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

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parasitic (Impavid; Zentaritis) 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 antiinflammatory agent can allow us to determine in animal models the effect of proliferation on cellular reactivity and possibly lead to new oculocutaneous therapies 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 27-gauge needle. On day 3 after detachment, the animals were perfused through the left ventricle with 2 N HCl for 20 minutes as an antigen retrieval step for overnight at 4°C. The following day, the sections were pretreated with 1:20 in PBS, 0.5% BSA, 0.1% Triton X-100, and 0.1% sodium azide (PBTA). 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-propl 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 [ErPCn]) and erucylphosphophospho(V,N,N-trimethyl)-propylammonium is very similar. In comparison to ErPC, ErPCn 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, ErPCn 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) 0.980 mg (2 millimoles) and cholesterol (MW 386.66) 810 mg (2.1 millimoles) were dissolved by warming in 60-ml ethanol.

ErPCn: Micellar Solution

ErPCn (MW 503.75), 1080 mg (2 millimoles) was tempered in 200 mL 0.15 M NaCl at 4°C for 15 minutes. Ultrasonication at 4°C for 15 minutes resulted in a water-clear micellar solution after 30°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.

Stock Solutions of ErPC and ErPCn

The liposomal dispersion of ErPC or the micellar solution of ErPCn 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 (ErPCn) to liposomes, was dissolved in 0.9% NaCl under sterile conditions and stored at 4°C (10 mM stock solution).

AEC and m-AEC were diluted in PS (pH 7.4) to the 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,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 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). 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 (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. (F, 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.

**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 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 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, 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 mics); 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 mics); L-APC on day 2: mucs, 3 ± 5; mics, 2 ± 3; and macs 1 ± 3 (mean ± SD; P < 0.05 for mics). 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 F-APC on day 1; Fig. 4D, attached region from a detached eye treated with L-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 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-fluorouracil 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. 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. 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

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. All regions appeared normal. (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. Magnification: (A) \( \times \) 6000; (B) \( \times \) 2400; (C) \( \times \) 5000; (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, 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