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 than when given on day 1. 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
parasitic (Impavid; 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 inducing 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 euthanatized 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, Warington, 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 treated 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 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 [ErPC₄]) and erucylphosphobis-(N,N,N-trimethyl)-propylammonium is very similar. In comparison to ErPC, ErPC₄ 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, ErPC₄ in water results in a clear micellar solution. To overcome the obvious difficulties with ErPC, we used a liposomal formulation of ErPC.

**ErPC₃: Liposomal Dispersion**

ErPC₃ (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.

**ErPC₄: Micellar Solution**

ErPC₄ (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 ErPC₄ was stored at +4°C to +8°C until use.

**Stock Solutions of ErPC and ErPC₄**

The liposomal dispersion of ErPC or the micellar solution of ErPC₄ 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 (ErPC₄) 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.
Statistical Analysis

All values are expressed as the mean ± 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, 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 into 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 μm.
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 OPL, 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, 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 ± SD; P < 0.05 for mucs); F-APC on day 2: mucs, 14 ± 9; mics, 1 ± 1; and macs 1 ± 0 (mean ± SD; P > 0.05 for all); L-APC on day 1: mucs, 4 ± 3; mics, 1 ± 0; and macs 0 ± 0 (mean ± SD; P < 0.05 for mucs); L-APC on day 2: mucs, 3 ± 5; mics, 2 ± 3; and macs 1 ± 3 (mean ± SD; P < 0.05 for mucs). In the saline control detachments, 21 ± 2 cells (mean ± 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 inhibiting 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. 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...
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 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-fluorouracil have been ineffective in the clinical setting as a PVR treatment strategy. 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. 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 ARPE19 cells and in a mouse model of PVR. To date, however, no effective agent has been identified that can inhibit the assembly of intermediate filament proteins in the detached retina.

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-μm-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.

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. All are from day 3 after the retinal 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) ×6000; (B) ×2400; (C) ×5000; (D) ×4700; (E) ×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,\textsuperscript{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.

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References