Alkylphosphocholines: A New Approach to Inhibit Cell Proliferation in Proliferative Vitreoretinopathy

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Abstract

Proliferative vitreoretinopathy represents the major complication in retinal detachment surgery and occurs in about 5–15\% of cases resulting in a significant loss of vision despite multiple surgical procedures. Although successful anatomical reattachment is usually achieved, the reduction in central vision often remains permanent due to the intraretinal changes induced by retinal detachment and the subsequent proliferative response within the retina. Retinal Müller glial cells play a pivotal role in this process together with retinal pigment epithelial (RPE) cells which are dispersed in the vitreous and stimulated by growth factors and serum in the vitreous after the breakdown of the blood-retinal barrier.

Alkylphosphocholines (APCs) are effective inhibitors of human ocular cell proliferation and currently in clinical use for the treatment of protozoan and malignant diseases. Previously, we have demonstrated that APCs can inhibit human retinal Müller glial cell proliferation and attenuate the hypoxia-induced actin filament expression in these cells. Moreover, retinal pigment epithelial (RPE) cell spreading and migration as well as proliferation and cell-mediated membrane contraction are effectively inhibited by APCs at nontoxic concentrations in vitro. The first in vivo toxicity studies in the rat eye also did not display any toxic effect of APCs to retinal tissue 7 days after intravitreal administration. Finally, in a well-established in vivo model for PVR, we were able to demonstrate that APCs can dramatically reduce intraretinal Müller glial cell proliferation induced by experimental retinal detachment in nontoxic concentrations. Thus, APCs are promising, novel pharmacologic substances that may be a useful adjunct to reattachment surgery by reducing the intraretinal proliferation induced by detachment and hence reduce the incidence of PVR.
Cell Biology of Retinal Detachment and Proliferative Vitreoretinopathy

Following retinal detachment, a cascade of events are set in motion: photoreceptor outer segments degenerate, some photoreceptors die by way of apoptosis [1] and synaptic terminals undergo significant remodeling. In response to changes in photoreceptors, in particular the loss of synaptic connections, second-order neurons extend neurites into the outer retina [2]. Ultimately, the ganglion cells respond to detachment by also elaborating new processes throughout the retina [3]. While loss of photoreceptors and ‘downstream’ neuronal changes most certainly contribute to a loss in visual acuity, non-neuronal cells, in particular the glial cells, are also thought to play a role in the continued reduction in vision following retinal reattachment surgery and contribute to the subsequent proliferative response that occurs within the retina [4, 5]. Following experimental retinal detachment, it has been shown that all non-neuronal cell types are stimulated to divide [6, 7]. These cell types include Müller cells, astrocytes, RPE cells, microglia and macrophages; however, the Müller glial cell appears to be the cell type undergoing the highest level of cell division. Indeed, Müller cell proliferation has been implicated in the pathogenesis of various human retinal diseases including retinal degeneration [8], retinal detachment and PVR [9], proliferative diabetic retinopathy (PDR) [10] and epiretinal membrane formation in idiopathic surface wrinkling maculopathy [11–13]. Moreover, Müller cells are thought to play a role in the degenerative events occurring in attached retinal regions within the detached eye [5, 14].

Intraretinal glial cell proliferation is one of the earliest responses to retinal detachment [6, 7]. It is accompanied by rapid glial hypertrophy and upregulation of intermediate filament proteins [15]. These events most likely contribute to the growth of these cells onto either the sub- or epiretinal surface. Indeed, Müller cells have been identified in both human retinal tissue [9, 11–13] and experimental animal models of retinal detachment [6, 8]. Importantly, the presence of these glial ‘membranes’ can have devastating consequences for the return of good vision. With subretinal growth, termed subretinal fibrosis, glial cells can prevent the regrowth of photoreceptor outer segments following reattachment surgery [16] while epiretinal growth can cause re-detachment of the retina [17]. The fact that these events occur rapidly soon after detachment indicates that treatment with antiproliferative agents should also begin at this time. In addition, since it has been shown that proliferation continues following retinal reattachment, albeit at low levels [18], successful inhibition of membrane formation may require not only early but also long-term delivery of the agent.

The stimulus for initiating the proliferation of cells is unknown; however, due to the breakdown of the blood-retinal barrier after retinal detachment, there is certainly an increased amount of serum and growth factors present in the vitreous cavity that may promote proliferation of Müller glial and RPE cells [19, 20]. Indeed, intravitreal injection of basic fibroblast growth factor into a normal feline eye, has been shown to induce proliferation of non-neuronal cells, upregulate intermediate filament proteins, and stimulate the formation of epiretinal membranes [21].
Another factor that is most certainly involved in the response of the retina following detachment is hypoxia of the outer retina as the neural retina becomes distanced from the choriocapillaris. Hypoxia is known to cause significant changes in the architecture of the actin cytoskeleton and thus plays a pivotal role in complex cellular events like migration and proliferation [22]. Moreover, treatment of animals with retinal detachment by hyperoxia (70% inhaled O₂) results in reduced photoreceptor deconstruction and fewer dividing Müller cells [23]. Finally, while PVR is generally considered a ‘disease’ of proliferation, cellular hypertrophy is also most certainly involved. Thus, the crucial pathophysiologic events to be influenced by a novel intravitreal pharmacotherapy are first, the serum/growth factor-induced proliferation/hypertrophy of Müller glial and RPE cells, and second the hypoxia-induced glial changes with deconstruction of photoreceptors.

**Current Therapeutic Concepts**

Surgical intervention in itself is insufficient to control the cellular responses of PVR and, therefore, a pharmacological concept is warranted as an adjuvant therapy in combination with complex retinal reattachment surgery. Current pharmacologic intervention focuses primarily on antiproliferative and anti-inflammatory agents to prevent PVR [24]. A number of antiproliferative pharmacologic substances such as colchicine, daunomycin and 5-fluorouracil have been tested for their effects on human retinal glial cells in vitro [25]. However, these substances are currently not part of any routine clinical treatment for PVR. One of the most promising candidates, 5-fluorouracil, combined with low-molecular heparin, has recently been tested in a large clinical trial; however, it proved to be ineffective in reducing the incidence of PVR [26, 27]. A number of factors most likely contribute to the failure of studies such as these, one of which certainly involves choosing the optimal treatment regime. Based on animal studies, the ideal treatment would involve early administration of the agent, perhaps at the time of reattachment surgery in high-risk cases, combined with sustained delivery without causing toxicity to the retina. We feel that alkylphosphocholines (APCs), encapsulated in liposomes meet these criteria.

**Alkylphosphocholines**

Alkylphosphocholines (APCs) are synthetic phospholipid derivatives (fig. 1) and known as effective inhibitors of cellular proliferation. Clinically, they are currently applied for the treatment of protozoan [28] and malignant diseases [29]. In addition, APCs have been shown to inhibit human ocular cell proliferation which may have therapeutic implications for a number of proliferative ocular conditions including
those secondary to retinal detachment [30] as well as the scar formation of Tenon fibroblasts after glaucoma filtration surgery [31].

Their mechanism of action involves binding to the membrane-bound G-protein PKC (protein kinase C) which is part of a major intracellular second-messenger systems that regulates cell attachment, spreading, migration and proliferation. In ocular cells (Tenon fibroblasts and retinal pigment epithelial cells) and in the leukemia cell line HL60, APCs are effective inhibitors of PKC [31–33]. Also, PKC has been shown to be associated with a downregulation of focal adhesion kinase (FAK) [34]. FAK is one of the key cytoplasmic tyrosine kinases and acts as a potential integrin effector. A high proliferation rate is associated with an increased FAK activity in malignant glioma cells [35]. Moreover, FAK directs signaling molecules like phosphatidylinositol 3-kinase-Akt/PKB (PI3K) to focal contacts between cells and decides if cells migrate or proliferate [36]. Since APCs have been reported to inhibit the PI3K pathway in epithelial carcinoma cell lines [37], actin organization and focal contact dynamics which are in part determined by a PKC [38] and a FAK-directed signaling cascade might be influenced by APCs via both pathways.

Previously, we have been able to demonstrate that APCs can inhibit RPE cell spreading and migration [39] as well as proliferation and cell-mediated membrane contraction [32] at nontoxic concentrations in vitro. Moreover, the first in vivo toxicity studies in the rat eye did not display any toxic effect of APCs to retinal tissue 7

![Chemical structure of the alkylphosphocholines](image-url)
APCs for PVR 5
days after intravitreal administration [40]. Recent in vitro [41] and in vivo [30] studies examining the effect of alkylphosphocholines on retinal Müller glial cells have also been performed to further elucidate their potential therapeutic role in retinal proliferative diseases. Thus, APCs are promising, novel pharmacologic substances which have been found to be effective in controlling both the Müller cell [36] and RPE-related component of PVR pathogenesis [32, 34].

In Vitro Effects

To examine the in vitro effects of APCs on retinal Müller glial cells, primary Müller glial cells were prepared from Long-Evans rats in accordance with applicable German laws and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Isolated cells were routinely evaluated by immunofluorescence microscopy using a monoclonal antibody against vimentin. Also, the spontaneously immortalized human Müller cell line, MIO-M1, was used to evaluate the role of the alkylphosphocholines oleyl-phosphocholine (C18:1-PC; fig. 1a) and erucyl-phosphocholine (C22:1-PC; fig. 1d) on cell proliferation and F-actin stress fiber expression under normoxic and hypoxic conditions. To determine the levels of Müller glial cell proliferation, the incorporation of 5-bromo-2-deoxyuridine (BrdU) was measured after assessment of toxicity by the trypan blue exclusion assay. For detection of F-actin stress fibers, the rhodamine-phalloidin staining protocol was applied.

With this in vitro study, we could demonstrate that C18:1-PC and C22:1-PC are able to inhibit the proliferation of primary rat retinal Müller glial cells (fig. 2a) as well as of the immortalized human retinal Müller glial cell line MIO-M1 (fig. 2b) in a dose-dependent manner. Effective concentrations were non-toxic and able to attenuate the content of F-actin stress fibers in these cells which were induced by hypoxia, a key feature in the pathogenesis of retinal diseases. Therefore, in conclusion, this study provides further evidence that APCs might offer a novel treatment strategy for the prevention of PVR: first, they inhibit Müller glial cell proliferation and second, they are able to diminish the hypoxia-related upregulation of F-actin stress fibers in vitro [41].

In Vivo Studies

A subsequent evaluation of the results obtained in vitro has been performed in a well established in vivo model for PVR, the rabbit eye, where APCs have been found to be effective inhibitors of intraretinal proliferation after experimental retinal detachment [30].

Retinal detachments were created in adult New Zealand Red pigmented rabbits by infusing a solution of sodium hyaluronate (0.25% in balanced salt solution; BSS)
via a glass pipette between the neural retina and RPE. Two experimental conditions were tested: (1) whether APC treatment was more effective given at day 1 or day 2 after detachment, and (2) whether liposome-bound APC was more effective than the free drug. Control eyes were injected with balanced salt solution 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. The eyes were bisected and half of each eye, containing both attached and detached regions, was fixed in 4% paraformaldehyde for immunocytochemical analysis. Anti-BrdU was used to detect dividing cells, antivimentin to determine the extent of Müller cell hypertrophy and isolectin B4, *Griffonia simplicifolia* to label microglia and macrophages. The other half was placed in 1% paraformaldehyde plus 1% glutaraldehyde in phosphate buffer for light and electron microscopic analysis to determine potential toxic effects of APC. 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, Calif., USA.

In control, nondetached retina, no anti-BrdU-labeled cells were detected (fig. 3a). In control detachments, Müller cells represented the largest subgroup of BrdU-labeled cells (fig. 3b, red) contributing to the massive intraretinal scar formation. The isolectin B4-labeled microglia within the retina formed the second largest subgroup and the isolectin B4-labeled macrophages in the subretinal space formed the third (fig 3a–e, both cell types in blue). In the APC injected eyes, the number of

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**Fig. 2.** C18:1-PC and C22:1-PC inhibit proliferation of retinal Müller glial cells. Primary rat Müller glial cells (a) or MIO-M1 cells (b) were cultured in 96-well microplates and incubated with different concentrations of the APCs. Proliferation of cells was assessed by measurement of BrdU incorporation (p < 0.05 for all concentrations >25 nM).
Fig. 3. Laser scanning confocal images of retinal sections labeled with probes to vimentin (green), BrdU (red) and isolectin B4 (blue). a Undetached control retina. Anti-vimentin labeled Müller cells extend from the ganglion cell layer (GCL) into the outer nuclear layer (ONL), and isolectin B4 labeled fine microglial cell processes in the inner plexiform layer (IPL). b Detached control retina 3 days after detachment and saline injection on day 1. Anti-vimentin labeling increased in Müller cells to the outer limiting membrane (OLM), anti-BrdU labeled dividing cells primarily in the inner nuclear layer (INL), and isolectin B4 labeled microglia that had rounded up and migrated throughout the retina. c Three days after retinal detachment and non-liposome-bound APCs injected on day 2. A slight reduction in the number of anti-BrdU-labeled Müller cells was observed; anti-vimentin and isolectin B4 labeling appeared similar to the control. d, e Three days after retinal detachment and liposome bound drug injected on day 1. There was a significant decrease in anti-BrdU-labeled Müller cell nuclei although anti-vimentin and isolectin B4 labeling appeared similar to controls. f RPE region, 3 days after retinal detachment and saline injection on day 1. A few anti-BrdU labeled cells were observed but this pattern was not significantly different from the drug-treated animals. Scale bars = 20 μm.
BrdU-labeled Müller cells was significantly reduced (fig. 3c–e) without effecting the number of microglia or macrophages. In both control and APC-treated animals, the anti-vimentin labeling increased in Müller cells to the outer limiting membrane and sometimes beyond indicating cellular hypertrophy in response to retinal detachment and hypoxia (fig. 3b, c). 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 (fig. 3b). Numerous anti-BrdU-labeled Müller cells and isolectin B4-positive cells were observed in retinas given free APC on day 2 (fig. 3c), whereas significantly fewer cells incorporated BrdU when the retinas were treated with the liposome-bound APC given on either day 1 (fig. 3d, e) or day 2. There was no evidence of toxicity detected in any of the treatment groups since the retinal morphology at the electron-microscopic level of treated animals appeared the same as in the control eyes [30]. The RPE was also stimulated to divide following detachment, as shown by BrdU incorporation (fig. 3f); however, the number of labeled cells was too low to detect a difference between treated and control animals.

Conclusions/Perspectives

In these recent in vitro and in vivo studies, we have been able to demonstrate the anti-proliferative effect of alkylphosphocholines on retinal Müller glial cells at nontoxic concentrations. By using a well-recognized animal model of experimental retinal detachment, we could show the safety and efficacy of APCs for inhibition of Müller cell proliferation after intravitreal injection in the rabbit. The effects on glial cell hypertrophy and expansion outside the retina, however, have yet to be determined. The in vitro assessment of F-actin stress fiber expression and distribution demonstrated a protective effect of APCs on human and rat Müller glial cells under hypoxic cell culture conditions.

The use of alkylphosphocholines may represent an important step towards the development of an adjuvant pharmacologic treatment strategy for PVR and clinical application of these compounds seems feasible. However, long-term toxicity studies in an in vivo model must be performed before APCs can be considered for clinical application.

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References


APCs for PVR