In Vitro Studies of Antiglaucomatous Prostaglandin Analogues: Travoprost with and without Benzalkonium Chloride and Preserved Latanoprost

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PURPOSE. With use of the Wong-Kilbourne derivative Chang conjunctival cell line, this study compared in vitro the ocular toxicity of three topical intraocular pressure (IOP)-lowering agents: travoprost 0.004% containing 0.015% benzalkonium chloride (BAK), travoprost Z 0.004%, a new formulation without BAK, and latanoprost 0.005% containing 0.02% BAK.

METHODS. Neutral red, Alamar blue, YOPRO-1, and annexin V/7-AAD assays were used to evaluate the effects of the IOP-lowering agents and BAK on cellular viability, membrane integrity, and apoptosis in the conjunctival cell line using microtitration fluorometric analysis and flow cytometry. All assessments were performed in a masked manner.

RESULTS. Assessment of cell viability and membrane integrity revealed a significant effect by latanoprost with BAK or BAK alone but no effect by travoprost Z without BAK or buffer alone (P < 0.0001). Latanoprost with BAK, travoprost with BAK, and BAK alone were cytotoxic in Chang conjunctival cells, whereas no cytotoxicity was observed in cells exposed to travoprost Z without BAK or in cells treated with buffer (P < 0.0001). No increase in apoptosis or necrosis was observed in cells treated with control or travoprost Z without BAK compared with BAK, travoprost with BAK, and latanoprost with BAK (P < 0.0001).

CONCLUSIONS. Latanoprost with BAK, travoprost with BAK, and BAK alone have significant cytotoxic effects on human conjunctiva-derived cells and are associated with apoptosis. These effects likely result from BAK used as a preservative. IOP-lowering agents with alternative preservatives instead of BAK will most likely have fewer ocular surface adverse effects than agents containing BAK. (Invest Ophthalmol Vis Sci. 2007;48: 4123–4128) DOI:10.1167/iovs.07-0266

MANAGEMENT OF GLAUCOMA TYPICALLY INVOLVES PHARMACOTHERAPY WITH TOPICAL OCULAR AGENTS, SUCH AS THE PROSTAGLANDIN ANALOGUES LATANOPROST AND TRAVOPROST. LONG-TERM USE OF TOPICAL OCULAR DRUGS IS OFTEN ASSOCIATED WITH OCULAR INFLAMMATION, ALLERGY, AND DRY EYE SYNDROME, AND FAILURE OF FILTRATION SURGERY.2–8 SEVERAL LINES OF EVIDENCE INDICATE THAT THESE AGENTS ARE ASSOCIATED WITH DISEASES SUCH AS TEAR FILM BREAK-UP TIME, INFILTRATION OF THE CONJUNCTIVA BY INFLAMMATORY CELLS AND FIBROBLASTS, AND OXIDATIVE STRESS INFLAMMATION MARKERS SUCH AS HLA-DR, INTERCELLULAR ADHESION MOLECULE (ICAM)-1, FAS ANTIGEN, OR THE APOPTOTIC MARKER APO 2.7,9–14 THE PRESERVATIVE BENZALKONIUM CHLORIDE (BAK), THE MOST COMMONLY USED OCULAR PRESERVATIVE, IS LARGELY RESPONSIBLE FOR THE OCULAR TOXICITIES AND INFLAMMATION ASSOCIATED WITH THE CHRONIC USE OF MANY OPHTHALMIC SOLUTIONS.9,15–1 BAK IS RETAINED IN TISSUES AND CAN BE FOUND 168 HOURS AFTER A SINGLE 30-µL DROP OF 0.01% BAK IN RABBITS.16 THE HALF-LIFE OF BAK FROM CORNEAL EPITHELIUM AND CONJUNCTIVAL TISSUES IS 20 HOURS.18 PROLONGED EXPOSURE TO BAK CAUSES INDIRECT AND DIRECT TOXIC EFFECTS TO THE OCULAR SURFACE, INCLUDING INFILTRATION BY INFLAMMATORY CELLS, REDUCED CELLULAR VIABILITY, AND COMPROMISED EPITHELIAL BARRIER FUNCTION THROUGH INCREASED CORNEAL EPITHELIAL CELL PERMEABILITY.9,10,19–22 THE TOXIC EFFECTS OF BAK ARE DOSE DEPENDENT, WITH PROGRESSIVE SLOW CELL GROWTH ARREST OCCURRING AT CONCENTRATIONS AS LOW AS 0.0001%, MORE RAPID APOPTOSIS AT 0.01%, AND IMMEDIATE EPITHELIAL CELL NECROSIS AT HIGHER CONCENTRATIONS OF 0.05%.22

The development of ocular preserves that provide a nontoxic alternative to BAK will likely become useful in the management of chronic diseases such as glaucoma. Using a human conjunctiva-derived cell line, we specifically investigated in vitro the first commercially available BAK-free prostaglandin solution, travoprost Z 0.004%, preserved without BAK, and compared the ocular toxicity of this topical solution with that of standard travoprost 0.004% containing 0.015% BAK, latanoprost 0.005% containing 0.02% BAK, and BAK alone. We evaluated the effects of commercial formulations of these IOP-lowering agents and BAK on cellular viability, membrane integrity, and apoptosis through microtitration fluorometric assays and flow cytometry.

Several types of assays were chosen to explore various mechanisms of cell damage caused by the preservative BAK on conjunctiva-derived cells. The neutral red cytotoxicity assay is a cell survival/viability assay that measures the integrity of the cell membrane.17 Alamar blue assay measures cell proliferation and cytotoxicity.23 YOPRO-1, a cell-impermeable nuclear dye that stains only cells that have lost membrane integrity, serves as an indicator of apoptosis.24 The double-staining annexin V/7-AAD assay discriminates cells that are undergoing early apoptosis, late apoptosis, and necrosis. These assays were used to provide a broader understanding of the cytotoxic effects of preservatives on the conjunctiva.

MATERIALS AND METHODS

Materials

The Wong-Kilbourne derivative of Chang conjunctiva, clone 1-5c-4, was obtained from American Type Culture Collection (CCL-20.2; Manassas, VA). Kanamycin and phosphate-buffered saline (PBS) without

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calcium and magnesium (PBS) were purchased from Eurobio, amoxi-
cillin from GlaxoSmithKline (London, UK), EDTA from Sigma-Aldrich
(Saint Quentin Fallavier, France), and fetal calf serum (FCS) from
Dominique Dutcher (Brunath, France). Dulbecco minimum essential
medium (DMEM 1×) with L-glutamine substitute (GlutaMAX-I), Alamar
blue, and YOPRO-1 were obtained from Invitrogen (Cergy-Pontoise,
France); annexin V/7-aminoactinomycin D (7-AAD) was obtained from
Immunotech (Luminy, France); and neutral red was obtained from
Fluka (Buchs, Switzerland). The cytotoxic effects of travoprost Z
0.004% preserved without BAK and travopropost 0.004% containing
0.015% BAK (Travatan Z and Travatan, respectively; Alcon, Fort Worth,
TX), and latanoprost 0.005% containing 0.02% BAK (Xalatan; Pfizer,
New York, NY) were compared with those of the culture medium,
using PBS as a negative control, and with those of BAK (Sigma-Aldrich)
dissolved in PBS to obtain the concentration of 0.02% as a positive
control of cytotoxicity.

Methods

Conjunctival Cell Culture. Chang conjunctiva-derived cells
were cultured under standard conditions (moist atmosphere of 5% CO2
at 37°C) in 75-cm² bottles in DMEM with L-glutamine substitute
(GlutaMAX-I; Invitrogen) containing 10% FCS, 4.5 g/L glucose, 1%
calcium and magnesium (PBS) were purchased from Eurobio, amoxi-
cillin from GlaxoSmithKline (London, UK), EDTA from Sigma-Aldrich
(Saint Quentin Fallavier, France); fetal calf serum (FCS) from
Dominique Dutcher (Brunath, France). Dulbecco minimum essential
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using PBS as a negative control, and with those of BAK (Sigma-Aldrich)
dissolved in PBS to obtain the concentration of 0.02% as a positive
control of cytotoxicity.

Assessment of Apoptosis and Necrosis with Annexin V/7-AAD. Annexin V binds to the membrane phosphatidyl-serine, which becomes exposed from the inner part of the cell membrane to the outer part during apoptosis and is an indicator of the first step of cell membrane alteration that occurs during the early phase of apoptosis. By reacting like propidium iodide, 7-AAD binds to DNA when the cell membrane is disrupted in the late phase of apoptosis and in necrosis. Conjunctiva-derived cells, cultured in six-well plates, were incubated for 30 minutes with 50-µL of undiluted test solution and were collected after incubation for 5 minutes in 2 mL of 1 mM EDTA. Cells were suspended in binding buffer at a concentration of 100,000 cells/mL and 50-µL of cell suspension were combined with 5 µL annexin V as a marker of early apoptosis and with 10 µL 7-AAD as a marker of necrosis, and were incubated for 15 minutes on ice. After the incubation period, 500-µL binding buffer was added, and the samples were analyzed by flow cytometry (Beckman Coulter XL-MCL, Miami, FL) with discrimination of annexin V and 7-AAD on a biparametric histogram giving four cell populations, cells negative to both markers (normal viable cells), cells positive only to annexin V (early apoptotic), cells positive to both annexin V and 7-AAD (late apoptotic), and cells positive only to 7-AAD (necrotic).

Statistical Analysis

All analyses were performed in a masked manner toward the drug
tested and were repeated in independent assays. Results were ex-
pressed as percentages of the untreated cell control and were the
means of 18 wells (six wells in three different assays). Mean values for
each concentration were analyzed with a one-way ANOVA test fol-
lowed by Bonferroni test using statistics software (Statview IV for
Windows; Abacus, Berkeley, CA); the level of significance was fixed at
0.05.

Results

Assessment of Cell Viability and Membrane Integrity with Neutral Red

There was no decrease in the viability of cells exposed to PBS
or travoprost Z 0.004% preserved without BAK to incorporate
neutral red compared with cells exposed to culture medium
(Fig. 1). By comparison, latanoprost 0.005% containing 0.02% BAK,
travoprost 0.004% with 0.015% BAK, and 0.02% BAK alone had significant effects on cell viability, as assessed by
neutral red uptake.

Assessment of Cytotoxicity with Alamar Blue

Travoprost Z preserved without BAK had no cytotoxic effects on
Chang conjunctival cells compared with cells treated with
PBS (Fig. 2). There was a significant cytotoxic effect of latano-
prost with BAK and 0.02% BAK alone. Travoprost with 0.015% BAK evoked an intermediate response that was significantly
different from that in the latanoprost group. The reduction of
resazurin to resorufin in the BAK- or latanoprost 0.004% preserved
without BAK to incorporate
neutral red.

Assessment of Apoptosis by YOPRO-1

There was no increase in apoptosis, measured by fluorescence
of YOPRO-1, in cells treated with travoprost Z without BAK
compared with control medium. In contrast, apoptosis in-
creased in cells treated with latanoprost with BAK, travoprost
with BAK, and BAK alone (P < 0.0001 compared with PBS and
travoprost Z without BAK). The degree of apoptosis for each
treatment was adjusted for cell viability by the ratio of
measured by fluorescence with excitation at 491 nm and emission at
509 nm. Results of the YOPRO-1 assay were expressed as a ratio to the
results of the neutral red assay to correlate apoptosis with cellular
viability.

Assessment of Apoptosis and Necrosis with Annexin V/7-AAD. Annexin V binds to the membrane phosphatidyl-serine, which becomes exposed from the inner part of the cell membrane to the outer part during apoptosis and is an indicator of the first step of cell membrane alteration that occurs during the early phase of apoptosis. By reacting like propidium iodide, 7-AAD binds to DNA when the cell membrane is disrupted in the late phase of apoptosis and in necrosis. Conjunctiva-derived cells, cultured in six-well plates, were incubated for 30 minutes with 50-µL of undiluted test solution and were collected after incubation for 5 minutes in 2 mL of 1 mM EDTA. Cells were suspended in binding buffer at a concentration of 100,000 cells/mL and 50-µL of cell suspension were combined with 5 µL annexin V as a marker of early apoptosis and with 10 µL 7-AAD as a marker of necrosis, and were incubated for 15 minutes on ice. After the incubation period, 500-µL binding buffer was added, and the samples were analyzed by flow cytometry (Beckman Coulter XL-MCL, Miami, FL) with discrimination of annexin V and 7-AAD on a biparametric histogram giving four cell populations, cells negative to both markers (normal viable cells), cells positive only to annexin V (early apoptotic), cells positive to both annexin V and 7-AAD (late apoptotic), and cells positive only to 7-AAD (necrotic).

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All analyses were performed in a masked manner toward the drug
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creased in cells treated with latanoprost with BAK, travoprost
with BAK, and BAK alone (P < 0.0001 compared with PBS and
travoprost Z without BAK). The degree of apoptosis for each
treatment was adjusted for cell viability by the ratio of
YOPRO-1 and neutral red (Fig. 3). The ratio was not significantly different in cells treated with travoprost Z and culture medium, but it was increased approximately 38-fold in cells exposed to either latanoprost or BAK \( (P < 0.0001) \) compared with medium (referred to as 100%), PBS, and travoprost Z.

**Assessment of Apoptosis and Necrosis with Annexin V/7-AAD**

In flow cytometry, the double-staining annexin V/7-AAD discriminates on a biparametric histogram cells that are undergoing early apoptosis (annexin positive, 7-AAD negative), late apoptosis (annexin positive, 7-AAD positive), and necrosis (annexin negative, 7-AAD positive). No significant apoptosis or necrosis occurred in cells treated with PBS or with travoprost Z preserved without BAK (Fig. 4). Cells treated with latanoprost with 0.02% BAK, travoprost with 0.015% BAK, or 0.02% BAK alone had significant late apoptosis and necrosis compared with cells treated with medium or travoprost Z \( (P < 0.0001) \). Significant early apoptosis occurred in cells treated with latanoprost with 0.02% BAK that was not observed with other treatments. The total toxic effect describes the damage to conjunctival cells caused by apoptosis and necrosis. Latanoprost with 0.02% BAK, travoprost with 0.015% BAK, and 0.02% BAK alone had similar total toxic effects on conjunctiva-derived cells (Fig. 5). There were no significant differences between cells treated with PBS representing spontaneous apoptosis in the cell line and travoprost Z.

**DISCUSSION**

Glaucoma, a chronic disease characterized by increased intraocular pressure that commonly leads to blindness, is often treated with topical prostaglandins or \( \beta \)-blockers. Unfortunately, the chronic use of most IOP-lowering medications is associated with some toxicity, such as allergic reactions, ocular pseudopemphigoid, allergic contact dermatitis, punctate corneal staining, and failure of filtration surgery. This toxicity has often been associated not with the active component of the medication but with the preservative BAK, which damages corneal epithelial cells, even at concentrations as low as 0.005\%. Samples et al. demonstrated that BAK caused a significant inhibition of the growth of trabecular meshwork cells at extremely low concentrations. Sherwood et al. reported in eyes chronically exposed to preservatives an increase in macrophages, lymphocytes, and fibroblasts in the conjunctiva and Tenon capsule and a decrease in the number of conjunctival goblet cells. Although there are subtle differences with primary cultures of human conjunctival cells, the Wong-Kilbourne derivative of the Chang conjunctiva-derived cell line has been used largely to determine the effects of toxic preservatives and IOP-lowering agents and is a well-recognized model. The present study used this conjunctival cell line to assess the direct cytotoxicity of travoprost Z, the first commercially available BAK-free prostaglandin analogue (which contains 0.004% travoprost and a preservative system [SofZia; Alcon Laboratories, Fort Worth, TX]), travoprost 0.004% with 0.015% BAK, and latanoprost 0.005% with 0.02% BAK and to assess the development of apoptosis after exposure to these.
agents. These agents are all prostaglandin analogues, but they differ in the concentration and the type of preservative used. The neutral red assay measures the ability of viable cells to incorporate dye in their lysosomes, and the Alamar blue assay assesses intracellular reduction of resazurin by intact enzymatic systems in viable cells. Both assays assess cell viability by different mechanisms and, when they are used in association, improve the analysis of cytotoxic effects. In both assays, latanoprost with 0.02% BAK, travoprost with 0.015% BAK, and BAK alone demonstrated deleterious effects on the viability of the Chang conjunctiva-derived cells, with travoprost 0.015% BAK the least toxic of the three, which is consistent with the well-demonstrated dose-dependent toxicity of BAK.22 Indeed previous studies comparing the three commercially available prostaglandin analogues found differences in their toxic profiles corresponding to their concentration in BAK (the least concentrated, the least toxic).17

YOPRO-1 is only taken up by membrane pores within cells undergoing apoptosis. Annexin V binds to phosphatidyl serine exposed on cell membranes only during apoptosis. Therefore, test results are positive only in cells undergoing apoptosis. However, the combined assay annexin V/7-AAD discriminates cells undergoing early apoptosis (annexin positive, 7-AAD negative), late apoptosis (annexin positive, 7-AAD positive), and necrosis (annexin negative, 7-AAD positive). As with the cytotoxicity assays, cells exposed to latanoprost with BAK, travoprost containing BAK, and BAK alone reached a significant level of apoptosis compared with media or travoprost Z without BAK. These results are consistent with previous studies in which the Chang cells were exposed to latanoprost with BAK, travoprost with BAK, and bimatoprost with BAK.17 In these studies, the development of cell cytotoxicity and apoptosis was clearly related to BAK.

As with any other experimental in vitro models, the present study did have some limitations. Evaluations were conducted in only one cell line, but these results are consistent with our previous results and with those previously published in other cell lines. In vitro studies remove the cells from the influence of circulating substances, such as hormones and inflammatory mediators, and from other cells present in the tissue, especially inflammatory cells. However, the present study is valuable for aiding our understanding of the effects of preservatives because the experimental stimulus can be well controlled, and the resultant cellular response can be quantitated. It would be of interest to extend these studies to other clones and to other cell types, such as corneal epithelial and trabecular meshwork cells. Additional studies evaluating the effects of BAK on inflammatory mediators such as prostaglandins and leukotrienes would be of value and interest.

Although the results of the present study cannot be directly extrapolated to humans, the toxicity to BAK in this human cell line was demonstrated to occur after exposure to the test solutions for only 30 minutes The half-life of BAK in the conjunctiva is nearly 12 hours, suggesting that the damage to conjunctival cells observed in vitro may also occur in vivo.18 Because of its complex structure and heterogeneous cell populations, tissue reaction after repeated contact with a topically administered drug differs from that of a cell monolayer ex-
posed to chemical compounds. Repeated administration over the long term of a weakly toxic drug would most likely cause increased renewal of epithelial structures and tissue stimulation on an inflammatory mode rather than destruction. Indeed, many reports show increased inflammatory responses in the conjunctiva of patients treated over the long term as a reaction to the chronic use of antiglaucoma drugs and especially to their preservatives; this was not found in patients treated with unpreserved beta blockers. Nevertheless, ocular surface involvement may be augmented after years or decades of treatment, or when patients have associated ocular surface diseases, such as dry eye disease or meibomian gland dysfunction. In such populations, the use of nontoxic compounds may be of critical importance, especially because the ocular surface has a major influence on compliance and filtering surgery outcome. In a series of in vitro assays, the absence of toxicity consistently observed with travoprost Z without BAK, compared with the high level of toxicity noted with exposure of conjunctival cells to BAK, suggests that use of topical prostaglandin analogues without BAK may reduce the topical opthalmic toxicity reported with chronic use of these agents. Future studies of these agents in humans will assist in further characterizing the presence or absence of topical opthalmic toxicity.

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