In Vitro Study of Inflammatory Potential and Toxicity Profile of Latanoprost, Travoprost, and Bimatoprost in Conjunctiva-Derived Epithelial Cells

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PURPOSE. Conjunctiva-derived epithelial cells were used to investigate, in vitro, the expression of various inflammation-associated markers known to be overexpressed in patients with glaucoma after contact with the three major commercially available eye drops containing prostaglandin analogues. The impact on cellular viability and apoptosis in the same cell line was evaluated, to address the possible proinflammatory and/or toxic origin of the most frequent clinical impairments induced by prostanoids (i.e., conjunctival hyperemia).

METHODS. Conjunctiva-derived cells were treated in vitro with the commercial solutions of latanoprost, travoprost, bimatoprost, prostaglandin (PG)F2α, tumor necrosis factor (TNF)-α, and different concentrations of benzalkonium chloride (BAC). Expressions of three inflammation- and immune-related markers, intercellular adhesion molecule (ICAM)-1, platelet-endothelial cell adhesion molecule (PECAM)-1 and HLA DR, were evaluated with flow cytometry after 24 to 72 hours of contact at low, subtoxic concentrations. Toxicological tests were also performed with cold-light cytofluorometry, in which cellular viability and apoptosis were evaluated with the neutral red and Hoechst/propidium iodide tests, respectively.

RESULTS. TNFαs induced or stimulated expression of the three inflammatory markers, whereas the PGF2α, latanoprost, travoprost, and bimatoprost solutions did not induce an increase in these markers and even produced a marked reduction of ICAM-1 and PECAM-1 expression in those solutions most concentrated in BAC, thus suggesting a toxic phenomenon in cellular membranes induced by the preservative rather than the medication itself. Cytotoxic assays confirmed this hypothesis and showed significant toxicity with prostaglandin analogues after prolonged contact, proportional to the concentration of BAC in the solution and similar to that of the corresponding concentration of BAC alone, bimatoprost having both the least concentration of BAC and the least cytotoxic in these experimental conditions.

CONCLUSIONS. The comparison of latanoprost, travoprost, and bimatoprost, in their commercial formulations, showed that of them appeared to induce direct stimulation of the inflammatory pathways involving adhesion molecules or class II antigens, although these markers have been found ex vivo in conjunctival specimens from patients treated with prostaglandins. In fact, their toxicity was mild and seemed to be primarily related to the concentration of BAC, their common preservative, which may be the major factor responsible for long-term ocular surface reactions in patients receiving topical prostaglandins, but most likely is not a factor in early and transient conjunctival hyperemia. (Invest Ophthalmol Vis Sci. 2005;46:2444–2450) DOI:10.1167/iovs.04-1331

The prostaglandin analogues latanoprost, travoprost, and bimatoprost have progressively become the most prominent topical treatments for ocular hypertension and glaucoma. They have shown their potent efficacy in lowering intraocular pressure and a favorable safety profile. Several side effects, however, have been described in clinical trials and postmarketing surveys that could suggest potential proinflammatory properties. Ocular hyperemia is a very common side effect, with an incidence that varies greatly among different studies and different molecules (5%–68%), and cases of macular edema or uveitis have been described in patients at high risk for inflammatory reactions or retinal vasculopathy, with each one of the three eye drops, including the recently marketed travoprost and bimatoprost. Indeed, hyperemia is currently the most frequent side effect and mainly occurs in the first few weeks of treatment, with a progressive but nonconstant decrease over time. This side effect may represent a cosmetic problem to the patient, possibly leading to non- or poor compliance. A study performed in rabbits indicated that PGF2α analogues cause ocular surface hyperemia by activating nitric oxide synthase. Also suggested that travoprost and bimatoprost could be responsible for a higher incidence of hyperemia, compared with latanoprost, because of a chemical difference in molecular structure (i.e., the double bond at the C13-C14 position on the latano-

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trations varying from 0.005% to 0.02%, we could therefore postulate proinflammatory and/or toxic effects of these solutions on the ocular surface. In a previous study, we showed on impression cytology specimens, by using a flow cytometry technique, that long-term use of unpreserved timolol would have almost no inflammatory or toxic effect on conjunctival epithelium compared with preserved timolol, whereas latanoprost eye drops only showed a slight proinflammatory effect, despite its analogy with PGF2α and its higher concentration of BAC. Epithelial expression of two major inflammatory markers, intercellular adhesion molecule (ICAM)-1 and the class II antigen HLA DR, was thus found at intermediate levels between those found with preservative-free and preserved β-blockers. This discrepancy was confirmed by an in vitro study in a human conjunctiva-derived cell line showing a lower level of toxicity of the preserved latanoprost solution after a short duration of contact compared with the same concentration of BAC alone and with preserved timolol.28

We therefore used the same cell line and the same methods to investigate three immune- or inflammation-related markers, HLA DR, ICAM-1 (CD54) and platelet-endothelial cell adhesion molecule (PECAM)-1 (CD31), as well as apoptosis-related markers, to assess and compare the proinflammatory potential of the three major prostaglandin analogues commercially available and their toxicologic profiles. Thus, using objective methods on a stable and reliable although nonclinical model, we investigated some of the pathways possibly involved in the most frequent clinical impairment of the ocular surface induced by prostaglandin analogues, conjunctival hyperemia.

**Materials and Methods**

**Conjunctival Cell Line**

Wong-Kilbourne human conjunctiva-derived epithelial cells from an established cell line (Wong-Kilbourne derivative of Chang conjunctiva, clone 1-5c-4; certified cell line [CCL], 20.2, American Type Culture Collection [ATCC], Manassas, VA) were cultured under standard conditions (humidified atmosphere of 5% CO2 at 37°C) in Dulbecco’s minimum essential medium (DMEM; Eurobio, Les Ulis, France) supplemented with 10% fetal calf serum, 1% glutamine, penicillin 50 IU/mL and streptomycin 50 IU/mL (Eurobio). Cells from passages 41 through 54 were used in all experiments. Normal culture development was assessed daily with phase-contrast microscopy (DMIRB; Leica, Wetzlar, Germany).

Confluent cultures were removed by gentle trypsin incubation, and the cells were counted. They were then seeded into 96-well culture plates (5000 cells per well; Corning BV, Schiphol-Rijk, the Netherlands) for microtitration analyses and into six-well culture plates (40,000 cells per well) for flow cytometric analyses. Cultures were kept at 37°C for 24 hours. After subconfluence was attained (culture surface covering nearly 70%), cells were exposed to the different formulations. Because this cell line spontaneously undergoes apoptosis at 100% of confluence (data not shown), 70% of confluence was chosen, to avoid any artifact in membrane integrity assays.

**Prostanoid and Preservative Treatment**

First, to evaluate the proinflammatory potential of prostaglandin analogues, we used flow cytometry analyses to compare latanoprost (Xalatan; Pfizer, New York, NY), travoprost (Travatan, Alcon, Fort Worth, TX), bimatoprost (Lumigan, Allergan, Irvine, CA), PGF2α at 10⁻⁴ M, TNFα at 1000 ng/mL, and BAC at 0.1%. This BAC concentration was chosen as a positive control for toxicity because of its well-known toxic effects. PGF2α was tested at a concentration of 10⁻⁴ M, corresponding to 35.5 μg/mL, which is comparable to the prostaglandin analogue concentration contained in the three eye drops. TNFα at a concentration of 1000 ng/mL is known to be a major proinflammatory molecule that induces overexpression of molecules such as CD54, CD31, or HLA DR and was also used in our experiments as a positive inflammatory control.

Secondly, based on the first results obtained with adhesion molecule expression, we evaluated the toxic effects of the three prostaglandin analogues in light of their corresponding BAC concentrations. For these cytotoxicity tests, we used the three concentrations of BAC present in the three eye drops. These were therefore lower than the dose used for flow cytometry analyses because we wanted to investigate more precisely whether BAC is directly involved in the toxicity of the three eye drops. Therefore, in cold-light cytometry experiments, we used eight different molecules: the three prostaglandin analogues in their commercial presentation, latanoprost, travoprost, and bimatoprost; three different concentrations of BAC—0.02%, 0.015%, and 0.005%—corresponding to the concentrations contained in latanoprost, travoprost, and bimatoprost, respectively; and PGF2α at 35.5 μg/mL and TNFα at 1000 ng/mL.

We examined each solution at the initial concentrations, diluted at 1:10, 1:100, and 1:1000 after 24, 48, and 72 hours of contact for flow cytometry analyses, and after a 24-hour contact for cold-light fluorometry measurements.

**Flow Cytometry**

Expressions of HLA DR, CD54 (ICAM-1) and CD31 (PECAM-1), three molecules known to be overexpressed during inflammatory episodes,29 were assessed by flow cytometry in direct and indirect immunofluorescence procedures. We used monoclonal antibodies directed against HLA DR (ε-chain, clone TAL185; Dako Cytomation, Glostrup, Denmark), CD54 (ICAM-1, clone 84H10; Beckman-Inmunotech, Mar-selles, France) at a 1:25 dilution. After 30 minutes of incubation in the dark at room temperature, the cells were washed in PBS (pH 7.2) and then centrifuged 5 minutes at 1600 rpm. A 1:25 dilution of FITC-conjugated goat anti-mouse immunoglobulins (Dako Cytomation) was added for 30 minutes. Phycoerythrin (PE)-conjugated anti-CD31 (PECAM-1, clone MEC 13.3, rat anti-human; Dako Cytomation) was incubated with the cells for 30 minutes at a 1:50 dilution. Corresponding isotypic negative controls were used. Mouse IgG1 (Immunotech) for HLA DR and CD54, and rat IgG2a-P (BD-PharMingen, San Diego, CA) for CD31. At the end of all incubations, cells were washed and suspended in PBS before flow cytometry processing. All analyses were performed on a flow cytometer (EPICS XL; Beckman Coulter, Miami, FL) equipped with an argon laser emitting at 488 nm, using software provided by the manufacturer (EPICS XL system II, Beckman Coulter) for data analysis.31 As previously described,32 cells were gated on a side scatter (cell size) versus forward scatter (cell granularity) histogram in logarithmic and linear modes, respectively. For each antibody, at least 1000 conjunctival cells were analyzed on a logarithmic fluorescence histogram, showing the number of cells as a function of fluorescence intensities. Results were obtained in mean fluorescence intensities (MFIs). Each experiment involving cell stimulation and immunostaining procedure was independently performed in triplicate.

**Microplate Cold-Light Fluorometry**

Fluorometry was performed on a microplate cytometer (Fluorolite 1000; Dynex; Cergy Pontoise, France). According to the recommendations of the European Center for the Validation of Alternative Methods (ECVAM), two cellular assays were used: neutral red for cellular viability and Hoechst-propidium iodide for apoptosis.33 Two different tests were used, according to previously validated methods in Chang’s cell line.35 Briefly, membrane integrity, closely correlating with cellular viability, was evaluated by using the neutral red test (Fluka, Ronkonkoma, NY) at a concentration of 50 μg/mL. In accordance with the validated protocol of Borenfreund and Puerner,34 200 μL per well of medium containing neutral red was added to living cells, and the microplates were incubated for 3 hours at 37°C in an atmosphere containing 5% CO2. The neutral red fluorescence was measured as previously described.35 Chromatin condensation was evaluated with the Hoechst 33342 dye (Molecular Probes, Eugene, OR). Hoechst
33342 is a specific UV fluorescent probe that specifically reacts with DNA at the adenine and thymine levels by intercalation after 30 minutes. This probe was used on cells at a final concentration of 10 μg/mL. One microliter of propidium iodide (Sigma-Aldrich, St. Louis, MO) at 0.5 mg/mL was added to the Hoechst 33342 solution to control cell necrosis. Supravital uptake of Hoechst combined with exclusion of propidium iodide has been proposed as an assay for apoptosis. To express results as a function of remaining cell density, we calculated the ratio between DNA condensation and cell viability (Hoechst33342-neutral red). Apoptotic cells therefore show an increase in the Hoechst-neutral red (Ho/NR) ratio (>1), whereas necrotic cells are characterized by a decreased Ho/NR ratio (<1). In all experiments, the background fluorescence was determined on wells without cells but containing the dye solution and was deduced from all control and treated wells. Wells containing cells with complete culture medium but without any treatment were used as the control. Microplate cold-light cytofluorometry results were obtained in fluorescence units and were expressed as a percentage of the control. Each drug was tested in six wells, and each experiment was performed in triplicate.

Statistical Analysis

Statistical comparisons were performed by analysis of variance (ANOVA) followed by the Tukey-Kramer test for cold-light cytofluorometry and the Bonferroni-Dunn test for flow cytometry (Statview V for Windows; SAS Institute, Cary, NC).

RESULTS

Expression of Inflammation Markers

Solutions tested in a 1:10 dilution appeared too toxic after prolonged contact to be analyzed, even at 24 hours. In contrast, the 1:1000 dilution did not change any marker with any drug (data not shown). We therefore focused our results on the 1:100 dilution, which showed significant changes over time with some of the drugs tested.

TNFα significantly increased CD54 expression at 24, 48, and 72 hours, compared with latanoprost (P < 0.001), travoprost (P < 0.001), bimatoprost (P < 0.001), and BAC (P < 0.002). Compared with cell control, this increase was only statistically significant at 24 (P < 0.05) and 48 hours (P < 0.001). It was not significant when compared with the values obtained with cells exposed to PGF2α, except at 48 hours (P < 0.001), probably because of the relatively large standard deviations (Fig. 1). Similar results were obtained with CD31, as TNFα increased CD31 expression at 24, 48, and 72 hours compared with PGF2α (P < 0.02 at 24 and 72 hours; P < 0.0001 at 48 hours), the control (P < 0.005), latanoprost (P < 0.0001), travoprost (P < 0.001), and bimatoprost (P < 0.02; Fig. 2).

BAC considerably decreased the expression of the two adhesion molecules, with statistically significant differences for...
CD54 compared with the control ($P < 0.05$) and TNFα ($P < 0.001$) at 24, 48, and 72 hours, and compared with PGF2α ($P < 0.05$) and bimatoprost ($P < 0.05$) at 24 and 72 hours. No statistically significant difference was observed for CD54 between BAC and latanoprost or travoprost. Concerning CD31, significant differences were found between BAC and cell control at 24 and 48 hours ($P < 0.001$); PGF2α at 24 ($P < 0.0001$) and 72 hours ($P < 0.02$); TNFα ($P < 0.0001$) and bimatoprost ($P < 0.02$) at 24, 48, and 72 hours; and latanoprost ($P < 0.02$) and travoprost ($P < 0.02$) at 24 hours.

CD54 expression tended to be reduced after prostaglandin treatment compared with the control, but this reduction was not statistically significant. Prostaglandin analogues decreased CD31 expression at 24, 48, and 72 hours compared with the control, but CD31 levels were only statistically significant for latanoprost ($P < 0.005$) and travoprost ($P < 0.05$) at 24 and 72 hours. In contrast, cells treated with bimatoprost did not show the same tendency as those with latanoprost or travoprost and were not significantly different from the control. Compared with PGF2α, each prostaglandin analogue induced a reduction in expression of both adhesion molecules but was statistically significant only for CD31 at 24 and 72 hours with latanoprost ($P < 0.05$) and travoprost ($P < 0.05$, $P < 0.02$; Figs. 1, 2).

HLA DR expression was not increased after 24 hours of treatment, to 2.24 ($P < 0.01$, compared with the control) and 1.78 ($P < 0.01$, respectively). There was no significant variation with BAC at 5 × $10^{-5}$% for all comparisons.

**DNA Condensation Evaluation**

BAC at the final concentrations of 2 × $10^{-4}$% and 1.5 × $10^{-4}$% induced a concentration-dependent increase in the Hoechst-neutral red ratio after 24 hours of treatment, to 2.24 ($P < 0.01$, compared with the control) and 1.78 ($P < 0.01$, respectively). There was no significant variation with BAC at 5 × $10^{-5}$% (Fig. 4). The effect of the three prostaglandin analogues seemed well correlated with their respective BAC concentrations. There was no difference between the control and the bimatoprost solution after 24 hours; latanoprost and travoprost solutions, in contrast, induced an increase in the fluorescence ratio to 1.94 and 1.69. ($P < 0.01$ for both drugs, compared with control values). A tendency toward lesser toxicity was found between the prostaglandin analogues and their corresponding BAC concentrations but with no statistically significant differences. Significant differences, however, were found between bimatoprost and latanoprost ($P < 0.01$) and between bimatoprost and travoprost ($P < 0.01$) but not between travoprost and latanoprost (Fig. 4). PGF2α (ratio, 0.93) and TNFα (ratio, 0.855) did not induce a significant difference in fluorescence ratios compared with control values.

**DISCUSSION**

Today, prostaglandin analogues are the first-line therapy in glaucoma and have overtaken β-blockers for this indication,
thanks to their efficiency in reducing intraocular pressure and the low rate and severity of adverse events, especially the absence of systemic side effects.\textsuperscript{1-4} Different molecules are available but only slight differences between these three medications are known in terms of efficacy and safety, except in the incidence of ocular hyperemia.\textsuperscript{4-9,20} In most clinical trials, conjunctival hyperemia is typically at its maximum the second day after starting treatment and then decreases after 1 week, and the ocular surface shows an almost normal aspect after 1 to 6 months of treatment.\textsuperscript{6-7} However, the incidence of hyperemia is highly variable, depending on the different clinical trials available. Thus, latanoprost induces a rate of hyperemia ranging between 5\% and 47\%,\textsuperscript{2,4,7-9} while bimatoprost is responsible for 8\% to 58\% of hyperemia.\textsuperscript{4-6,9-11} and travoprost 28\% to 68\%.\textsuperscript{4,6-8} Even though these results are similar from one molecule to another, all studies in which comparisons were made found a slightly lower incidence in patients treated with latanoprost.\textsuperscript{4,6-9} Furthermore, in a recent study concerning nearly 2000 patients treated with bimatoprost for 2 months, the authors found hyperemia in only 8\% of patients, which is one of the lowest overall incidences reported, but bimatoprost was not compared with the other prostanoids.\textsuperscript{10}

This side effect could be caused by a vasodilatation related to the delivery of nitric oxide (NO), resulting from an overproduction of NO synthase induced by prostaglandin analogues, but the precise mechanism is not fully understood, and this theory should be tested in further studies, for a full assessment of the mechanisms of hyperemia.\textsuperscript{17} Recently, Leal et al.\textsuperscript{17} performed conjunctival biopsies on patients with bimatoprost-induced hyperemia and found no more frequent histopathologic signs of inflammation in conjunctival specimens from bimatoprost-treated patients than in those from untreated control subjects. It is important to note that even though latanoprost contains the highest concentration of BAC, it seems to cause a lower incidence of hyperemia, thus suggesting that hyperemia is not a direct consequence of BAC toxicity.

However, PGF2α is considered as a potent proinflammatory agent, responsible for recurrence of uveitis or cystoid macular edema. We therefore found it interesting to evaluate the expression of some of the cellular inflammatory markers previously found to be overexpressed in the conjunctival epithelium of patients receiving latanoprost using flow cytometry in a human conjunctiva-derived cell line, to determine whether treatments with prostaglandin analogues would induce or potentiate an immunologic or inflammatory mechanism. In control experiments, (1) for prostaglandin analogues, we examined the action of PGF2α; (2) for inflammation, the action of TNFα; and (3) for toxicity, the action of BAC. As expected, TNFα induced substantial overexpression of inflammatory markers CD31, CD54, and HLA DR, consistent with the potent proinflammatory properties of this cytokine.\textsuperscript{29} BAC, in contrast, decreased the expression of the two adhesion molecules CD31 and CD54, most likely after a toxic phenomenon leading to apoptonecrosis of conjunctival cells. Indeed, cultured cells submitted to BAC, even at very low concentrations, showed a dramatic decrease in cell size, a characteristic of apoptotic cell death (data not shown). We have shown that BAC has potent dose-dependent toxic effects resulting from the combination of the quaternary ammonium with cell membranes and interaction with major cell-defense mechanisms.\textsuperscript{24} In impression cytology specimens, CD54 (ICAM-1) was found to be increased in eyes receiving preservative-containing drugs for long periods, most likely by chronic subclinical inflammatory reactions related to repeated eye drop instillations. To our knowledge, this is the first report to investigate BAC effects in vitro on adhesion molecules. We hypothesize that the low levels of CD54 and CD31 found with BAC in the present study may result mainly from toxic effects to the cell culture and alterations to cell membranes.

In contrast with TNFα, the three prostaglandin analogues did not induce an increase in the expression of adhesion molecules but on the contrary caused a significant reduction, more pronounced with latanoprost, followed by travoprost and bimatoprost. This phenomenon was not found with PGF2α, which had no significant effect on adhesion molecules. In fact, expression of adhesion molecules after treatment with any of the three prostaglandin analogues seemed to be consistent with their BAC concentrations, as the higher the concentration of BAC, the lower the expression of CD31 and CD54. Indeed, the commercial preparations of latanoprost, travoprost, and bimatoprost, even diluted to 1:100, still contained significant doses of BAC (2 × 10^{-7}%, 1.5 × 10^{-7}%, and 0.5 × 10^{-7}%, respectively); thus, the decrease in CD54 and CD31 may be primarily related to the toxic action of BAC they contain. Bimatoprost contains less BAC than do travoprost and latanoprost, and the expression levels of CD54 and CD31 were thus higher than those obtained with the two other prostaglandin analogues. In addition, HLA DR, another inflammatory marker, was not found to be overstimulated by treatments with any prostaglandin analogues, even after 3 days of low-dose treatments, in contrast with TNFα, which highly stimulated it. Thus, in our in vitro experiments, neither PGF2α nor the three prostaglandin analogues activated the inflammatory pathways involving these three inflammation-related mediators. This find-
The absence of direct proinflammatory effects of the three prostaglandin analogues and PGF2α and the reduction profile that seemed to correlate with the BAC concentration led us to undertake additional toxicological tests to evaluate comparatively the three prostaglandin analogues using our validated apoptosis assay techniques. Our results showed significant differences in conjunctiva-derived cells in vitro in the three prostaglandins. Again, the effects on membrane integrity and apoptosis correlated highly with the concentrations of BAC contained in the three commercial solutions. Moreover, toxicological tests (neutral red and Hoechst 33342) showed no statistical differences between prostaglandin analogue preparations and their respective BAC used alone. Thus, the BAC contained in the three prostaglandin preparations seemed to be the principal factor responsible for toxic effects on conjunctival cells, although the total absence of toxic effects of the active compounds could not be eliminated, because the prostaglandin analogues alone (i.e., preservative-free), were not available at the time of this study. In a previous study, Goto et al. found similar results of latanoprost toxicity in lens epithelial cells in vitro and showed that the BAC contained in the commercial solution of latanoprost was responsible for the damage induced in lens epithelial cells. We found that the commercial preparation of bimatoprost, which contained the lowest BAC concentration (5 × 10⁻⁶M), was less cytotoxic than our experimental conditions than BAC concentrations in latanoprost and travoprost for both tests (neutral red and the Hoechst 33342/neural red ratio). In our study we even observed with prostanoids a tendency to a slightly lower toxicity compared with the corresponding concentration of BAC used alone as a control. This suggests a possible protective effect of the prostanoids toward BAC-induced toxicity, as we previously compared with the corresponding concentration of BAC used.

In conclusion, this in vitro study, conducted according to a series of previous toxicological investigations performed in the same human conjunctiva-derived cell line, was aimed at comparing the three major commercial preparations of prostaglandins used in glaucoma treatment. None of them induced direct stimulation of the inflammatory pathways involving major inflammation-related markers (i.e., adhesion molecules and HLA class II antigens). Moreover, the comparison between latanoprost, travoprost, and bimatoprost, in their commercial presentations, showed that their toxicity was mild and primarily related to the concentration of the preservative BAC. Obviously, these results obtained in this in vitro model cannot be fully extrapolated to in vivo conditions, but all our previous experimental results were in good agreement with data from the literature assessing toxic side effects induced in the ocular surface by preservatives and in some cases by active compounds. This first preliminary comparative study of three major prostaglandin analogues thus suggests that hyperemia is most likely not an inflammatory response directly caused by drugs and that it appears too early after onset of treatment to be the inflammatory consequence of long-term treatments. In addition, despite the relatively low toxicity of all three drugs, this study again argues for the development of preservative-free solutions for long-term use in patients with glaucoma.

References


