

The Effect of Alkylphosphocholines on Intraretinal Proliferation Initiated by Experimental Retinal Detachment

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PURPOSE. To determine the effect of alkylphosphocholines (APCs) on intraretinal proliferation induced by experimental retinal detachment in the rabbit.

METHODS. Retinal detachments were created in adult pigmented rabbits. APCs, either liposome bound (liposome, L-APC) or unbound (free, F-APC), were injected intravitreally on either day 1 or day 2 after detachment. BrdU was injected on day 3, 4 hours before death. After fixation, retinas were triple labeled with anti-BrdU, anti-vimentin, and the isolectin B4. The number of anti-BrdU-labeled cells was counted per millimeter of retina from sections imaged by laser scanning confocal microscopy. Toxicity was examined using toluidine blue-stained sections imaged by light microscopy and by electron microscopy for ultrastructural evaluation.

RESULTS. Retinal detachment initiated proliferation of all non-neuronal cells. After intravitreal injection on day 1 or 2 after experimental induction of retinal detachment, APCs significantly reduced the number of dividing cells at day 3. Liposome-bound drug given on day 2 was more effective on Müller cell proliferation than was unbound drug. Injection of F-APC on day 1 was more effective than when given on day 2. No apparent effect was seen on Müller cell hypertrophy as indicated by vimentin expression. In addition, no evidence of toxicity was observed in the retina at day 3 for any of the conditions.

CONCLUSIONS. APCs significantly reduce the number of Müller cells that are stimulated to divide as a result of retinal detachment. The preliminary results indicate no evidence of significant toxicity; however, further studies are needed. APCs have the potential to be used as part of a therapeutic approach if they can be combined with other agents that can suppress the fibrosis that is also a critical event in the pathogenesis of proliferative vitreoretinal diseases such as proliferative vitreoretinopathy (PVR). (*Invest Ophthalmol Vis Sci.* 2007;48:1305-1311) DOI:10.1167/iovs.06-0591

Retinal detachment is a relatively common retinal condition often resulting in severe impairment of visual function. Early surgical reattachment of the detached region is crucial for visual recovery with an anatomic success rate of up to 90%, depending on extent and duration of the condition.^{1,2} Functional recovery, however, can be compromised due to apparent long-term intraretinal changes induced by the detachment.³⁻⁶

Apart from photoreceptor degeneration and synaptic remodeling of the detached retina,⁷⁻⁹ non-neural retinal cells, in particular, Müller glia cells, are thought to play a major role in the persistent reduction in visual acuity even after successful retinal reattachment.¹⁰⁻¹³ A glial cell-mediated mechanism of retinal degeneration even in nondetached retina adjacent to detached regions has also been proposed.¹⁴ Moreover, there are ample data to suggest that Müller cell hypertrophy and intraretinal proliferation play a crucial role in subretinal and epiretinal membrane formation.^{15,16} Such cellular membranes on the vitreal surface of the retina constitute proliferative vitreoretinopathy (PVR) and can promote subsequent tractional redetachment of the retina,¹⁷⁻²⁰ whereas glial subretinal membranes can block photoreceptor outer segment regeneration after successful reattachment surgery.²¹

The exact role of cell proliferation in these diseases is unknown; however, effective antiproliferative agents have long been sought as adjuncts to surgical reattachment because of the belief that they may decrease the likelihood of PVR with subsequent traction detachment or subretinal fibrosis. Current pharmacologic intervention focuses on treating PVR once the process is well established.²²⁻²⁷ PVR, however, most certainly has its beginnings soon after the detachment occurs. Indeed, it has been shown in animal models that proliferation of non-neuronal cells begins within a day after the detachment has formed.^{10,28} Most of these substances, however, have not been tested for their ability to influence the early stages of intraretinal proliferation in vivo. For those substances that have been tried, there is a paucity of data supporting efficacy and knowledge of potential toxic side effects. Thus, there is no routine, safe pharmacological agent for preventing or treating PVR (Wickham C et al. *IOVS* 2006;47:ARVO E-Abstract 4201).

Alkylphosphocholines (APCs) are effective inhibitors in vitro of human retinal pigment epithelial (RPE) cell attachment, spreading, migration, proliferation and cell-mediated membrane contraction at nontoxic concentrations.^{29,30} In addition, a study in the rat eye did not reveal any toxic effect of APCs on retinal tissue based on ultrastructural evaluation and electrophysiologic examination 7 days after intravitreal administration.³¹ Their action is mediated via binding to protein kinase C (PKC), a membrane-bound G-protein involved in the intracellular cascade of second-messenger systems that regulate these events.^{30,32-35} Thus, the inhibition of this pathway by APCs could offer a new perspective on PVR prevention. APCs are currently in routine clinical use due to their antitumoral (Miltex; Zentaris GmbH, Frankfurt, Germany) and anti-

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Supported by National Eye Institute Grant EY00888 (SKF), National Science Foundation Grant 0331697 (SKF), and an Alcon Retina Scholarship (KHE).

Submitted for publication June 1, 2006; revised October 21 and November 14, 2006; accepted January 19, 2007.

Disclosure: **K.H. Eibl**, Alcon Retina Scholarship (R); **G.P. Lewis**, None; **K. Betts**, None; **K.A. Linberg**, None; **A. Gandorfer**, None; **A. Kampik**, None; **S.K. Fisher**, None

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parasitic (Impavido; Zentaris) properties³⁶⁻³⁸ but have so far not been introduced into the field of ophthalmology.

The purpose of this study was to investigate the effect of APCs on intraretinal proliferation that occurs during the early stages of retinal detachment. The availability of a safe antiproliferative agent can allow us to determine in animal models the effect of proliferation on cellular reactivity and possibly lead to new ocular therapeutics for these diseases.

METHODS

Tissue Preparation

Retinal detachments were created in adult New Zealand Red pigmented rabbits by infusing a solution of sodium hyaluronate (Healon, 0.25% in physiologic saline solution; Pharmacia, Piscataway, NJ) via a glass pipette between the neural retina and RPE. The sodium hyaluronate is necessary to prevent spontaneous reattachment of the retina. The pipette was inserted into the eye via an incision that was made several millimeters below the pars plana to prevent the pipette from touching the lens. Approximately one half of the inferior retina was detached in the right eye, leaving the superior attached regions as internal controls. The left eyes served as the noninjected control.

Two experimental conditions were tested: (1) whether APC treatment was more effective given at day 1 or day 2 after detachment, (2) whether liposome-bound APC was more effective than the free drug. Three animals were used for each experimental paradigm: group 1, free drug given on day 2; group 2, free drug given on day 1; group 3, liposome-bound drug given on day 2; group 4, liposome-bound drug given on day 1; group 5, saline (physiologic saline (PS); Balanced Salt Solution [BSS]; Alcon Laboratories, Fort Worth, TX) injected on day 1. In treated eyes, 100 μM of APC in 50 μL BSS was injected intravitreally via a 30-gauge needle. On day 3 after detachment, the animals were injected with 10 μg BrdU (Sigma-Aldrich, St. Louis, MO) in 50 μL PS, 4 hours before being euthanized with sodium pentobarbital (120 mg/mL, IV). After enucleation, the eye was bisected and half of each eye, containing both attached and detached regions, was fixed in 4% paraformaldehyde (in 0.1 M sodium cacodylate buffer, pH 7.4; Electron Microscopy Sciences, Fort Washington, PA) for immunocytochemical analysis. The other half was placed in 1% paraformaldehyde plus 1% glutaraldehyde in phosphate buffer for light and electron microscopic analysis. After dehydration in graded ethanol series and infiltration with propylene oxide/Spurr resin mixture, the eyes were embedded in Spurr resin. All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Resource Center of the University of California, Santa Barbara.

Immunocytochemistry

After at least 24 hours of fixation, retinal pieces approximately 3 mm² were excised from three detached regions from within each eye. The tissue was rinsed in phosphate-buffered saline (PBS), embedded in low-melting-point agarose (5%; Sigma-Aldrich), and sectioned at 100 μm on a vibratome (Technical Products International-Polysciences, Warrington, PA). Sections were incubated in normal donkey serum (1:20) in PBS, 0.5% BSA, 0.1% Triton X-100, and 0.1% azide (PBTA) overnight at 4°C on a rotator. The following day, the sections were pretreated with 2 N HCl for 20 minutes as an antigen retrieval step for the BrdU. After the sections were rinsed in PBTA, the primary antibodies and lectin were added and incubated overnight at 4°C on a rotator in PBTA. Anti-BrdU (1:200, Accurate Chemical and Scientific Corp., Westbury, NY) was used to detect dividing cells, anti-vimentin (1:500; Dako, Carpinteria, CA) was used to determine the extent of Müller cell hypertrophy, and isolectin B4, *Griffonia simplicifolia* (1:50; Vector Laboratories, Burlingame, CA) was used to label microglia and macrophages. After the primary antibodies were rinsed in PBTA, the secondary antibodies (streptavidin CY5, donkey anti-rat CY3, and donkey anti-mouse CY2; Jackson ImmunoResearch, West Grove, PA) were

added together, each at 1:200 in PBTA, overnight at 4°C on a rotator. On the final day, the sections were rinsed in PBTA, mounted on glass slides using 5% *n*-propyl gallate in glycerol, and viewed on a laser scanning confocal microscope (Fluoview 500; Olympus, Tokyo, Japan).

To count BrdU-labeled cells, images of the retinas were collected as single-plane pictures from at least four sections taken from three different regions within each eye. Labeled cells were then counted and tabulated per millimeter of retina from the stored images with a magnification bar embedded in the image. Higher-quality images to show the morphology of the retina were collected as a *z*-series of five planes and collapsed as a projection of the images.

To check for potential toxic effects of APC, 1- μm -thick sections were cut from retinas embedded in Spurr resin, stained with toluidine blue, and imaged (BX60; Olympus). Attached and detached regions were both examined. In addition, electron microscopy was performed for ultrastructural evaluation of APC-injected eyes.

Alkylphosphocholines

Alkylphosphocholines (erucyl-homo-phosphocholine [ErPC₃]) and erucylphosphocholine [ErPC] were synthesized and kindly provided by Hansjoerg Eibl, PhD (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany). All reagents were of analytical grade, as determined by high-performance liquid chromatography.

The molecular structure of the two phospholipids, erucylphosphocholine (ErPC) and erucylphospho-(*N,N,N*-trimethyl)-propylammonium is very similar. In comparison to ErPC, ErPC₃ has an increased distance between phosphate and (*N,N,N*-trimethyl)-propylammonium. The smallest possible difference of only one methylene group, however, results in different physical properties. For instance, ErPC in water forms gel-like structures, so-called interdigitated bilayer systems. This could make a systemic application difficult. In comparison, ErPC₃ in water results in a clear micellar solution. To overcome the obvious difficulties with ErPC, we used a liposomal formulation of ErPC.

ErPC: Liposomal Dispersion

ErPC (MW 489.72) 980 mg (2 millimoles) and cholesterol (MW 386.66) 810 mg (2.1 millimoles) were dissolved by warming in 60-mL ethanol. The solvent was removed by evaporation. The dried residue was tempered at 60°C with 180 mL H₂O and then dispersed by ultrasonication at 60°C for 15 minutes. Then, sodium oleate, 20 g of a 5 mM solution in water (0.1 millimoles) was added and the mixture again treated by ultrasonication at 60°C for 15 minutes. To achieve physiologic conditions, 1.76 g NaCl was added. After ultrasonication at 60°C for 10 minutes, the size of the liposomes was approximately 80 nm. The dispersion was sterile filtered through a 0.2- μm filter (Millipore, Bedford, MA) and stored at +4°C to +8°C until use.

ErPC₃: Micellar Solution

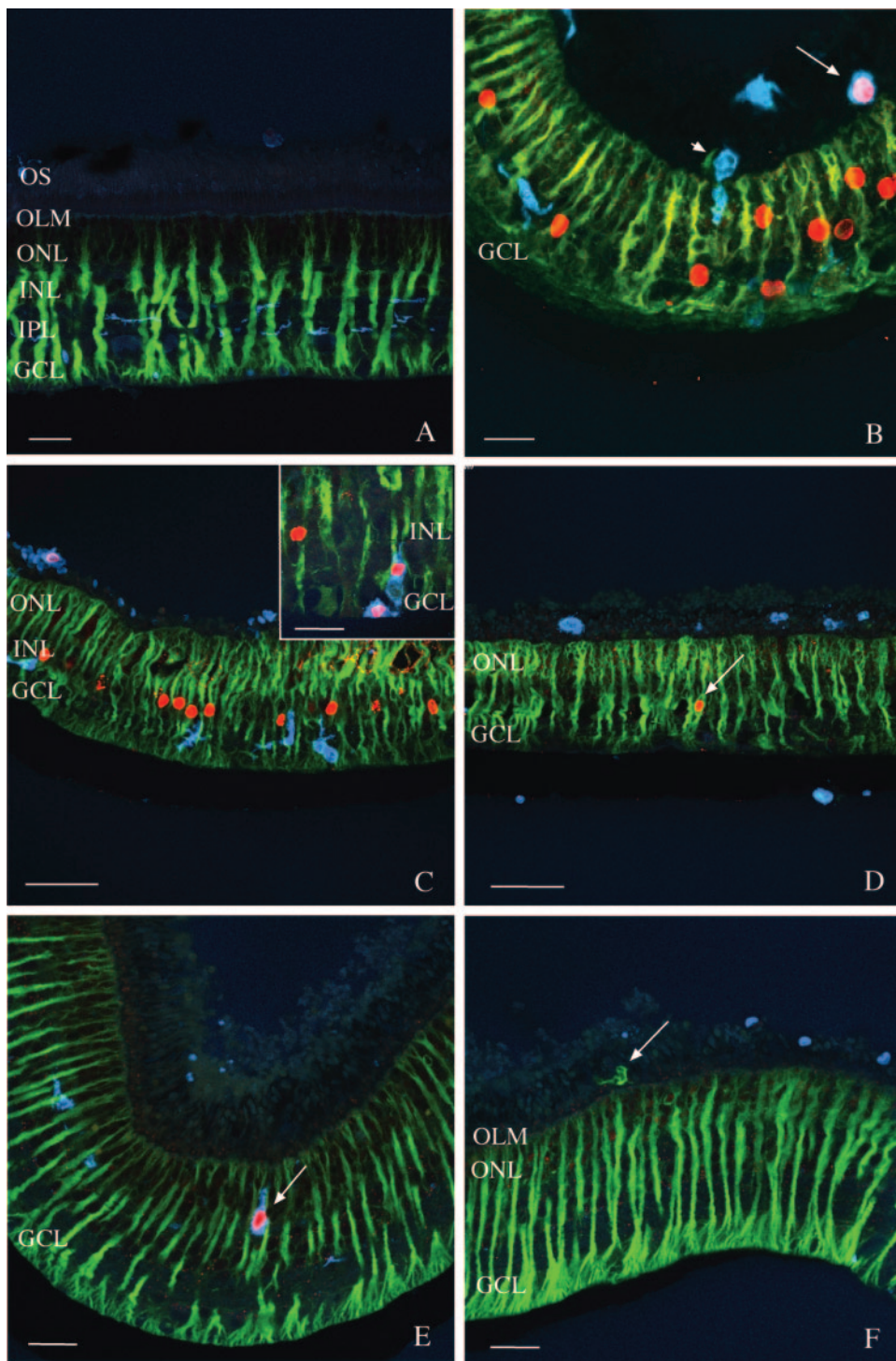
ErPC₃ (MW 503.75), 1080 mg (2 millimoles) was tempered in 200 mL 0.15 M NaCl at 60°C for 15 minutes. Ultrasonication at 60°C for 15 minutes resulted in a water-clear micellar solution. After sterile filtration through a 0.2- μm filter, the micellar solution of ErPC₃ was stored at +4°C to +8°C until use.

Stock Solutions of ErPC and ErPC₃

The liposomal dispersion of ErPC or the micellar solution of ErPC₃ was prepared in a 10-mM concentration in 0.15 M NaCl. The concentration applied in the biological experiment was obtained by dilution with 0.15 M NaCl.

APC, either bound (ErPC) or unbound (ErPC₃) to liposomes, was dissolved in 0.9% NaCl under sterile conditions and stored at 4°C (10 mM stock solution). Independent dilution series in PS (pH 7.4) were used to obtain a final concentration of 100 μM each, in equal volumes of PS. PS was also injected into control eyes as a control for the injection procedure.

FIGURE 1. Localization of proliferating cells in rabbit retina after a 3-day experimental retinal detachment, with and without APCs. Laser scanning confocal images of a 3-day detached rabbit retina labeled with antibodies to vimentin (green) and BrdU (red) and isolectin B4 (blue). (A) Undetached control retina. Anti-vimentin stained Müller cells extending into the ONL; the lectin labels fine microglia cell processes in the inner retina. (B) Detached control retina 3 days after experimental retinal detachment and saline injection on day 1. Müller cells hypertrophied to the outer limiting membrane (OLM; anti-vimentin; green), proliferated (anti-BrdU; red) and migrated into the ONL; microglia rounded up, migrated throughout the retina, and divided. Presumptive macrophages were observed in the subretinal space (arrowhead) some of which underwent division (isolectin B4; blue, arrow). (C) Three days after retinal detachment and injection of F-APC (free APC) on day 2 after the detachment procedure, there were proliferating (anti-BrdU; red) Müller cells (anti-vimentin; green) and microglia (lectin; blue; inset). Also, there was significant Müller cell hypertrophy. (D) After injection of L-APC (liposome bound APC) on day 1 after the experimental retinal detachment, only a few anti-BrdU-positive cells (arrow) were detectable throughout the retina and in the subretinal space; many microglia migrated out of the retina so there is no labeling present. (E, F) After injection of L-APC on day 2 after the detachment procedure, few proliferating cells were detectable. (E, arrow, dividing microglia; anti-BrdU, red; lectin, blue; F, arrow: small subretinal Müller cell growth without active proliferation). IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 20 μ m.



Statistical Analysis

All values are expressed as the mean \pm SD. To determine significant differences among groups for cell counts, statistical analysis was performed using the paired Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of APCs on Intraretinal Proliferation

Based on previous data showing that cell proliferation peaks at 3 days after detachment in the rabbit,²⁸ we injected BrdU 4

hours before death on day 3 to determine the effects of APCs administered 1 and 2 days earlier. In control nondetached retina, no anti-BrdU-labeled cells could be detected. In addition, anti-vimentin labeling of Müller cells extended from the inner limiting membrane (ILM) into the outer nuclear layer (ONL)—the typical pattern found in rabbit retina—and the isolectin B4-labeled microglial cells and their fine processes were restricted to the inner retina (Fig. 1A). Three days after detachment, in both control and APC-treated animals, anti-vimentin labeling increased in Müller cells to the outer limiting membrane and sometimes beyond, whereas the isolectin B4-labeled cells rounded up and migrated throughout the retina

and into the subretinal space (Figs. 1B-F). Anti-BrdU labeled Müller cells were observed both in the inner nuclear layer (INL), their normal location in the retina, and in the ONL, as a result of migration into the outer retina (Figs. 1B-D). Double-labeled anti-BrdU and isolectin B4-labeled cells were observed within the retina and in the subretinal space (Figs. 1B-E). Examples of APC-treated triple-labeled retinas are shown in Figures 1C-F. Numerous anti-BrdU-labeled Müller cells and isolectin B4-positive cells were observed in retinas given F-APC on day 2 (Fig. 1C, inset). Significantly fewer cells incorporated BrdU when the retinas were treated with the liposome-bound APC given on either day 1 (Fig. 1D) or day 2 (Figs. 1E, 1F). Although the APCs were effective at reducing cellular proliferation, Müller cell hypertrophy, as evidenced by the upregulation of vimentin, did not appear to be affected (Fig. 1C-E). Occasionally, vimentin-labeled processes extended into the subretinal space (Fig. 1F, arrow). Proliferation of retinal pigment epithelial cells was observed after detachment, but the levels were extremely low in the untreated retinas and therefore were not included in the study.

Figure 2 shows the number of BrdU-labeled cells per millimeter of retina. The total number of labeled cells was also broken down into three subgroups: Müller cells, microglia, and macrophages (Fig. 3). Müller cells were identified by retinal location and vimentin expression, microglia were identified as isolectin B4-positive cells within the retina, and macrophages were identified as isolectin B4-positive cells in the subretinal space. The discrimination between microglia and macrophages came from a previous study,³⁹ where it was shown that cells in the subretinal space were most likely macrophages and those within the retina were either resting or activated microglia. The exact numbers are as follows: F-APC on day 1: Müller cells (mucs), 4 ± 3 ; microglia cells (mics), 1 ± 1 ; and presumptive macrophages (macs), 1 ± 1 (mean \pm SD; $P < 0.05$ for mucs); F-APC on day 2: mucs, 14 ± 9 ; mics, 1 ± 1 ; and macs 1 ± 0 (mean \pm SD; $P > 0.05$ for all); L-APC on day 1: mucs, 4 ± 3 ; mics, 1 ± 0 ; and macs 0 ± 0 (mean \pm SD; $P < 0.05$ for mucs); L-APC on day 2: mucs, 3 ± 5 ; mics, 2 ± 3 ; and macs 1 ± 3 (mean \pm SD; $P < 0.05$ for mucs). In the saline control detachments, 21 ± 2 cells (mean \pm SD) were counted per linear millimeter of retina (mucs, 19 ± 1 ; mics, 1 ± 1 ; and macs 1 ± 0). A statistically significant decrease in proliferation was observed in all treated groups except the F-APC given on day 2. In general, giving APCs on day 1 was more effective in inhib-

Effect of APCs on total intraretinal proliferation

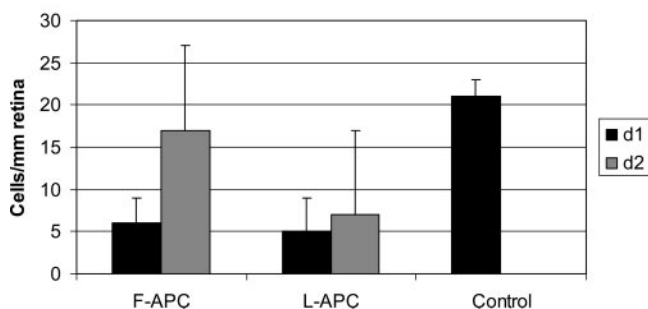


FIGURE 2. Effect of APCs on total intraretinal proliferation after a 3-day retinal detachment. Retinal detachments were created in adult pigmented rabbits. APCs, either bound to liposomes (L-APC) or unbound (F-APC), were injected intravitreally on either day 1 or 2 after detachment. To detect proliferating cells, BrdU was injected on day 3, 4 hours before death. The number of anti-BrdU-labeled cells were counted per millimeter of retina from sections imaged by laser scanning confocal microscopy.

Effect of APCs on intraretinal proliferation: Subgroup analysis

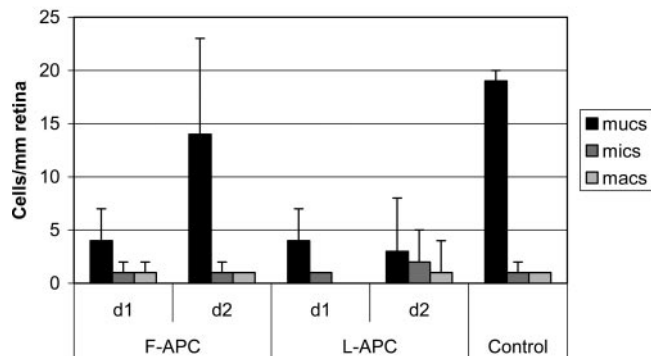


FIGURE 3. Effect of APCs on intraretinal proliferation after a 3-day retinal detachment: subgroup analysis. Retinal detachments were created in adult pigmented rabbits. APCs, either bound to liposomes (L-APC) or unbound (F-APC), were injected intravitreally on either day 1 or day 2 after detachment. To detect proliferating cells, BrdU was injected on day 3, 4 hours before death. Retinas were triple labeled with anti-BrdU, anti-vimentin and the isolectin B4. The anti-BrdU-positive cells were identified as Müller cells, microglia or presumptive macrophages and counted per millimeter of retina from sections imaged by laser scanning confocal microscopy.

iting proliferation than when given on day 2. In addition, the liposome-bound drug given on day 2 was more effective than the free drug given on day 2.

In control detachments, Müller cells made up the largest subgroup of BrdU-labeled cells (Fig. 3). Isolectin B4-labeled microglia within the retina formed the second largest subgroup, and isolectin B4-labeled macrophages in the subretinal space formed the third. In the treated retinas, APCs had a statistically significant effect on the number of labeled Müller cells but had little effect on the number of microglia or macrophages.

To determine whether APCs are toxic to the retina, tissue was embedded in Spurr resin and sectioned for light microscopy (Fig. 4) and electron microscopy (Fig. 5). Because detachment itself induces retinal degeneration, it was difficult to discern the effects of the APCs from the effects of detachment. Therefore, we examined attached retinal regions from the eyes with a detachment and found no evidence of cellular toxicity in any of the treatment paradigms, that is, the retinal morphology from all the treatment groups appeared the same as in the control eyes. In addition, there was no evidence of ultrastructural abnormalities in any of the specimens examined by EM (Fig. 4A, normal untreated control; Fig. 4B, attached region from a detached eye injected with saline on day 1; Fig. 4C, attached region from a detached eye treated with L-APC on day 1; Fig. 4D, attached region from a detached eye treated with F-APC on day 1; Figs. 5A-C, attached regions from detached eyes treated with L-APC on day 1; Figs. 5D, 5E, attached regions from detached eyes treated with F-APC on day 1).

DISCUSSION

Retinal detachment induces changes beyond the degeneration of outer segments which might explain in part the imperfect visual recovery that can occur even after successful reattachment surgery. Within just a few days after detachment, there is photoreceptor terminal retraction and neurite sprouting from second- and third-order neurons.^{17,40,41} In addition, intraretinal proliferation, especially the proliferation of Müller cells, may play a crucial role in this process, since Müller cells are thought to be involved in the development of subretinal fibrosis and

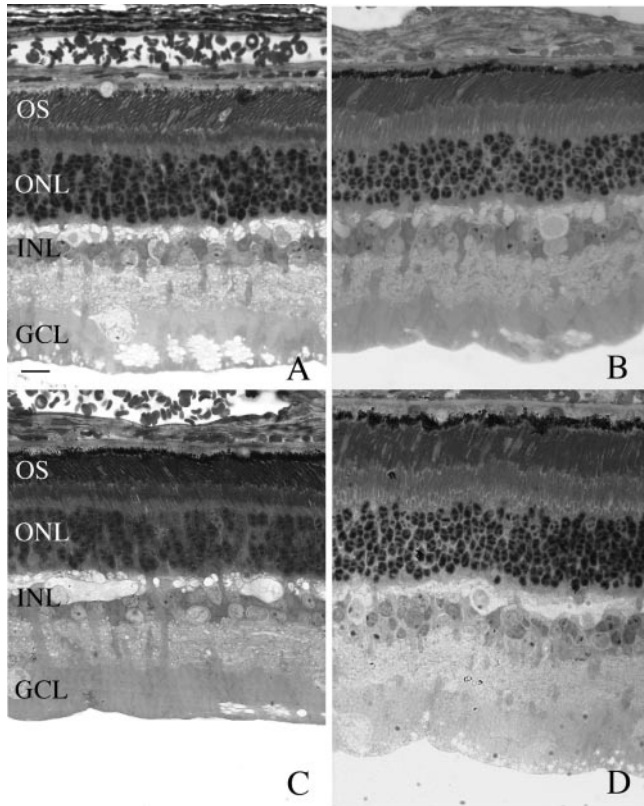


FIGURE 4. Light microscopy of rabbit retina after intravitreal administration of APCs. For detection of potential toxic effects of F- or L-APCs after intravitreal injection, 1- μ m-thick sections were cut from retinas embedded in Spurr resin and stained with toluidine blue. Attached regions from within the detached (treated) eyes were examined to avoid the degenerative effects caused by the detachment. All are from day 3 after the retinal detachment. All regions appeared normal. (A) Attached control retina from an eye that did not have a retinal detachment. (B) Attached region from an eye with a detachment that had a saline injection on day 1 after the detachment procedure. (C) Attached region from an eye that had a detachment and an injection of L-APC on day 1. (D) Attached region from an eye that had an injection of F-APC injection on day 1 after the experimental detachment.

PVR.^{13,17,30,42} Thus, the inhibition of Müller glial cell proliferation *in vivo* by APCs represents an important step toward control of glial cell reactivity during the course of a retinal detachment. Moreover, these data indicate that if the intraretinal proliferation that occurs within a few days after detachment plays a role in PVR, the early administration of antiproliferative agents such as APCs may be more effective at reducing proliferation than later, after PVR is well under way.

Glial cell hypertrophy, as measured by the increase in vimentin expression within the cells, in response to retinal detachment remains unaffected by the intravitreal injection of APCs in this animal model of experimental retinal detachment. These data suggest that proliferation and hypertrophy are mechanistically separate cellular events. This lack of effect on Müller cell reactivity and hypertrophy may explain why pure antiproliferative strategies like 5-fluorouracil²³ have been ineffective in the clinical setting as a PVR treatment strategy. Thus, it may be necessary to find therapeutic agents that will control the hypertrophy of glial cells as well as prevent their proliferation to prevent or treat fibrotic diseases of the retina.^{43,44} A possible strategy to control Müller cell hypertrophy could be to inhibit the upregulation of the intermediate filament proteins GFAP and vimentin. Recently, a p38MAP kinase inhibitor has been identified that is supposed to inhibit the fibrotic reaction

in ARPE19 cells and in a mouse model of PVR.⁴⁵ To date, however, no effective agent has been identified that can inhibit the assembly of intermediate filament proteins in the detached retina.

It has been shown previously that retinal detachment causes the activation of microglia and the influx of macrophages into the subretinal space.³⁹ In this study, the data suggest that APC treatment did not affect retinal microglia nor subretinal macrophage activation by the induced detachment. However, the small number of dividing cells in these two categories makes it difficult to determine this effect with confidence.

In this *in vivo* study, by using a well-recognized animal model of experimental retinal detachment, we were able to demonstrate partially the safety and efficacy of APCs for inhibition of Müller cell proliferation after intravitreal injection in

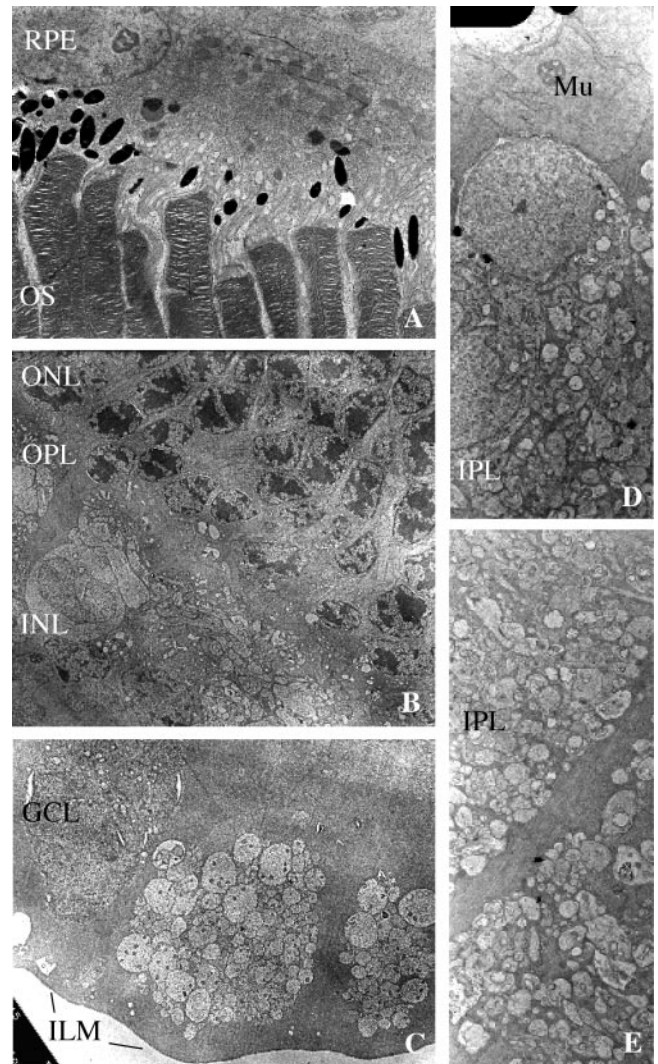


FIGURE 5. Electron microscopy of rabbit retina after intravitreal administration of APCs. Attached regions from within the detached (treated) eyes were examined to avoid the degenerative effects caused by the detachment. No structural abnormalities were observed in any of the specimens examined. All are from day 3 after the retinal detachment. Example micrographs are shown from L-APC (A–C) and F-APC (D, E) injections on day 1 after retinal detachment. (A) Photoreceptor outer segments (OS) and retinal pigment epithelium (RPE). (B) ONL, OPL, and INL. (C) Ganglion cell layer (GCL) showing ganglion cell axons and the ILM. (D) Müller cell nucleus (Mu) and IPL. (E) IPL. Magnification: (A) $\times 6000$; (B) $\times 2400$; (C) $\times 3000$; (D) $\times 4700$; (E) $\times 4200$.

the rabbit. This represents an important step toward a potential clinical application of these compounds. Since a stage-specific expression of growth factors in human PVR membrane samples has recently been proposed,⁴⁶ stage-specific therapies targeting either the early proliferative and/or late tractional stages of PVR should be found. Thus, APCs may be part of a combined strategy directed against Müller cell proliferation and hypertrophy. However, long-term toxicity studies in an in vivo model must be performed before they can be considered for clinical application.

Acknowledgments

The authors thank Nicholas Reese for expert technical assistance and C. Albrecht May, MD, for fruitful discussions.

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