Published in final edited form as:

Cornea. 2017 June ; 36(6): 719–724. doi:10.1097/ICO.0000000000001181.

Influence of pilocarpine and timolol on human meibomian gland epithelial cells

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Abstract

Purpose—Investigators have discovered that topical anti-glaucoma drugs may induce meibomian gland dysfunction. This response may contribute to the dry eye disease commonly found in glaucoma patients taking such medications. We hypothesize that drug action involves a direct effect on human meibomian gland epithelial cells (HMGEs). To test this hypothesis, we examined the influence of the antiglaucoma drugs, pilocarpine and timolol, on the morphology, survival, proliferation and differentiation of HMGEs.

Methods—Immortalized (I) HMGEs (n = 2–3 wells/treatment/experiment) were cultured with multiple concentrations of pilocarpine or timolol for up to 7 days. Experiments included positive controls for proliferation (epidermal growth factor [EGF], and bovine pituitary extract, [BPE]) and differentiation (azithromycin; AZM). Cells were enumerated with a hemocytometer, and evaluated for morphology, neutral lipid staining and lysosome accumulation.

Results—Our results demonstrate that pilocarpine and timolol cause a dose-dependent decrease in the survival of IHMGEs. The clinically-used concentrations are toxic, and lead to cell atrophy, poor adherence or death. In contrast, drug levels that are known to accumulate within the conjunctiva, adjacent to meibomian glands, do not influence IHMGE survival. These latter concentrations also have no effect on IHMGE proliferation or differentiation, and do not interfere with the ability of AZM to stimulate cellular neutral lipid and lysosome accumulation. This dose of pilocarpine, though, did suppress the EGF+BPE-induced proliferation of IHMGEs.

Conclusions—Our results support our hypothesis and demonstrate that these antiglaucoma drugs, pilocarpine and timolol, have direct effects on HMGEs that may influence their morphology, survival and proliferative capacity.

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Conflict of interest: The authors have no conflict of interests

Disclosure of funding: This research was supported by NIH grant R01EY05612, the Margaret S. Simon Scholar in Ocular Surface Research Fund, the Chinese Scholar Council, and the Guoxing Yao Research Fund.

Keywords

human meibomian gland epithelial cells; anti-glaucoma drugs; pilocarpine; timolol; meibomian gland dysfunction; dry eye disease

Introduction

Recently, investigators have discovered topical anti-glaucoma drugs induce meibomian gland dysfunction (MGD)^{1–8}. This response is clearly a significant adverse effect. Normally, meibomian glands secrete a lipid and protein mixture (i.e. meibum) that provides a clear optical surface for the cornea, interferes with bacterial colonization and retards tear overflow⁹. Moreover, these glandular secretions promote the stability and prevent the evaporation of the tear film, thereby playing an essential role in the health and wellbeing of the ocular surface^{9–11}. Conversely, MGD destabilizes the tear film, increases its evaporation and osmolarity, and is the key trigger for the induction of evaporative dry eye disease (DED). DED is characterized by a vicious cycle of tear film hyperosmolarity and corneal stress, leading to increased friction, inflammation, ocular surface damage and visual impairment^{9,12,13}.

This MGD-inducing effect of topical anti-glaucoma drugs could account for and/or contribute to the decreased tear volume, tear film instability, posterior blepharitis, loss of visual acuity and symptoms of ocular burning, stinging, discomfort, redness, photophobia and foreign-body sensation commonly found in glaucoma patients taking such medications^{4,14–19}. Further, this MGD reaction may help to explain why topical anti-glaucoma treatment often leads to, or worsens, DED in particular and ocular surface disease in general^{8,17,20–23}. It is estimated that 40 to 59% of glaucoma patients suffer from ocular surface disease²⁰.

However, the precise mechanisms underlying these adverse effects of topical anti-glaucoma drugs on the meibomian gland are unclear, and whether they involve drug-related pathways as in other exocrine tissues are also unknown. We hypothesize that the action of these drugs involves a direct effect on human meibomian gland epithelial cells (HMGEs). To begin to test this hypothesis, we examined the influence of the antiglaucoma drugs, pilocarpine and timolol, on the morphology, survival, proliferation and differentiation of HMGEs.

Materials & Methods

Cell cultures

Immortalized (I) HMGEs were cultured in keratinocyte serum-free medium (KSFM), supplemented with 5 ng/ml epidermal growth factor (EGF) and 50 µg/ml bovine pituitary extract (BPE) (Life Technologies, Grand Island, NY). Cells (n = 2–3 wells/treatment/experiment) were treated with various doses of preservative-free pilocarpine hydrochloride (Santa Cruz Biotechnology, Dallas, TX), timolol maleate salt (Sigma-Aldrich, St. Louis, MO) or vehicle for varying time points in the presence or absence of EGF+BPE. Drug doses were selected based on commercially-available product concentrations, previous publications studying the effects of anti-glaucoma drugs on various immortalized cells lines,

and/or animal studies investigating the physiologic concentrations in ocular surface tissues (e.g. conjunctiva) after long term administrations. Cells were counted using a hemocytometer and cellular morphology was examined with a phase-contrast microscope.

Lipid analysis

IHMGEs were grown in a mixture of Dulbecco's Modification of Eagle's Medium and Ham's F-12 (DMEM/F12, Mediatech, Inc., a Corning Subsidiary, Manassas, VA) containing 10% fetal bovine serum (FBS, Life technologies) and 10 ng/ml EGF (Sigma-Aldrich). Azithromycin (AZM, 10 µg/mL, Santa Cruz Biotechnology) was selected as a positive control for its influence on lipid accumulation in IHMGECs²⁴. After treatment with drugs and vehicles, in the presence or absence of AZM for 7 days, cells were stained for lysosomes (LysoTracker Red DND-99, Life technologies) and neutral lipids (LipidTox Neutral Green, Life technologies). The lysosome protein biomarker, Lamp-1 was analyzed by immunoblot. In brief, cells were lysed in Laemmli buffer (Bio-Rad, Hercules, CA) supplemented with 1% protease inhibitor (Sigma-Aldrich) and 5% beta-mercaptoethanol (Sigma-Aldrich), denatured at 95°C for 10 minutes, separated on 4%–20% Tris-glycine gels (Invitrogen Corp.), and transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies specific for lamp-1 (H4A3, 1:500; Developmental Studies Hybridoma Bank, Iowa City, IA) and β -actin (1:10,000; Cell Signaling Technology) were used, followed by incubation with HRP-conjugated secondary antibodies (1:5,000; Sigma-Aldrich). Proteins were visualized with commercial Western blotting substrate (Pierce ECL Western Blotting Substrate; Thermo Fisher Scientific) and band densitometry was performed by using GeneSys software (Syngene).

Statistical Analyses

One-way ANOVA and Student's t-test were performed using Prism 5 (GraphPad Software, Inc., La Jolla, CA). Each experiment was performed in triplicate under the same conditions and repeated at least three times.

Results

Influence of commercially-available doses of pilocarpine or timolol on IHMGECs survival

To test our hypothesis that commercially-available doses of pilocarpine or timolol impair IHMGEC survival, we treated cells ($n = 3$ wells/treatment/experiment) with vehicles, various doses of drugs or EGF+ BPE for 24 hours. As illustrated in Figure 1, 4% pilocarpine, 0.4% pilocarpine and 0.5% timolol significantly decreased IHMGEC proliferation, while 0.05% timolol had minimum effects. Of particular interest, both 4% pilocarpine and 0.5% timolol, which are used clinically, were toxic and led to death of most of the cells. Morphologically, 4.0% pilocarpine induced cellular atrophy and detachment within 3 hours. 0.4% pilocarpine stimulated the accumulation of perinuclear vesicles and the size of these vacuoles appeared to increase by 24 hours. HMGEs treated with 0.5% timolol exhibited star-like shrinkage after 3 hours and cell rounding and poor adherence by 24 hours, whereas the 0.05% dose did not cause obvious cell morphological changes, as compared to cells cultured in KSFM with or without EGF+BPE.

Effects of physiological doses of anti-glaucoma drugs on IHMGECs survival and proliferation

Following topical exposure to pilocarpine or timolol, the 'physiological' concentrations within the palpebral conjunctiva reportedly equal 0.001% and 0.0002 %, respectively^{25,26}. Given that meibomian gland acini would likely be exposed to these amounts, as compared to the topical clinical doses, we determined whether these physiological concentrations modulate IHMGEC survival and proliferation. We exposed IHMGECs (n = 3 wells/treatment/experiment) to vehicle or drugs in the presence or absence of EGF+BPE for various time periods. As demonstrated in Figure 2, neither 0.001% pilocarpine nor 0.0002% timolol affected IHMGEC survival after 24 hours treatment, as compared to vehicle exposure. After 5 days of treatment with vehicle or drugs in KSFM or KSFM+EGF/BPE (Figure 2), IHMGECs were not significantly influenced by 0.0002% timolol, relative to control. In contrast, while the 0.001% pilocarpine did not impact cellular proliferation in KSFM, this concentration did suppress the ability of EGF+BPE to promote IHMGEC proliferation.

Impact of physiologic doses of pilocarpine and timolol on neutral lipid and lysosome accumulation in IHMGECs

To observe whether physiological levels of pilocarpine or timolol alter IHMGEC differentiation, we cultured the cells (n = 2 wells/treatment/experiment) in DMEM/F12 supplemented with serum, vehicle, drugs, AZM or drugs in combination with AZM for 7 days. We then processed the cells for LipidTox staining (for neutral lipids), LysoTracker staining (for lysosomes) and western blot analysis (for Lamp-1). Neither 0.001% pilocarpine nor 0.0002% timolol altered the neutral lipid or lysosome accumulation in IHMGECs, whether in the presence or absence of AZM (Figure 3). Similarly, these drugs did not influence the expression of Lamp-1 protein, as compared to that of controls (data not shown).

Discussion

Our findings show that pilocarpine and timolol cause a dose-dependent decrease in IHMGEC survival. The topical concentrations of these drugs used clinically are toxic, and lead to cell atrophy, poor adherence or death. In contrast, drug levels equivalent to those that reportedly appear within the palpebral conjunctiva following topical application do not influence IHMGEC survival. These latter levels also have no effect on IHMGEC proliferation or differentiation, and do not interfere with the AZM's ability to stimulate cellular neutral lipid and lysosome accumulation. The 0.001% dose of pilocarpine, though, does suppress the EGF+BPE-induced proliferation of IHMGECs. Overall, our results support our hypothesis and demonstrate that the antiglaucoma drugs, pilocarpine and timolol, have direct effects on HMGECS that may impact their morphology, survival and proliferative capacity.

Our data demonstrate that clinical doses of pilocarpine and timolol, as well as a 10-fold lower dose of pilocarpine, are toxic to IHMGECs. The 4.0% pilocarpine amount caused cell atrophy and death. The 0.4% pilocarpine concentration induced perinuclear vesicle

accumulation, impaired cellular adherence, and reduced IHMGEC survival. The development of intense vacuolation in the perinuclear cytoplasm is known to be a harbinger of cell death²⁷. Such vacuolation and cell shrinkage have also been found following exposure of corneal endothelial cells to 4.0% pilocarpine²⁸. As concerns timolol, near clinical concentrations (i.e. 0.1 to 0.4%) have also been shown, after only 15 minutes of treatment, to decrease the viability of conjunctival epithelial cells²⁹.

The initial mechanism(s) by which pilocarpine and timolol act on IHMGECs is likely through specific receptors. Pilocarpine, an acetylcholine analogue, binds to the muscarinic acetylcholine receptor 3, which we have demonstrated is transcribed, translated and functional in IHMGECs³⁰. Our finding that doses between 0.4 to 4% pilocarpine were detrimental to the cells is analogous to results we obtained with rat lacrimal gland epithelial cells (LGECs)⁴⁰. In those studies, similar concentrations of carbachol, another acetylcholine analogue, caused a dramatic suppression of LGEC function³¹. Timolol, in turn, is a beta-adrenergic receptor antagonist, and associates with beta 1, 2 and 3 adrenergic receptors³². We have identified beta 3 adrenergic receptor mRNA in mouse meibomian glands^{9,33}, and these receptors reportedly mediate an increase lipolysis, fat oxidation, energy expenditure and insulin action³⁴. Interference with insulin activity, such as might occur with a high concentration of a beta-adrenergic receptor antagonist, would have a negative impact on IHMGECs³⁵.

In contrast to the effects with clinical doses, concentrations of pilocarpine and timolol analogous to those within the palpebral conjunctiva following topical application do not influence IHMGEC survival, proliferation or differentiation. Similarly, these drug levels do not interfere with AZM's ability to stimulate IHMGEC accumulation of neutral lipids and lysosomes. Our findings are not unlike those of another investigation²⁸, which found that toxic clinical doses of pilocarpine do produce tissue concentrations sufficient to present a danger to corneal endothelial cells in vivo.

However, we observed that the 0.001% dose of pilocarpine did suppress the EGF+BPE-induced proliferation of IHMGECs. This effect, if present in vivo, would limit meibomian gland function and detract from ocular surface health. The reason is that meibomian gland secretion is very dependent upon the active proliferation of glandular epithelial cells³⁶. Once generated, these cells undergo a maturation process towards terminal differentiation, lipid synthesis and holocrine secretion. This secretion involves the death and disintegration of mature, lipid-rich epithelial cells, their release into lateral ducts, and ultimately, delivery to the ocular surface^{9,36}. Given this continual loss of cells, stimulation of epithelial cell proliferation is critically important and promotes not only MGEC replenishment, but also the elaboration of meibum. Any interference with such stimulatory activity could lead to MGD.

For comparison, we recently discovered that Bimatoprost, a prostaglandin F2 α analogue, also has a direct effect on IHMGECs³⁷. This compound is used clinically to promote eyelash lengthening, as well as to treat glaucoma. We found that exposure of IHMGECs to an amount (10 μ M) of Bimatoprost 24-times smaller than the topical clinical 0.01% (i.e. 240 μ M) concentration significantly decreased the intracellular phosphorylation of AKT, a

mediator of cell survival³⁷. Our finding may help explain why Bimatoprost has been reported to induce MGD and DED^{2,4,18,38–40}.

MGD is the leading cause of DED in the world¹³. An antiglaucoma drug-induced development of MGD could be a major contributing factor to the increased prevalence of DED in glaucoma patients^{17,22,41–45}. This response could lead to patient dissatisfaction with topical anti-glaucoma medications and to non-compliance with prescribed therapies^{44,46–48}. In consequence, as C. Everett Koop, MD, a past Surgeon General said, “Drugs don’t work in patients who don’t take them.” We believe, as stated by Servat and Bernardino¹⁸, that reducing iatrogenic events with topical glaucoma drugs can lead to higher levels of adherence to antiglaucoma therapy and help save people’s vision. We also believe that testing the influence of such drugs on IHMGECs, and determining how to circumvent any negative effects, will help with this effort.

Acknowledgments

This research was supported by NIH grant R01EY05612, the Margaret S. Sinon Scholar in Ocular Surface Research Fund, the Chinese Scholar Council, and the Guoxing Yao Research Fund.

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Figure 1.

Influence of clinical doses of pilocarpine or timolol on IHMGEC survival. IHMGECs ($n = 3$ wells/treatment/experiment) were treated with various concentrations of anti-glaucoma drugs, or EGF+BPE or vehicle for 24 hours. (a) Pilocarpine and timolol induce time- and dose-dependent morphological changes in IHMGECs. Images shown are representative of three separate experiments. All images are 200X magnification. (b) Cells were cultured for 24 hours with different dosages of pilocarpine or timolol, or EGF+BPE or vehicle. Pooled data from three experiments are shown. ** indicates $p < 0.001$, * indicates $p < 0.01$. Mean $\pm$ standard error are shown.

Figure 2.

Effect of physiologic doses of pilocarpine or timolol on IHMGEC survival and proliferation. (a,b) IHMGECs ($n = 3$ wells/treatment/experiment) were treated with 0.001% pilocarpine, 0.0002% timolol, or vehicle for 24 hours. A representative of three separate experiments is shown. (c,d) Cells were cultured with 0.001% pilocarpine or 0.0002% timolol for 5 days in KSFM with or without EGF+BPE. A representative example of three experiments is shown. ** indicates $p < 0.001$, * indicates $p < 0.01$. Mean $\pm$ standard error are shown.

Figure 3.

Impact of physiologic doses of pilocarpine or timolol on neutral lipid and lysosome accumulation in IHMGECs. Cells ($n = 2$ wells/treatment/experiment) were treated for 7 days with 0.001% pilocarpine or 0.0002% timolol, in the presence or absence of AZM (10 μ g/ml) in media containing DMEM, 10% FBS and 10ng/ml EGF. AZM is a positive control for the accumulation of both neutral lipids and lysosomes. Cells were stained for neutral lipids (LipidTox Neutral Green) and lysosomes (LysoTracker Red). Images shown are representative of three separate experiments. (a) Cells were treated with vehicle, AZM, 0.001% pilocarpine, or 0.001% pilocarpine in combination with AZM. (b) Cells were treated with vehicle, AZM, 0.0002% timolol, or 0.0002% timolol in combination with AZM. All images are 200X magnification