Alkylphosphocholines: A New Therapeutic Option in Glaucoma Filtration Surgery

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PURPOSE. To investigate the effect of alkylphosphocholines (APCs) on human Tenon fibroblast (HTF) proliferation, migration, and cell-mediated collagen gel contraction.

METHODS. HTFs were isolated from tissue samples of three patients obtained during surgery and cultured in DMEM and 10% fetal calf serum (FCS). HTFs (passage 3–6) were treated with one APC in different concentrations spanning the 50% inhibitory concentration (IC50), as determined previously. Inhibition of cell proliferation was assessed by the trypan blue exclusion assay. Migration was determined in chemotactic chambers with fibronectin-coated polycarbonate membranes. For inhibition of contraction, three-dimensional collagen gels were seeded with HTFs, and the gel size was measured. Cell viability was determined by the trypan blue exclusion assay. For analysis of the mechanism of action, protein kinase C (PKC) activity was measured.

RESULTS. The IC50 varied between 7.0 and 10.5 μM for all APCs tested. At this concentration, all four APCs inhibited HTF migration and cell-mediated collagen gel contraction in the presence of serum. The inhibitory effects on HTF proliferation, migration, and contraction were observed at nontoxic concentrations. PKC activity was reduced to 50% of control level at the IC50 of all APCs applied.

CONCLUSIONS. APCs are effective inhibitors of HTF proliferation, migration, and cell-mediated contraction of collagen gels at nontoxic concentrations. Their mechanism of action seems to involve the inhibition of the PKC pathway. (Invest Ophthalmol Vis Sci. 2004;45:2619–2624) DOI:10.1167/iovs.03-1351

Glaucoma is a major cause of blindness worldwide. Despite effective antiglaucoma medication, a large number of patients with primary open-angle or closed-angle glaucoma have to undergo trabeculectomy to preserve vision during the course of the disease.1–3 The main cause of failure in glaucoma filtration surgery is the scarring of the filtering bleb site.4 Fibroblasts from the subconjunctival space play a crucial role in this process through proliferation, migration, production, and subsequent contraction of the extracellular matrix.5 Current concepts of therapy are intended to limit the scarring process and include the perioperative administration of antimetabolites such as 5-fluorouracil (5-FU) and mitomycin C (MMC).6–8 These agents allow for better control of the scarring complications but are accompanied by severe side effects, such as keratitis, bleb leakage, chronic hypotony with maculopathy, and endophthalmitis.9–13 Despite the use of these antiproliferative agents, surgery can still fail in some patients, probably because of the tendency of the growth-arrested fibroblasts to migrate and interact with surrounding untreated fibroblasts.14–16 Recently, anti-TGF-β2 antibodies have been launched as a new antiscarring treatment option in glaucoma filtration surgery.4,19–21 Clinical studies are under way, but neutralizing anti-TGF-β2 antibodies are not yet available for routine clinical use.

Alkylphosphocholines (APCs) represent a new class of substances with antiproliferative properties. Hexadecylphosphocholine (miltefosine; Cayman Chemical Co., Ann Arbor, MI) is known as an effective treatment of cutaneous breast cancer metastasis due to its low toxicity to the surrounding tissue compared with 5-FU.22,23 In a recent study, we were able to show an inhibitory effect of APCs on proliferation and matrix contraction in cultured retinal pigment epithelial cells.24 As these cellular mechanisms are also involved in the scarring process after glaucoma surgery, APCs could be an interesting alternative for antiscarring therapy in glaucoma filtration surgery without the disadvantages of 5-FU and MMC.

In our study, we used four APCs in different concentrations in the presence of serum to test their ability to inhibit proliferation, migration, and contraction of human Tenon fibroblasts (HTFs) at nontoxic concentrations. We also investigated their mechanism of action with the main focus on protein kinase C (PKC), because this has been proposed in other cell systems.25–27

MATERIALS AND METHODS

Alkylphosphocholines

The APCs oleylphosphocholine (C18:1-PC), (Z)-10-eicosenyl-phosphocholine (C20:1-PC), (Z)-12-heneicosenyl-phosphocholine (C21:1-PC), and erucylphosphocholine (C22:1-PC), 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. All substances were dissolved in ethanol and stored at 4°C. Independent dilution series in ethanol were used to obtain final concentrations of APCs in equal volumes of ethanol.

HTF Isolation and Cell Culture Conditions

HTF samples were obtained from tissue explants of three white male patients (age 20, 40, and 60 years) without any topical eye treatment, who underwent routine cataract or strabismus surgery. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the procedure. The HTFs were cultured as previously described2 and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) FCS, 2 mM l-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL.
gentamicin, and 0.25 mg/mL amphotericin B (all from Invitrogen-Gibco, Paisley, Scotland, UK) at 37°C with 5% (vol/vol) CO₂ in air. Cultures were used between passages 3 and 6 for all experiments. The guidelines of the Declaration of Helsinki were followed, and institutional human experimentation committee approval was granted.

**Cell Proliferation Assay**

The tetrazolium dye-reduction assay (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich, St. Louis, MO) was used to determine cell survival and proliferation rate. HTFs (passages 3–6) were seeded in 96-well plates (150 μL/well) at a density of 5 × 10³ cells/well in DMEM and 10% fetal calf serum (FCS) and exposed to five concentrations of APCs chosen to span the 50% inhibitory concentration (IC₅₀), as determined by preliminary assays. The IC₅₀ is defined as the concentration of a drug that produces a 50% reduction in the number of cells. The calculation of the IC₅₀ from the curves was performed as follows: Two concentrations closest to the IC₅₀ were identified and selected for a linear interpolation, which connected the mean response values of the upper and lower concentrations by a straight line. The value at the center of this interval represented the IC₅₀. The corresponding 95% confidence interval was calculated.

The MTT test was performed as described by Mosmann with some modifications. In brief, after incubation with APCs for 48 hours, the cell culture medium was removed, and the cells were washed with PBS. The MTT solution (1.5 mL MTT stock [2 mg/mL in PBS] plus 28.5 mL DMEM) was added (200 μL/well). HTFs were incubated at 37°C for 1 hour. Formazan crystals that formed after dissolution by the addition of DMSO (200 μL/well). The final concentration of DMSO in the cell culture medium was found to have no antiproliferative effect on HTFs. Absorption was measured by a scanning multwell spectrophotometer at 550 nm (Molecular Probes, Garching, Germany). Results from the wells are expressed as the mean percentage of control proliferation (control OD 0.7 at 550 nm assigned as 100% proliferation). Experiments were performed in triplicate and repeated five times. HTFs of the same passage number incubated with 5% (vol/vol) ethanol without addition of APCs served as the control.

**Trypan Blue Staining of Proliferating HTFs after Treatment with APCs**

HTFs (passages 3–6) were seeded in 24-well plates (Nunc, Wiesbaden, Germany) at a density of 2 × 10⁴ cells per well in 1 mL DMEM and 10% FCS. The medium was changed after 24 hours, and APCs were added in concentrations spanning the IC₅₀ interval between 0.1 and 42.0 μM (to 5% [vol/vol] ethanol in DMEM and 10% FCS). After 48 hours, HTFs were trypsinized and stained with 2% trypan blue (1:1 vol/vol) for 5 minutes. Viable (unstained) and dead (stained) cells were counted in each well with a hemocytometer (Neubauer chamber). Experiments were performed in triplicate and repeated five times. HTFs of the same passage number incubated with 5% (vol/vol) ethanol without addition of APCs served as the control. At least 400 cells from each well were counted.

**Cell Migration Assay**

HTFs were trypsinized and stained with 2% trypan blue (1:1 vol/vol) for 5 minutes. The migrated cells on the other side of the filter were scraped off with a cotton tip. The migrated cells on the other side of the filter were fixed in methanol and stained with hematoxylin and eosin. Five randomly chosen fields were counted at a 200X magnification with a phase-contrast microscope (Leica, Wetzlar, Germany). Experiments were performed in triplicate and repeated at least three times. HTFs of the same passage number incubated with 5% (vol/vol) ethanol without addition of APCs served as the control (number of control cells, 218 ± 12). Results are expressed as the percentage of the number of control cells.

**Collagen Lattice Contraction Assay**

For analysis of cell-populated, three-dimensional collagen matrix contraction, the method of Mazure and Grierson was modified. Rat tail type I collagen (Sigma-Aldrich) was dissolved in 0.1% (vol/vol) acetic acid in sterile distilled water and stored at 4°C overnight. The 24-well plates were preincubated with 2% bovine serum albumin in PBS overnight to block unspecific binding. HTFs (passages 3–6) were counted and resuspended in modified Eagle’s medium (MEM; Biochrom, Berlin, Germany) at a volume of 1 mL containing 1 × 10⁵ cells, sufficient for one 24-well plate. The cell suspension was mixed with 5.0 mL of 3 mg/mL collagen and with 3.0 mL concentrated serum-free MEM containing glutamine, antibiotics, and 391 μL 1 M NaOH. The collagen-cell mixture was then transferred in 500-μL aliquots to a 24-well plate to cover the bottom of the wells. The solution polymerized within 1 hour when incubated at 37°C. After incubation of the enzyme sample with the PKC biotinylated substrate (Molecular Probes, Garching, Germany), the amount of protein per enzyme sample was determined as described. The migrated cells on the other side of the filter were scraped off with a cotton tip. The migrated cells on the other side of the filter were fixed in methanol and stained with hematoxylin and eosin. Five randomly chosen fields were counted at a 200X magnification with a phase-contrast microscope (Leica, Wetzlar, Germany). Experiments were performed in triplicate and repeated at least three times. HTFs of the same passage number incubated with 5% (vol/vol) ethanol without addition of APCs served as the control (number of control cells, 218 ± 12). Results are expressed as the percentage of the number of control cells.

**Measurement of PKC Activity**

For determination of PKC activity, a radioactive assay was applied to investigate the mechanism of action of APCs on RPE cells in vitro (SignaTECT PKC Assay System; Promega, Madison, WI). This assay is based on the measurement of ³²P-labeled phosphate transfer to a PKC-specific peptide that can be captured on phosphocellulose filters. It is known to be PKC specific and reliable for measurement of enzyme activity in crude tissue extracts. HTFs (passages 3–6) were seeded in 35-mm Petri dishes at a density of 1 × 10⁵ cells/dish in DMEM and 10% FCS, exposed to one of the four APCs for 24 hours, and processed as indicated by the manufacturer’s protocol. In brief, after incubation with one APC per well at its IC₅₀ (to 5% [vol/vol] ethanol) determined in preliminary assays (Fig. 2), the medium was removed, and the cells were washed with PBS, resuspended, and homogenized (40 strokes with a Dounce homogenizer; Bellco Glass Co., Vineland, NJ) in cold extraction buffer (25 mM Tris·HCl [pH 7.4], 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 1 mM benzamidine, 1 μg/mL leupeptin, and 1 μg/mL aprotinin). Cell lysates were passed over a 1-mL column of DEAE cellulose (DE52; Whatman, Kent, UK) prepared previously. To elute the PKC-containing fraction, 5 mL of extraction buffer containing 200 mM NaCl was used. The amount of protein per enzyme sample was determined as described by Bradford (Bio-Rad, Mannheim, Germany). After incubation of the enzyme sample with the PKC biotinylated peptide substrate and [³²P]ATP in the appropriate reaction volume at
30°C for 5 minutes, the reaction was terminated, and 10 μL of the reaction volume spotted on a streptavidin-labeled membrane (SAM² Membrane) which was supplied with the PKC assay system (Promega). Membranes were washed and dried according to the manufacturer’s protocol before analysis by scintillation counting. The test was performed in triplicate and repeated five times. HTFs of the same passage number incubated with equal volumes of ethanol without addition of APCs served as the control. Results were expressed as the mean percentage of control proliferation. Data are the mean of result in five experiments, each performed in triplicate. Error bars, SEM.

Statistical Evaluation

Statistical analysis was performed on computer (SPSS V 11.0; SPSS Science, Inc., Chicago, IL). All results are expressed as the mean ± SEM or ± 95% confidence interval (CI), as indicated. For determination of the significance of differences, an ANOVA was performed. Differences with P < 0.01 were considered statistically significant.

RESULTS

Inhibition of HTF Proliferation by APCs In Vitro

APCs inhibited proliferation of HTFs in a dose-dependent manner in vitro (Fig. 1A). A single application of each APC and continuous exposure of HTFs over 48 hours prevented an increase in the counted number of cells (ANOVA analysis; P < 0.001). This effect was observed with all APCs applied, regardless of different alkyl chain lengths. Consequently, the IC_{50} did not differ significantly among the four APCs applied (P > 0.01; Table 1). The concentration inhibiting control cell growth by 50% (IC_{50}) was determined from each of the dose-response curves.

Oleyl-phosphocholine (C18:1-PC) showed an inhibition of HTF cell proliferation at concentrations between 1 and 42 μM (Fig. 1). Cells exposed to C18:1-PC at concentrations below 1 μM were not compromised in their growth compared with the control. The IC_{50} was 10.5 ± 1.2 μM (Table 1) and did not differ significantly from the IC_{50} of all other APCs applied (P > 0.01). However, the dose-response curve of C18:1-PC lay slightly above the ones for the other APCs with an alkyl chain length of C20 and more.

Eicosanyl-phosphocholine (C20:1-PC)-treated HTFs decreased in number at concentrations above 0.1 μM (Fig. 1). This corresponds well to the dose–response curves of hecicosanyl-phosphocholine (C21:1-PC) and erucyl-phosphocholine (C22:1-PC). For these APCs, concentrations below 0.1 μM yielded quantities of cells similar to the control. Their IC_{50} values were as follows: 7.0 ± 1.1 μM for C20:1-PC, 8.0 ± 0.9 μM for C21:1-PC, and 8.5 ± 1.2 μM for C22:1-PC (Table 1).

Cell Viability Study

Proliferating HTFs were treated with the four APCs at concentrations between 0.1 and 42 μM. This interval was chosen to span the IC_{50} of each APC and to determine cell viability at effective antiproliferative and anticontractile concentrations in vitro. Cell morphologic changes in phase-contrast microscopy and toxicity as determined by the trypan blue exclusion test did not differ from the control, which corresponded to a maximum toxicity (blue staining) of 5% in APC-treated RPE cells (Table 1).

Inhibition of HTF Migration by APCs

HTF migration was assessed using microchemotaxis chambers with fibronectin-coated polycarbonate filters placed on the TGF-β2/DMEM-filled lower half of the chamber. HTFs were placed in the upper half of the chamber with APCs added in different concentrations and incubated under standard cell culture conditions for 15 hours.

All APCs applied inhibited HTF migration under serum conditions at concentrations above 0.1 μM. This corresponds to the concentration interval of the antiproliferative effect of these substances. At their IC_{50} (Table 1), however, all APCs inhibited HTF migration completely (Fig. 2). Fifty-percent inhibition of HTF migration was achieved at concentrations between 0.1

![Figure 1](http://iovs.arvojournals.org/pdfsaccess.ashx?url=/data/journals/iovs/932928/)
and 1 μM which is by a factor 10 to 100 lower than proliferation inhibition.

**Inhibition of Contraction of HTF-Populated Collagen Matrices by APCs**

HTFs were used to populate collagen gel matrices. All four APCs caused a significant concentration-dependent inhibition of HTF cell-mediated collagen gel contraction in the presence of 10% FCS (Fig. 3). The baseline HTF-mediated collagen gel contraction was 57% (control). Incubation with APCs at their IC₅₀ decreased collagen gel contraction markedly. After treatment with C18:1-PC, C21:1-PC, and C22:1-PC, collagen gel contraction was reduced to 7% (12% of control level). Incubation with C20:1-PC at its IC₅₀ reduced HTF-mediated collagen contraction to 12% (21% of control level). Thus, baseline HTF-mediated collagen contraction was reduced by 79% (C20:1-PC) and even more effectively by up to 88% (C18:1-PC, C21:1-PC, and C22:1-PC).

**DISCUSSION**

Scarring is the reason for the failure of surgery in many visually disabling and blinding conditions. Successful filtration surgery for the treatment of glaucoma depends directly on an individual’s wound-healing response.² Maintenance of intraocular pressure in the low teens can prevent long-term progression of glaucoma.¹,¹⁸ However, scarring of the filtering bleb site leads to an increase in intraocular pressure with further progression of glaucoma, resulting in surgical failure.¹⁶,¹⁷ HTFs play a crucial role in this process. Studies have shown that subconjunctival scarring of the filtering bleb site is mainly mediated by HTF proliferation, migration, and contraction.⁵,¹⁴

In the present study, for the first time APCs were found to have an antiproliferative effect on HTFs. In addition, other features of the scarring process, such as HTF migration and contraction of scarlike tissue, were significantly inhibited by APCs. Viability testing showed that this was not due to HTF cell toxicity in the concentration interval tested.

APCs are known to have antiproliferative effects in other cell systems.²⁵,²⁶,²⁷ Based on these findings APCs are successfully used for the treatment of cutaneous breast cancer metastasis and visceral leishmaniasis in humans (hexadecylphosphocholine; miltefosine; Cayman Chemical).²⁵,²⁶,²⁷ Because inhibition of the proliferation of HTFs is the reason for the subconjunctival injection of such toxic antimetabolites as MMC and 5-FU after glaucoma filtration surgery,⁷,⁸ the application of APCs in vivo could be a favorable alternative. However, ocular toxicity cannot be excluded, since there are no data available on toxicity so far. As far as general toxicity is concerned, gastrointestinal side effects have been observed after oral ad-
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In summary, our results demonstrate that APCs inhibit HTF proliferation, migration, and contraction at concentrations that are nontoxic to these cells. The mechanism of action of APCs on HTFs seems to involve the inhibition of the PKC pathway. Thus, APCs may be promising for prevention of bleb failure in glaucoma filtration surgery. Further experiments are needed to determine effectiveness and tolerance in vivo.

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References


