Conjunctival Proinflammatory and Proapoptotic Effects of Latanoprost and Preserved and Unpreserved Timolol: An Ex Vivo and In Vitro Study

Pierre-Jean Pisella,[1,2] *Caroline Debbasch,*[2] *Pascale Hamard,*[3,4] *Catherine Creuzot-Garcher,*[5] *Patrice Rat,*[2] *Françoise Brignole,*[1,2] *and Christophe Baudouin*[3,4]

Purpose. To compare the toxicity of latanoprost and preserved and unpreserved timolol on conjunctival cells. Expression of inflammatory markers and MUC5AC-related mucin production were evaluated by impression cytology in a case–control ex vivo study. The proapoptotic effect of the same drugs was also evaluated in vitro in a conjunctival cell line and compared with that of benzalkonium chloride (BAC).

Methods. Impression cytology (IC) specimens were obtained from a series of normal subjects and from patients with glaucoma treated for at least 1 year with latanoprost eye drops or preserved or unpreserved timolol. All groups were comparable in age and duration of treatment. Expression of HLA-DR, intercellular adhesion molecule (ICAM)-1, and mucin was evaluated in a masked manner by flow cytometry. For the in vitro study, a human conjunctiva–derived cell line was treated with 0.02% BAC-containing latanoprost or timolol, unpreserved timolol, or 0.02% BAC alone for 15 minutes, followed or not by 4 or 24 hours of cell recovery in normal medium. Cell viability and chromatin condensation were evaluated using microplate cold light cytofluorometry with the neutral red and the Hoechst 33342 tests, respectively. The Hoechst–neutral red ratio was defined for the apoptosis assay, and cytoskeleton changes were assessed by confocal microscopy.

Results. No difference was found between normal eyes and those receiving unpreserved timolol. Preserved latanoprost and timolol significantly increased the inflammatory marker expression and decreased MUC5AC expression, but to a significantly higher extent in the preserved timolol group compared with latanoprost. In vitro, 0.02% BAC-containing timolol and latanoprost triggered conjunctival cell apoptosis— however, to a significantly lesser extent than did 0.02% BAC alone. Unpreserved timolol did not cause any cell toxicity.

Conclusions. These ex vivo and in vitro studies demonstrate that BAC-containing latanoprost and timolol exhibit higher proinflammatory and proapoptotic effects on conjunctival cells than does unpreserved timolol. Latanoprost caused less toxicity, however, than preserved timolol, and both drugs were less toxic than BAC alone. These results suggest a potential protective effect of the prostaglandin analogue and to a lesser extent of timolol against the toxicity of BAC in conjunctival cells. (*Invest Ophthalmol Vis Sci.* 2004;45:1360–1368) DOI: 10.1167/iovs.03-1067

In the past decade, new hypotensive molecules have been developed for the medical treatment of glaucoma, such as the prostaglandin analogue latanoprost, which reduces intraocular pressure by increasing the uveoscleral outflow. Prostaglandins have the theoretical potential to stimulate inflammatory pathways, and the hypothesis that latanoprost may induce ocular surface changes therefore cannot be excluded, even though pivotal multicenter clinical studies have clearly demonstrated that ocular discomfort after treatment with the prostaglandin analogue was low and similar to the discomfort reported with preserved timolol and possibly related to the preservative benzalkonium chloride (BAC).[1–3]

The toxicity of preservative-containing antiglaucomatous drugs on the conjunctival epithelium has therefore been widely described in patients treated for a long period, either through an indirect deleterious effect on the lacrimal film or through a direct toxic effect on epithelial cells.[4–6] In several clinical and experimental studies, it has been shown that long-term use of ophthalmic solutions associated with a preservative can induce conjunctival stroma infiltrates and overexpression of inflammation- or apoptosis-related molecules such as HLA-DR, intercellular adhesion molecule (ICAM)-1, Fas antigen, or the apoptotic marker Apo2.7.[7–10] BAC is the most commonly used preservative in ophthalmic solutions, especially in antiglaucoma drugs. Its potential responsibility in filtering surgery failure has been suggested,[11–14] and its cellular toxicity, even at low concentrations, has been experimentally demonstrated in in vitro studies of a continuous human conjunctiva-derived cell line.[6,15,16]

The purpose of the present ex vivo case–control study was to investigate the conjunctival profile of three groups of patients with glaucoma, treated for a long period with latanoprost or preserved or unpreserved timolol, in comparison with a series of normal eyes. We used a well-validated flow cytometry technique of impression cytology,[10,17,18] to evaluate the expression of inflammatory markers by conjunctival cells as well as the presence of soluble mucins of the *MUC5AC* gene. We also conducted an in vitro study on a continuous human conjunctival cell line[16,19] to evaluate the proapoptotic effect of these antiglaucoma drugs and of BAC.

Materials and Methods

Ex Vivo Study Design

Three groups of patients with glaucoma were included in this case–control study conducted in compliance with the Declaration of Hel-

From the [1]Department of Ophthalmology, University Hospital of Tours, Tours, France; the [2]Department of Toxicology, Faculty of Biological and Pharmacological Sciences, University of Paris 5, Paris, France; the [3]Department of Ophthalmology, Quinze-Vingts National Ophthalmology Hospital and INSERM U598, Paris, France; [4]Ambroise Paré Hospital, APHP, Paris Ouest School of Medicine, Paris, France; and the [5]Department of Ophthalmology, University Hospital of Dijon, Dijon, France.

Supported by a grant from University Paris 6 (EA3123).

Submitted for publication September 26, 2003; revised November 25, 2003; accepted January 10, 2004.

Disclosure: **P.-J. Pisella**, None; **C. Debbasch**, None; **P. Hamard**, None; **C. Creuzot-Garcher**, None; **P. Rat**, None; **F. Brignole**, None; **C. Baudouin**, None

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Corresponding author: Christophe Baudouin, Quinze-Vingts National Ophthalmology Hospital, 28 rue de Charenton, 75012 Paris, France; baudouin@quinze-vingts.fr.

TABLE 1. Demographic Characteristics of Normal Subjects and the Three Groups of Patients with Glaucoma

	Timolol,BAC+	Timolol,BAC−	Latanoprost	Normal Subjects
Eyes (*n*)	15	17	21	18
Age (ys)				
Mean ± SD	53.7 ± 24.1	57.5 ± 21.5	57.7 ± 12.7	48.1 ± 27.7
Range	23–91	16–77	42–70	22–68
Sex (*n*, %)				
Male	6 (40)	8 (47)	10 (48)	8 (44)
Female	9 (60)	9 (53)	11 (52)	10 (56)
Race (*n*, %)				
White	15 (100)	17 (100)	21 (100)	18 (100)
Duration of treatment (months)				
Mean ± SD	17.5 ± 11.4	18.7 ± 9.2	19.6 ± 8	NA
Range	6–36	7–36	6–36	NA

No statistical difference between each group for age and duration of treatment; Kruskal-Wallis test. Timolol,BAC+, timolol preserved with 0.01% BAC; timolol,BAC−, timolol without preservative; NA, not applicable.

sinki, Scotland amendment, 2000. All patients had primary open-angle glaucoma but no other ocular disease, as assessed by complete ocular examination. A group of 18 normal subjects with no ocular abnormality or topical treatments was also investigated after approval of the Ethics Committee of Dijon University, France. All patients had received specific explanations of impression cytology and had given informed consent for the procedure. The main inclusion criterion was a clinically well-tolerated topical antiglaucoma monotherapy given for at least 1 year with the same drug. None of them had received other antiglaucoma drugs before the ones investigated in this study. Exclusion criteria were a history of chronic ocular surface diseases; signs of ocular surface toxicity, as expressed by moderate to severe hyperemia or superficial punctate keratitis; and the use of topical ocular drugs other than the hypotensive monotherapy. The first group (timolol,BAC+) included 15 eyes of 15 patients treated with 0.5% timolol preserved with 0.01% BAC, (Timoptol; MSD, Rahway, NJ.), the second group (timolol,BAC−) included 17 eyes of 17 patients treated with preservative-free 0.5% timolol (Timabak; Laboratoires Théa, Clermont-Ferrand, France), and the third group included 21 eyes of 21 patients treated with latanoprost preserved with 0.02% BAC (Xalatan; Pharmacia, Kalamazoo, MI). Timolol (BAC+ or BAC−) was applied twice daily, whereas latanoprost was applied once a day. All patients were white. As shown in Table 1, all groups were comparable in age and duration of treatment.

Sample Collection and Handling. After a 1-drop instillation of 0.04% oxybuprocaine (Novartis Ophthalmics, Bulach, Switzerland), two pieces of filter, 13 × 6.5 mm in size (polyethersulfone filter, 0.20-μm pores, 13 mm in diameter; Supor; Gelman Sciences, Ann Arbor, MI), were applied to neighboring areas of the superior and supertemporal bulbar conjunctiva without exerting any pressure, as previously described.[17,18] Specimens were collected at least 15 minutes after clinical examination that required the use of fluorescein eye drops, to avoid interference with immunofluorescence analyses. Membranes were removed immediately after contact and were dipped in tubes containing 1.5 mL of cold phosphate-buffered saline (PBS, pH 7.4) and fixative (0.05% paraformaldehyde) and were kept at 4°C before cell extraction. Conjunctival cells were extracted by gentle agitation for 30 minutes and then centrifuged at 1600 rpm for 5 minutes.

Antibodies and Immunofluorescence Procedures. Three antibodies and an isotypic negative control were used for assaying. Indirect immunofluorescence (IF) was performed with two sets of antibodies. To determine the inflammatory profile of immunocytology (IC) specimens, primary antibodies were mouse IgG1 anti-HLA-DR α chain (clone TAL.1B5, 50 μg/mL; Dako SA, Glostrup, Denmark), and mouse IgG1 anti-ICAM-1 (CD 54, clone 6.5B5, 50 μg/mL; Dako). For mucin detection, the primary antibodies used were mouse IgG anti-M1 antibodies, composed of a mixture of monoclonal antibodies reacting with the peptidic core of gastric mucin and recognizing products of the *MUC5AC* gene.[20,21] Secondary antibodies were FITC-conjugated goat anti-mouse immunoglobulins (Dako) for all the assays. A nonimmune mouse IgG1 (Dako) was used as a negative isotypic control. HLA-DR and ICAM-1 antibodies were diluted in 1% bovine serum albumin (BSA) containing PBS, to a final dilution of 1:50. Anti-M1 antibody dilution was 1:500. After 30 minutes of incubation with the primary antibodies, cell suspensions were washed in PBS and centrifuged for 5 minutes. Cells were then incubated for 30 minutes with the secondary anti-mouse immunoglobulins in a 1:50 dilution, centrifuged in PBS (1600 rpm, 5 minutes), resuspended in 500 μL of PBS, and analyzed on a flow cytometer (Coulter Epics-XL; Beckman-Coulter, Hialeah, FL), according to previously validated methods.[10,17,18]

Flow Cytometry Processing. Specimens containing less than 10,000 cells were discarded. For each antibody investigated, a minimum of 2500 conjunctival cells was acquired on a biparametric histogram showing side scatter (cell size) versus forward scatter (cell granulometry), both on linear modes. Analytic gates were set around this population to exclude cellular debris. A logarithmic fluorescence histogram gated on the main cell population was obtained, giving the number of cells as a function of fluorescence intensities. The highest level of fluorescence intensity obtained for the isotypic control antibody was considered to be the limit of background fluorescence and the threshold of positivity for the tested antibodies.[10,17] For each of the antibodies tested, results were given in percentages of positive cells and in mean fluorescence intensities. Mean fluorescence intensity levels of HLA-DR and ICAM-1 were further quantified by a commercial system (Quantitative Indirect Fluorescence Intensity [QIFI]; Dako). Calibrated beads coated with five different levels of monoclonal antibody (CD5) were included in each technical procedure and were reacted with the secondary FITC-conjugated goat anti-mouse antibody at the same time and in the same manner as were conjunctival cell samples. A calibration curve was then obtained, giving mean fluorescence intensities of each bead versus the number of molecules of antibody bound, thus defining antibody-binding capacity (ABC) units (Dako). This curve provided quantification of the fluorescence expressed by conjunctival cells, after conversion into ABC units of the mean fluorescence intensity observed for each monoclonal antibody. The actual number of ABC units for a specific marker was further obtained by subtraction of the number of ABC units found for the isotypic negative control. This method objectively compared the different samples and improved the reliability and quality of fluorescence measurements.[10] All flow cytometric analyses were performed in a masked manner for treatment groups and patient characteristics. Statistical comparisons were performed using the nonparametric Mann-Whitney test, with $P < 0.05$ considered significant (StatView IV for Windows; Abacus, Berkeley, CA).

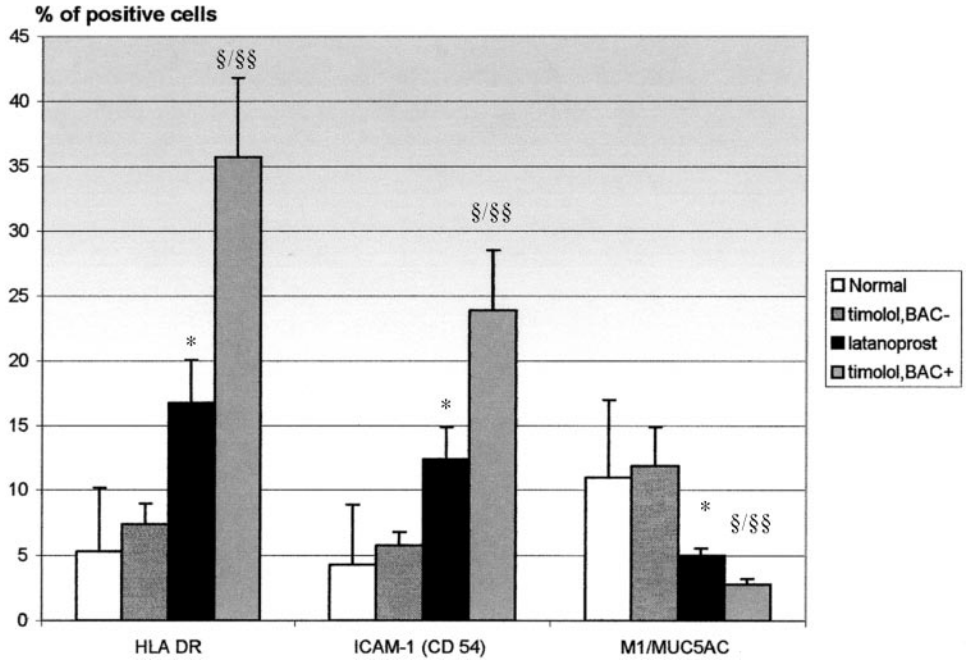

FIGURE 1. Results of flow cytometry of impression cytology specimens. The percentage of positive cells in normal eyes and eyes with glaucoma. §$P \leq 0.01$, compared with the latanoprost group, §§$P \leq 0.001$, compared with the other groups. *$P \leq 0.03$; compared with timolol,BAC− and normal control. Timolol,BAC+, timolol preserved with 0.01% BAC; timolol,BAC−, timolol without preservative.

Conjunctival Cell Line

Wong Kilbourne–derived human conjunctival epithelial cells, an established cell line (Wong Kilbourne derivative of Chang conjunctiva, clone 1-5c-4, CCL-20.2; American Type Culture Collection [ATCC], Manassas, VA), were cultured under standard conditions (humidified atmosphere of 5% CO_2 at 37°C) in Dulbecco minimum essential medium (DMEM; Eurobio, Les Ulis, France) supplemented with 10% fetal bovine serum (Dominique Dutscher, Brumath, France), 1% glutamine (Eurobio), 0.1% ampicillin (Panpharma, Fougères, France), and 2% kanamycin (Bristol-Myers Squibb, Paris, France), as previously described.[16,17] Normal culture development was assessed daily by phase-contrast microscopy. Confluent cultures were removed by gentle trypsin incubation, and cells were counted. They were then seeded into 96-well culture plates (5000 cells per well; Nunc, Roskilde, Denmark). When cells reached confluence (culture surface covering nearly 70%), they were exposed to the different formulations.

Cell Treatment. Cells were treated with 0.5% timolol (Laboratories Théa), either unpreserved or prepared with 0.02% BAC (Sigma-Aldrich, St. Louis, MO), 0.005% latanoprost in its commercial preparation (i.e., associated with 0.02% BAC; Xalatan, Pharmacia), or 0.02% BAC alone. Pure BAC and BAC-containing timolol were tested with a final concentration of 0.02% BAC, so that it could be compared with latanoprost solution preserved with 0.02% BAC. Unpreserved latanoprost was not available, and therefore only the commercial preparation was tested. Untreated cells were used as a negative control. Three protocols of incubation times were applied to treated cells: (1) 15 minutes of treatment; (2) 15 minutes of treatment and 4 hours of cell recovery in normal culture medium; and (3) 15 minutes of treatment and 24 hours of cell recovery in normal culture medium. The 4 and 24 hours of cell recovery in the culture medium without drugs were chosen to allow cells to undergo the apoptotic process, as performed in our previous studies on the same cell line.[15,16]

Microplate Cold Light Fluorometry. For the in vitro study, we used a microplate cold light cytofluorometry technique,[16,19] a procedure that appeared to be a reliable complement for ex vivo analyses in IC, as it makes it possible to work on living cells with labile markers and has been shown to be useful for drug-induced apoptosis assays. Cytotoxicity tests were performed on a microplate cytofluorometer (Fluorolite 1000 Thermobioanalysis; Dynex, Issy-Les-Moulineaux, France). Two cellular markers—cell viability and chromatin condensation—were evaluated in Chang's cell line,[16,19] to establish the Hoechst/neutral red ratio for apoptosis assessment. Cellular viability was evaluated with a neutral red test (Fluka, Ronkonkoma, NY) at a concentration of 50 μg/mL, with fluorometric detection after exposure of cells to the different drugs.[16] Chromatin condensation was evaluated with the intercalating dye Hoechst 33342 (Molecular Probes, Eugene, OR) at a concentration of 10 μg/mL, associated with 0.05% propidium iodide (Roche Diagnostics, Mannheim, Germany) to control necrotic cells. Supravital uptake of Hoechst combined with exclusion of propidium iodide has been proposed as an assay for apoptosis.[22] Apoptotic cells therefore increase the Hoechst/neutral red (Ho/NR) ratio (>1), whereas necrotic cells decrease the Ho/NR ratio (<1).[16] For each experiment, the background fluorescence level was evaluated from wells containing the dye solution alone and was expressed in fluorescence units. The fluorescence level of control or treated cells was determined after deducting this background fluorescence level. Results were then expressed in percentages of the control values calculated from wells seeded with untreated cells. Each concentration of drug was tested in six wells, and each experiment was performed in triplicate. Statistical comparisons were performed using a Student's *t*-test for unpaired data (Statview IV for Windows; Abacus).

Immunocytology. Confocal immunofluorescence was performed to assess morphologic patterns of cells. Cells were cultured on slides (Laboratory-Tek II chambered coverslips; Nalge Nunc International, Naperville, IL) and treated with the same four drugs, according to the contact and recovery times previously described. Cells were washed in PBS and fixed with 95% ethanol in PBS at −20°C for 10 minutes. Alexa 488-conjugated phalloidin (200 U/mL, Molecular Probes) was then added to detect F-actin. After 30 minutes of incubation, cells were washed in PBS. Propidium iodide was added to mark cell nuclei before examination with a confocal epifluorescence microscope (E800, PCM 2000; Nikon, Tokyo, Japan). All examinations were conducted in a masked manner.

RESULTS

Flow Cytometry Analysis from IC Specimens

For each marker, flow cytometry results were expressed as a percentage of positive cells and in ABC units (Figs. 1, 2). The mean percentage of HLA-DR–positive conjunctival cells was higher in both the timolol,BAC+ and latanoprost groups

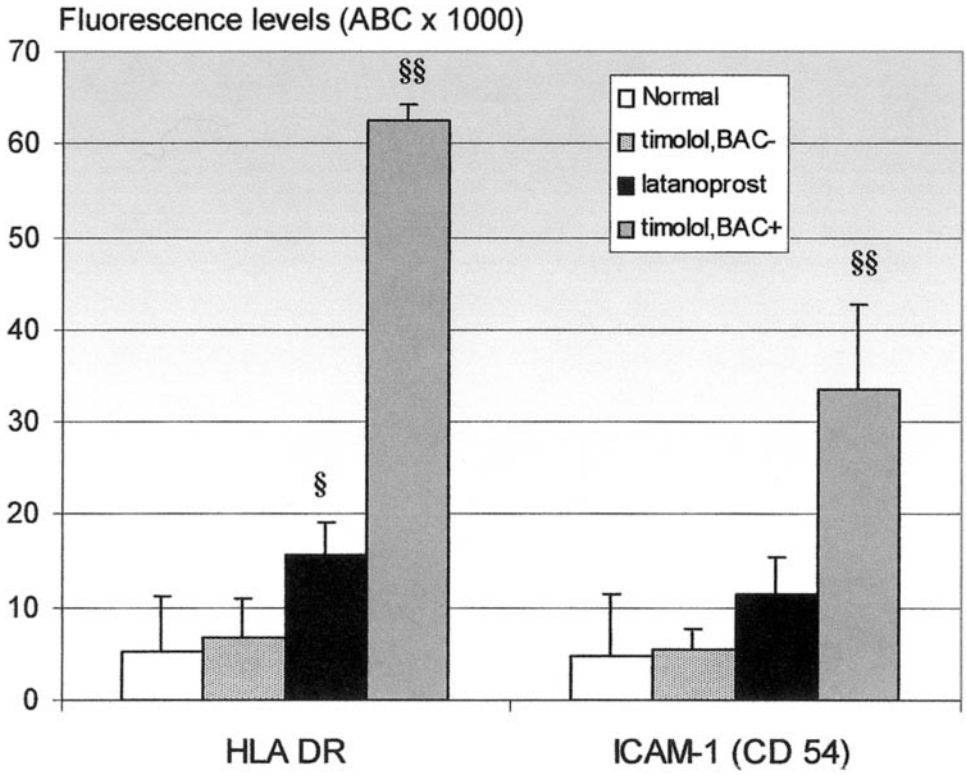

FIGURE 2. Fluorescence results in flow cytometry of impression cytology specimens. Levels of fluorescence in the four groups are expressed in ABC units. §$P = 0.01$, compared with the normal control; §§$P \leq 0.009$ compared with the three other groups. Timolol,BAC+, timolol preserved with 0.01% BAC; timolol,BAC−, timolol without preservative.

(mean ± SD, 35.7% ± 24.6%, and 16.8% ± 15.3%, respectively) than in the timolol,BAC− group (7.4 ± 6.4%; $P < 0.001$ and $P = 0.02$, respectively) and the normal eyes (5.3% ± 4.9%; $P < 0.001$ for both groups). No difference was found between the two latter groups, but the mean percentage of HLA-DR–positive cells was higher in the timolol,BAC+ group than in the latanoprost group ($P = 0.007$). The mean levels of fluorescence expressed in ABC units showed similar results, with significantly higher values in the timolol,BAC+ group (62,541 ± 75,720 ABC units) than in the timolol,BAC− group (6,768 ± 12,542 ABC units; $P = 0.005$) and the normal eye group (5,119 ± 6,079 ABC units; $P < 0.001$). There was also a significant difference between the timolol,BAC+ and latanoprost groups (15,529 ± 17,033 ABC units; $P = 0.009$). The difference between the latanoprost group and normal eyes was significant ($P = 0.01$) but did not reach significance between the latanoprost and timolol,BAC− groups ($P = 0.08$) and between normal eyes and the timolol,BAC− group.

ICAM-1 (CD 54) expression was very similar to that of HLA-DR antigens, with the percentage of ICAM-1–positive cells higher in both the timolol,BAC+ and latanoprost groups (23.9% ± 18.8% and 12.4% ± 11.5%, respectively) than in the timolol,BAC− group (5.8% ± 4.2%; $P = 0.006$ and $P = 0.03$, respectively) and normal eyes (4.3% ± 4.6%; $P = 0.003$ and $P = 0.01$, respectively). The difference was also significant between the timolol,BAC+ and latanoprost groups ($P = 0.01$), but not between the normal eye and the timolol,BAC− groups. The mean number of ABC units in the timolol,BAC+ group (33,524 ± 37,676 ABC units) was also significantly higher than in the normal eye (4,734 ± 6,785 ABC units; $P < 0.001$), the timolol,BAC− (5,559 ± 8,236; $P = 0.005$), or the latanoprost (11,467 ± 18,823; $P = 0.02$) groups. However, the difference in mean ABC units was not significantly different between the latanoprost group and the timolol,BAC− and normal eye groups.

Percentages of cells positive to the mucin marker, related to MUC5AC-expressing goblet cells,[23,24] ranged from 1% to 18%, with significantly lower values in the timolol,BAC+ group than in the latanoprost group (2.8% ± 1.4% and 5% ± 2.7%, respectively; $P = 0.008$), in the timolol,BAC+ and latanoprost groups than in the timolol,BAC− group (11.9 ± 12%; $P = 0.007$ and $P = 0.01$, respectively), and in the normal eyes (11% ± 6%; $P < 0.001$ for both groups), but with no difference between normal eyes and the timolol,BAC− group.

Microplate Cold Light Fluorometry

Cellular Viability. After 15 minutes of cell treatment with the four different drugs, cellular viability remained unchanged with unpreserved timolol (101% of control cells), whereas it significantly decreased after treatment with 0.02% BAC-containing latanoprost, 0.02% BAC-containing timolol, and 0.02% BAC alone, compared with that of the control and unpreserved timolol (mean fluorescence: 30%, 32%, and 21% of control cell level, respectively; $P < 0.001$; Fig. 3). Latanoprost and preserved timolol were similarly and significantly less toxic than BAC alone, despite the same concentration of preservative ($P = 0.01$ compared with BAC for both drugs).

After 15 minutes of cell treatment followed by 4 hours of recovery in normal medium, similar effects on cellular viability were observed, with no significant change after treatment with unpreserved timolol (99% of control cells), but a significant cell loss after treatment with 0.02% BAC-containing latanoprost, 0.02% BAC-containing timolol, and 0.02% BAC, alone compared with the control and unpreserved timolol (mean fluorescence: 40%, 32%, and 32%, respectively; $P < 0.001$). Preserved latanoprost, however, induced significantly less decrease in cells than did 0.02% BAC-containing timolol and BAC alone ($P = 0.02$ for both drugs compared with latanoprost).

After 15 minutes of cell treatment followed by 24 hours of recovery, no significant change in cellular viability was observed with unpreserved timolol (97% of control cells), whereas a significant decrease in cellular viability was reported with 0.02% BAC-containing latanoprost, timolol, and BAC treat-

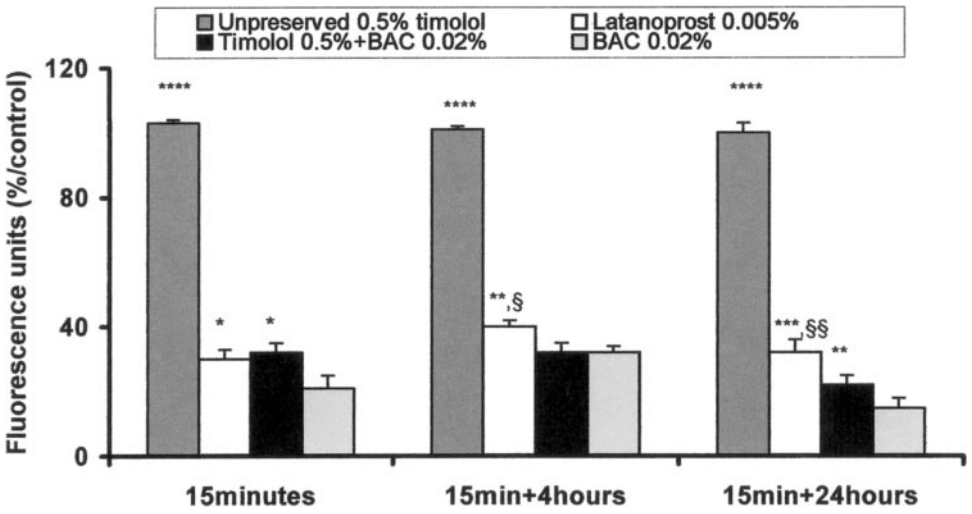

FIGURE 3. Cold light cytofluorometry in Chang's conjunctiva-derived cells. Cell viability evaluations after 15 minutes of treatment and after 15 minutes of treatment followed by 4 and 24 hours of cell recovery. $^{*}P = 0.01$, $^{**}P = 0.02$, $^{***}P = 0.003$, $^{****}P < 0.001$ compared with BAC; $\S P = 0.02$, $\S\S P = 0.009$ compared with preserved timolol. Preserved timolol, latanoprost, and BAC values were always found significant ($P < 0.001$) compared with control. Results are expressed as percentages of control value (100%).

ments compared with the control and unpreserved timolol (mean fluorescence: 32%, 22%, and 15%, respectively; $P < 0.001$). Latanoprost and BAC-containing timolol caused significantly less alteration in cellular viability than did BAC alone ($P = 0.003$ and $P = 0.02$, respectively). Latanoprost also appeared to be significantly less toxic than BAC-containing timolol ($P = 0.009$).

Hoechst 33342/NR Ratio Analysis. After 15 minutes of cell treatment with the different drugs, unpreserved timolol did not significantly modify the Ho/NR ratio (0.85) compared with the control (Fig. 4). Preserved timolol and latanoprost induced a similar and significant increase in the Ho/NR ratio compared with no treatment and unpreserved timolol (11.3 and 11.6, respectively; $P < 0.001$). The Ho/NR ratio was significantly higher after BAC alone (18.1) than after treatment with BAC-containing timolol and latanoprost ($P < 0.01$ for both drugs compared with BAC alone), and with unpreserved timolol and the control ($P < 0.001$). No difference was observed between latanoprost and preserved timolol.

After 15 minutes of cell treatment followed by 4 hours of recovery in normal culture medium, the Ho/NR ratio in cells treated with unpreserved timolol was similar to that of untreated cells (0.88; not significant), whereas it significantly increased with preserved timolol (9.3; $P < 0.001$), latanoprost (7.4; $P < 0.001$), and BAC (10.9; $P < 0.001$). Preserved timolol and BAC alone induced a similar Ho/NR ratio increase (9.3 and 10.9, respectively, not significant) whereas the Ho/NR ratio after latanoprost treatment was significantly lower than after BAC treatment (7.4 and 10.9, respectively; $P < 0.01$), although the difference from the ratio in cells treated with preserved timolol did not reach significance.

After 15 minutes of cell treatment followed by 24 hours of recovery, unpreserved timolol did not modify the Ho/NR ratio compared with untreated cells (0.88; not significant). The Ho/NR ratio was significantly increased after treatment with preserved timolol and latanoprost (8.1 and 4.3, respectively; $P < 0.001$ compared with the control and unpreserved timolol). BAC treatment induced a significantly higher increase in the Ho/NR ratio than did BAC-preserved timolol or latanoprost (12.2, 8.1, and 4.3, respectively; $P < 0.01$ between BAC and the two other drugs). Moreover, the increase in the Ho/NR ratio with latanoprost treatment was significantly lower than with preserved timolol treatment ($P < 0.001$).

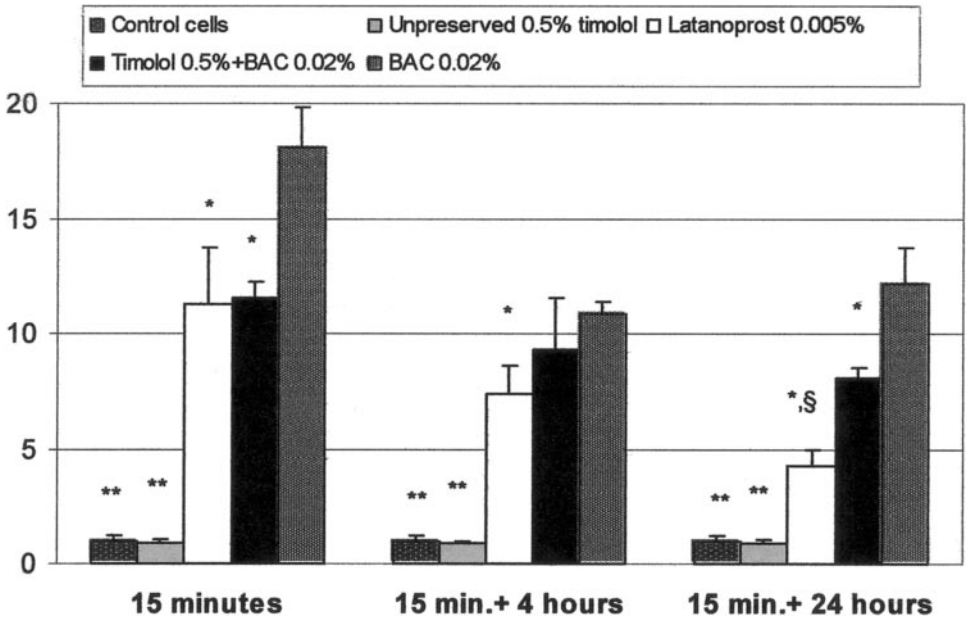

FIGURE 4. Cold light cytofluorometry in Chang's conjunctiva-derived cells: Ho/NR fluorescence ratios in the different treatment groups. Apoptosis was present at a Ho/NR ratio higher than 1. $^{*}P < 0.01$ compared with BAC−; $^{**}P < 0.001$ compared with BAC; $\S P < 0.001$ compared with preserved timolol.

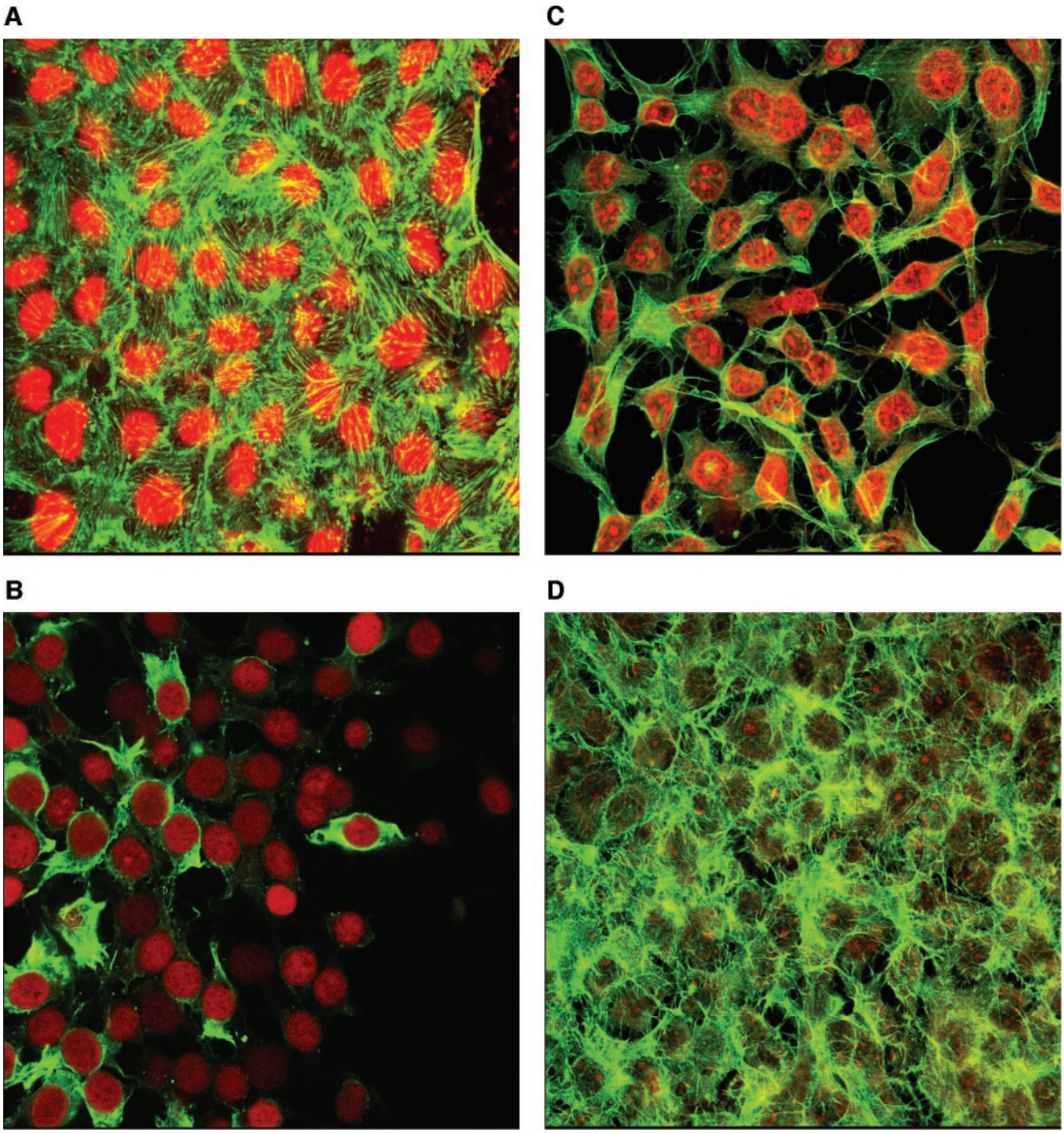

FIGURE 5. Confocal microscopy of conjunctiva-derived cell line, using phalloidin and propidium iodide stains. (**A**) Control cells. Effects of (**B**) BAC 0.02%, (**C**) timolol 0.5% preserved with BAC 0.02%, and (**D**) latanoprost after 15 minutes of treatment and 4 hours of cell recovery in normal culture medium. Note the intense cytotoxicity with shrinkage of cell cytoplasm after BAC treatment (**B**) and the partial cell retraction with timolol compared with control cells (**B**). In contrast cells treated with latanoprost (**D**) could not be distinguished from normal control cells at this time point. Magnification, ×800.

Morphologic Changes. Morphologic analysis by confocal microscopy of treated cells demonstrated cell shrinkage with all tested drugs except with unpreserved timolol (Fig. 5). As shown in Figure 5, cell retraction after latanoprost treatment was less marked than after treatment with preserved timolol or BAC, especially after 4 and 24 hours of cell recovery when latanoprost-treated cells appeared indistinguishable from untreated cells.

DISCUSSION

The long-term use of antiglaucoma drugs has been reported to induce inflammatory ocular surface changes, causing progressive ocular discomfort on instillation, tear film instability, corneal surface impairment, and potential risk for failure of further glaucoma surgery.[7,9,12,13] However, to our knowledge, comparative investigations on the inflammatory status of the ocular

surface after treatments with preserved and preservative-free antiglaucoma drugs have not yet been performed. We used a technique of flow cytometry on impression cytology specimens that we had previously validated in ocular surface disorders such as keratoconjunctivitis sicca[10,17] and ocular rosacea.[18] This technique reliably assesses and quantifies inflammation in the ocular surface by investigating the expression of inflammatory markers by conjunctival epithelial cells. In the present study, we used flow cytometry to evaluate the effects of preserved or preservative-free timolol and the prostaglandin analogue latanoprost on the conjunctival epithelium, by investigating HLA-DR, ICAM-1, and the MUC5AC-related mucin production. We therefore demonstrated that the β-blocker, when preserved with BAC, induced significantly higher inflammatory levels in the conjunctival epithelium than did its preservative-free counterpart, which by itself did not stimulate significant expression of the inflammatory marker compared with normal untreated eyes. This study was not prospective, because of the long duration most likely required for topical treatments to induce inflammatory reactions.[9,12,13] However, we conducted a case–control study, using an objective, highly reliable tool to analyze in a masked manner conjunctival inflammatory reactions, and it is noteworthy that all patient groups were fully comparable in age and duration of treatment, with no clinically relevant inflammation in any treated eye.

Moreover, our results showed that in patients treated with preserved antiglaucoma drugs, the subclinical inflammation was associated with a highly significant decrease in mucin-expressing cells compared with normal eyes and patients treated with preservative-free timolol. The alteration of this major mucin system, strongly associated with goblet cells,[23,24] has already been described in inflammatory ocular surface diseases, such as ocular rosacea[18] and keratoconjunctivitis sicca.[25] In the present study, the lowest levels of MUC5AC-positive cells were found in the group with the highest HLA-DR and ICAM-1 levels and were therefore associated with conjunctival inflammation, thus emphasizing the negative correlation between inflammation and mucin production by the conjunctival epithelium. This decrease in goblet cell density and/or mucin production by the conjunctival epithelium could be explained at least partly by the chronic inflammatory reaction of the ocular surface induced by the preservative. However, it can also be hypothesized that BAC itself may trigger a direct toxicity on conjunctival goblet cells, as previously demonstrated in the conjunctival epithelium. [4]

It has thus been reported that chronic instillation of preservative-containing antiglaucoma drugs induces goblet cell loss,[26] increases subepithelial collagen deposition,[27] and causes infiltration of the substantia propria by inflammatory cells.[9,12,13] These modifications involving the ocular surface could be responsible in part for the high frequency of adverse functional and clinical signs previously described in patients treated with preserved antiglaucoma drugs compared with those receiving preservative-free β-blockers.[28,29] BAC has therefore been considered to be responsible for major impairment of ocular structures, with significant squamous metaplasia in impression cytology after 3 months of treatment, whatever the active compound associated,[30] loss of corneal epithelium barrier function,[31] severe loss of endothelial cells if accidentally introduced into the anterior chamber,[32] and blood–aqueous barrier disruption, resulting in subsequent angiographic cystoid macular edema in the early phase of pseudophakia.[33]

Our results from impression cytology specimens also demonstrate that latanoprost induced a higher conjunctival inflammation than did unpreserved β-blocker, but a lower one than that induced by preserved timolol. Moreover, even though the number of MUC5AC-positive cells was lower after treatment with latanoprost than with unpreserved timolol, it remained significantly higher with latanoprost than with preserved timolol treatment. These results demonstrate that the effects of latanoprost on conjunctival cells, as evaluated by an increase in inflammatory marker expression and a decrease in mucin production, were less substantial than expected for a prostaglandin analogue, and lower than the toxic and/or inflammatory levels caused by prolonged treatments with preserved timolol.

A previous study,[34] using flow cytometry in impression cytology, was conducted in patients receiving monotherapy with latanoprost or two preserved β-blockers for at least 3 months and showed that conjunctival epithelium overexpresses HLA-DR antigens. There was no difference in the intensity of HLA-DR expression between the three treatments investigated, which was hypothesized to be the result of a subclinical inflammation caused by the preservative. However, the latanoprost group seemed to display HLA-DR expression after a shorter duration of treatment than did the two β-blockers, irrespective of HLA-DR levels. Another study,[35] using standard IC, a method less sensitive than flow cytometry, found a significantly higher level, although it remained low, of HLA-DR expression in patients treated with latanoprost compared with a control group treated with 0.02% BAC-containing substitute eye drops. Analyses were performed in this study 4 months after the beginning of treatment and should not be directly compared with our results recorded after at least 1 year of treatment. These two studies, however, could indicate that inflammation may be an early phenomenon, especially after treatment with a prostaglandin analogue, but its intensity would not considerably increase over time and could therefore stabilize, while longer durations of treatments with preserved β-blockers would finally reach higher inflammatory levels.

Our results are therefore in accordance with a study reporting that latanoprost induced fewer morphologic changes in the substantia propria of the conjunctiva than did preserved timolol[36]—namely, lower collagen density, showing that latanoprost might prevent excessive fibrous tissue formation, potentially through upregulation of MMP-3 and TIMPs in the conjunctival epithelium. Other studies conducted to evaluate latanoprost in the ocular surface concluded that there were only minor effects due to the prostaglandin analogue on the corneal epithelial function[37] or the corneoconjunctival tissues[38] unless also impaired by allergic disorders. A prospective comparative study conducted by Thygesen et al.[39] found a better preservation of tear break-up time and tear secretion in the latanoprost group than in the timolol group after a short-duration treatment.

In addition to our results obtained in impression cytology, we undertook an in vitro cytotoxicity assay based on cellular viability and chromatin condensation with the same drugs as used in patients with glaucoma. The potent proapoptotic effects of 0.01% BAC and timolol when preserved with 0.01% BAC have been reported in vitro in a human conjunctiva-derived cell line, whereas unpreserved timolol displayed no toxicity.[15,19] As latanoprost in its commercial preparation is preserved with BAC in a 0.02% concentration, we tested the hypothesis, based on the ex vivo study, of a possible protective effect of latanoprost on ocular surface cells by comparing in the same conjunctival cell line the proapoptotic effects of unpreserved timolol, BAC, and the two active compounds timolol and latanoprost when associated with the same 0.02% BAC concentration. Unfortunately, preservative-free latanoprost, to our knowledge, is still not available, possibly because of chemical formulation concerns, and could not directly be tested. Our results showed that timolol and latanoprost associated with BAC in a 0.02% concentration, displayed a similar toxic effect on conjunctival cells after 15 minutes of treatment,

with a dramatic cell retraction, a decrease in cell viability and an increase in chromatin condensation and the Hoechst/NR ratio, related to an apoptotic mechanism. At each time point of the experiments, this toxic effect remained; however, it was significantly lower than that induced by 0.02% BAC alone. Moreover, after 15 minutes of treatment followed by 4 or 24 hours of cell recovery, the apoptosis and cell viability decreases observed with latanoprost were significantly lower than those induced by preserved timolol. As expected, unpreserved timolol did not show cell toxicity at any time point, consistent with all previous in vitro[15,19] and in vivo[8,11] studies. Our results in a conjunctiva-derived cell line cannot be fully extrapolated to the in vivo situation in which drug concentrations may be different, and it cannot be excluded that the absence in a cell line of a preocular mucin layer, normally present at the apex of conjunctival cells, may induce a higher susceptibility of cells to undergo apoptosis when exposed to xenobiotics.

Our ex vivo and in vitro studies suggest that the toxicity of preserved drugs is mainly related to BAC used as preservative and that latanoprost, and to a lesser extent timolol, may exhibit a relative protective effect toward the toxicity of this preservative on conjunctival cells. As latanoprost exhibited less toxic and/or inflammatory effects on conjunctival epithelial cells than did the preserved β-blocker, the hypothesis of a specific protective effect of the prostaglandin analogue, through a mechanism that remains to be determined, cannot be ruled out. Further investigations are needed to offer fully safe drugs in the future for the ocular surface in patients with glaucoma who must undergo chronic treatment.

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