Objective: To investigate the effects of latanoprost, timolol maleate, and benzalkonium chloride on cell damage and induction of the secretion of chemical mediators of stress and wound healing by human lens epithelial cells in culture.

Methods: Cells from a human lens epithelial cell line (SRA01/04) were cultured in Dulbecco minimum essential medium supplemented with 5% fetal bovine serum. The amounts of latanoprost (50 µg/mL), timolol maleate (5 mg/mL), or benzalkonium chloride (200 µg/mL) used in eyedrops, and 1/10 to 1/1000 dilutions thereof, were added to the medium. After 7 days’ culture, cell morphological changes were assessed using phase-contrast microscopy, and cell-free culture supernatants were collected for prostaglandin E2 (PGE2), interleukin 1β (IL-1β), and interleukin 6 (IL-6) iodine I125 radioimmunoassay, enzyme-linked immunosorbent assay, and chemiluminescent enzyme immunoassay, respectively.

Results: All cells that were cultured with the concentrations of latanoprost, timolol, or benzalkonium chloride used in eyedrops detached from the culture dish and died within 3 days. At a 1/10 dilution of latanoprost or timolol or a 1/100 dilution of benzalkonium chloride, no proliferation or elongation of the cells was observed. Secretions of PGE2, IL-1β, and IL-6 at 1/10 dilutions of latanoprost or timolol were 3 to 77 times higher than in controls, whereas they were 190 to 305 times higher at a 1/180 dilution of benzalkonium chloride. The amounts of these soluble mediators in culture supernatants depended on the dose of latanoprost, timolol, or benzalkonium chloride added.

Conclusion: Our results indicate that benzalkonium chloride, used as the preservative in eyedrops containing latanoprost or timolol, is the agent most damaging to lens epithelial cells and most strongly stimulates the expression of soluble chemical mediators in these cells.

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Many investigators have reported that the preservative in eyedrops causes inflammatory changes to the ocular surface. Benzalkonium chloride, one of the most common preservatives for eyedrops, has antibacterial effects, especially on gram-positive bacteria. However, benzalkonium chloride induces cytotoxic damage in conjunctival cells and is implicated in stromal damage and thinning of the cornea. It also decreases the stability of the tear film.

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Recently, it has been reported that antiglaucoma eyedrops such as those containing latanoprost and timolol maleate induce inflammatory disruption of the blood-aqueous barrier and increase the incidence of angiographic cystoid macular edema (CME) in patients with early postoperative pseudophakia. These antiglaucoma eyedrops contain the preservative benzalkonium chloride, and it is unclear whether the adverse effects of antiglaucoma eyedrops in early postoperative pseudophakia are due to the agents themselves, the preservative, or both. One clinical investigation has shown that the administration of timolol eyedrops or the vehicle containing benzalkonium chloride induces greater disruption of the blood-aqueous barrier and a higher incidence of angiographic CME than the vehicle alone, not containing benzalkonium chloride. It is therefore suggested that the addition of benzalkonium chloride to antiglaucoma agents contributes to these adverse effects.
operative pseudophakia is accelerated by antiglaucoma agents or the preservative, and that they disrupt not only the blood-aqueous barrier but also the blood-retinal barrier following the onset of CME.

Here, we investigate the morphological changes in human lens epithelial cells and the increased production of chemical mediators such as PGE2, IL-1α, and IL-6 by such cells cultured with the antiglaucoma agents latanoprost and timolol or the preservative benzalkonium chloride alone.

**METHODS**

**CELL CULTURE**

Human lens epithelial cell line (SRA01/04) cells transfected with an immortalizing gene (large T antigen of simian virus 40) were used throughout. The culture methods were as described previously. Briefly, a mean ± SD of 70 ± 5 cells/mm² of SRA01/04 were seeded into 25-mm² culture flasks with culture media at 37°C, 5% carbon dioxide, and 100% humidity. Dulbecco minimum essential medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (Gibco) without any antibiotics or antifungal agents was used as the standard medium.

**ANTIGLAUCOMA AGENTS AND PRESERVATIVE**

The concentrations of latanoprost (50 µg/mL; Cayman Chemical, Ann Arbor, Mich), timolol maleate (5 mg/mL; Sigma, St Louis, Mo), or benzalkonium chloride (200 µg/mL; Sigma) used in eyedrops, and ×10 to ×100 dilutions thereof, were added to the standard medium. After 7 days, cell shape was monitored by phase-contrast microscopy, and cell-free culture supernatants were analyzed by iodine 125 radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), or chemiluminescent enzyme immunoassay (CLEIA) for PGE2, IL-1α, and IL-6, respectively. Cultures in standard medium without any additives were used as controls.

**RADIOIMMUNOASSAY**

The level of PGE2 in the culture media was measured using an RIA kit (NEN Life Science Products, Boston, Mass), according to the manufacturer’s instructions. Briefly, 100 µL of sample mixed with 100 µL of 125I-PGE2 tracer and 100 µL of PGE2 antibody were incubated for 24 hours at 4°C; 1 mL of cold precipitating reagent was then added to the sample, and it was incubated for a further 30 minutes. After centrifugation at 1000g for 30 minutes, all liquid was decanted, and the sample was counted in a gamma counter. The same procedure was followed to generate a standard curve. The standard concentrations of PGE2 were in the range of 1 to 500 pg/0.1 mL.

**CHEMILUMINESCENT ENZYME IMMUNOASSAY**

The level of IL-1α was quantified using an ELISA kit (Jimro, Gunma, Japan), according to the manufacturer’s instructions. Briefly, 200 µL of the sample was incubated in a microtiter plate with anti–IL-1α–coated wells. After overnight incubation, the sample was reacted with 100 µL of rabbit anti–IL-1α antibody for 2 hours, 100 µL of anti-rabbit IgG–horseradish peroxidase conjugate for 2 hours, and 100 µL of chromogenic solution for 10 minutes. The reaction was then stopped with stop solution. The absorbance of the plate was measured at 490 nm using a plate reader. All procedures were performed at room temperature, and the plate was washed 3 times with washing solution between each reaction. The same procedure was followed for the standard curve. The standard concentrations of IL-1α were between 0 and 250 pg/mL.

**RESULTS**

**MORPHOLOGICAL CHANGES OF THE CELLS**

There were no surviving cells in cultures carried out in medium supplemented with the eyedrop concentrations of latanoprost, timolol, or benzalkonium chloride, even after only 3 days (Figure 1A and B). When latano-
prostaglandin E2 (PGE2) with latanoprost or timolol maleate (n=6) (A) and benzalkonium chloride (n=6) (B). This occurs in a dose-dependent manner. *Test vs control: asterisk indicates P<0.01; dagger, P<0.05.

The effects of latanoprost and timolol were examined at 1/10, 1/18, 1/30, 1/60, and 1/100 dilutions, and those of benzalkonium chloride at 1/180, 1/300, 1/600, and 1/1000 dilutions, because there were no or very few living cells at the eyedrop concentration of latanoprost or timolol, and for benzalkonium chloride, even at the 1/100 dilution, only a few cells could attach to the wells, and no proliferation or elongation was observed (Figure 1G). The cell shape was the same at 5 and 7 days of culture.

SECRETION OF CHEMICAL MEDIATORS

The effects of latanoprost and timolol were examined at 1/10, 1/18, 1/30, 1/60, and 1/100 dilutions, and those of benzalkonium chloride at 1/180, 1/300, 1/600, and 1/1000 dilutions, because there were no or very few living cells at the eyedrop concentration of latanoprost or timolol, and for benzalkonium chloride, even at the 1/100 dilution. The amounts of chemical mediators present in culture supernatants were calculated per 10^5 cells because cell numbers varied at each dilution of the antiglaucoma agents and benzalkonium chloride, due to the different degrees of cell growth over the 7-day culture period.

In the standard medium, the mean±SD PGE2 level was 8.4±2.3 pg/10^5 cells. At the 1/10 dilutions of latanoprost and timolol, the mean±SD levels of secreted PGE2 were increased to 45.7±8.0 and 143.2±10.7 pg/10^5 cells, respectively, in a dose-dependent manner (Figure 2A). At the 1/180 dilution of benzalkonium chloride, the mean±SD amount of PGE2 was increased to the much higher level of 2566.7±723.4 pg/10^5 cells, also in a dose-dependent manner (Figure 2B).

Production of IL-1α and IL-6 showed similar tendencies. Figure 3A depicts the amount of IL-1α in supernatants of SRA01/04 cells cultured in medium supplemented with latanoprost and timolol. IL-1α was secreted by cells in the standard medium (mean±SD, 10.4±4.5 pg/10^5 cells), but this increased to 35.0±6.0 and 802.8±152.6 pg/10^5 cells in 1/10 dilutions of latanoprost and timolol, respectively. It increased to a much greater level (mean±SD, 2598.9±322.2 pg/10^5 cells) with the 1/180 dilution of benzalkonium chloride (Figure 3B). The mean±SD level of IL-6 was 359.8±10.8 pg/10^5 cells in the standard medium, rising to 3383.7±913 and 121.5±453.6 pg/10^5 cells with 1/10 dilutions of latanoprost and timolol, respectively (Figure 4A). Again, secretion increased to a much greater level (mean±SD, 7311.1±27797.6 pg/10^5 cells) with the 1/180 dilution of benzalkonium chloride (Figure 4B).

Using human lens epithelial cells in culture, we investigated the effects of latanoprost, timolol, and benzalkonium chloride on cell morphological changes and secretion of soluble mediators. It may be instructive to consider these results in conjunction with the clinical data reported by Miyake et al10,20 on the effects of latanoprost or timolol, with or without additional benzalkonium chloride, on inflammation following cataract or intraocular lens surgery. Recent reports suggest that lens epithelial cell wound healing processes play significant roles in the onset of
postoperative inflammation following cataract or intraocular lens surgery. In vivo, lens epithelial cells synthesize chemical mediators such as PGE₂ during the wound healing process, and nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to suppress this in baboons. Similarly, in vitro, cultured lens epithelial cells obtained from lenses during cataract surgery in elderly patients synthesize PGE₂, IL-1, and IL-6 during pseudometaplasia. Again, NSAIDs reportedly suppress this synthesis.

The present study is the first to report increased synthesis of PGE₂, IL-1α, and IL-6 by lens epithelial cells in the presence of latanoprost, timolol, or benzalkonium chloride in vitro. This result suggests that these drugs or the additive benzalkonium chloride contribute to an intensified early postoperative inflammatory response. The present results also demonstrate that the effect of benzalkonium chloride is considerably stronger than that of either latanoprost or timolol in terms of morphological damage and increased synthesis of PGE₂, IL-1α, and IL-6 in a cell line in vitro. This suggests that benzalkonium chloride makes a significantly greater contribution than latanoprost or timolol to an increased inflammatory response. The results of this basic study support the earlier clinical observations that the additive benzalkonium chloride acts more strongly than the main active agents latanoprost or timolol in increasing the incidence of CME and postoperative aqueous flare following cataract or intraocular lens surgery.

Recently, it has been reported that the use of antiglaucoma agents, especially β-blockers, is one of the risk factors for the incidence of nuclear lens opacity or the requirement for cataract surgery. Our results also suggest that cell stress associated with the chronic use of benzalkonium chloride might induce cataract.

In general, the intraocular migration of antiglaucoma eyedrops is low. For example, timolol is reported to be diluted 1000 in healthy eyes. With 1000 dilutions of latanoprost or timolol in the present investigation, there was either no or extremely little morphological change or production of chemical mediators. On the other hand, although there are no data on the intraocular migration of benzalkonium chloride, the present investigation showed that chemical mediators were stimulated at even a 1000 dilution of benzalkonium chloride. The intraocular penetration of drugs generally increases following eye surgery, and the intraocular penetration of latanoprost, timolol, and benzalkonium chloride described in this study would seem to strongly suggest that benzalkonium chloride plays a significantly greater role than the 2 drugs themselves in postoperative intraocular cell damage and the expression of mediators.

In conclusion, 2 recent clinical studies and the present in vitro study all suggest that the preservative benzalkonium chloride plays a much greater role than active agents such as latanoprost or timolol in the aggravated early postoperative inflammation caused by antiglaucoma eyedrops. Expression of PGE₂ is seen both as an early phenomenon in general cell damage and as an accompaniment to pseudometaplasia of lens epithelial cells. It is reportedly suppressed by NSAIDs. The results of the present study indicate that this PGE₂ expression is increased by the presence of latanoprost, timolol, and particularly the additive benzalkonium chloride. In the clinical setting, it has also been reported that NSAIDs can suppress increased damage to the blood-aqueous barrier and the occurrence of CME caused by antiglaucoma eyedrops. This suggests that prostaglandins that are aggravated by benzalkonium chloride are one cause of postoperative inflammatory conditions such as CME. It should be noted this does not contradict the prostaglandin theory put forward to explain the general occurrence of aphakic or pseudophakic CME.

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