be atrophic. Atrophy of photoreceptor cells over the transplanted area was also observed in both techniques, probably in response to the detachment, because photoreceptor cell death and necrosis in the outer nuclear layer has been found in detachments of only a few days' duration. The aforementioned strong adhesive forces observed in the rabbit may also contribute to the damage to the photoreceptors. Photoreceptor atrophy was not reported in rabbits when the transplanted cells were injected beneath the RPE (which remained attached to the retina); however, the experiment was also terminated only 24 hr after the cells were transplanted, perhaps too soon to see atrophy. Atrophy was not reported in transplants of longer duration in RCS rats. Flattening the bleb immediately after transplantation could reduce the atrophy. However the amount of damage introduced during the separation of the RPE and retina could be a species-dependent variable influencing the success of these transplants.

Hypothetically, because oxygen is supplied to the outer retina from the choroid in all species, the type of retinal vasculature found in the retina should not influence the success of the RPE transplant. However, the avascular retina of the rabbit could increase the probability of photoreceptor degeneration in some undefined way. In any species, displacement from the choroid-RPE could cause deprivation of glucose or essential growth factors, such as fibroblast growth factor. It is unlikely that lower oxygen itself would be damaging because photoreceptors and the outer retinal layers have relatively high glycolytic capacity that is similar in monkey and rabbit retinas; however, glycolysis in the avascular inner retina would increase the pyruvate, lactic acid, and other glycolytic byproducts in the microenvironment.

Not all injected cells remained at the desired position on Bruch's membrane. Transplanted cells were observed in the choroid (external approach), on the internal limiting membrane (internal approach), and in the vitreous (internal approach, once; external approach, once), especially when a retinal hole was present. Over- or underpenetration with the pipette or needle in either technique caused subretinal hemorrhage or separation within the sensory retina (retinal schisis). Iatrogenic retinal schisis was found in both approaches, although this complication has not been previously reported. Retinal or subretinal hemorrhage occurred only occasionally with the internal approach. Subretinal hemorrhage associated with penetration of the choriocapillaris occurred often with the posterior approach when using a 27 G needle. Replacement with a finer 33 G needle avoided bleeding in almost all cases. Cell survival (as determined by the trypan blue exclusion test) was not influenced by needle gauge.

### Transplantation Surgery

Three different approaches have been used previously for RPE cell transplantation: internal (anterior transvitreal), external (posterior transscleral), and open sky. This last approach allowed researchers to place cultured human RPE cells onto a denuded Bruch's membrane; histologic examination of the eye fixed 1 hr later demonstrated the xenograft on Bruch's membrane. However, this technique was not considered for our study because it can cause trauma to the retina, vitreous, and anterior chamber.

The internal approach is a closed-eye technique using a micromanipulated micropipette inserted into the eye at the pars plana. Compared with hand-held methods, the micromanipulators reduced the risk of over- or underpenetration, which could cause subretinal hemorrhage or retinal schisis, both of which were occasionally still observed in this study. Transplanted RPE cells were sometimes found inserted into the original RPE monolayer. The direct visualization of the placement of the micropipette into the subretinal space allowed greater success in placing the transplantation bleb. Limitations of this technique include damage to the photoreceptors and nerve fiber layer and the presence of a retinal hole that can leak RPE cells into the vitreous where they might eventually support PVR. Although a retinal hole is clearly undesirable, it is possible to place it away from the macula so that the consequences would be less significant, assuming PVR can be avoided.

The external approach for RPE cell transplantation had been successfully used to prevent photoreceptor degeneration in RCS rats. In these earlier studies and in ours, cultured cells were injected with a needle introduced through the sclera into the subretinal space. We found layers of transplanted cells or mixed layers of preexisting and transplanted RPE cells on Bruch's membrane, which was completely or partially denuded at the local injection site. Multilayered foci of transplanted cells were also occasionally observed.
An advantage of the external approach was the reduction of leakage of transplanted RPE cells because of the tangential angle of injection and the watertight suturing of the flap. Moreover, any leakage along the injection path brought RPE cells into deeper layers of choroid, rather than into the vitreous. The low angle of entry permitted the injection site and diathermy to be located 400–600 μm from the intended transplantation area; this would be important in clinical application. A major disadvantage of the external approach was the disinsertion of a rectus muscle in the posterior pole surgery. In addition, because the globe was rotated, direct monitoring of the formation of the retinal bleb during injection of the transplanted cells was not possible. A recent report describes a modification of the external technique that permits rotation of the eye to its normal position and direct monitoring of bleb formation. 51

A limitation in both techniques was the adequate removal of sufficient host RPE cells to prepare Bruch’s membrane for the transplant. Even when the RPE cells were dislodged, bare regions of Bruch’s membrane were observed several days later in the transplantation site, perhaps indicating that the basal lamina itself may have not been accessible to the transplanted cells. Considering that shearing of RPE cells from Bruch’s membrane might leave some basal cytoplasm that could interfere with attachment of the transplanted cells, we modified the external approach. A brief treatment with EDTA facilitated RPE removal and exposed larger areas of Bruch’s membrane. (Recently, the basal plasmalemma of RPE cells was found still covering Bruch’s membrane 20 min after surgical removal of the RPE. 36) Incorporating an equivalent EDTA treatment into the internal method was not possible in our study because it required removal and then replacement of the pipette; redirecting the pipette to the original insertion point was difficult.

We found successfully transplanted cells more frequently in eyes that underwent the external approach. We attributed this to several characteristics of this technique. The smaller hole at the injection site may have minimized the loss of cells before their attachment to Bruch’s membrane. The tightly sutured scleral flap provided a watertight closure of the injection site not available with the internal approach. Finally, the use of EDTA permitted more effective removal of the RPE cells from Bruch’s membrane and may have increased the probability for the transplanted cells to find a suitable matrix to which they could attach.

RPE cells were successfully placed into the subretinal space in approximately 80% of all our transplants, both internal and external. Surgical complications (no transplantation bleb formation noted after injection of cells from over- or underpenetration into the choroid or the vitreous, cell leakage through the needle or pipette insertion site, intraretinal or subretinal hemorrhage, retinal schisis, and iatrogenic holes in the external approach) occurred in the remaining 20%.

Clinical Relevance

These studies demonstrate the feasibility of transplanting autologous RPE cells from the periphery to the macula. As elective macular surgery 37,38 is becoming more common, mechanisms for immediate repair of damage to the RPE gain importance. Transplantation of autologous RPE could offer a significant advantage over allografts and associated problems of tissue matching and immunosuppression. For patients with hereditary disorders expressed primarily in the macula, autologous transplants could provide a means of delaying degeneration. Using molecular biology to correct the defect in autologous cells could make prevention a reasonable goal for diseases such as Stargardt’s disease. In such cases, the reduced risk for subsequent proliferative retinopathy would justify the use of the external approach, routinely employed for several clinical problems, eg, decompression of the optic nerve in papillary edema and trauma near the optic nerve. The diathermy could be done easily 0.5 mm from the area of transplantation; lower angles of injection could increase this distance. Issues that need to be resolved include the effects of: (1) flattening the bleb on atrophy, (2) regional differences in the RPE, 39–41 and (3) changes in the biochemical and physical properties structure of Bruch’s on the phenotypic expression of the transplanted cells. 43,44

The primary defect in AMD is assumed to reside in the RPE and to be expressed preferentially in the macula. Replacement of the effete posterior RPE with less damaged peripheral RPE cells might slow the progression of AMD and extend the period of useful vision by years. However, age- and disease-related changes in the composition or lipid content of Bruch’s membrane may have a profound influence on the success of transplants. Differentiation of RPE cells in vitro is strongly influenced by the chemical and physical characteristics of the matrix on which they are plated. 41 The Bruch’s membrane in AMD is extensively altered by basal linear deposits and/or various types of drusen. Moreover, the ability (or inability) of transplanted cells to restore permeability to a lipid-laden Bruch’s membrane is likely to have a determining influence on the utility of transplantation.

In conclusion, these experiments have documented the feasibility of autologous transplantation of RPE
cells. The clear advantage of autologous transplantation is that it obviates the need for immunosuppression and may provide a readily available source for RPE cells in selected disease states.

Key words: biopsy, macular degeneration, rabbit, retinal pigment epithelium (RPE), transplantation autologous

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References


