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Human Growth Hormone Promotes Corneal Epithelial Cell Migration in Vitro

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Abstract

Purpose—Corneal wound healing is a highly regulated process that requires the proliferation and migration of epithelial cells and interactions between epithelial cells and stromal fibroblasts. Compounds that can be applied topically to the ocular surface and that have the capability of activating corneal epithelial cells to proliferate and/or migrate would be useful to promote corneal wound healing. We hypothesize that human growth hormone (HGH) will activate Signal Transducer and Activators of Transcription-5 (STAT5) signaling and promote corneal wound healing by enhancing corneal epithelial cell and fibroblast proliferation and/or migration *in vitro*. The purpose of this study is to test these hypotheses.

Methods—We studied cell signaling, proliferation and migration using an immortalized human corneal epithelial cell line and primary human corneal fibroblasts *in vitro*. We also examined whether insulin-like growth factor-1 (IGF-1), a hormone known to mediate many of HGH's growth promoting actions, may play a role in this effect.

Results—We show that HGH activates STAT5 signaling and promotes corneal epithelial cell migration *in vitro*. The migratory effect requires an intact communication between corneal epithelia and fibroblasts, and is not mediated by IGF-1.

Conclusion—HGH may represent a topical therapeutic to promote corneal epithelial wound healing. This warrants further investigation.

Keywords

corneal wound healing; growth hormone; IGF-1

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Conflict of Interest Juan Ding and David A. Sullivan: none.

Barbara Wirostko is the co-founder and CSO of Jade Therapeutics Inc., receives financial support, has personal financial interest, holds patent, and is on the board of Jade, a biotech start up working on the development of an rHGH ophthalmic therapeutic indicated for corneal wound healing.

Introduction

Corneal wound healing is a highly regulated process that requires the proliferation and migration of epithelial cells¹ and interactions between epithelial cells and stromal fibroblasts.^{2,3} When the process is altered either in conditions of ocular surface disease, trauma, systemic disease and/or from surgical intervention, delays in corneal epithelial wound healing can result thus leading to corneal defects that will not “close”. This impaired corneal wound healing can lead to persistent corneal epithelial defects (PCED), which result in corneal scarring, ulceration, opacification and neovascularization, and ultimately visual compromise and loss of vision.¹ Pharmacological acceleration of epithelial closure has the potential to reduce the risk of these vision-threatening complications in affected patients. Compounds that can be applied locally to the ocular surface and that have the capability of activating corneal epithelial cells to proliferate and/or migrate would be useful for the treatment of PCED, as can be associated with recurrent corneal erosions, severe dry eye, neurotrophic keratopathy, diabetic corneal defects, chemical and thermal burns, and surgical and non-surgical trauma.

We hypothesize that human growth hormone (HGH) is such a compound. HGH is a pituitary hormone belonging to the somatotropin/prolactin family, and is required for normal human tissue growth and development.⁴ HGH is known to act on a variety of target organs and tissues, but little is known about its possible action on the cornea. Recently several human studies have shown alterations in the thickness and biomechanics of the cornea in acromegalic patients and GH deficient children,⁵⁻⁷ suggesting a role of GH in the cornea. GH exerts its effect by binding to pre-dimerized GH receptors (GHRs) located on cell plasma membranes, leading to activation of intracellular Janus-kinase 2 (JAK2).⁸ This kinase activates Signal Transducer and Activators of Transcription-5 (STAT-5), which, upon tyrosyl phosphorylation, dimerizes and enters the nucleus to regulate the transcription of GH-specific genes including insulin-like growth factor-1 (IGF-1).⁹ It is well established that GH induces the expression of IGF-1, which mediates the anabolic actions of GH on muscle and bone via endocrine, paracrine and/or autocrine actions. However, GH is also known to have actions independent from IGF-1.¹⁰⁻¹³ IGF-1 signals by binding to the membrane bound IGF-1 receptor (IGF-1R), which activates insulin receptor substrate (IRS)-1, leading to the intracellular cascade of phosphoinositide 3-kinase (PI3K)/Akt pathway activation.^{14,15} This pathway has also been demonstrated to play a key role in corneal healing.¹⁶⁻²⁰

Recombinant (r) HGH, which has been approved by the United States Food and Drug Administration to treat dwarfism and adult GH deficiency, is beneficial in the clinic and used off-label for epidermal wound healing associated with surgery and burns.²¹ rHGH has been shown to be effective in closing cutaneous defects of the epithelium, accelerating wound healing,²¹⁻²³ activating epidermal and epithelial cell migration in burn victims, and assisting wound healing in diabetic rats.^{24,25} However, it is not known whether these effects of GH are mediated by IGF-1, and conflicting data have been reported regarding the roles of GH versus IGF-1 on the mechanism of increased cutaneous healing. For example, GH was reported to enhance human keratinocyte migration, promote human foreskin fibroblast expression of IGF-1 and stimulate fibroblast proliferation *in vitro*.²⁶ However,

another study found that IGF-1, but not GH, promoted neonatal foreskin fibroblast proliferation.²⁷ To this day, no peer-reviewed publications have explored the possible effect of HGH on corneal wound healing. On the other hand, IGF-1 has been shown to successfully increase primary corneal epithelial cell migration *in vitro*,¹⁹ and promote cornea healing when used together with substance P in animal models as well as in the clinic in PCED patients.²⁸⁻³⁰ Consequently, rHGH is attractive as a possible agent to promote corneal epithelial wound healing as is IGF-1 and clinical studies are currently being completed to assess their role systemically for cutaneous epithelial wound healing ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00673309) identifier: NCT00673309). Clearly understanding the underlying mechanism of HGH and the involvement of IGF-1 mechanistically would be of significant value for various types of epithelial defects.

We hypothesize that HGH will activate phospho (p)-STAT5 signaling and promote corneal wound healing by enhancing corneal epithelial and fibroblast proliferation and/or migration. To test this hypothesis, we studied cell signaling, proliferation and migration using an immortalized human corneal epithelial cell line (abbreviated as epithelial cells) and primary human corneal fibroblasts (abbreviated as fibroblasts) *in vitro*. We also examined whether IGF-1 may be a mediator of HGH effects on the cornea.

Materials and Methods

Cell culture and hormonal treatment

Immortalized human corneal epithelial cells, developed, characterized and authenticated in Dr. James Jester's laboratory,³¹ were maintained 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 were cultured in DMEM/F12 (50:50; Corning Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) to allow differentiation and stratification. The EGF+BPE and serum-containing media are known to promote human corneal epithelial cell proliferation and differentiation, respectively.³² Primary human corneal fibroblasts were kindly provided by Dr. James Zieske (Boston, MA) and cultured in DMEM/F12 supplemented with 10% FBS. Recombinant human (rh) IGF-1 and HGH were purchased from the National Hormone and Peptide Program (Torrance, CA), dissolved in phosphate-buffered saline (PBS) and filter-sterilized. Human IGF-1R blocking monoclonal antibody and the corresponding mouse IgG1 isotype control were purchased from R&D Systems (Minneapolis, MN).

Immunoblotting

Cells were directly lysed in SDS Laemmli buffer (Bio-Rad, Hercules, CA) supplemented with 1% protease inhibitor cocktail, 5% β-mercaptoethanol and 20 µM sodium vanadate (all from Sigma-Aldrich, St. Louis, MO). Lysates were heated at 95°C for 10 minutes, separated by SDS-PAGE on 10% Tris-glycine precast gels (Life Technologies), and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Rabbit or mouse antibodies specific for p-Akt (Ser473), p-STAT5, β-actin (all from Cell Signaling Technology) and STAT5 (from Santa Cruz, Dallas, TX) were used. Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.01% Tween-20 (TBS/T). All primary

antibodies were diluted 1:1000 in blocking buffer except for p-Akt (1:4,000) and β -actin (1:10,000). HRP-conjugated secondary antibodies were goat anti-rabbit IgG and Fc-specific goat anti-mouse IgG diluted 1:5,000 (Sigma-Aldrich). Proteins were visualized with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL).

Scratch test

Human corneal epithelial cells were grown to confluence, and a scratch wound was made in the center of the well using a 10 μ l pipet tip, followed by phase contrast microscopy imaging at baseline and various time points up to 30 h post scratch. Scratch tests were performed in corneal epithelial cells grown alone or in co-culture with corneal fibroblasts with two co-culture methods: fibroblasts grown on 24 mm polyester membrane transwell inserts (0.4 μ m pore size; Corning Incorporated, Corning, NY) while epithelial cells were grown on 6-well plates; or confluent fibroblast layer with epithelial cells grown on top.

Cell proliferation

Epithelial cells and fibroblasts were cultured in 12-well plates, exposed to IGF-1 or GH for defined time periods, followed by cell trypsinization and counting using a hemocytometer.

Statistical analyses

One-way ANOVA, two-way ANOVA, Tukey test as a post-hoc test after ANOVA, Student's t-test and F test were performed when appropriate using Prism 5 software (GraphPad Software, Inc., La Jolla, CA). Error bars represent standard error of the mean (SEM) in all graphs.

Results

To determine whether HGH signaling is present in human corneal epithelial cells and fibroblasts, we treated both cells with various doses of HGH in their native media after overnight starvation (no growth factor supplements or serum). We found a dose-dependent increase of p-STAT5 in both cell types, with a maximum response to HGH at 10 nM (Figure 1 A and B). Therefore our subsequent experiments were carried out with this dose. To further study whether IGF-1 plays a role in these cells, we analyzed IGF-1 signaling p-Akt and found a maximum response at 10 nM IGF-1 in both cell types (Figure 1 C and D).

To test the hypothesis that HGH promotes corneal epithelial cell migration, we applied an *in vitro* scratch test on confluent epithelial cells, which were cultured together with fibroblasts in the serum-containing medium in a transwell system. We found that HGH significantly increased corneal epithelial cell migration, as compared to the vehicle-treated control (Figure 2 A and B). In contrast, HGH did not facilitate migration when corneal epithelial cells were cultured alone (Figure 2 C and D). This indicates that real-time communication between epithelial cells and fibroblasts is required for the HGH stimulation of wound healing. Further, in the transwell system, the epithelial cells and fibroblasts were not in physical contact, suggesting that soluble factor(s) may be mediating the effect of HGH. We hypothesized that IGF-1 may be such a factor. To test this, we cultured epithelial cells on top of a confluent layer of fibroblasts in KSFM, and treated cells with HGH and/or IGF-1 in

scratch tests. We found that while HGH significantly increased migration, IGF-1 did not have such an effect (Figure 3). To further test the possible effect of IGF-1 on migration, we pretreated cells with an IGF-1R blocking antibody or corresponding IgG1 control in this co-culture system. While IGF-1R antibody markedly diminished IGF-1-induced p-Akt signal (Figure 4 A), it did not have much effect on HGH-induced faster migration in scratch tests (Figure 4 B). This showed that the HGH effect on scratch healing was not mediated by IGF-1, and that IGF-1 did not modulate corneal epithelial cell migration.

To test whether HGH or IGF-1 promotes cell proliferation, we treated corneal epithelial cells and fibroblasts with HGH and/or IGF-1 for 2 days and found no effect on cell proliferation of either hormone in either cell type (Figure 5).

Discussion

Delays in corneal epithelial wound closure as occur in PCED can be due to a variety of acute and chronic conditions such as herpetic infection, exposure keratopathy, neurotrophic keratopathy, limbal stem cell deficiency, diabetes, trauma, surgery, and severe dry eye.³³ Development of PCED can lead to corneal ulceration, scarring, perforation, and loss of vision and thus a tremendous need exists to attempt to heal the cornea in a timely manner. Although general methods exist to treat PCED include patching, lubricants, ointments, and even in severe cases amniotic membrane, corneal grafting and lid tarsorrhaphy, no approved pharmacotherapy exists.³³ One successful therapy to date appears to be the use of topical autologous serum. However, this therapy, although effective, is not easy to acquire, process, and dose.^{34,35} The fact that human serum contains such proteins as GH and IGF-1 may help explain why this therapy has been successful. Agents that can accelerate wound closure by increasing the migration and/or proliferation of corneal epithelial cells are of great importance because of their potential benefit for patients with acute and/or chronic epithelial damage. In this report, we explored the efficacy of HGH, a well-studied and naturally occurring anabolic protein, in corneal epithelial wound healing both *in vitro* and *in vivo*. We found that HGH activated the STAT5 signaling pathway in human corneal epithelial cells and human corneal fibroblasts and promoted epithelial cell migration in co-culture models of these two cell types. These effects were not mediated by IGF-1 but perhaps by other soluble factor(s).

Our data that HGH enhanced corneal epithelial wound healing, most likely via increasing cell migration and not cell proliferation, are consistent with a report in which GH promotes skin keratinocyte migration.²⁶ We showed that HGH enhanced corneal epithelial healing *in vitro*, and required co-culture of corneal epithelial cells and fibroblasts. Inconsistent results were seen when corneal epithelial cells were cultured alone, or treated with conditioned media from fibroblasts exposed to HGH. This indicates that HGH does not act on corneal epithelial cells directly to stimulate migration, but rather acts through an intact epithelial-fibroblast communication system. This duplicates the situation *in vivo*, where epithelia and stromal keratocytes (fibroblasts) coexist. Our findings may help to explain why rHGH promoted corneal wound healing using a rabbit cornea debridement model in a pilot study [Rafii MJ, et al., IOVS 2013; 5048: ARVO E-Abstract B0006].

A well-known mediator of HGH action is IGF-1. However, we found that IGF-1 did not have a significant effect in the scratch test. Further, blocking IGF action using an IGF-1R antibody did not affect the ability of HGH to promote wound closure. This is consistent with a previous study demonstrating that IGF-1 promotes cornea epithelial migration when used together with substance P but not alone. These observations were made in an organ culture, in rabbits, and in humans with PCED.^{30, 36-38} The signals mediating GH action on the corneal epithelial cell – fibroblast communication remain to be elucidated.

We found that neither HGH nor IGF-1 has an effect on corneal epithelial or fibroblast proliferation *in vitro*. This is analogous to a report showing no effect of IGF-1 on cornea epithelial cell proliferation in the organ culture,³⁶ but different from literature where GH promotes human foreskin fibroblast proliferation via increasing IGF-1.^{26, 27} These differences may be due to the type of epithelia and fibroblasts (cornea versus skin) used.

Given the lack of IGF-1 influence on human corneal epithelial and fibroblast proliferation and/or migration, we ask the question does IGF-1 have an effect on corneal wound healing? IGF-1R has been identified in human corneal and conjunctival cells,³⁹ and we have shown p-Akt signal in response to IGF-1 in both corneal epithelial cells and corneal fibroblasts. Clearly IGF-1 acts on the cornea. IGF-1 promotes corneal limbal stem cell differentiation into epithelial like cells in a mouse model of mechanical corneal damage.⁴⁰ Also, when combined with substance P, IGF-1 promotes corneal epithelial migration and has shown efficacy in treating patients with PCED.^{30, 36, 38} Therefore the role of IGF-1 may be in differentiation and depend on other relevant factors and cellular interactions for corneal epithelial migration.

Conclusion

We show that HGH activates STAT5 signaling and promotes corneal epithelial cell migration *in vitro*. The migratory effect requires an intact communication between corneal epithelia and fibroblasts, and is not mediated by IGF-1. HGH therefore may represent a topical therapeutic to promote corneal epithelial wound healing, an area of unmet medical need, which warrants further investigation.

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Figure 1.

GH and IGF-1 signaling in corneal epithelial cells and fibroblasts. GH activates p-STAT5 in corneal epithelial cells (A) and fibroblasts (B), and IGF-1 activates p-Akt in corneal epithelia (C) and fibroblasts (D), dose-dependently. A, corneal epithelial cells were cultured in medium containing 10% FBS for 5-7 days, or in KSFM supplemented with EGF/BPE for 2 days, then starved (serum or supplement withdraw) overnight and treated with HGH of increasing doses for 15 min, followed by cell lysis and immunoblotting. B, corneal fibroblasts were cultured in medium containing 10% FBS for 2 days, starved overnight and treated with HGH for 15 min. C, corneal epithelial cells were cultured in medium containing 10% FBS for 5-7 days, then starved overnight and treated with IGF-1 for 15 min. D, corneal fibroblasts were cultured in medium containing 10% FBS for 2 days, starved overnight and treated with IGF-1 for 15 min.

Figure 2.

GH promotes cornea epithelial cell migration in scratch tests when epithelial cells are co-cultured with fibroblasts but not alone *in vitro*. (A) and (B), human corneal epithelial cells were co-cultured with human corneal fibroblasts in 6-well transwell plates (fibroblasts on the transwell insert and epithelia on the well below) for 5 days in serum-containing medium, pretreated with 10 nM HGH for an additional day, before a mechanical scratch was made and observed after 30 hours. Blue dotted line shows the edge of the scratched wound at baseline, and red line shows the edge of the migrated epithelial cells after 30 hours (A). Quantification was shown in (B). In contrast, when corneal epithelial cells were cultured alone, either in KSFM as shown in (C), or serum-containing medium (not shown), no significant effect of HGH was seen (D). The migrated distance was quantified using Image J and data were analyzed with Student's t test, unpaired, two-tailed. These experiments were performed in triplicates and repeated at least three times with similar results, with one representative experiment shown.

Figure 3.

GH, but not IGF-1, promotes corneal epithelial cell migration in epithelial cells co-cultured with fibroblasts *in vitro*. Epithelial cells were cultured on top of confluent corneal fibroblasts in KSFM supplemented with EGF/BPE. After reaching confluence, the medium was switched to KSFM with 10 nM GH and/or IGF-1 for an additional day, and mechanical scratches were made. Images were taken at baseline and 6 h after the scratch (A). The migrated distance was quantified using Image J and data were analyzed with one-way ANOVA and Tukey's post-hoc test (B). This experiment was performed in triplicates and repeated three times with similar results, with one representative experiment shown. *p<0.05 compared to control; **p<0.01 compared to control.

Figure 4.

IGF-1 did not mediate GH effect on corneal epithelial cell migration *in vitro*. A, IGF-1R antibody blocked IGF-1-induced p-Akt signaling in a dose-dependent manner. Corneal epithelial cells were cultured in KSFM/EGF/BPE (2 d) or serum-containing media (5 d), followed by overnight starvation and with IgG1 control or IGF-1R antibody, before treatment with 10 nM IGF-1 for 15 min. B, Epithelial cells were cultured on top of confluent corneal fibroblasts in KSFM/EGF/BPE. After reaching confluence, the medium was switched to KSFM with 10 nM GH and/or IGF-1 and 10 nM IgG1 or IGF-1R antibody for an additional day, and mechanical scratches were made. Scratch healing after 6 hours was quantified using ImageJ and data were analyzed as following: Two-way ANOVA revealed a significant effect of GH, IGF-1 or GH+IGF-1 treatment, but not of IGF-1 receptor antibody versus IgG1 control; the data of the IGF-1 receptor antibody versus IgG1 control were then pooled and subjected to one-way ANOVA with Tukey's post hoc test, which showed significant difference between GH versus control and GH+IGF-1 group versus control. *p<0.05 compared to control. This experiment was performed in triplicates and repeated twice with similar results, with one representative experiment shown.

Figure 5.

Neither GH nor IGF-1 has an effect on the proliferation of corneal epithelial cells or fibroblasts. Corneal epithelial cell number was not changed by GH of various doses (A), or by 1 nM GH and/or 10 nM IGF-1 (B) in KSFM after 2 d. Corneal fibroblast number was not altered by 10 nM GH and/or IGF-1 in either serum-free (C) or serum-containing medium (D) after 2 d. One-way ANOVA revealed no significant ($p > 0.05$) effect in any of the experiments. This experiment was performed in triplicates and repeated three times with similar results, with one representative experiment shown.