Short-Term Study of Allogeneic Retinal Pigment Epithelium Transplants onto Debrided Bruch's Membrane

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PURPOSE. To investigate the survival and behavior of retinal pigment epithelium (RPE) microaggregates transplanted onto hydraulically debrided Bruch's membrane and to compare results of using three different vehicles for cell delivery.

METHODS. RPE microaggregates obtained from male cats were transplanted onto the tapetal area of female cats after native RPE was debrided. For the control, one of three vehicles was introduced into the debridements. Each transplant or control specimen was analyzed histologically and immunohistochemically. Transplanted male RPE cells were identified by in situ labeling of the cat Y chromosome.

RESULTS. Histologically, significant numbers of condensed, darkly stained RPE nuclei were observed in all transplants compared with few TUNEL-positive RPE cells. Cellular retinaldehyde-binding protein was present up to day 7 in all RPE cells in transplants. In both transplant and control specimens, the antibody against the Ki-67 nuclear antigen labeled some RPE cells at day 3. TUNEL-positive outer nuclear layer nuclei were most frequently observed at day 1, but were much less frequent at 7 days in both transplant and control specimens.

CONCLUSIONS. Transplanted RPE appeared to retain at least some markers of differentiation up to 7 days after surgery. Some proliferation of transplanted RPE cells was also seen. Apoptotic cell death of transplanted RPE, as judged by TUNEL staining was observed rarely. RPE transplants imposed no adverse effect on the overlying retina. RPE survival appeared to be similar with each of the three vehicles for cell delivery. (*Invest Ophthalmol Vis Sci.* 2001;42:2990–2999)

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Age-related macular degeneration (AMD) is the leading cause of blindness in the United States among persons more than 55 years old.[1-4] Most cases of severe visual loss in AMD are due to the growth of choroidal new vessels (CNVs) under the retinal pigment epithelium (RPE) and retina, which causes subretinal bleeding, secondary retinal detachment, subretinal scarring, and retinal degeneration.[5] Laser photocoagulation and photodynamic therapy,[6] the only known effective treatments for CNVs, are often associated with poor visual outcome and a high rate of recurrent CNV growth,[7-12] and a substantial number of patients are not eligible for these treatments.[13,14] Surgical excision of CNVs, as an alternative to photocoagulation, usually removes RPE in addition to the abnormal blood vessels.[15-18] Incomplete RPE resurfacing can lead to photoreceptor and choriocapillaris atrophy, which would compromise the surgical outcome.[18-21] One way to replace iatrogenically removed RPE is to transplant healthy RPE to the dissection bed.

RPE transplantation appears to be effective in the Royal College of Surgeons (RCS) rat.[22-28] RPE transplants in humans with AMD have limited effectiveness, however.[29] In contrast to the RCS rat, humans with AMD have abnormal Bruch's membrane, induced both by the disease and by CNV excision.[17,30] These abnormalities may contribute to differing results in humans and rats. Thus, understanding how to optimize RPE transplantation on different types of Bruch's membrane surfaces may be important. In addition, determining an optimal way to deliver RPE transplants is of importance. Several animal models, such as the rat,[22-28] monkey,[31-33] rabbit,[33-42] and miniature pig,[43] have been used for RPE transplantation studies. In the current study, we used a well-characterized cat model of localized RPE debridement[21] to investigate the survival and behavior of RPE microaggregates that were transplanted onto hydraulically debrided Bruch's membrane. We compared three different vehicles (PBS, DMEM, and 10% cat serum in PBS), for their capacity to deliver healthy RPE cells to the debrided areas. Transplants were analyzed histologically and immunocytochemically to identify transplanted cells and to assess the degree of RPE proliferation, differentiation, and apoptotic cell death in transplant areas.

MATERIALS AND METHODS

Cats

Male domestic short-haired cats (2–5 kg) were used as RPE donors, and female domestic short-haired cats (2–5 kg) served as hosts for the RPE transplantation studies. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RPE Cell Preparation

Microaggregates of pigmented male cat RPE were prepared by isolating a full-thickness biopsy specimen consisting of retina, RPE, and choroid.

Specimens were incubated in collagenase type IV (0.04 mg/ml) for 1 hour and 15 minutes at 37°C. After incubation, RPE sheets were separated from the choroid and retina. RPE microaggregates were formed by triturating a $6 \times 6\text{-mm}^2$ RPE sheet in 25 μl of one of the vehicles: PBS (Cellgro; Mediatech, Herndon, VA), DMEM (Gibco BRL, Grand Island, NY), or 10% normal cat serum (Accurate Chemical & Scientific Corp., Westbury, NY) diluted in PBS.

Transplantation Surgery

Hydraulically debrided retinal detachments (blebs) were made according to a procedure described previously.[21] After pars plana vitrectomy, a 33-gauge cannula was introduced through a sclerotomy and placed on the surface of the retina, overlying the tapetum. Forceful injection of balanced salt solution (BSS) through an attached syringe, simultaneously created a localized retinal detachment and removed native RPE in the central four fifths of the bleb area. Most blebs measured 2 to 3 mm in diameter. A 30-gauge cannula attached to a syringe (Hamilton, Reno, NV) was introduced into the subretinal space through the original retinotomy, and 45,000 RPE cells/5 μl vehicle were injected under the detached retina. Some RPE efflux occurred after the injection; this efflux, judged under the operating microscope, appeared to be minimal. The sclerotomies and conjunctiva were closed with a 7-0 Vicryl suture (Ethicon, Piscataway, NJ). The canthotomy was closed with a 6-0 black silk suture. Dexamethasone (8 mg) and gentamicin (20 mg) were injected subconjunctivally, and the eye was dressed with bacitracin and 1% atropine ointment.

Fluorescein Angiography and Fundus Photography

Fluorescein angiograms (FAs) and color fundus photographs were performed with a fundus camera (model CF-60UVi; Canon USA, Lake Success, NY) within 24 hours of surgery and again 3 and 7 days after surgery. FAs of experimental eyes were obtained by injecting fluorescein intravenously (10% AK fluor [Alcon, Fort Worth, TX], 0.3–0.5 ml) after sedation with ketamine (10 mg/kg) and xylazine (0.5 mg/kg).

Experimental Design

RPE transplantation was performed on 17 eyes from 17 animals (5 eyes for 1-day survival and 6 eyes each for 3- and 7-day survival). In 16 eyes, four blebs were made in the tapetal area; in the 17th eye, only three blebs were made. RPE microaggregates, suspended in one of the three vehicles, were delivered to each transplant bleb. Twenty-seven transplants were analyzed (three transplants from three different eyes for each vehicle at each time point). In addition, 15 eyes from 15 animals were used as control specimens (5 eyes for day 1, 4 eyes for day 3, and 6 eyes for day 7). In each of these blebs, one vehicle but no RPE was injected into the subretinal space after hydraulic debridement. Twenty-seven control experiments were included in this study (three control specimens from three different eyes for each vehicle at each time point).

For both transplant and control specimens, groups of consecutive slides were selected from one end of the bleb to the other end, separated by approximately 200 μm. The consecutive slides were treated by immunolabeling of nuclear antigen Ki-67 (MIB-1; Immunotech, Westbrook, ME) and antibody against cellular retinaldehyde binding protein (CRALBP, a gift from John C. Saari, University of Washington, Seattle), TUNEL staining (Intergen Co., Purchase, NY), toluidine blue staining, or Y chromosome in situ labeling[44] (transplant only). Y chromosome in situ hybridization also was performed on male control retina to monitor the sensitivity of the labeling and on female control retina peripheral to each transplant bleb to monitor the specificity of the labeling.

Tissue Processing

At 1, 3, or 7 days after surgery, animals were injected with an intravenous overdose of pentobarbital sodium (200 mg/kg) after sedation

with ketamine (10 mg/kg) and xylazine (0.5 mg/kg). Eyes were enucleated immediately and fixed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 minutes to 1 hour to harden the tissue. The anterior segment of the eye was then removed, and the posterior eyecup was immersed in fresh fixative at 4°C for 16 hours.

To study each bleb, a piece of tissue approximately $4 \times 4 \text{ mm}^2$ was trimmed off and washed in 0.1 M phosphate buffer for 30 minutes with three changes. The tissue was then dehydrated in ascending ethanol concentrations (30%, 50%, 70%, 85%, 95%, and 100%). After dehydration, the tissue was cleared in butanol-ethanol (1:1) for 30 minutes, in pure butanol for 1.5 hours with three changes at room temperature, and in pure butanol for 30 minutes at 60°C in a vacuum oven. The tissue was then infiltrated in diethylene glycol disterate (DGD; Polysciences Inc., Warrington, PA) with 0.3% dimethyl sulfoxide (DMSO)/butanol (1:1) for 45 minutes at 60°C and DGD with 0.3% DMSO for 2 hours at 60°C with four changes in a vacuum oven. The tissue was oriented with the retina facing up in a cryomold (Miles Laboratory, Inc., Elkhart, IN), and a piece of parafilm was put on top of the mold to eliminate air from the DGD during polymerization. Two-micrometer-thick sections were cut on a ultramicrotome (Utracut UCT; Leica Microsystems Inc., Deerfield, IL), transferred to slides (ProbeOn Plus; Fisher Scientific, Fairlawn, NJ), and baked at 55°C overnight.

Histology

Selected sections were dewaxed in xylene and rehydrated in descending ethanol (100%, 95%, 85%, 70%, and 50%) through water. Sections were selected as described in the experimental design. Sections were stained with 0.125% toluidine blue and coverslipped with mounting medium (DePex; BDH Laboratory Supplies, Poole, UK).

In Situ Hybridization with Cat Y Chromosome Probe to Identify Transplanted RPE Cells

Preparation of Cat Female Genomic DNA. Cat female genomic DNA was extracted from cat liver as described by Sambrook et al.[45] with the following changes: Tissue was digested overnight at 45°C in extraction buffer and proteinase K. After phenol extractions, 24:1 chloroform-isoamyl alcohol was used to remove phenol from the aqueous phase. Isopropanol was used to precipitate DNA from the final aqueous phase, and the resultant pellet was washed in 70% ethanol before resuspension in filtered deionized water.

Probe Preparation. The cat Y chromosome-specific probe was labeled with digoxigenin-dUTP by polymerase chain reaction (PCR), as described previously.[44] Each 100-μl PCR reaction mixture contained $1\times$ PCR buffer (50 mM KCl, 20 mM Tris [pH 8.4]; Gibco BRL); 1.5 mM $MgCl_2$; 100 pmol Jun_1 primer; 200 μM each of dATP, dCTP, and dGTP; 190 μM dTTP; 10 μM digoxigenin-11-dUTP; 390 ng DNA template; and 5 U *Taq*I polymerase (Gibco BRL). The DNA was denatured at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, a 30-second annealing at 57°C, and extension at 72°C for 1 minute. After final extension at 72°C for 5 minutes, the reaction mixture was held at 4°C until removal. The PCR product was aliquoted and kept at -20°C until use.

To make 100 μl hybridization mixture, 20 μl salmon sperm DNA (10 mg/ml, Gibco BRL), 20 μl yeast transfer (t)RNA (10.2 mg/ml; Sigma Chemical Co, St. Louis, MO), 10 μl cat female genomic DNA (100–250 ng/μl), and 20 μl probe were ethanol precipitated at -80°C for 2 hours. After centrifugation at 14,000 rpm for 10 minutes, the pellet was resuspended in hybridization solution containing 50% formamide (Gibco BRL), 10% dextran sulfate (Gibco BRL), and $2\times$ SSC (0.3 M sodium chloride and 0.03 M sodium citrate [pH 7.4]; probe-hybridization solution: 1:5). The hybridization mixture was denatured at 78°C for 6 minutes immediately before use.

Tissue Processing. The in situ hybridization procedure was performed on RPE transplants, on female control retina peripheral to each transplant bleb, and on male control retina. Tissue sections were dewaxed in xylene through 100% ethanol. Endogenous peroxidase was quenched with 1% hydrogen peroxide in methanol for 10 minutes.

After rehydration in descending ethanol, antigenicity was increased by microwaving sections in 240 ml 0.1 M sodium citrate (pH 6).[46] Sections were microwaved three times for 5 minutes each at high power. After the first two microwave treatments, the sodium citrate volume was restored to 240 ml. After the final microwave treatment, slides were allowed to cool for 20 minutes in the same solution. To decondense DNA, sections were incubated in $2\times$ SSC (pH 7.4) for 5 minutes at 80°C and rinsed with filtered deionized water for 5 minutes with three changes. The sections were then digested with pepsin (4 mg/ml) in 0.2 N HCl for 2.5 minutes at room temperature. Slides were rinsed with filtered deionized water very briefly, followed by two 5-minute rinses with PBS (pH 7.4). DNA was denatured by incubating sections in 70% formamide in $2\times$ SSC for 6 minutes at 80°C. Sections were dehydrated immediately in cold graded ethanol (70%, 95%, and 100%) and air dried. Five microliters of denatured hybridization mixture was applied to each slide. A negative control was generated by omitting the Y chromosome probe in the hybridization mixture. Sections were coverslipped immediately, sealed with dental wax, and incubated in a moist chamber at 37°C overnight.

After hybridization, the sections were rinsed initially in 50% formamide in $2\times$ SSC for 10 minutes at 43°C, followed by three more rinses for 10 minutes each in $2\times$ SSC at 43°C. Nonspecific binding sites were blocked by incubation in 0.5% bovine serum albumin (ICN Biomedicals, Inc., Costa Mesa, CA) and 0.1% Triton X-100 in PBS for 30 minutes at room temperature in a moist chamber. Sections were incubated initially in sheep anti-digoxigenin antibody (1:100, Boehringer Mannheim Corp., Indianapolis, IN) for 1 hour at room temperature, rinsed in PBS for 15 minutes with three changes, incubated in biotinylated donkey anti-sheep antibody (1:100, Sigma) for 1 hour, and rinsed in PBS for 15 minutes with three changes. Signal was amplified by incubating sections in avidin-biotin complex (ABC) reagent (Vector Laboratories, Burlingame, CA) and visualized by incubating the sections with 0.01% 3,3-diaminobenzidine (DAB; Research Genetics, Huntsville, AL). Male and female control tissues (which were taken from the nonpigmented tapetal area of the cat) were dehydrated immediately, cleared, and mounted. To bleach the pigment in transplanted cells, transplant slides were incubated in 2.5% hydrogen peroxide in 1% dibasic sodium phosphate for 16 hours at room temperature. Slides were then dehydrated, cleared, and mounted.

Immunocytochemistry

CRALBP, a protein that may participate in the reactions of the visual cycle,[47] is present in both neural retina and the RPE.[48] A polyclonal antibody made in rabbits against bovine CRALBP was used as a marker for RPE differentiation.[49] The monoclonal MIB-1 antibody against the Ki-67 nuclear antigen (which is associated with cell proliferation and is present in every proliferative phase—G_1, S, G_2, M— of the cell cycle) was used as a marker for RPE proliferation.[50,51]

Selected sections were dewaxed and rehydrated as described earlier. For Ki-67 labeling, antigenicity was increased by microwaving slides in 240 ml 0.01 M sodium citrate (pH 6) two times for 5 minutes each at high power. For CRALBP, the microwave treatment was one time for 5 minutes at low power.[52] After the microwave treatment, sections were cooled for 20 minutes in the same solution. For both Ki-67 and CRALBP labeling, nonspecific binding sites were blocked by incubating sections in 2% normal goat serum (Vector), 0.5% bovine serum albumin, and 0.1% Triton X-100 in PBS for 45 minutes at room temperature. Primary antibodies (rabbit anti-CRALBP 1:40,000; mouse anti-Ki-67 1:100) were applied to sections overnight at 4°C before rinsing with PBS for 30 minutes with three changes. For negative controls no primary antibodies were applied. Sections were then incubated in linker solution (biotinylated anti-mouse-rabbit polyclonal antibody) from a kit (LSAB2; Dako Corp., Carpinteria, CA) for 30 minutes at room temperature and rinsed with PBS for 30 minutes with three changes. Sections were then incubated in alkaline phosphatase–conjugated streptavidin for 30 minutes at room temperature and rinsed for 30 minutes with PBS. 5-Bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT; Dako Corp.) was used as the phosphatase substrate. After incubating in BCIP/NBT for 10 minutes at room temperature, sections were dehydrated and permanently mounted. For Ki-67 labeling, sections were counterstained with methyl green before dehydration and mounting.

Terminal Transferase dUTP Nick End Labeling

Apoptotic figures in RPE transplants were identified by TUNEL using a kit (ApopTag In Situ Apoptosis Detection; Intergen Co.). Staining procedures were modified based on the manufacturer's recommendations. Sections were dewaxed and rehydrated as described earlier. Endogenous peroxidase was inactivated by incubating the sections in 2% hydrogen peroxide at room temperature for 5 minutes followed by two 5-minute rinses with PBS. Sections were pretreated in equilibrium buffer for 10 minutes at room temperature and incubated in 54 μl terminal deoxynucleotidyl transferase (TDT) enzyme in reaction buffer (1:12) for 1 hour at 37°C. Reactions were stopped by transferring the sections to a buffer (Stop/Wash, Intergen Co.) for 10 minutes at room temperature. Sections were rinsed three times in PBS for 5 minutes each and were then incubated in peroxidase-conjugated antidigoxigenin antibody for 30 minutes at room temperature and rinsed in PBS for 30 minutes with three changes. One 15-minute incubation in 0.01% stable DAB was used to visualize the signal. To bleach the RPE pigment, sections were treated with 1% hydrogen peroxide and 1% dibasic sodium phosphate solution at room temperature for 15 hours. After depigmentation, sections were rinsed briefly with filtered deionized water and counterstained with 1% to 4% methyl green (Vector Laboratories) before being dehydrated and mounted. Positive controls were prepared in slides that were ready for equilibrium buffer pretreatment by nicking DNA with 10 U/ml DNase 1 (Roche Molecular Biochemicals) in DNase buffer (0.1 M sodium acetate and 5 mM $MgSO_4$ [pH 5.0]) for 15 minutes at room temperature. These slides were then processed as described earlier. Negative controls were generated by substituting filtered deionized water for the TdT enzyme.

Statistical Analysis

RPE Nuclei. The numbers of Y chromosome[+], Ki-67^{+}, TUNEL[+], and darkly stained RPE nuclei in each bleb were counted in bleached, treated, or stained sections for all selected regions throughout each transplant. Ki-67^{+} RPE nuclei were also counted for each vehicle control bleb. The frequencies were calculated by dividing the counts by the total number of RPE cells in the section, estimated by counting RPE in adjacent toluidine blue–stained sections. Criteria used to mark the bleb border included determination of the area of retinal detachment, evidenced by shortening of the photoreceptor outer segments and presence of retinal folds. Average frequencies for each transplant or control specimen were calculated by dividing the sum of frequencies from all regions by the number of regions in each transplant or control specimen. Finally, the average frequencies for each time point were calculated by dividing the sum of average frequencies from all transplants or control specimens by the number of transplants or control specimens at each time point. Before combining the average frequencies from all transplants or control specimens at each time point, single-factor analysis of variance (ANOVA) was used to detect whether significant differences occurred among the average frequencies.

Outer Nuclear Layer Nuclei. To determine whether the frequency of apoptotic nuclei in the outer nuclear layer (ONL) over the debridement changes over time and to compare the change between transplants and control specimens, frequency of TUNEL[+] ONL nuclei was calculated by dividing the number of positive nuclei by the total number of ONL nuclei (visualized by methyl green counterstaining) in the same section. Average frequency for each transplant or control specimen was calculated by dividing the sum of frequencies from all regions by the number of regions in each transplant or control specimen. The average frequency of positive cells for each time point was obtained by dividing the sum of frequencies from all transplants or control specimens by the number of transplants or control specimens at each time point.

TABLE 1. Average Frequency of Y Chromosome[+], Ki-67^{+}, TUNEL[+], and Darkly Stained RPE Nuclei at Different Time Points after Surgery

	Y Chromosome[+]*	Ki-67[+]†	TUNEL[+]‡	Darkly Stained§
Day 1	43.25 ± 1.26 ($n = 9$)	0.32 ± 0.24 ($n = 9$)	0.11 ± 0.07 ($n = 9$)	6.25 ± 1.51 ($n = 9$)
Day 3	31.72 ± 1.17 ($n = 9$)	13.54 ± 2.46 ($n = 9$)	0.44 ± 0.19 ($n = 9$)	2.89 ± 0.80 ($n = 9$)
Day 7	24.55 ± 1.60 ($n = 9$)	1.13 ± 0.28 ($n = 9$)	0.74 ± 0.38 ($n = 9$)	6.10 ± 1.40 ($n = 9$)
Male control	46.58 ± 3.08 ($n = 20$)			

Darkly stained RPE cells significantly outnumbered TUNEL^{+} RPE cells at each time point (Student's *t*-test, $P < 0.05$). Data are mean percentage of positive cells $\pm$ SE.

* Days 1, 3, and 7 are significantly different from one another. Both day 3 and day 7 are significantly different from male control. Tukey multiple comparison tests show calculated q values $> q_{0.05,30,4}$ for (=0.05). Day 1 is not significantly different from male control. Calculated q value is less than $q_{0.05,30,4}$ for (=0.05).

† Day 3 is significantly different from day 1 or 7. Tukey multiple comparison tests show calculated q value $> q_{0.05,24,3}$ for (=0.05). Days 1 and 7 are not significantly different. Calculated q value is less than $q_{0.05,24,3}$ for (=0.05).

‡ Days 1, 3, and 7 are not significantly different from one another (ANOVA, $P > 0.05$).

§ Days 1, 3, and 7 are not significantly different from one another (ANOVA, $P > 0.05$).

The observer was blinded to whether Y chromosome probe, TdT enzyme, or primary antibody was applied in the process of counting Y chromosome[+], TUNEL[+], or Ki-67^{+} cells. ANOVA and the Tukey multiple comparison tests were used to compare average frequencies within and between each time point for both transplants and control specimens. At each time point, Student's *t*-test was used to compare average frequencies of TUNEL-positive and darkly stained RPE in transplants. Student's *t*-test also was used to compare, at each time point, the average frequencies of TUNEL-positive nuclei in transplants and vehicle-treated control specimens.

Because the average frequencies regarding RPE nuclei or ONL nuclei did not vary in a statistically significant way among vehicles, the values presented in Tables 1 and 2 in the Results section are averages of all transplants or control specimens at each time point.

RESULTS

Fundus Photography and FA

Color fundus photographs showed that in all (9/9) cat serum–RPE transplants, eight of nine DMEM-RPE transplants, and six of nine PBS-RPE transplants, most of the RPE pigment was concentrated toward the inferior margin of the blebs. In one DMEM-containing and three PBS-containing transplants, RPE pigment appeared to be symmetrically distributed throughout the bleb (Fig. 1A). FA of transplants at all three time points showed hypofluorescence in areas of clinically evident pigment clumping, which are areas presumed to contain transplanted RPE. Dye leakage at day 1 was observed in hydraulically debrided areas with no pigment clumps. At day 3, smaller circular or patchy leakage was observed in pigment-free areas

in the transplant bleb. At day 7, minimal punctate or no leakage was seen in pigment-free areas (Fig. 1).

Control hydraulic debridements (into which one of the three vehicles but no RPE cells were injected) at day 1 showed intense hyperfluorescence due to dye leakage into the subretinal space. Dye leakage throughout the whole bleb indicates that native RPE cells were fully debrided.[21] Control blebs showed smaller circular leakage at day 3, with minimal to no leakage at day 7.

Histologic Analysis

In all the transplants at day 1, multilayers of pigmented RPE were found in the subretinal space beneath the detached retina. Most of the transplants were asymmetrically distributed in the bleb, as the fundus photographs indicate. Sometimes, an acellular zone was present between transplants and native RPE at the bleb edges. At day 3, eight transplants were multilayered. One (in cat serum) was predominantly bilayered and/or monolayered. Flattened RPE cells were usually seen at the bleb edges on the host side. At day 7, six transplants were multilayered, and three transplants (1 in each vehicle) were predominantly bilayered and/or monolayered. RPE monolayers were also found at the bleb edges. For all vehicle-treated control specimens at day 1, no RPE cells were observed in the debrided areas. By day 7, Bruch's membrane was completely resurfaced by an RPE monolayer, although RPE multilayers were seen occasionally beneath retinal folds.

For both transplants and control preparations, no mitotic figures were seen at day 1 or day 7. A few were observed at day 3 at an average frequency of $1.65\% \pm 0.73\%$ (mean $\pm$ SE; $n =$

TABLE 2. Comparison of Ki-67^{+} RPE and TUNEL[+] ONL Nuclei between Transplants and Vehicle-Treated Control Specimens at Different Time Points after Surgery

	Ki-67[+] RPE*		TUNEL[+] ONL Nuclei†	
	Control	Transplant	Control	Transplant
Day 1	0.58 ± 0.58 ($n = 9$)	0.32 ± 0.24 ($n = 9$)	0.55 ± 0.12 ($n = 9$)	0.64 ± 0.18 ($n = 9$)
Day 3	16.13 ± 2.30 ($n = 9$)	13.54 ± 2.46 ($n = 9$)	0.47 ± 0.10 ($n = 9$)	0.34 ± 0.09 ($n = 9$)
Day 7	1.09 ± 0.43 ($n = 9$)	1.13 ± 0.28 ($n = 9$)	0.02 ± 0.01 ($n = 9$)	0.01 ± 0.01 ($n = 9$)

Data are mean percentage of positive nuclei $\pm$ SE.

* For both transplant and control, day 3 is significantly different from days 1 and 7 (Tukey multiple comparison tests, q values $> q_{0.05,24,3}$ for (=0.05)), whereas days 1 and 7 are not significantly different from one another: q value $< q_{0.05,24,3}$ for (=0.05). Transplant is not significantly different from vehicle-treated control specimens at any time point (*t*-test, $P > 0.05$).

† For both transplant and control, day 1 is significantly different from day 7: q values $> q_{0.05,24,3}$ for (=0.05). For transplants, day 3 is not significantly different from day 1 or day 7: q values $< q_{0.05,24,3}$ for (=0.05). For control specimens, day 3 is not significantly differently from day 1: q values $< q_{0.05,24,3}$ for (=0.05); but it is significantly different from day 7: q values $> q_{0.05,24,3}$ for (=0.05). Transplant is not significantly different from vehicle-treated control at any time point (*t*-test, $P > 0.05$).
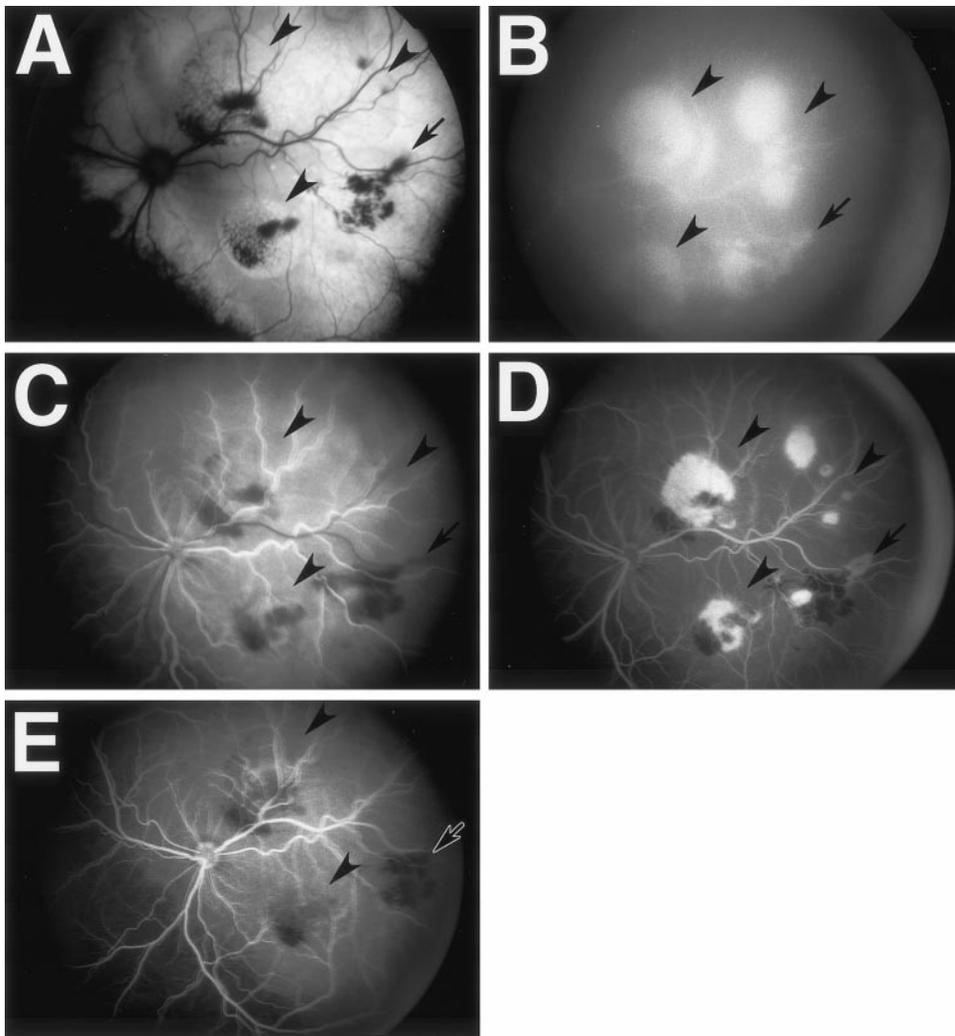
FIGURE 1. A 7-day survival transplant. (**A**) Fundus photograph. Four blebs are observed in the posterior pole. Three of them are transplant blebs. Clumping of pigment in areas presumed to contain transplanted RPE is asymmetrically located in two blebs. In the third bleb (*arrow*), pigment is distributed more uniformly. (**B**) FA at day 1. Dye leakage is observed in hydraulically debrided areas without pigment clumps. (**C, D**) FA at day 3. Smaller circular or patchy leakage is observed in pigment-free areas at the late stage (**D**). Less intense leakage is seen at the early stage (**C**). (**E**) FA at day 7. Minimal punctate or no leakage is seen in pigment-free areas. The control bleb is not shown.

9 transplants from six animals) for the transplants and 1.79% ± 0.48% (mean ± SE; $n = 9$ controls from four animals) for the control specimens. In transplants, although mitotic figures were located mostly at bleb edges and along edges of RPE multilayers, a few also were seen between RPE multilayers (Fig. 2B). In control specimens, mitotic figures were observed exclusively at bleb edges.

In transplants, condensed RPE nuclei that were darkly stained by toluidine blue (Fig. 2B) were observed occasionally in the RPE multilayers with all vehicles and at all three time points. The average frequencies of these nuclei for the three time points are not significantly different from one another (Table 1). No darkly stained RPE nuclei were observed in any of the control specimens.

In general, the retina overlying each transplant appeared similar to control blebs and was fairly well preserved, with photoreceptor outer segments shortened or absent in all transplants and inner segments shortened in seven transplants (one in DMEM and one in cat serum at day 1; one in each vehicle at day 3; and two each in PBS and DMEM at day 7). Foci of ONL nuclei displaced into the subretinal space were seen in a total of 14 transplants: 4 transplants at day 1 (one in DMEM and three in cat serum); 4 transplants at day 3 (one in PBS, two in DMEM, and 1 in cat serum); and 6 transplants at day 7 (two in each vehicle). Foci of displaced ONL nuclei were also observed in 10 vehicle-treated control specimens: 5 at day 3 (two in PBS and three in cat serum) and 5 at day 7 (one in PBS and two each in DMEM and cat serum). Retinal folds were present in three

transplants at day 1 (one PBS and two cat serum), in five transplants at day 3 (two in PBS, two in DMEM, and one in cat serum), and in all nine transplants at day 7. RPE pigment infiltration into the neural retina was seen in three transplants at day 7 (two in PBS and one in cat serum; Fig. 2C). Few inflammatory cells were seen in the retina or choroid at any time point.

Identification of Transplanted RPE

In these experiments, the presence of intracellular pigment can sometimes be used to help identify transplanted RPE cells, because pigmented RPE from male animals was transplanted into the nonpigmented tapetal region of female animals. However, pigment is not a very reliable marker, primarily because stressed RPE cells are known to expel their pigment into the subretinal space, and surrounding RPE may take up expelled pigment.[42,53,54] This expelled pigment, especially in multilayer clumps of RPE, can make it very difficult to identify transplanted cells with confidence.

For this reason, the cat Y chromosome probe was used to identify transplanted RPE cells with better precision. However, because the Y chromosome occupies such a small domain within the nucleus, the Y chromosome probe does not label 100% of the male cells in any one section. In previous work we estimated that, at most, only 70% of the cells in the retina were positively labeled by this probe.[44] The frequency of RPE labeling is lower, and in our present study it averaged 46.58% ±
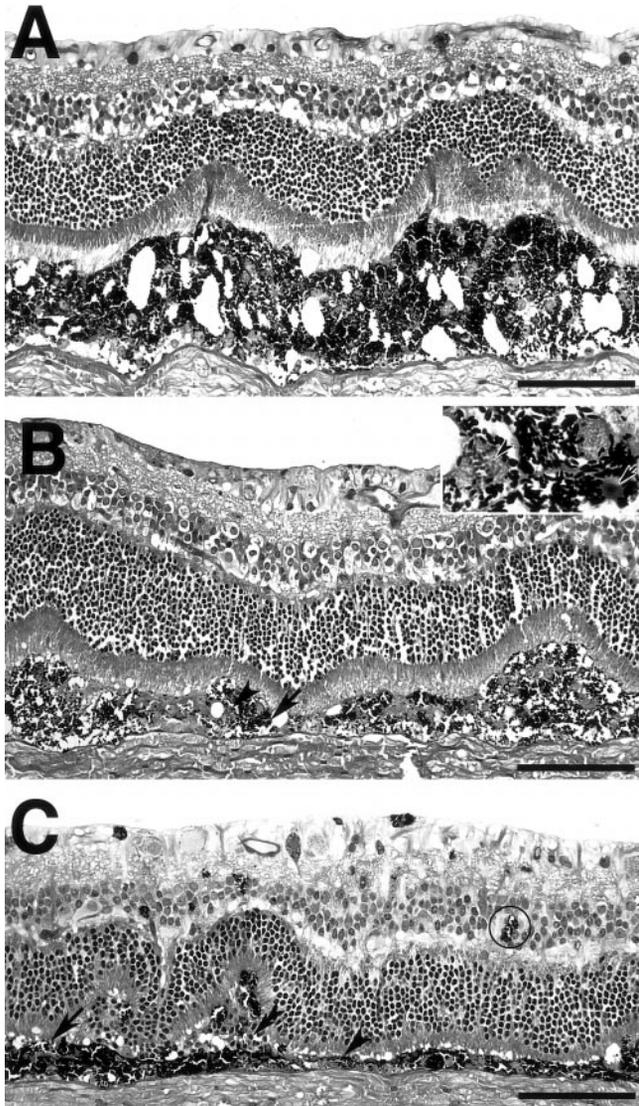
Figure 2. Toluidine blue staining in transplants: (**A**) 1-day survival. Pigmented RPE multilayers are present in the subretinal space. Retina is fairly well preserved, with photoreceptor outer segments shortened in some areas; (**B**) 3-day survival. Mitotic figures are seen in RPE multilayers (*arrowhead*). Darkly stained RPE cells also are present (*arrow*). *Inset*: mitotic nuclei (*arrowhead*) and a darkly stained RPE nucleus (*arrow*); (**C**) 7-day survival. RPE transplant is present in the subretinal space as monolayers (*arrowhead*) and multilayers (*arrow*). Retinal folds are present. Focal displacement of ONL nuclei (*double arrowhead*) and pigment infiltration into the neural retina (*circle*) also are observed. Scale bar, 100 µM.

3.08% (*n* = 20 sections from one animal). Therefore, although this technique allows unambiguous identification of many transplanted RPE cells, because no specific labeling was observed in female control tissues, the number of Y chromosome–positive cells probably underestimates the total number of transplanted RPE cells at each time point.

At day 1, almost all the Y chromosome–positive RPE cells were present in the pigmented RPE multilayer areas. The average frequency of the positive signals at day 1 was very close to that in the male control tissue. This suggests that the pigmented RPE cells in the blebs are mainly transplanted RPE. At days 3 and 7, most Y chromosome–positive RPE cells still appeared in the multilayers or monolayers in the bleb center, with a few also present in the bleb periphery. The average

frequencies of the positive signals decreased significantly over time (Fig. 3, Table 1).

Immunocytochemistry

In transplants, all the RPE cells in the bleb center were labeled by CRALBP in the cytoplasm at all three time points with no obvious change in label intensity up to 7 days after surgery (Fig. 4A).

Abundant Ki-67^{+} nuclei were observed in 3-day transplants. Although most positive cells were seen at bleb edges and at edges of RPE multilayers, some were noted deep within multilayers as well. Very few positive nuclei were seen at days 1 and 7. The average frequency of positive cells was significantly
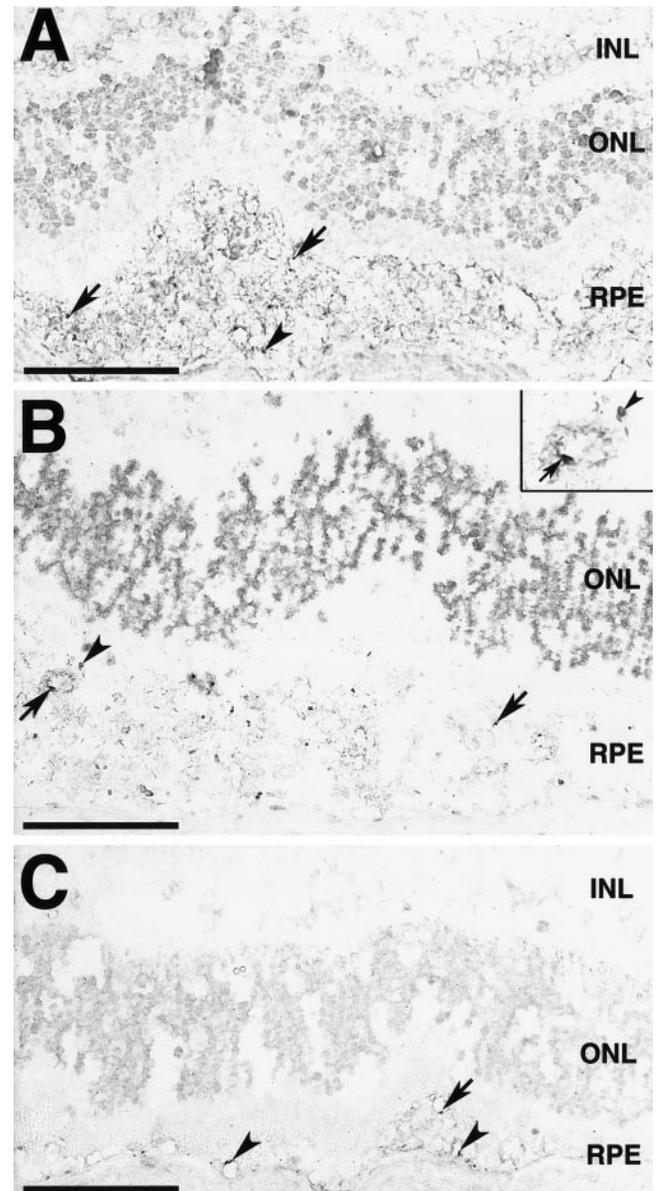

Figure 3. Y chromosome labeling: (**A**) 1-day transplant. Y chromosome signals are mostly in the bleb center (*arrows*). A positive cell is present on Bruch's membrane (*arrowhead*); (**B**) 3-day survival. Signals are scattered in the bleb center (*arrows*). Residual pigment granules (*arrowhead*) can be distinguished from signals based on their location, labeling intensity, and shape (*inset*); (**C**) 7-day survival. Y chromosome–positive cells are in the RPE monolayers, apposed to Bruch's membrane (*arrowheads*), as well as in the RPE multilayer (*arrow*). Scale bar, 100 µM.
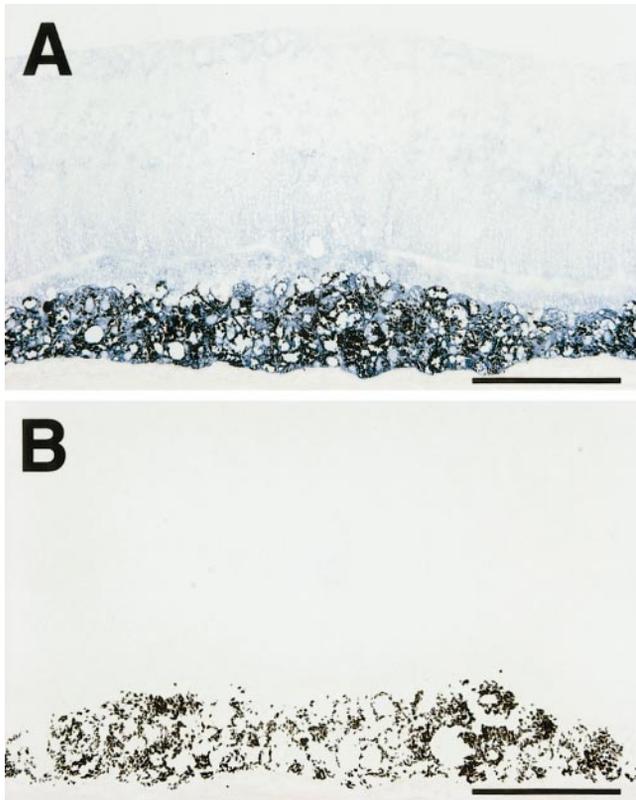
Figure 4. (A) CRALBP labeling in a 7-day transplant at the bleb center. Labeling is present in the cytoplasm of most, if not all, RPE cells in pigmented RPE multilayers. (B) A companion negative control of (A). No labeling is observed in RPE layers. Scale bar, 100 μM.

higher at day 3 than at either day 1 or day 7. No statistically significant difference between days 1 and 7 is present. In vehicle-treated control specimens, Ki-67$^+$ RPE cells were most abundant at 3 days and were located along the edges of blebs. The average frequency at 3 days was significantly higher than at either day 1 or 7. No statistically significant differences were observed between transplant and vehicle control blebs at any single time point (Fig. 5, Table 2).

No specific labeling was observed in any of the negative controls for either CRALBP or Ki-67 staining (Fig. 4B).

TUNEL Staining

Very few TUNEL$^+$ nuclei in RPE layers were seen at any time point (Fig. 6). RPE cells with darkly stained, condensed nuclei significantly outnumbered TUNEL$^+$ RPE cells at each of the three time points (Table 1). No TUNEL$^+$ RPE nuclei were present in the subretinal space in any of the control specimens.

TUNEL$^+$ ONL nuclei were seen, usually over RPE multilayers in transplants and over the RPE debridement in control specimens at day 1. In both conditions, the number of TUNEL$^+$ ONL nuclei decreased significantly at 7 days after surgery. TUNEL$^+$ ONL nuclei frequencies in transplants and vehicle-treated control specimens are not significantly different at any single time point (Fig. 6, Table 2).

In TUNEL$^+$ control specimens generated by treating the retina with DNase I, most, if not all, cells were labeled (Fig. 6A), whereas in TUNEL$^-$ control specimens (no TdT enzyme applied), there was no observable specific labeling (not shown).

Discussion

Seven days after injecting RPE microaggregates into the subretinal space, RPE multilayers and occasionally RPE monolayers were formed, and they appeared to be attached to the hydraulically debrided Bruch's membrane. Among the transplanted RPE cells were native RPE, as indicated by the dilution of the average frequency of the Y chromosome–positive signals over time. In a prior study, we have shown that centripetal ingrowth of native RPE cells resurfaces hydraulically debrided Bruch's membrane within 7 days after debridement.[21] However, the presence of Y chromosome–positive RPE, particularly those attached to Bruch's membrane at this time point, suggests that transplanted RPE may also be involved in the resurfacing. The surviving RPE transplants retained their identity as differentiated RPE, which is indicated by the continued presence of CRALBP, a protein that is specific for differentiated RPE cells.[47-49]

The presence of Ki-67$^+$ RPE cells and mitotic figures in the areas where most Y chromosome–positive signals were found (i.e., the bleb center) at day 3 indicates that some transplanted cells were proliferating, although the degree of this proliferation is hard to estimate because native RPE cells were also proliferating as seen in control specimens. Mitosis has been noted in transplanted cultured adult RPE.[32,55] In our experiments, a more sensitive marker (Ki-67) was used to detect proliferating cells not only in the mitotic phase, but also in other proliferating phases (G$_1$, S, and G$_2$).[50,51] Our data suggest that transplanted freshly harvested RPE also can proliferate to some degree. The causes of proliferation are not well understood. However, the appearance of RPE proliferation after retinal detachment[32,56] and the cessation of RPE proliferation after retinal reattachment[56] suggest that the close apposition of the RPE to the neural retina may help to keep the RPE in a mitotically inactive state. The dramatic decline of proliferating RPE cells at day 7 in our study may be because resurfaced RPE were well apposed to the overlying retina and/or because of cell–cell contact-induced inhibition of proliferation.
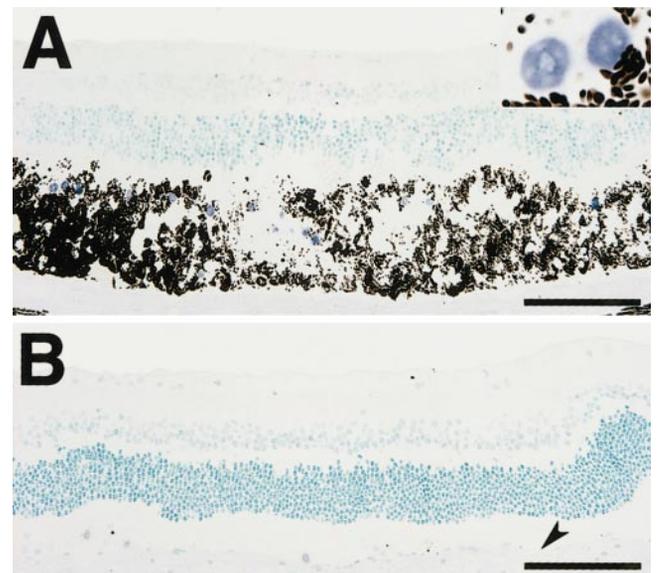

Figure 5. (A) Ki-67 labeling in a 3-day transplant. Positive RPE nuclei (*blue-purple* stain) mostly are present along edges of pigmented RPE multilayers. *Inset*: two Ki-67$^+$ RPE nuclei. (B) Ki-67 labeling in a 3-day vehicle-treated control specimen. Some positive nuclei are present at the bleb edge. *Arrowhead*: edge of the RPE debridement. Scale bar, 100 μM.
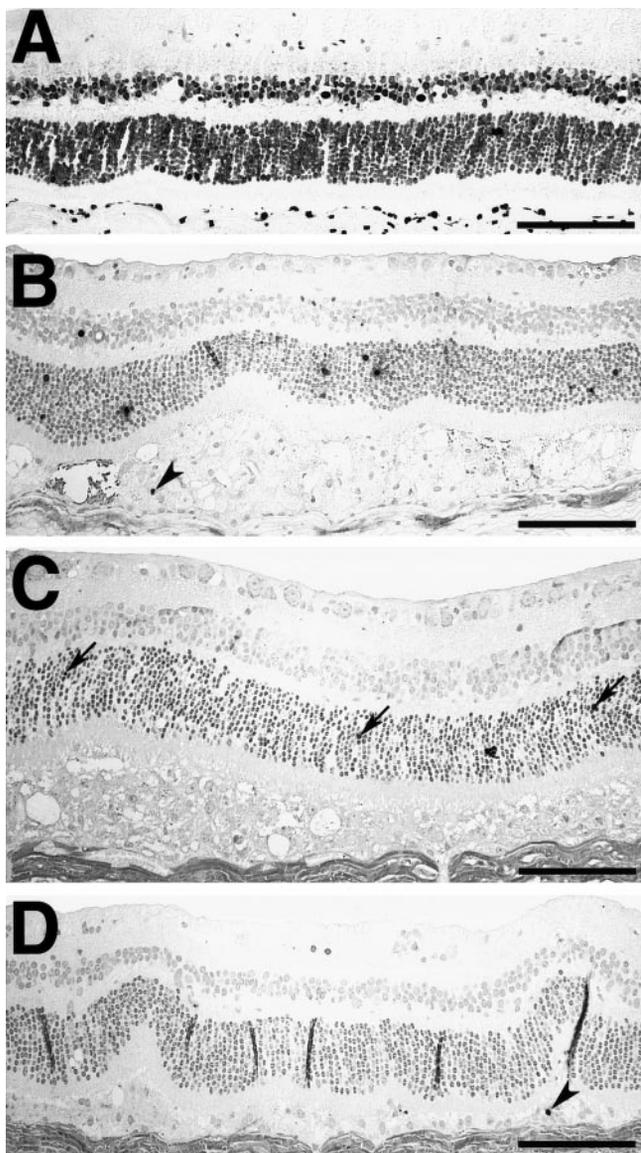
FIGURE 6.   TUNEL staining. (**A**) In a positive control (retina from a cat with no RPE transplant, section treated with DNase I before being processed with the TUNEL staining), all nuclei are labeled with variation in label intensity in different cells. (**B**) A 1-day survival transplant. Few positive nuclei are present in the subretinal space (*arrowhead*). Some positive nuclei are noted in the ONL over the RPE multilayer. (**C**) A 3-day survival transplant. No positive nuclei are seen in the subretinal space. A few positive nuclei are noted in the ONL over the RPE multilayers (*arrows*). (**D**) A 7-day survival transplant. One positive nucleus is noted in the subretinal space in RPE multilayers (*arrowhead*). No positive nuclei are present in the ONL over the RPE multilayers or monolayers. Sections in (**B**), (**C**), and (**D**) counterstained with methyl green. Scale bar, 100 μM.

Although some transplanted RPE lived up to 7 days after surgery, a few, especially those that were not attached to Bruch's membrane, appeared to be dying. These cells, with very condensed and darkly stained nuclei, were present in RPE multilayers where most Y chromosome–positive RPE cells were located. The absence of these cells in vehicle-treated control specimens indicates that they originated from the donor. Death of transplanted cells may be one reason for the thinning of transplants over time. If the darkly stained RPE cells were in fact dying, the mechanism of cell death is unclear. These cells did not appear to be injured or located in an area of damaged tissue, and death due to cell trauma therefore seems unlikely. An in vitro study has shown that RPE cells that are not attached to Bruch's membrane within 24 hours undergo apoptosis.[57] However, in the present study, darkly stained RPE cells greatly outnumbered TUNEL+ cells, but they were not also labeled by the TUNEL technique for apoptosis. The TUNEL technique detects 3'-OH ends of fragmented internucleosomal DNA, which is a characteristic feature of cells undergoing apoptosis.[58-60] The TUNEL technique is not foolproof, however, because internucleosomal DNA fragmentation can also be detected in cells exhibiting necrotic morphology,[61] even as it can be absent in induced apoptosis.[62] Thus, the failure of the TUNEL technique to label the darkly stained RPE cells does not necessarily mean that these cells are not dying by an apoptotic mechanism.

Allogeneic RPE grafts implanted into the immunologically privileged subretinal space can survive for 1 month to 1 year before graft rejection takes place.[25,36,42,63,64] In this short-term study, it is therefore not surprising that no obvious graft rejection was observed in any of the transplants. The absence of obvious inflammatory cell infiltration, disruption of the transplant, and severe photoreceptor degeneration at day 7 after surgery was interpreted as evidence that immune rejection had not occurred. Some ONL degeneration occurred, as indicated by the presence of TUNEL+ nuclei in this layer at day 1 after surgery and by foci of displaced ONL nuclei at all three time points. However, the frequency of TUNEL+ ONL nuclei decreased significantly 7 days after surgery in both transplants and control specimens with no statistically significant difference between the two. This result indicates that transplanted RPE have no adverse effect on the retina.

Different vehicles used to deliver RPE transplants (i.e., PBS, DMEM, or 10% cat serum in PBS) did not appear to affect the survival of transplanted RPE differently. This conclusion is indicated by similar degrees of RPE cell differentiation, proliferation, and death observed with different vehicles. Despite the use of three different vehicles, most transplanted RPE cells accumulated in the inferior portion of each bleb. This distribution of cells was likely due to gravity, which prevented the spread and uniform attachment of the cells to the debrided Bruch's membrane surface. This phenomenon has been observed by others[42] and may be one of the pitfalls of transplanting an RPE cell suspension. Some investigators have noted that RPE sheet transplants appear to distribute more uniformly in the subretinal space.[3,63] Others have proposed that a single-cell suspension is more likely to be immune-rejected than an intact RPE sheet.[65] It remains unclear whether transplanting RPE sheets or RPE cell suspensions is better. Further studies comparing RPE sheet transplantation and RPE microaggregate transplantation are in progress.

In short, RPE microaggregates that survived retained their differentiation for at least 7 days after subretinal transplantation. Transplanted RPE cells also showed a certain degree of proliferation after surgery. Apoptotic cell death of transplanted RPE, as judged by TUNEL staining was seen rarely. Cell death by other mechanisms cannot be ruled out. No signs of graft rejection were observed. RPE transplants imposed no adverse effect on the overlying retina. RPE survival and behavior appeared to be the same regardless of the vehicle used.

Due to technical limitations, we are neither able to estimate the fraction of transplanted cells that survived or proliferated, nor could we know whether the transplant had a rescue effect. Ideally, double staining of the Y chromosome probe and other markers applied in this study (i.e., Ki-67, CRALBP, and TUNEL) would have provided a more precise way to investigate the survival and effectiveness of transplanted RPE. However, because the Y chromosome probe labels only a fraction of donor cells, and because of the technical difficulties involved in

combining the complex and harsh Y chromosome labeling technique with the other labeling strategies, separate experiments were performed instead.

We believe this study is the first to use various immunomarkers to assess the short-term survival of microaggregate RPE transplants. The quantitative approaches used in this study may allow comparison of RPE microaggregates and sheets in a more precise way in future experiments.

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