The Combined Effect of Azithromycin and Insulin-Like Growth Factor-1 on Cultured Human Meibomian Gland Epithelial Cells

Yang Liu and Juan Ding

Schepens Eye Research Institute, Massachusetts Eye and Ear, Harvard Medical School, Boston, Massachusetts, United States

Correspondence: Juan Ding,
Schepens Eye Research Institute, 20
Stanford Street, Boston, MA 02114,
USA;
Juan_ding@meei.harvard.edu.

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PURPOSE. Meibomian gland dysfunction (MGD) is the leading cause of dry eye disease, a prevalent disorder severely affecting patients' quality of life but has no cure. We have discovered that azithromycin, a topical antibiotic used off-label to treat MGD-associated posterior blepharitis, directly acts on the human meibomian gland epithelial cells (HMGEs) to promote their differentiation, and in doing so, reduces cell proliferation. We have also found that insulin-like growth factor-1 (IGF-1), a drug approved by the Food and Drug Administration primarily used to treat dwarfism, stimulates the proliferation and lipid accumulation in these cells. We hypothesize that the combination of azithromycin and IGF-1 will promote cellular differentiation and lipid accumulation, while preserving the normal proliferation of HMGEs.

METHODS. We cultured immortalized HMGEs with vehicle, 10 nM IGF-1, 10 µg/mL azithromycin, or a combination of IGF-1 and azithromycin for 5 to 13 days. Cells were evaluated for intracellular neutral lipids and lysosome accumulation by different staining methods; lipid composition of cell lysates were analyzed using high-performance thin-layer chromatography; proteins of interest (sterol regulatory element binding protein-1 [SREBP-1], cyclins B1 and D1) were measured by immunoblotting, and cell numbers were counted using a hemocytometer.

RESULTS. Our findings demonstrate that the combination of azithromycin and IGF-1 promotes the differentiation and lipid accumulation of HMGEs, while preserving their normal proliferation rate. This combined treatment also increased the levels of neutral lipids, phospholipids, and SREBP-1, and restored cyclin B1 content to control amounts.

CONCLUSIONS. Our results support our hypothesis, and this combination regime may represent a unique and effective treatment of MGD.

Keywords: meibomian gland, azithromycin, IGF-1

Meibomian gland dysfunction (MGD) is the leading cause of dry eye disease (DED), which afflicts tens of millions of Americans, severely affecting their quality of life.^{1–4} It has been reported that MGD counts for approximately 78% of dry-eye patients.⁵ The healthy meibomian gland secretes via a holocrine manner a mixture of lipids and proteins (called meibum) that provides a clear and smooth optical surface for the cornea, slows evaporation of the tear film, and interferes with bacterial colonization, thereby playing an essential role for the normal homeostasis of the ocular surface.⁶ In contrast, MGD destabilizes the tear film, increases its osmolarity, and accelerates tear evaporation, resulting in increased friction on the ocular surface. MGD also may lead to bacterial growth at the lid margin and inflammation in the adjacent conjunctiva (e.g., posterior blepharitis).¹

The most common pharmaceutical management of MGD in the United States is the off-label use of topical azithromycin (AZM).⁷ This macrolide antibiotic is presumed to be effective because of its anti-inflammatory and antibacterial actions on suppressing MGD-associated posterior blepharitis and growth of lid bacteria.⁸ We have recently shown that AZM can act directly on human meibomian gland epithelial cells (HMGEs),

promoting their differentiation and lipid accumulation.⁹ This effect is accompanied by an increased lysosome formation, a decreased cell proliferation, and a holocrine-like lipid secretion, which are the major characteristics of HMGE differentiation.^{9–11} In addition, we have discovered that insulin-like growth factor-1 (IGF-1) is essential for meibomian gland health and promotes both cell proliferation and lipid accumulation.¹² Given these findings, we hypothesize that the combination of AZM and IGF-1 will promote cell differentiation and lipid accumulation while preserving the normal cell proliferation in HMGEs. We further hypothesize that the combination will modulate lipid composition, and the expression of a key lipogenesis factor (sterol regulatory element-binding protein 1 [SREBP-1]). The SREBP-1 regulates the synthesis and uptake of cholesterol, triglyceride (TG) and phospholipids, and is activated by cleavage of the precursor form into a mature form,¹³ which is a cluster of protein bands between 59 and 68 kDa.^{14,15} In addition, we hypothesize that IGF-1 and AZM will affect cell-cycle-related proteins cyclins B1 and D1, members of cyclins that control the cell cycle by promoting G2/M transition and mitosis, and G1/S transition, respectively.^{16,17} The purpose of this study was to test these hypotheses.

FIGURE 1. Effect of IGF-1, AZM, and IGF-1+AZM combination on intracellular accumulation of lipids and lysosomes. Cells were treated with vehicle, 10 nM IGF-1, 10 μ M AZM, or IGF-1+AZM combination for 13 days. (a) The green color represents LipidTOX green neutral lipid staining, and red color LysoTracker staining for lysosomes. (b) The fluorescence intensity of LipidTOX staining was quantified using ImageJ. Two-way ANOVA showed significant effect of AZM (**** $P < 0.0001$). (c) The fluorescence intensity of LysoTracker staining was quantified using ImageJ. Two-way ANOVA showed significant effect of AZM ($P < 0.0001$). The experiments were repeated four times with similar results; data shown here are from a single experiment.

MATERIALS AND METHODS

Immortalized human meibomian gland epithelial cells (IHMGEs), generated in our laboratory,¹⁸ were cultured in the presence of 10% fetal bovine serum according to published protocols.¹⁹ Cells were treated with ethanol vehicle, 10 μ M AZM (Santa Cruz Biotechnology, Dallas, TX, USA), 10 nM IGF-1 (National Hormone and Peptide Program, Torrance, CA, USA), or a combination of 10 μ M AZM and 10 nM IGF-1 for 5 to 13 days.

Cellular neutral lipid and lysosome were examined by LipidTOX green neutral lipid stain (1:800; Invitrogen, Grand Island, NY, USA) and LysoTracker Red DND-99 (50 nM; Invitrogen), respectively, as previously reported.¹⁰ The lipid extractions from samples containing equivalent amount of cells were developed on a high-performance thin-layer chromatography (HPTLC) plate (Silica Gel 60; Merck, Darmstadt, Germany). For nonpolar lipid analysis, the plate was developed in benzene:hexane (65:35, vol/vol) alone. For polar lipid analysis, the plate was developed sequentially in chloroform:methanol:water (65:25:4 vol/vol/vol) and then benzene:hexane (65:35, vol/vol).²⁰ Cholesterol oleate (Nu-Chek Prep, Inc., Elysian, MN, USA), triolein, free cholesterol (FC; Sigma-Aldrich Corp., St. Louis, MO, USA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC) (Avanti Polar Lipids, Inc., Alabaster, AL, USA) were used as standards. Bands were visualized according to published protocols.²¹ Cell

numbers were counted with a hemocytometer. The SREBP-1 and cyclins B1 and D1 were analyzed by immunoblot as described earlier.¹² Primary antibodies were diluted at the concentration of 1:500 (SREBP-1, H-160; Santa Cruz), 1:1000 (cyclins B1 and D1; Cell Signaling Technology, Danvers, MA, USA) and 1:10,000 (β -actin; Cell Signaling Technology). Horseradish peroxidase-conjugated secondary antibodies, goat anti-rabbit IgG, and Fc-specific goat anti-mouse IgG were diluted 1:5000 (Sigma-Aldrich). All experiments were repeated at least three times. Intensities were quantified using ImageJ (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). For statistical evaluation, two-way ANOVA was performed using Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

We observed significant increases in neutral lipid and lysosome staining in IHMGECs by AZM alone or in combination with IGF-1 (Fig. 1A). The combination treatment produced a similar effect on neutral lipid accumulation and lysosome formation as AZM alone (Figs. 1B, 1C). The result of HPTLC showed that for lipid composition, AZM alone significantly increased the content of cholesterol ester (CE; $P < 0.0001$), FC ($P < 0.01$), PE ($P < 0.0001$), and PC ($P < 0.0001$), but significantly decreased TG ($P < 0.0001$); statistically significant increases in CE ($P < 0.05$) and TG ($P < 0.0001$) also were observed in the

FIGURE 2. Effect of IGF-1, AZM, and IGF-1+AZM combination on the accumulation of CE, TG, FC, PE, and PC. (a) Cells were treated with vehicle, 10 nM IGF-1, 10 μ g/mL AZM, or IGF-1+AZM combination for 7 days before performing chromatographic analyses of total lipid extracts. (b) Band intensity was quantified using ImageJ. Control band intensity was set to 1, and data (mean $\pm$ SE) were reported as fold-change compared with control values. The IGF-1 showed a significant effect on CE (* P < 0.05) and TG (**** P < 0.0001). The AZM showed a significant effect on CE, TG, PE, PC (P < 0.0001 for all four), and FC (** P < 0.01). Other bands are unidentified lipids. Band intensity analysis included data from three independent experiments. STD, lipid standard.

IGF-1 group, with little effect on FC or polar lipids. The combination treatment significantly increased CE, FC, PE, and PC to the same extent as AZM alone, and restored the TG content to control level (Fig. 2). Consistent with increased lipid accumulation, by immunoblotting, we observed a significant increase in SREBP-1 by IGF-1 (both precursor and mature forms, P < 0.05) and AZM (mature form, P < 0.01) alone, or in combination (both forms) (Figs. 3A–C). Because of the inconsistency in the separation of PI, the quantification of PI is not included in the results.

For cell proliferation, AZM induced approximately 50% decrease in cell proliferation, whereas IGF-1 increased cell proliferation approximately 3-fold (Fig. 4). The combination treatment maintained the cell proliferation to a level similar to the control group. Consistent with this, cyclin B1 was significantly upregulated by IGF-1 (P < 0.0001), downregulated by AZM (P < 0.0001), and the expression was similar to control levels in the combination treatment (Fig. 3D). In contrast, no effect was seen on cyclin D1 expression (Fig. 3A).

DISCUSSION

Our study supports the hypothesis that the combination of AZM and IGF-1 significantly promotes IHMGEC differentiation and lipid accumulation, while preserving the normal cell proliferation rate.

The combination of AZM and IGF-1 induces both neutral and polar lipid accumulation, and promotes lysosome formation in IHMGECs. In this regard, the increase in CE, FC, and phospholipids, as well as lysosome formation in IHMGECs are similar in the combination treatment compared with AZM alone, as we previously reported,¹⁰ whereas TG is restored to control level even though AZM alone decreases it. The lipid-

promoting effect of AZM and IGF-1 involves the upregulation of SREBP-1. Both AZM and IGF-1 upregulate SREBP-1 expression, although in different ways: AZM increases only the mature form, whereas IGF-1 promotes both mature and precursor forms. The combination treatment again shows an additive action of IGF-1 and AZM, with both SREBP-1 forms maximally upregulated. This result is consistent with previous reports.^{12,22} Our data indicate that AZM and IGF-1, although both promoting lipid accumulation in IHMGECs, may actually act through separate mechanisms, thereby allowing an additive effect on lipid accumulation and composition. The increase in these neutral and phospholipids, which are major components of meibum, is highly significant, because MGD is characterized by a reduced quantity and quality of meibum.^{23–27} For example, CE, FC, and phospholipids are decreased in patients with MGD,^{2,21} and we show that AZM increases CE, FC, and phospholipids in meibomian gland epithelial cells in vitro, consistent with the report from a clinical trial that AZM restores the lipid composition and the tear film stability in meibum of MGD patients.⁸

The effect of IGF-1 on lipid accumulation is quite distinct from that of AZM. We have previously reported significant increase in overall neutral lipid accumulation by treatment of AZM^{9,10} and IGF-1¹² of approximately 7 days, which is a representative time point when IHMGECs have undergone differentiation in the serum-containing medium.¹⁸ However, it is not known whether they act similarly on lipid composition changes at this point. We found in the present study that IGF-1 did not affect any of the polar lipids that we tested, but stimulated TG content remarkably and to a lesser extent, CE, after 7 days. This is consistent with studies in other cell types.^{28,29} We went on to examine SREBP-1 (lipogenic transcription factor) and cyclins at 5 days, which is similar to 7 days in terms of differentiation, and without significant cell

FIGURE 3. Effect of IGF-1, AZM, and IGF-1+AZM combination on the expression of SREBP-1, cyclins B1 and D1. Cells were incubated with vehicle, 10 nM IGF-1, 10 μ M AZM, or IGF-1+AZM combination for 5 days. Cell lysates were evaluated on immunoblots for precursor and mature forms of SREBP-1, cyclins B1 and D1 (a), and protein band intensities quantified using ImageJ (b). The IGF-1 significantly affected the expression of pre-SREBP-1 and mature SREBP-1 (* P < 0.05) and cyclin B1 (**** P < 0.0001). The AZM showed significant effect on mature SREBP-1 (** P < 0.01) and cyclin B1 (P < 0.0001). These experiments were repeated at least three times with similar results.

loss induced by AZM⁹ that often occurs at later time points (e.g., beyond 10 days), which potentially influences the quantitative analysis of lipids and proteins. We found complementary actions of AZM and IGF-1 on increasing SREBP-1, as discussed above. We also examined overall neutral lipid at 13 days of IGF-1 and/or AZM treatment, because this is a clinically relevant time point (AZM is usually administered for 2–4 weeks in clinics³⁰). Interestingly, in the present study, IGF-1 alone did not induce a significant increase in the overall neutral lipid after 13 days of treatment. These combined data suggest that IGF-1 accelerates neutral lipid accumulation (evident at 6–7 days), but this effect may reach a plateau after prolonged treatment and becomes insignificant after 13 days. We did not perform further lipid or protein analyses at 13 days because of significant cell loss and marked disparity in cell number among AZM and IGF-1 treatments.

Our results showed that after differentiating for 13 days in the serum-containing medium, AZM significantly reduced the cell number of meibomian gland epithelial cells, which could reflect cell loss due to holocrine secretion. The effect of AZM and IGF-1 allows a normal cell proliferation, which appears to involve the modulation of cyclin B1 but not D1. Cyclin B1 is a

member of cyclins, a family of regulatory proteins that control the cell cycle,¹⁷ and promotes G2/M transition and mitosis.¹⁶ Whereas AZM downregulates cyclin B1 and IGF-1 upregulates it, the combination treatment group shows the same levels as the control group, in agreement with the proliferation pattern. In contrast, cyclin D1, which controls G1/S transition, is not affected by either AZM or IGF-1. These results are consistent with our previous studies.^{9,12} Azithromycin and IGF-1 also have been reported to influence cell proliferation and cell-cycle-related proteins, including cyclins D1 and B1 in the opposite directions.^{9,12,22,31} The normal proliferation is important for meibomian gland, which undergoes constant renewal due to holocrine secretion of the acinar epithelial cells. Therefore, the combination of AZM and IGF-1 is ideal in maintaining normal proliferation while promoting lipid accumulation. We previously showed that IGF-1 activates Akt signaling, which is a survival and pro-proliferation pathway in these cells¹²; therefore, it is possible that other hormones or drugs, such as insulin, that activate the same pathway may produce a similar effect in meibomian gland epithelial cells.

In summary, our study has demonstrated that the combination of AZM and IGF-1 promotes lipid accumulation including

FIGURE 4. Effect of IGF-1, AZM, and IGF-1+AZM combination on the proliferation of IHMGECs. Cells were seeded (50,000 cells/well in 12-well plates, $n = 3$ wells/group) and treated with vehicle, 10 nM IGF-1, 10 μ g/mL AZM, or IGF-1+AZM combination for 13 days before cell counting. Results were reported as mean $\pm$ SE. The IGF-1 and AZM both exerted a significant but opposite effect on cell proliferation (**** $P < 0.0001$ for both). Data from one experiment were shown as a representative of three studies performed under the same conditions.

both neutral and polar lipids, while preserving a normal cell proliferation in IHMGECs. These effects involve the modulation of SREBP-1 and cyclin B1, respectively. We believe this combination regime may represent a unique and effective treatment of MGD when used topically on the ocular surface or eyelid.

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Disclosure: **Y. Liu, P; J. Ding, P**

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