EFFECTS OF ANTIGLAUCOMA DRUGS ON CELLULAR PROLIFERATION IN CULTURED HUMAN CORNEAL KERATOCYTES

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The purpose of this study was to investigate the effects of various antiglaucoma drugs, including timolol, betaxolol, carteolol, levobunolol, dipivefrin, and pilocarpine, on cellular proliferation in cultured human corneal keratocytes. Human corneal keratocytes were cultured with RPMI-1640 medium containing 10% fetal bovine serum. Antiglaucoma drugs were prepared from original concentrations to dilutions of 1/10, 1/100, and 1/1,000. After exposure to drugs for 100 minutes, cellular proliferation was estimated by [3H]thymidine uptake methodology. It was found that cellular proliferation in corneal keratocytes was inhibited by only a 1/10 dilution of various drugs including timolol, betaxolol, carteolol, levobunolol, dipivefrin, and pilocarpine. The [3H]thymidine uptake values were significantly inhibited to 63%, 18%, 87%, 68%, 55%, and 67% by a 1/10 dilution of the above drugs. However, the cellular proliferation was also significantly suppressed by 0.01 mg/mL of benzalkonium chloride preservative. It is shown that the inhibition of cellular proliferation by high concentrations of antiglaucoma drugs may result from the benzalkonium chloride preservative contained in these drugs.

Key Words: antiglaucoma drugs, cellular proliferation, corneal keratocytes (Kaohsiung J Med Sci 2006;22:120–5)

It is now recognized that glaucoma is a chronic disease caused by the death and subsequent loss of ganglion cells and their axons in the optic nerve [1]. Patients with glaucoma must administer antiglaucoma drugs for a long period of time before their elevated intraocular pressure is well controlled by alternatives such as laser therapy or surgery. Under these circumstances, the cornea is continuously soaked in antiglaucoma drugs over a long period of time; thus, the physiologic function of the cornea may be changed gradually in the process of drug administration. Some evidence indicates that timolol suppresses cellular proliferation of corneal epithelial cells [2,3]. Other reports also show that loss of corneal endothelial cells occurs with long-term topical epinephrine therapy in glaucoma patients [4]. After 1 year of treatment, the mean age percent loss in endothelial cell density of the human cornea from baseline was 4.5% and 4.2% for timolol- and betaxolol-treated groups, respectively [5]. Thus, antiglaucoma drugs may influence corneal cells. However, the effects of antiglaucoma drugs

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on corneal keratocytes have been poorly investigated, even though ever more antiglaucoma drugs are becoming available on the market. In the present study, we investigated the effects of various commercially available antiglaucoma drugs, including timolol, betaxolol, carteolol, levobunolol, dipivefrin, and pilocarpine, on cellular proliferation in cultured human corneal keratocytes.

**Materials and Methods**

**Materials**

Culture materials, including trypsin, minimal essential medium, glutamine, gentamicin, and fetal bovine serum, were obtained from GIBCO (Grand Island, NY). [3H]thymidine (specific activity 15.0 Ci/mmol) was purchased from New England Nuclear (Du Pont, Boston, MA). Commerically available preparations of antiglaucoma drugs were prepared with serum-free medium to make three dilutions of these drugs of 1/10, 1/100, and 1/1,000 for all experiments. The drugs obtained were: 0.25% (5.8 mM) timolol (Timoptol; Merck, Sharp & Dohme-Chibret, containing 0.048 mg/mL benzalkonium chloride), 0.5% (16.2 mM) betaxolol (Betoptic; Alcon, containing 0.1 mg/mL benzalkonium chloride), 1% (68 mM) carteolol (Arteoptic; Otsuka, containing 0.005 mg/mL benzalkonium chloride), 0.5% (17.1 mM) levobunolol (Bunolgan; Allergan, containing 0.04 mg/mL benzalkonium chloride), 0.1% (2.8 mM) dipivefrin (Propine; Allergan, containing 0.04 mg/mL benzalkonium chloride), and 1% (40.8 mM) pilocarpine (Spersacarpine; Dispersa, containing 0.1 mg/mL benzalkonium chloride). All other chemicals were obtained from Merck (Darmstadt, Germany).

**Culture of human corneal keratocytes**

Human corneal keratocyte primary cultures were obtained and cultured using human donor corneas that had been discarded after transplantation, as described in an earlier publication [6,7]. The endothelial and epithelial layers were removed, then the corneal stroma was minced into cubes of about 1 mm and plated in a culture flask for culture of keratocytes. The culture medium for cells consisted of RPMI-1640 medium containing 10% fetal bovine serum, 3.8 mM L-glutamine, and 50 μg/mL gentamicin. Culture conditions were maintained in a humidified chamber containing 5% CO2 at 37°C, and the medium was changed every 2 or 3 days. The cells usually appeared within 3–7 days. The cells used in this experiment were from passage 3 to passage 5.

**Statistics**

The values are presented as mean ± SEM from three experiments with triplicate determinations. All data were analyzed with analysis of variance followed by comparison with the Dunnett test. The values were considered significantly different from corresponding controls at p < 0.05.

**Results**

To estimate the effects of antiglaucoma drugs on cellular proliferation, [3H]thymidine uptake was performed after treating the cells with various antiglaucoma drugs at 1/10, 1/100, and 1/1,000 dilutions for 100 minutes. We found that the [3H]thymidine uptake in cultured human corneal keratocytes was significantly inhibited by 580 μM timolol to 63% ± 3% when compared with the control group (which was assumed as 100% response) (Figure 1). However, 58 and 5.8 μM timolol had no effect on cellular proliferation. After treatment with a selective β1-adrenergic antagonist, betaxolol, at the concentrations of 1,620, 162, and 16.2 μM, the cellular [3H]thymidine uptake was to 18% ± 5%, 95% ± 4%, and 101% ± 3%, respectively (Figure 2). Only the 1,620 μM concentration of betaxolol showed an inhibitory effect on cellular proliferation. In the presence of nonselective β-adrenergic antagonists including levobunolol and carteolol, the cellular [3H]thymidine uptake was significantly inhibited by both drugs only at the 1/10 diluted concentration. When cells were treated with 6,800 μM carteolol for 100 minutes, the value of [3H]thymidine uptake was significantly inhibited to 87% ± 4% in comparison with the control group (Figure 3). Again, only 1,710 μM and not 171 and 17.1 μM levobunolol significantly inhibited cellular proliferation to 68% ± 4% when compared with the control value (Figure 4). After addition of the sympathomimetic drug dipivefrin with 280, 28, and 2.8 μM for 100 minutes, the [3H]thymidine uptake value was significantly inhibited only by 280 μM and not by 28 and 2.8 μM concentrations to 55% ± 3% in comparison with the control group (Figure 5). In the case of the parasympathomimetic drug pilocarpine, only the 1/10 dilution (4,080 μM) and not the 408 and 40.8 μM drug concentrations significantly inhibited [3H]thymidine uptake to 67% ± 3% in comparison with the control value (Figure 6).

All of the commercial antiglaucoma drugs described above contain benzalkonium chloride as a preservative. To estimate the effect of benzalkonium chloride on corneal keratocytes, we tested various concentrations of...
benzalkonium chloride alone, at 0.01, 0.001, and 0.0001 mg/mL in $[^3]$Hthymidine uptake experiments. The results demonstrated that only at 0.01 mg/mL concentrations did benzalkonium chloride significantly inhibit $[^3]$Hthymidine uptake to 51% ± 3% in comparison with the control group (data not shown).

**Figure 1.** Dose-dependent effects of timolol on cellular $[^3]$Hthymidine uptake in cultured human corneal keratocytes. Cells were exposed to timolol at the concentrations of 580, 58, or 5.8 μM for 100 minutes. All data are presented as percentage of control cells. Data are presented as mean ± SEM (n = 3; triplicates averaged from three different experiments). *p < 0.05 compared with control.

**Figure 2.** Dose-dependent effects of betaxolol on cellular $[^3]$Hthymidine uptake in cultured human corneal keratocytes. Cells were exposed to betaxolol at concentrations of 1,620, 162, or 16.2 μM for 100 minutes. All data are presented as percentage of control cells. Data are presented as mean ± SEM (n = 3; triplicates averaged from three different experiments). *p < 0.05 compared with control.

**Figure 3.** Dose-dependent effects of carteolol on cellular $[^3]$Hthymidine uptake in cultured human corneal keratocytes. Cells were exposed to carteolol at concentrations of 6,800, 680, or 68 μM for 100 minutes. All data are presented as percentage of control cells. Data are presented as mean ± SEM (n = 3; triplicates averaged from three different experiments). *p < 0.05 compared with control.

**Figure 4.** Dose-dependent effects of levobunolol on cellular $[^3]$Hthymidine uptake in cultured human corneal keratocytes. Cells were exposed to levobunolol at concentrations of 1,710, 171, and 17.1 μM for 100 minutes. All data are presented as percentage of control cells. Data are presented as mean ± SEM (n = 3; triplicates averaged from three different experiments). *p < 0.05 compared with control.
DISCUSSION

Our experimental data show that the antiglaucoma agents betaxolol, timolol, levobunolol, carteolol, dipivefrin, and pilocarpine at high concentrations have potent effects on inhibiting cellular proliferation in cultured human corneal keratocytes.

Corneal keratocytes are a type of fibroblast and are found throughout the stroma between the stroma lamellae. They play a crucial role in producing ground substance and collagen fibrils during embryogenesis and after corneal injury [8]. Although several studies have demonstrated that timolol suppresses cellular proliferation in corneal epithelial cells [2,3] and that loss of corneal endothelial cells occurs with long-term topical epinephrine therapy in glaucoma patients [4], as yet, little is known about whether antiglaucoma drugs influence the physiologic functions of corneal keratocytes, even though increasing numbers of glaucoma patients have used the antiglaucoma drugs for a very long time. When the corneal stroma is damaged or during the wound healing process, such as following corneal injury or the refractive surgeries, photorefractive keratectomy (PRK) and laser-assisted in situ keratomileusis (LASIK), the corneal keratocytes transform from quiescent keratocytes to mitotic fibroblasts [9]. Under these circumstances, cellular proliferation ability plays a crucial role in the regulation of corneal physiologic function. In the present study, we found that high concentrations of various antiglaucoma drugs inhibited cellular proliferation in corneal keratocytes. Thus, careful monitoring of the process of wound healing in stroma after corneal injury or refractive surgery is needed, especially in patients on long-term treatment with antiglaucoma drugs.

According to our previously published data, pilocarpine stimulates cellular proliferation in cultured human Tenon’s fibroblast cells as a result of its methylcellulose content [10]. However, in the present study, we found that pilocarpine inhibited cellular proliferation with three diluted concentrations in cultured human corneal keratocytes, although both cell types are fibroblasts [8,10].

Benzalkonium chloride is one of the most commonly used preservatives in ophthalmic preparations, especially in antiglaucoma drugs. High concentrations of benzalkonium chloride may result in corneal damage [11]. In the present study, we found that all 1/10 diluted concentrations of antiglaucoma drugs suppressed cellular proliferation. Moreover, benzalkonium chloride alone at 0.01 mg/mL significantly inhibited [3H]thymidine uptake in corneal keratocytes. This toxic effect of benzalkonium chloride at high concentration may interfere with normal physiologic function, especially with respect to cellular proliferation of corneal stromal cells. Evidence also exists that the use of benzalkonium chloride-preserved viscoelastic material in cataract surgery leads to penetrating keratoplasty and striae keratopathy [12].

Figure 5. Dose-dependent effects of dipivefrin on cellular [3H]thymidine uptake in cultured human corneal keratocytes. Cells were exposed to dipivefrin at concentrations of 280, 28, or 2.8 μM for 100 minutes. All data are presented as percentage of control cells. Data are presented as mean ± SEM (n = 3; triplicates averaged from three different experiments). *p < 0.05 compared with control.

Figure 6. Dose-dependent effects of pilocarpine on cellular [3H]thymidine uptake in cultured human corneal keratocytes. Cells were exposed to pilocarpine at concentrations of 4,080, 408, and 40.8 μM for 100 minutes. All data are presented as percentage of control cells. Data are presented as mean ± SEM (n = 3; triplicates averaged from three different experiments). *p < 0.05 compared with control.
In summary, cellular proliferation of corneal keratocytes were significantly inhibited by many commercial antiglaucoma drugs, especially at high concentrations. Thus, patients on long-term therapy with antiglaucoma drugs must be carefully monitored with respect to changes in the corneal stroma.

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REFERENCES

抗青光眼藥對人類角膜纖維母細胞增生之影響

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本實驗主要是研究不同的抗青光眼藥包括 timolol，betaxolol，carteolol，levobunolol，dipivefrin and pilocarpine 對人類角膜纖維母細胞生長的影響。人類角膜纖維母細胞經 RPMI-1640 及 10% 胎牛血清培養後加入經 1/10，1/100 及 1/1,000 倍稀冊的抗青光眼藥，經過 100 分鐘培養後測試細胞對 [³H]thymidine 的攝取量。結果發現細胞的生長只被 1/10 倍稀冊的 timolol，betaxolol，carteolol，levobunolol，dipivefrin 及 pilocarpine 抑制到只有控制組的 63%，18%，87%，68%，55% 及 67%。而且 0.01 mg/mL 的 benzalkonium chloride 防腐劑也會明顯抑制人類角膜纖維母細胞的生長。因此本實驗顯示高濃度的抗青光眼藥可能因為含 benzalkonium chloride 防腐劑而會抑制人類角膜纖維母細胞的增生。

關鍵詞：抗青光眼藥，細胞增生，角膜纖維母細胞
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