The Effects of Insulin-like Growth Factor 1 and Growth Hormone on Human Meibomian Gland Epithelial Cells

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IMPORTANCE A phase 1 study has reported that dry eye disease is the most common adverse effect of human exposure to the antibody figitumumab, an anticancer drug that prevents insulin-like growth factor 1 (IGF-1) from binding to its receptor. We hypothesized that the mechanism underlying this effect is the inhibition of IGF-1 action in epithelial cells of the meibomian gland.

OBJECTIVES To test the hypothesis that IGF-1 stimulates meibomian gland function in vitro and to examine whether growth hormone, a closely related hormone of IGF-1, has the same effect.

DESIGN, SETTING, AND MATERIAL Immortalized human meibomian gland epithelial cells were cultured in the presence or the absence of IGF-1, growth hormone, and an IGF-1 receptor–blocking antibody. Signaling pathways, cell proliferation, neutral lipid staining, and a key protein involved in lipid biogenesis were evaluated.

INTERVENTION Application of IGF-1 and growth hormone to human meibomian gland epithelial cells.

MAIN OUTCOMES AND MEASURES Immunoblotting, cell counting, and neutral lipid staining.

RESULTS Insulin-like growth factor 1 activated the phosphoinositol 3-kinase/Akt and forkhead box O1 pathways (showing a dose-dependent effect on immunoblotting), stimulated cellular proliferation (about 1.8-fold increase in cell number), increased sterol regulatory element-binding protein 1 expression (about 3-fold increase on immunoblotting), and promoted lipid accumulation in human meibomian gland epithelial cells (about 2-fold increase in lipid staining). These IGF-1 actions, which may be blocked by cotreatment with the anti-IGF-1 antibody, were accompanied by inconsistent effects on extracellular signal-regulated kinase phosphorylation. We were not able to demonstrate activation of Akt, forkhead box O1, extracellular signal-regulated kinase, Janus kinase 2, or signal transducers and activators of transcription 5, induced cell proliferation, or lipid accumulation in these cells by growth hormone application.

CONCLUSIONS AND RELEVANCE Our results support the hypothesis that IGF-1 acts on human meibomian gland epithelial cells and may explain why treatment with figitumumab, the IGF-1 inhibitor, causes dry eye disease. Ophthalmic care for dry eye disease may be needed when patients with cancer undergo treatment with drugs that inhibit IGF-1 action.
The hormone insulin-like growth factor 1 (IGF-1) plays a very important role in human growth and development. Insulin-like growth factor 1 is a potent activator of the phosphoinositide 3-kinase (PI3K)/Akt pathway, which stimulates cell proliferation and differentiation and inhibits programmed cell death. The potency of IGF-1 action is such that alterations in its signaling pathway components may promote the development of a variety of malignant diseases. This recognition, in turn, has led to the generation of pharmaceuticals to block the IGF-1 receptor (IGF-1R) and serve as anticancer treatments.

One such drug is figitumab, a human IgG2 monoclonal antibody that prevents the binding of IGF-1 to its receptor, blocks IGF-1 downstream signaling, and induces IGF-1R degradation. Of particular interest, the most common adverse event of figitumab in a recent phase 1 trial was dry eye disease (DED). Dry eye disease is one of the most frequent causes of patient visits to eye care practitioners and affects tens of millions of people in the United States. This condition is characterized by a vicious cycle of tear film hyperosmolarity and ocular surface stress, leading to increased friction, inflammation, and damage to the eye. The impact of moderate to severe DED is comparable to that of conditions such as dialysis and severe angina and is associated with significant pain and role limitations, low vitality, and poor general health.

We hypothesized that the mechanism by which figitumab induces DED is inhibition of IGF-1 action in the epithelial cells of the meibomian glands. These large sebaceous glands typically secrete lipids that increase the stability and decrease the evaporation of the tear film, thereby promoting the health and well-being of the ocular surface. However, their dysfunction, termed meibomian gland dysfunction, leads to lipid insufficiency, tear film hyperosmolarity and instability, and evaporative DED. Meibomian gland dysfunction is the primary cause of DED worldwide.

In support of this hypothesis, investigators have reported that IGF-1 stimulates the function of epithelial cells in other sebaceous glands. More specifically, IGF-1 activates the PI3K/Akt pathway, enhances proliferation, and augments lipid biosynthesis in rat and human sebaceous gland cells. The lipid effect appears to be linked to an upregulation of sterol regulatory element–binding protein 1 (SREBP-1), a key transcription factor that impels lipogenesis. However, given that considerable differences exist in the control of sebaceous glands between species and between different types of sebaceous glands, IGF-1 may or may not exert similar actions in the epithelial cells of human meibomian glands.

The purpose of this study was to test the hypothesis that IGF-1 acts on human meibomian gland epithelial cells. Toward that end, we sought to determine whether IGF-1 activates the PI3K/Akt pathway, stimulates proliferation, increases SREBP-1 expression, and promotes lipid accumulation in these cells. As a corollary to this study, we examined whether IGF-1 (1) activates the extracellular signal-regulated kinase (ERK, also known as mitogen-activated protein kinase) pathway, which is involved in IGF-1 signaling in other cell types; (2) modulates the phosphorylation of forkhead box O1 (FoxO1), a transcription factor that, when phosphorylated by Akt, is inhibited from reducing cell proliferation, SREBP-1 expression, and lipogenesis; and (3) elicits effects analogous to those of growth hormone (GH). Growth hormone and IGF-1 are known to act in concert to influence sebaceous gland function and dysfunction.

**Methods**

**Cell Culture and Treatment**

Immortalized human meibomian gland epithelial cells, which were developed in our laboratory, were maintained in keratinocyte serum-free medium supplemented with 5 ng/mL of epidermal growth factor (EGF) (Life Technologies) and 50 μg/mL of bovine pituitary extract (BPE) (Life Technologies), as previously described. When indicated, cells were cultured in basal keratinocyte serum-free medium alone or a supplemented serum-free medium (Dulbecco modified Eagle medium/Ham F-12 nutrient mixture (DMEM/F12 mixture; Sigma-Aldrich) supplemented with 1% or 10% fetal bovine serum (FBS). The EGF–BPE– and 10% FBS–containing media are known to promote epithelial cell proliferation and differentiation, respectively, in human meibomian gland epithelial cells. The human prostate cancer cell line, LNCaP, was obtained from American Type Culture Collection and cultured in the DMEM/F12 mixture supplemented with 10% FBS. Recombinant human IGF-1 and human GH were purchased from the National Hormone and Peptide Program, dissolved in phosphate-buffered saline, and filter sterilized. The IGF-1 and GH were applied at a final concentration of 10 nM to cells. A PI3K inhibitor, LY294002, was purchased from Cell Signaling Technology, Inc. Human IGF-1R–blocking monoclonal antibody and the corresponding mouse IgG1 isotype control were purchased from R&D Systems.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblots**

Cells were directly lysed in Laemmli buffer (Bio-Rad) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich) and 5% β-mercaptoethanol (Sigma-Aldrich). Lysates were heated at 95°C for 10 minutes, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% Tris-glycine precast gels (Novex; Life Technologies), and transferred to polyvinylidene difluoride membranes. We used rabbit or mouse antibodies specific for phosphorylated Akt (p-Akt) (Ser473), pan-Akt, p-FoxO1 (Ser256), FoxO1, p-JAK2, JAK2, and β-actin (Cell Signaling Technology); for p-ERK, ERK-2, STAT5, and the precursor form of SREBP-1 (Santa Cruz Biotechnology); and for p-STAT5 (Millipore). Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.01% polysorbate 20 (Tweens-20; Sigma Aldrich). All primary antibodies were diluted to 1:1000 in blocking buffer except for p-Akt (1:4000), p-ERK (1:2000), ERK-2 (1:4000), SREBP-1 (1:200), and β-actin (1:10 000). Horseradish peroxidase–conjugated secondary antibodies were goat anti-rabbit IgG and FC-specific goat anti-mouse IgG diluted to 1:5000 (Sigma-
Proteins were visualized with enhanced chemiluminescent substrate (Pierce ECL; ThermoFisher Scientific) or with ultrasensitive chemiluminescent substrate (SuperSignal West Femto; Thermo Fisher Scientific). Densitometry was performed with publicly available image-processing software (ImageJ; http://rsb.info.nih.gov/ij).

Cell Proliferation Assay

Human meibomi gland epithelial cells and LNCaP cells were cultured in 12-well plates in designated media and then exposed to IGF-1 or GH for defined periods. At experimental termination, cells were trypsinized and counted with a hemocytometer.

Green Neutral Lipid Staining

Immortalized human meibomian gland epithelial cells were cultured in slides treated to facilitate cell growth (Lab-Tek II Chamber Slide System; Nalge Nunc International) in FBS-containing medium in the presence or absence of IGF-1, anti-IGF-1R, and/or GH. Media were replaced every 2 to 3 days for a total of 6 days, at which time cells were washed and fixed in 4% paraformaldehyde for 30 minutes. After additional washes, cells were stained with green neutral lipid stain (1:800; LipidTOX; Life Technologies) in a humid chamber for 30 minutes. Coverslips were mounted on slides with an antifade reagent that contains 4′,6-diamidino-2-phenylindole nuclear stain (Pro-Long Gold; Life Technologies) and permitted to dry overnight before imaging with a microscope (Nikon Eclipse E800; Nikon Instruments). Fluorescence intensity was quantified using the image-processing software.

Statistical Analysis

We performed 1- and 2-way analysis of variance, the Tukey test as a post hoc test after analysis of variance, and the unpaired 2-tailed t test. All statistical analysis used commercially available software (Prism 5; GraphPad Software, Inc).

Results

Influence of IGF-1 and GH on Signaling Pathways

To determine whether IGF-1 promotes the phosphorylation of Akt in human meibomian gland epithelial cells, we cultured cells under proliferating or differentiating conditions and then exposed them to IGF-1 for 15 minutes. As demonstrated in Figure 1A, IGF-1 induced a striking, dose-dependent expression of p-Akt. This effect could be detected after exposure to a 1 nM concentration of IGF-1 and became maximal at a 10 nM concentration; this latter dose was used for all further studies. The stimulatory influence of IGF-1 on Akt was paralleled by an increased phosphorylation of FoxO1 (Figure 1B), and these actions occurred in differentiating but not proliferating cells. The differentiated effects were blocked by preincubation with the PI3K inhibitor LY294002 (Figure 1B). In contrast, IGF-1 had an inconsistent effect on ERK. In 2 studies with differentiating cells, IGF-1 enhanced the phosphorylation of ERK, whereas in 3 other experiments (eg, Figure 1A), IGF-1 had no activating influence on ERK.

To assess whether GH activates Akt, FoxO1, ERK-2, or its classic (ie, JAK2 and STAT5) signaling pathways in human meibomian gland epithelial cells, we cultured cells in proliferat-
ing or differentiating media, exposed them to a 10nM concentration of GH for 15 minutes, and processed them for immunoblot analyses. As demonstrated in Figure 1B and C, we were not able to show that GH elicited effects on these signaling components. The same negative results were also found at GH doses of 0.05 to 50 nM (data not shown). For comparison, GH activated the JAK2/STAT5 pathway in the positive control LNCaP cells (Figure 1C).

Cell Proliferation of IGF-1 and GH
To evaluate whether IGF-1 stimulates proliferation of human meibomian gland epithelial cells, cells were cultured in basal, proliferating, or differentiating medium and treated with IGF-1 for 2 days. As shown in Figure 2A, IGF-1 induced an increase in cell number in the 10% FBS-containing medium but not under basal conditions (ie, keratinocyte serum-free medium) or in media already primed to promote cellular proliferation (ie, keratinocyte serum-free medium and the EGF-BPE mixture). This stimulatory effect of IGF-1 on cell proliferation continued throughout a 6-day course (Figure 2B). In contrast, GH had no influence on the proliferation of human meibomian gland epithelial cells in any of the media at doses ranging from 0.1 to 100 nM (data not shown). The proliferation of LNCaP control cells was increased by IGF-1 and GH (Figure 2C).

Influence of IGF-1 and GH on SREBP-1 Expression and Lipid Accumulation
To examine whether IGF-1 stimulates SREBP-1 expression and lipid accumulation in human meibomian gland epithelial cells, we cultured cells for 6 days in differentiation media with IGF-1 and then processed the cells for protein and histologic analysis. As demonstrated in Figure 3A, IGF-1 induced a rise in the amount of the precursor form of SREBP-1 protein. This stimulatory effect was accompanied by an increase in the accumulation of neutral lipids, as shown in Figure 3B. These IGF-1 actions were not duplicated by cellular treatment with GH (Figure 3).

Inhibition of IGF-1 Action by Anti–IGF-1R
To determine whether anti–IGF-1R is able to inhibit IGF-1 action on human meibomian gland epithelial cells, cells were cultured in differentiating conditions and exposed to anti–IGF-1R for varying periods. As demonstrated in Figure 4A, anti–IGF-1R suppressed the ability of IGF-1 to phosphorylate Akt. This inhibitory effect of anti–IGF-1R was found at a 1nM dose, became maximal at 10nM, and could not be duplicated by the use of an irrelevant IgG antibody (Figure 4A). In addition to this interference with IGF-1–mediated signaling, the anti–IGF-1R also specifically blocked the IGF-1–linked accumulation of lipids in human meibomian gland epithelial cells (Figure 4B).

Discussion
Our study demonstrates that IGF-1 exerts a marked influence on the function of human meibomian gland epithelial cells. Insulin-like growth factor 1 activates the PI3K/Akt and FoxO1 pathways, stimulates proliferation, increases SREBP-1 expres-
The stimulation of cell proliferation, SREBP-1 expression, and lipid production by IGF-1 may be mediated through its activation of the PI3K/Akt and FoxO1 pathways. Other researchers have found that phosphorylation of Akt induced by IGF-1 promotes mitogenesis, SREBP-1 upregulation, and lipid synthesis. Further, IGF-1 increased the levels of p-FoxO1, which prevents the FoxO1 suppression of cell proliferation and lipogenesis. It is likely that PI3K/Akt is the intermediate of the IGF-1 effect on FoxO1, given that IGF-1 action was considerably reduced in the presence of the PI3K inhibitor LY194002.

The ability of IGF-1 to stimulate cell proliferation in media containing 10% FBS but not EGF-BPE is not surprising. The FBS-containing medium, which primes human meibomian gland epithelial cells for differentiated functions, is known to support IGF-1–associated proliferation. However, EFG-BPE–containing medium promotes the proliferation of human meibomian gland epithelial cells, a process involving a significant increase in the expression of cell cycle genes (eg, cyclins B2, D1, D2, and D3; cyclin-dependent kinases 4 and 6; and E2F transcription factor). Given that IGF-1 also upregulates cyclins D1 and D3 to stimulate cell proliferation, this hormonal effect may be undetectable because before IGF-1 treatment, EGF and BPE have already maximally stimulated the major molecular components driving cell cycle progression.

Our data indicate that IGF-1 inconsistently activates ERK and that GH does not elicit detectable effects in human meibomian gland epithelial cells. The lack of a consistent IGF-1 activation of ERK suggests that this pathway is not the...
major one for IGF-1 in these cells. We were not able to detect activations of Akt, FoxO1, ERK, JAK2, and/or STAT5 or changes in cell proliferation or lipid accumulation by GH treatment, suggesting that these signaling systems and responses may not be susceptible to GH influence in human meibomian gland epithelial cells. Growth hormone increases the percentage of lipid-forming colonies of rat preputial sebaceous gland cells, but this effect is relatively small in the absence of exogenous insulin. Growth hormone does not modulate DNA synthesis in these preputial cells. A question, then, is what does GH regulate in the meibomian gland? A previous study identified the messenger RNA for the GH receptor in mouse meibomian gland tissue. However, whether such transcripts exist in human meibomian gland epithelial cells and are translated into functional receptors awaits the demonstration of a GH action in these cells.

Conclusions

Our results are consistent with the hypothesis that the mechanism by which figitumumab induces DED is inhibition of IGF-1 action in human meibomian gland epithelial cells. Such an inhibition could reduce glandular lipid accumulation, lead to a lipid insufficiency on the ocular surface, and ultimately cause evaporative DED. Another possible consequence of IGF-1 blockade might be a loss of androgen influence in this tissue. A decrease in downstream IGF-1 receptor signaling is known to attenuate the expression and function of androgen receptors in human prostatic cells. Androgen receptor dysfunction, in turn, is a significant risk factor for the development of meibomian gland dysfunction and DED. Future investigations should determine whether IGF-1 and androgen signaling pathways are interrelated in the human meibomian gland.
developed the idea and generated the supportive data for this provisional patent.

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