Suppression of Th1-Mediated Keratoconjunctivitis Sicca by Lifitegrast

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Abstract

Purpose: Increased interferon gamma (IFN- γ) expression in dry eye causes ocular surface epithelial disease termed keratoconjunctivitis sicca (KCS). The purpose of this study was to investigate the effects of the LFA-1 antagonist, lifitegrast, in a mouse desiccating stress (DS) dry eye model that develops KCS similar to Sjögren syndrome.

Methods: Mice were treated with vehicle or lifitegrast twice daily for 5 days and expression of Th1 family genes (IFN- γ , CXCL9, and CXCL11) was evaluated by real-time polymerase chain reaction. Cornea barrier function was assessed by Oregon Green dextran staining and goblet cell number and area were measured.

Results: Compared to the vehicle-treated group, the lifitegrast-treated group had significantly lower expression of Th1 family genes, less corneal barrier disruption, and greater conjunctival goblet cell density/area.

Conclusions: These findings indicate that lifitegrast inhibits DS-induced IFN- γ expression and KCS. This suggests that ICAM-LFA-1 signaling is involved with generation of Th1 inflammation in KCS.

Keywords: keratoconjunctivitis sicca, Sjögren's syndrome, dry eye, interferon gamma, goblet cells

Introduction

AQUEOUS TEAR DEFICIENCY (ATD) initiates an immune cycle that results in ocular surface epithelial disease, termed keratoconjunctivitis sicca (KCS). The most severe ATD and KCS develop in the autoimmune condition Sjögren syndrome. Increased expression of interferon gamma (IFN- γ) inversely correlates with goblet cell density in ATD.¹ Studies performed in the desiccating stress (DS) dry eye model that develops Sjögren-like KCS have found that IFN- γ causes secretory dysfunction and loss of mucin producing conjunctival goblet cells and apoptosis of the corneal epithelium.²⁻⁵ Both cyclosporine A, a T cell immunomodulatory agent, and IFN- γ neutralization have been shown to prevent goblet cell loss in this model.⁵⁻⁷ In human clinical trials, cyclosporine A was found to increase goblet cell number in eyes with KCS.⁸⁻¹⁰

The environmental stressors in the mouse DS model have been found to initiate innate and adaptive immune pathways with recruitment of IFN- γ -producing CD4⁺ T cells that cause corneal epithelial disease and loss and dysfunction of conjunctival goblet cells.^{2-6,11,12} In addition, there is increased interleukin-17 (IL-17) expression that contributes to cornea barrier disruption.^{13,14} Expression of the Th2 cytokine does not change in this model, but the ratio of IL-13 to

IFN- γ has been found to decrease.^{2,6} Furthermore, the DS model created by systemic cholinergic blockage with scopolamine and exposure to a drafty, low humidity environment, used in this study and numerous previously published studies by our group and others,^{2-4,6,12,13,15-26} recapitulate several features of human dry eye, including corneal barrier disruption (seen in humans as increased fluorescein staining and loss of conjunctival goblet cells).^{7,10,27-36} A similar pattern of increased production of disease-relevant cytokines, chemokines, and matrix metalloproteinases that contribute to corneal and conjunctival epithelial disease has been found in the mouse model and humans.^{18,37-45}

Lifitegrast is an FDA-approved dry eye therapy that competitively inhibits ICAM-1 binding to its ligand LFA-1.⁴⁶ Prevention of LFA-1-ICAM-1 adhesion by lifitegrast has been found to inhibit the formation of the immune synapse between dendritic and T cells.⁴⁶ A lifitegrast concentration of 1 μ M significantly inhibited IFN- γ production by stimulated lymphocytes *in vitro*.⁴⁷ Efficacy of lifitegrast for treatment of irritation symptoms and corneal fluorescein staining in dry eye has been documented in clinical trials,^{48,49} but its effects on expression of Th1-associated genes and conjunctival goblet cell number and mucus production in a dry eye model have not been investigated.

The purpose of this study was to compare the effects of lifitegrast and its vehicle on expression of Th1 family genes (IFN- γ , CXCL9, and 11), cornea barrier function, and goblet cell number and mucus production in a murine dry eye model.

Results

The effects of lifitegrast were evaluated in a well-validated DS model of dry eye where tear production is inhibited pharmacologically and mice are placed in a dry drafty environment.^{2,6,11,13,15,17–19,21,29,30,50–56} To determine if lifitegrast inhibited expression of Th1-associated genes *in vivo*, we measured expression levels of IFN- γ and the Th1 chemokines CXCL9 and CXCL11 in the conjunctiva by polymerase chain reaction (PCR) obtained from mice subjected to DS, treated with either lifitegrast or saline vehicle. We found significantly lower levels of IFN- γ and CXCL9 transcripts in the lifitegrast group compared to the vehicle group (Fig. 1).

The effects of lifitegrast on ocular surface epithelial disease were determined by measuring corneal staining with the fluorescent molecule 70 kDa Oregon Green dextran (OGD) as a marker of cornea barrier disruption. Corneal OGD staining was significantly lower ($P < 0.02$) in the lifitegrast group compared to the vehicle group (Fig. 2).

IFN- γ has been found to cause loss and secretory dysfunction of conjunctival goblet cells.^{2,11,12,57} To compare the effects of vehicle and lifitegrast on conjunctival goblet cell loss that develops in the DS model, the density and area of conjunctival goblet cells normalized by length of the goblet cell zone were evaluated (Fig. 3A–C). Compared to vehicle-treated eyes, there was a 39% increase in the number of conjunctival goblet cells and a 22% increase in the normalized goblet cell area in the lifitegrast group (Fig. 3D–F; $P < 0.05$ for both).

Discussion

This study evaluated the effects of lifitegrast on expression of Th1 family genes and the ocular surface epithelial disease that develops in a murine DS model of dry eye, which develops KCS with features similar to that observed in SS. Lifitegrast was found to decrease expression of Th1 chemokines, as well as the signature Th1 cytokine, IFN- γ . This was accompanied by improvement in markers of epithelial disease, including corneal barrier function and goblet cell number and area.

These findings are consistent with improved clinical disease that was observed in human clinical trials of lifitegrast.⁴⁸ IFN- γ has been found to cause apoptosis of the

conjunctival and corneal epithelium and loss and dysfunction of conjunctival goblet cells.^{2,5,12} It is possible that suppression of IFN- γ by lifitegrast contributed to the observed improvement in ocular surface epithelial disease in human clinical trials.⁴⁸ Conjunctival goblet cell density was not measured in any of the clinical trials performed for registration of the drug, but significant improvement in conjunctival lissamine green staining was noted in the Opus 1 study, and lissamine green staining has been reported to worsen as conjunctival goblet cell density decreases.^{1,48}

As opposed to human trials of lifitegrast, where a heterogeneous population of dry eye patients with different chronicity, severity, and prior treatment history were evaluated, the mouse model used in these studies induces a similar level of ocular surface inflammation and KCS.⁵⁸ Therapeutic improvement of ocular disease with lifitegrast was observed within 5 days of induced dry eye in this model, which is consistent with observed improvement in clinical findings following 10 days of treatment in the human clinical trials.⁴⁸

There is increasing recognition of the importance of goblet cells in maintaining ocular surface immune tolerance. An increased number of antigen-presenting cells producing the Th1-inducing cytokine IL-12, accompanied by an increased Th1 polarizing activity, was found in the SAM pointed domain containing ETS transcription factor knockout mouse strain (Spdef KO) that lacks conjunctival goblet cells.⁵⁹ These findings suggest that the loss of conjunctival goblet cells in KCS may create a self-amplifying Th1-inducing cycle on the ocular surface.⁶⁰ Studies are needed to investigate if lifitegrast also improves conjunctival goblet cell number/function in human KCS as part of its therapeutic effects.

The results of these studies provide justification to explore the mechanism by which lifitegrast interferes with generation of the Th1 response in dry eye. There are several potential points in the ocular surface immune cycle of dry eye where inhibition of LFA-1-ICAM-1 binding by lifitegrast could have suppressive effects.⁶¹ In the afferent arm, it could suppress conjunctival antigen-presenting cell migration to the draining lymph nodes, while in the efferent arm, it could inhibit adherence of activated T cells to vascular endothelial or epithelial cells, migration of these cells into the conjunctiva, or secondary stimulation by resident antigen-presenting cells. The pathogenesis of dry eye is very complex and involves multiple pathways, a plethora of mediators and cytokines and a self-amplifying vicious circle.⁶² In this study, we opted to investigate the effects of lifitegrast on features of KCS that are mediated by the Th-1 cytokine IFN- γ . Further studies are necessary to determine if lifitegrast treatment is also efficacious in inhibiting other

FIG. 1. Comparison of relative fold expression of Th1 cytokine IFN- γ and chemokines CXCL9 and CXCL11, measured by RT-PCR, in conjunctiva of mice subjected to DS and treated with lifitegrast or vehicle to ocular surface 2 times daily for 5 days ($n = 5$ per group). Results are expressed as mean $\pm$ SEM, $**P = 0.003$. DS, desiccating stress; IFN- γ , interferon gamma; RT-PCR, real-time polymerase chain reaction.

FIG. 2. Uptake of 70 kDa OGD by cornea epithelium was compared in mice subjected to DS for 5 days and treated with either lifitegrast or vehicle 2 times daily for 5 days ($n=15/\text{group}$). *Left*, representative images of OGD-stained corneas in vehicle (*left*)- and lifitegrast (*right*)-treated groups. *Right*, mean $\pm$ SEM of fluorescent intensity in corneas of vehicle (veh)- and lifitegrast (lifi)-treated groups. OGD, Oregon Green dextran.

factors, such as IL-17 and MMPs, which are also involved in the pathogenesis of dry eye disease.^{13,14,18,19,52,63} There are differences between the mouse and human lacrimal functional unit and the findings in this mouse model remain to be confirmed in a human clinical trial.

In summary, these studies provide new insight into the mechanism of action of lifitegrast on suppressing dry eye-induced ocular surface inflammation and epithelial disease and have direct implications for treatment of SS-associated KCS.

Methods

Animals and DS

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine, and it

conformed to the standards in the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Female C57BL/6J mice aged 6–8 weeks, were purchased from Jackson Laboratories (Bar Harbor, ME) and allowed to rest for a week before the experiment. Fifty female mice were treated, 15 per group to measure corneal barrier disruption, 5 per group to measure goblet cell density/area, and 5 per group to measure gene expression.

DS was induced by subcutaneous injection of scopolamine hydrobromide (0.5 mg/0.2 mL; Sigma-Aldrich, St. Louis) 4 times daily (08:00, 12:00, 14:00, and 17:00 h), alternating between the left and right flanks of animals, as previously described.^{2,11,17,29,54} Mice were placed in modified cages with a perforated plastic screen on 1 side to allow airflow from a fan placed 6 inches in front of it for 16 h/day. Room humidity was maintained at $\leq 30\%$. Mice were subjected to DS for 5 consecutive days and received bilateral

FIG. 3. (A–C). Goblet cell density and area were measured in the areas spanning the goblet cell-rich zone of the conjunctiva using Nikon NIS Elements software. The length of a line drawn over the conjunctival surface (A) in the goblet cell zone was measured in microns and the goblet cell number was manually counted by placing a mark in each mucin-filled goblet cell (B) and the area was measured by outlining mucin-filled goblet cells (C) in PAS-stained paraffin sections. Normalized goblet cell density and area were expressed as cells/mm and $\mu\text{m}^2/\mu\text{m}$, respectively. (D) Normalized goblet cell density and area (mean $\pm$ SEM) in conjunctiva of mice subjected to DS and treated with vehicle (veh) or lifitegrast (lifi) to ocular surface 2 times daily for 5 days ($n=5$ per group) * $P < 0.05$. (E, F) Representative PAS-stained paraffin-embedded conjunctival sections from mice subjected to DS for 5 days and treated with either vehicle (E) or lifitegrast (F). PAS, periodic acid Schiff.

topical treatment with 1 drop per eye (2 μ L volume), 2 times per day (BID), initiated on day 1 concurrently with DS and continued through day 5. Mice received either lifitegrast 5% (Shire, Lexington, MA) or vehicle (balanced salt solution; Alcon, TX).

Measurement of corneal barrier function

Corneal epithelial permeability to Oregon Green dextran 488 (OGD; 70,000 molecular weight [MW]; Invitrogen, Eugene, OR) was assessed as previously described.^{13,15,55,64} Briefly, 1 μ L of 50 mg/mL OGD was instilled onto the ocular surface 1 min before euthanasia, then rinsed with PBS, and photographed with a high-resolution digital camera (Coolsnap HQ2; Photometrics, Tucson, AZ) attached to a stereoscopic zoom microscope (SMZ 1500; Nikon, Melville, NY), under fluorescence excitation at 470-nm. The severity of corneal OGD staining was graded in digital images using NIS Elements (version 3.0; Nikon) within a 2-mm diameter circle placed on the central cornea by 2 masked observers. The mean fluorescence intensity measured by the software inside this central zone was transferred to a database, and the results were averaged within each group.

Measurement of goblet cell density and area

Following euthanasia, eyes and ocular adnexa were excised ($n=5$ /group), fixed in 10% formalin, paraffin embedded, and 5- μ m sections were cut with a microtome (Microm HM 340E; Thermofisher Wilmington, DE). Sections were stained with periodic acid Schiff (PAS) reagent. Sections from 5 left eyes in each group were examined and photographed with a microscope (Eclipse E400; Nikon) equipped with a digital camera (DXM1200; Nikon).^{2,11,21} Using the NIS Elements software, goblet cells were counted manually, then outlined, and the area measured by the software “auto-detect” tool. To determine the length of the conjunctival goblet cell zone, a line was drawn on the surface of the conjunctiva from the first to the last PAS⁺ goblet cell. Results are presented as PAS⁺ goblet cells/mm and area of goblet cells/length (μ m²/mm).

RNA isolation and quantitative PCR

Following euthanasia, conjunctiva was excised on day 5 and total RNA was extracted using a QIAGEN RNeasy Plus Micro RNA isolation kit (Qiagen) following the manufacturer’s protocol.^{64–67} The concentration of RNA was measured, and cDNA was synthesized using the Ready-To-Go™ You-Prime First-Strand kit (GE Healthcare). Quantitative real-time PCR was performed with specific minor groove binder (MGB) probes as previously published.¹³ Murine MGB probes were IFN- γ (*ifn- γ* , Mm00801778), Chemokine (C-X-C motif) ligand 9 (CXCL9, Mm00434946_m1), CXCL11 (Mm00444662_m1), and hypoxanthine phosphoribosyltransferase (HPRT1, Mm00446968). The HPRT-1 gene was used as an endogenous reference for each reaction. The results of real-time PCR were analyzed by the comparative CT method, and the results were normalized by the CT value of HPRT-1.

Statistical analysis

The sample size was calculated using StatMate 2 (GraphPad Software, Inc., San Diego, CA) based on pilot

studies to have at least 90% power to detect differences with an alpha of 0.05. Based on normality, parametric student T or nonparametric Mann–Whitney U tests were performed for statistical comparisons with an alpha of 0.05 using GraphPad Prism 7.0 software (GraphPad Software, Inc.).

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Authors’ Contributions

S.C.P., C.D.P., and M.E.S. designed the experiments. C.D.P., Z.Y., and R.G.D.S. performed the experiments. All the authors analyzed the data and contributed to the article that was reviewed and approved by all authors.

Author Disclosure Statement

S.C.P. and M.E.S. are consultants for Shire, Inc. C.S.D.P. received a research grant to perform the studies. The sponsor did not influence the design of the study. R.G.D.S. and Z.Y. declare no conflict of interests.

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