Evidence has been found suggesting that long-term therapy with topical antiglaucoma medications may decrease the success of glaucoma filtering surgery. To investigate this question further, the antiproliferative effects of the preservative benzalkonium chloride and three pure and commercially available β-adrenergic antagonist preparations (timolol, betaxolol, and levobunolol) were studied on tissue cultures of human Tenon's capsule fibroblasts. Each drug preparation was tested on three different cell lines. Fibroblast growth was measured with tritiated thymidine uptake and hexosaminidase assays. Trypan blue uptake was used to assess cell viability microscopically. The commercially available preparations containing benzalkonium chloride and those of betaxolol and levobunolol without the preservative had similar inhibitory doses for 50% of cells. The timolol preparation without preservative was significantly less toxic than its commercially available one. The three tested β-adrenergic blockers did not stimulate fibroblast proliferation directly in this in vitro model. Even when the cultures were washed free of the drugs, growth continued to be suppressed, suggesting that the inhibition was not reversible. An increase in fibroblasts and inflammatory cells after long-term antiglaucoma medical therapy thus may be caused not by a direct stimulation of cell proliferation but by chronic inflammation from the irritating effects of antiglaucoma medications and/or their preservatives. Invest Ophthalmol Vis Sci 33:2233–2241, 1992

Glauc going is a group of ocular diseases characterized by increased intraocular pressure (IOP), optic nerve atrophy, and visual field loss. One category of medications used to lower IOP in primary open-angle glaucoma and ocular hypertension is the topical β-adrenergic blockers—nonselective timolol and levobunolol and β1-selective betaxolol. These medications lower IOP by decreasing aqueous humor production by the ciliary body epithelium (possibly by blocking β-receptor mediated isoproterenol-stimulated cyclic adenosine monophosphate synthesis).

If IOP lowering sufficient to prevent further optic nerve damage is not achieved medically, laser therapy or filtering surgery is indicated. The most common cause of filtering surgery failure is external scarring of the bleb from wound healing. Fibroblast attachment, proliferation, and migration are essential to this process.

Recently, evidence was found indicating that long-term therapy with topical antiglaucoma medications may increase filtering surgery failure by increasing inflammation and proliferation of fibroblasts. A comparison of the effects of pure and commercially available topical β-adrenergic blockers and the preservative benzalkonium chloride on human ocular fibroblasts could elucidate the mechanism of how these medications affect fibroblast proliferation.

Materials and Methods

Drug Preparation

Each commercially available preparation of β-adrenergic blockers—Timoptic (timolol maleate 0.5%, benzalkonium chloride 0.01%; Merck, Sharp and Dohme, West Point, PA), Betoptic (betaxolol HCl 0.5%, benzalkonium chloride 0.01%; Alcon, Fort Worth, TX), and Betagan (levobunolol HCl 0.5%, benzalkonium chloride 0.004%; Allergan, Horninguero, Puerto Rico)—was diluted in Eagle's modified minimal essential medium with Earle salts and glutamine (MEM; Flow, McLean, VA) to make eight serial dilutions of these drugs in MEM at concentrations ranging from 4.3 × 10^{-3} mol/l to 5.6 × 10^{-8} mol/l.

Solid forms of each drug were reported to be greater than 99% pure by certificates of analysis from the manufacturers. Each of these pure preparations was di-
aliquoted into each well of the 96-well plates.

A 17% concentration of benzalkonium chloride (Zephran chloride, also containing ethyl alcohol 2% and water 81%; Winthrop-Breon, New York, NY) was diluted in MEM to make eight concentrations ranging from $2.7 \times 10^{-4}$ mol/l to $4.0 \times 10^{-10}$ mol/l.

All drugs were buffered between pH 7.0 and 7.4 and were filtered before they were added to the culture plates.

### Tissue Culture

Human Tenon’s capsule fibroblast tissue cultures were established using specimens obtained at the time of strabismus surgery with the approval of the UCLA Human Subject Protection Committee. The cell lines originated in patients who had not been treated previously with any antiglaucoma medications. Before each experiment, the medium was discarded from the cultures, and the cells were washed with 25 ml of Dulbecco’s calcium- and magnesium-free phosphate-buffered saline (PBS), incubated in trypsin 0.25% for 10 min at 37°C, centrifuged at 1000 rpm for 10 min after the addition of MEM and fetal bovine serum 10% (FBS), resuspended in PBS, and recentrifuged. The supernatant was discarded, and the cells were resuspended in 2 ml of PBS. The live cell number per milliliter was counted on a hemacytometer using the trypan blue exclusion method. One thousand viable fibroblasts in 100 μl of MEM with FBS 10% were aliquoted into each well of the 96-well plates.

### Acute Effects on Fibroblast Attachment and Proliferation

The drugs were added immediately after plating the cells to assess their effect on cell attachment. We added 100 μl of each drug per MEM concentration to each well, resulting in a final volume of 200 μl of MEM with FBS 5% per well. Twenty-four hours after cell plating and drug addition, the plates were washed with PBS to remove unattached cells. The number of remaining cells was determined by hexosaminidase assay. For proliferation studies, the cells were allowed to attach to the substratum for 24 hr at 37°C in CO2 5% before drug addition. Cell proliferation was quantified by ³H-thymidine uptake into the DNA and hexosaminidase assay after 24 and 48 hr of incubation with the drugs, respectively.

### Delayed Effects on Fibroblast Proliferation

Similarly, drugs were added after fibroblast attachment was established. After 24 hr of incubation, the culture medium was discarded, the cells were washed twice with 200 μl of PBS to remove the drug, resuspended with 200 μl of fresh MEM supplemented with FBS 2%, and reincubated at 37°C in CO2 5%. Before determining the cell number, the fibroblasts were allowed to grow for at least two complete cell cycles (approximately 24 hr/cycle) to eliminate any effects that might have persisted from any remaining drug. In addition, to eliminate possible cell growth stimulation by growth factors in the serum that might mask those possibly caused by drugs, the fibroblasts were maintained in a state of low proliferation using FBS 2%. Delayed responses on cell proliferation then were determined by ³H-thymidine uptake and hexosaminidase assays on days 7, 8, 9, and 14.

To differentiate the acute effects that various components of the β-adrenergic blockers might have on fibroblast attachment and proliferation, benzalkonium chloride and commercially available and pure preparations of these drugs were tested. To test for possible delayed stimulation of cell growth, commercially available forms of β-adrenergic blockers were assayed. Control specimens were run under identical conditions except that the drugs were not added. To prevent evaporation losses, the wells located on the periphery of each plate received 200 μl of MEM. All serial dilutions of each drug were done in quadruplicate during each experiment using the same cell line with the same number of passages under identical experimental conditions. These experiments then were repeated twice using different patient cell lines.

Cell viability was assessed microscopically at both 24 and 48 hr by adding trypan blue. Cell death was confirmed by trypan blue uptake into the cell. All photographs were taken using a Zeiss (Oberkochen, Germany) microscope with a 32X objective lens.

All figures in this article that show the acute and delayed effects of these drugs on Tenon’s fibroblasts are representative of the three experiments.

### Statistical Analysis

All values obtained by the two assays were normalized by dividing the experimental mean of each drug concentration by the control mean to yield percent control values. The inhibitory dose in 50% of cells (ID50) for each drug in each experiment was calculated using an empiric method. Paired student t-tests were used to ascertain the statistical significance of differences in ID50 values. A level of $P < 0.05$ was accepted as statistically significant. Two-factor analy-
sis of variance was done to compare changes in the drug response (indicated by curve shifts or changes in the dose effects and average ID$_{50}$).

Results

Acute Effects on Fibroblast Attachment

The effects of pure and commercially available β-adrenergic blockers on fibroblast attachment are shown in Figure 1. All three pure preparations prevented attachment above $4.0 \times 10^{-3} \text{ mol/l}$. There was little difference among the pure preparations of timolol, betaxolol, and levobunolol. All three commercially available preparations prevented human ocular fibroblast attachment above $1.0 \times 10^{-3} \text{ mol/l}$. Benzenonium chloride prevented attachment above $1.0 \times 10^{-6} \text{ mol/l}$. There was also little difference among the commercially available preparations in their effects on fibroblast attachment.

Acute Effects of Pure β-Blockers on Fibroblast Attachment (Hexosaminidase Assay)

Fig. 1. Acute effects of β-blockers on cell attachment, based on hexosaminidase assay. Commercial drugs prevent attachment above $1.0 \times 10^{-3} \text{ M}$. Pure drugs prevent attachment above $4.0 \times 10^{-3} \text{ M}$. BAK prevents attachment above $1.0 \times 10^{-6} \text{ M}$.

Acute Effects of Pure and Commercial Timolol on Fibroblast Proliferation (Thymidine Assay)

The dose–response curve for pure timolol was shifted to the right of the curve for the commercially available preparation in both the thymidine and hexosaminidase assays, indicating pure timolol was less toxic (Fig. 2). Two-factor analysis of variance showed this difference to be statistically significant (F ratio, 23.5 in the hexosaminidase assay; \(P < 0.0001\); F ratio, 41.3 in the thymidine assay; \(P < 0.0001\)).

The dose–response curves of pure and commercially available betaxolol and pure and commercially available levobunolol were nearly identical in both assays (Figs. 3, 4). This result indicated that both preparations of each drug had similar toxicities.

The effects of the three pure preparations on fibroblast proliferation, based on thymidine and hexosaminidase assays, are compared in Figure 5. The finding that timolol's dose–response curve was the most...
Acute Effects of Pure and Commercial Betaxolol on Fibroblast Proliferation
(Thymidine Assay)

Molar Concentration

% Control

120-
100-
80-
60-
40-
20-
0

Fig. 3. Acute effects of pure and commercial betaxolol on fibroblast proliferation, based on thymidine uptake and hexosaminidase assays. Similarities in dose-response curves indicate similar toxicities in both assays.

right shifted in both assays seemed to indicate it was less toxic than betaxolol and levobunolol, but paired Student’s t-tests on timolol, betaxolol, and levobunolol showed this difference was not statistically significant. The P values for both assays were greater than 0.15, with a 0.3 power of comparison.

The dose–response curves of commercially available preparations of these drugs, based on thymidine and hexosaminidase assays, are compared in Figure 6. Their similarity indicated little difference in toxicity.

The dose–response curve of benzalkonium chloride (Fig. 6) was the most left shifted of all, indicating it was the most toxic agent tested. The concentrations at which the preservative caused cell growth inhibition were the same concentrations at which it was present in commercial preparations of timolol and betaxolol.

Delayed Effects on Fibroblast Proliferation

Figures 7–9 show that when cultures were grown in the presence of commercially available timolol, betaxolol, and levobunolol, respectively, and then washed free of these drugs and recultured, normal growth did not resume. For these commercially available preparations, except levobunolol, toxic effects were found during subsequent cultures (delayed phase) at levels similar to those found during the acute phase. The dose–response curve of levobunolol in the delayed phase (Fig. 9) was shifted to the right of the curve during the acute phase, indicating that it became less toxic. The change in potency was statistically significant (F ratio, > 15; P < 0.001). We did not detect any increase in toxicity or fibroblast growth stimulation during the delayed phase over 2 weeks of incubation.

Microscopic Observations

Because observations at 48 hr were identical to those at 24 hr, only 24-hr observations are reported. At the highest commercially available preparation
concentration (1.6 × 10⁻² mol/l), all three drugs caused trypan blue uptake into the cell with preservation of cell size and morphology. At 4.0 × 10⁻³ mol/l, cell lysis, fragmentation, and a hazy grainy background were observed. At 1.0 × 10⁻³ mol/l, the cells were rounded and dead, as shown by trypan blue uptake into the cell. At 6.25 × 10⁻³ mol/l, the cells were shrunken with some loss of spindle structure, but without trypan blue uptake. The same cell destruction sequence at the same concentrations was seen with pure preparations of betaxolol and levobunolol.

By microscopic assessment, at any given concentration, pure timolol during the acute phase was less toxic than the other two pure and commercially available drugs and commercially available timolol. At a 5.0 × 10⁻⁴ mol/l concentration of pure timolol, there was total cell viability with normal size and morphology. At this concentration, the other drugs caused the fibroblasts to shrink and lose some of their spindle shape.

Benzalkonium chloride caused a similar cell destruction sequence in the following concentrations: (1) 2.7 × 10⁻⁴ mol/l—death with preservation of cell morphology, (2) 5.4 × 10⁻⁵ mol/l—death with lysis and fragmentation, (3) 1.1 × 10⁻⁵ mol/l—death with loss of cell size and spindle shape, and (4) 4.3 × 10⁻⁷ mol/l—viable with normal morphology.

Data from acute effects on fibroblast proliferation are summarized in Table 1, which compares the ID₅₀ values expressed as a mean of the three experiments. With the exception of timolol, all β-adrenergic blockers tested had similar toxicities. The graphic data showed pure timolol was less toxic than commercially available timolol, all the other tested drugs, and benzalkonium chloride. These data were consistent with the results of microscopic assessment. Statistical analysis showed a significant difference only between pure and commercially available timolol. Paired Stu-
Delayed Effects of Commercial Timolol on Fibroblast Proliferation (Thymidine Assay)

![](delayedEffectsTimololThymidine.png)

Fig. 7. Delayed effects of commercial timolol on fibroblast proliferation, based on thymidine uptake and hexosaminidase assays. Similarities in dose-response curves indicate similar toxicities in both assays.

Student's t-tests of the ID$_{50}$ values showed pure timolol to be significantly different from commercially available timolol ($P = 0.04$ in both proliferation assays). There were no significant differences between pure and commercially available preparations of betaxolol or levobunolol.

Discussion

We found both pure and commercially available preparations of three ophthalmic β-adrenergic blockers did not stimulate fibroblast growth in vitro. These drugs were toxic to fibroblasts at concentrations below those in clinically used preparations. These results do not support the hypothesis that antiglaucoma medications stimulate fibroblast growth, thus enhancing glaucoma filtering surgery failure. This question had been raised after increased numbers of inflammatory cells and fibroblasts were found on histologic specimens from eyes of patients at the time of glaucoma filtering surgery. These patients had all received antiglaucoma medical treatment for more than 2 yr.²

It is possible, however, that long-term therapy with these medications irritated the cornea and surrounding tissues. Such irritation might cause a chronic low-grade inflammatory response that could account for these findings. The combinations of antiglaucoma agents received by the patients before surgery were not specified.² It also is possible that combination therapy effects on fibroblast viability and proliferation may differ from those of the individual agents alone. These two hypotheses are supported by a study showing that in some instances long-term topical antiglaucoma medication use can affect the results of fistulizing surgery adversely.⁸

During their administration, antiglaucoma medications may block fibroblast growth by interacting with growth receptors. After cessation of medical therapy,
Delayed Effects of Commercial Levobunolol on Fibroblast Proliferation (Thymidine Assay)

Delayed Effects of Commercial Levobunolol on Fibroblast Proliferation (Hexosaminidase Assay)

Fig. 9. Delayed effects of commercial levobunolol on fibroblast proliferation, based on thymidine uptake and hexosaminidase assays. Similarities in dose-response curves indicate similar toxicities in both assays.

A "rebound" phenomena may cause fibroblast growth when stimulators interact with previously blocked receptors.

We considered whether the mechanism of β-adrenergic blocker toxicity may be related to the interaction of these drugs with β-adrenoceptors. However, the lowest concentrations of the drugs we used were much higher than physiologic concentrations of timolol (5-10 ng/ml), β levobunolol (1.2 ng/ml), and betaxolol necessary to saturate the receptors. Therefore, a receptor-mediated physiologic mechanism of drug toxicity is unlikely.

Caution must be exercised in comparing in vitro studies with in vivo states. Others elucidated the problem of comparing in vitro corneal or Tenon's fibroblasts toxicity studies with in vivo toxicity of topical preparations. The corneal contact time of a solution of drug instilled into the eye is usually 8-11 min, depending (to some extent) on the viscosity of the vehicle. Thus, instillation of eye drops twice daily exposes the cornea to the drug for a short time; in tissue culture, there is continuous exposure. Also, a time comparison cannot consider the recuperative properties and natural replacement of damaged tissue during intervals between instillations of the drug. Nevertheless, drug-induced pathologic responses can be studied conveniently by tissue culture methods, and some comparisons can be drawn with respect to their toxicities on tissues.

We found pure preparations of β-adrenergic blockers prevented attachment at a higher concentration than did commercially available ones of these drugs. Benzalkonium chloride may be related to this difference. This preservative is toxic to many tissues in vivo. Such toxicity may have occurred when the fibroblasts were attaching to the plates.

In regard to acute effects on cell proliferation, we found a significant difference in toxicity between pure and commercially available timolol. We did not observe a significant difference between the commercially available and pure preparations of betaxolol and levobunolol. Although some of the experimental graphs and microscopic observations indicated a possible difference in toxicity between pure timolol and pure and commercially available betaxolol and levo-

Table 1. Mean ID_{50} determinations (with 95% confidence interval) of three β-blockers on fibroblast proliferation as determined by hexosaminidase and 3H-thymidine uptake assays

<table>
<thead>
<tr>
<th>Drug</th>
<th>Hexosaminidase ID_{50}(×10^{-4} M) (95% C.I.)*</th>
<th>3H-thymidine uptake ID_{50}(×10^{-4} M) (95% C.I.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betaxolol, commercial</td>
<td>1.5 (0.4-5.6)</td>
<td>2.0 (0.4-10.0)</td>
</tr>
<tr>
<td>Betaxolol, pure</td>
<td>2.0 (0.9-5.0)</td>
<td>1.7 (0.16-20.0)</td>
</tr>
<tr>
<td>Levobunolol, commercial</td>
<td>2.0 (0.7-4.8)</td>
<td>1.6 (0.1-25.0)</td>
</tr>
<tr>
<td>Levobunolol, pure</td>
<td>2.0 (0.8-5.7)</td>
<td>0.8 (0.5-1.0)</td>
</tr>
<tr>
<td>Timolol, commercial†</td>
<td>1.5 (1.2-1.8)</td>
<td>0.2 (0.05-8.0)</td>
</tr>
<tr>
<td>Timolol, pure†</td>
<td>6.0 (1.7-18.0)</td>
<td>2.7 (2.0-3.0)</td>
</tr>
<tr>
<td>Benzalkonium chloride</td>
<td>0.007 (0.004-0.009)</td>
<td>0.0006 (0.0004-2.0)</td>
</tr>
</tbody>
</table>

* C.I.: Confidence interval.
† Note: Paired t-tests showed the only significant difference in ID_{50} was that between pure and commercial timolol, with P = 0.04 for both assays. All other differences between the ID_{50} values were not statistically significant, with P > 0.05.
bunol, these observations were not confirmed by Student's t-tests between their ID\textsubscript{50} values.

From the study of delayed effects on cell proliferation, we found levobunolol to be the only drug that became less toxic after the cells were washed free of drug and recultured. Because benzalkonium chloride might contribute to the toxicity and the commercially available preparation of levobunolol contained 2.5-fold less preservative than did those of betaxolol or timoptic, the cells that were exposed to levobunolol might have been more liable to recuperate from the initial injury than those exposed to the other drugs. Our study did not show any stimulation of fibroblast growth in subsequent cultures after they were exposed to these drugs.

Our results do not agree with those of an earlier study\textsuperscript{16} that investigated the toxic effects of Betagan, Betoptic, and Timoptic on regenerating rabbit corneal epithelium. This study found that Timoptic was more toxic than either Betagan or Betoptic, despite the fact that Timoptic had the same benzalkonium chloride concentration as Betoptic (0.01%) and a greater one than Betagan (0.04%). These discrepancies with our study may be caused by differences between an in vivo rabbit model and our in vitro tissue culture system, species differences between human and rabbit tissues, the fact that the other authors were studying regenerating corneal epithelium after it was damaged and we were studying healthy fibroblasts grown from human Tenon's capsule, or the possibility that an in vitro system studying sustained effects of drugs on tissue over many hours may not be sensitive enough to pick up differences manifested in vivo.

The thymidine assay showed a greater range of ID\textsubscript{50} determinations for the various \(\beta\)-adrenergic blockers than did the hexosaminidase assay. A more direct and sensitive assay, it may have been influenced more by differences in cell lines, number of passages, and experimental variability.

At the highest concentrations of commercially available preparations, we found cell death with preservation of cell morphology. At lower concentrations, we observed cell fragmentation and lysis. This was an unusual and unexpected finding. We postulate that cell death may have occurred so rapidly at these higher drug concentrations that cell structure was preserved and fixed to the bottom of the wells without the shrinkage and shape deformation expected to occur during a slower cell death process at lower drug concentrations.

In summary, there was a statistically significant difference in toxicity between a pure and commercially available preparation of timolol. The other pure preparations of \(\beta\)-adrenergic blockers had similar toxicities. Except for levobunolol, which showed decreased potency after washing, the commercially available preparations also had similar toxicities. Pure preparations affected attachment of fibroblasts at a slightly higher concentration than did commercially available ones. These effects may have been caused by the presence of benzalkonium chloride in commercially available preparations.

None of the tested drugs stimulated fibroblast growth. They were toxic to fibroblasts at clinically used concentrations. By creating a tissue inflammatory response from their irritating effects, topical antiglaucoma medications may still contribute to an increase in fibroblasts and tissue inflammatory cells found at the time of glaucoma surgery. Additional studies that examine the effects of other antiglaucoma medications (eg, pilocarpine or dipivefrin) on fibroblast growth both during and after administration of these drugs are needed.

**Key words:** \(\beta\)-adrenergic blockers, benzalkonium chloride, betaxolol, levobunolol, timolol, tissue culture

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