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Research Article

The Effect of Human Growth Hormone on Corneal Wound Healing in Mice

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Abstract

Purpose: We hypothesize that HGH promotes corneal wound healing by facilitating corneal epithelial migration, and that HGH induces IGF-1 expression in corneal epithelial cells and corneal fibroblasts.

Methods: Male wild type C57BL/6J mice of 3 months of age were subjected to 1.5 mm central corneal epithelial debridement in both eyes, with one eye treated with HGH dissolved in carboxymethylcellulose (vehicle) and the fellow eye with vehicle. Corneal fluorescein imaging was taken at various time points post debridement to monitor re-epithelialization. Physiological (10 nM) and pharmacological (6.7 μM) doses of HGH were tested in separate experiments. Human corneal epithelial cells (HCECs) and human corneal fibroblasts (HCFs) were treated with 10 nM HGH, and IGF-1 was assessed from whole cell lysates via Western Blot.

Results and Conclusion: We found that HGH of both doses were well-tolerated in these mice without adverse reactions. However, we found no significant difference in epithelial wound healing rate in HGH treated vs control eyes. Further, we found that HCECs and HCFs did not produce detectable levels of IGF-1 with or without HGH treatment *in vitro*. Future study will focus on dose- and time-dependent studies in females.

Keywords: *Growth Hormone; Cornea; Corneal Wound Healing; Corneal Epithelium; Hormone; Growth Factor*

Abbreviations

GH: Growth Hormone; HGH: Human Growth Hormone; (r) HGH: Recombinant Human Growth Hormone; IGF-1: Insulin-Like Growth Factor-1; STAT-5: Signal Transducer and Activator of Transcription 5; HCECs: Human Corneal Epithelial Cells; iHCECs: Immortalized Human Corneal Epithelial Cells; HCFs: Human Corneal Fibroblasts; CEDs: Corneal Epithelial Defects; PBS: Phosphate-Buffered Saline; BSA: Bovine Serum Albumin; CMC: Carboxymethylcellulose; KSFM: Keratinocyte Serum-Free Medium; EGF: Epidermal Growth Factor; BPE: Bovine Pituitary Extract; DMEM/F12: Dulbecco's Modified Eagle's Medium and Ham's F-12; FBS: Fetal Bovine Serum; PVDF: Polyvinylidene Difluoride; EGF: Epidermal Growth Factor; TGF-β: Transforming Growth Factor-β; HGF: Hepatocyte Growth Factor

Introduction

Growth hormone (GH) is a pituitary hormone belonging to the somatotropin/prolactin family, and is required for normal human growth and development. Growth hormone stimulates the secretion of another hormone called insulin-like growth factor (IGF)-1, which

mediates many of GH's activity via endocrine, paracrine and/or autocrine actions. Together the GH/IGF-1 axis is a major determinant of longitudinal growth. GH and IGF-1 are also known to promote cell survival and proliferation [1,2].

Human GH (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 [3-5], suggesting a role of GH in the cornea. 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 [6]. Recombinant HGH has been shown to be effective in closing cutaneous defects of the epithelium, accelerating wound healing [6-8], activating epidermal and epithelial cell migration in burn victims, and assisting wound healing in diabetic rats [9,10].

Currently there lacks an effective product to promote the healing of corneal epithelial defects (CEDs) [11]. Whether caused by injury or ocular diseases (e.g. severe dry eye, diabetes and herpes), CEDs can persist for weeks to months, be recalcitrant to treatment, and may lead to corneal ulcers and endophthalmitis, corneal scarring and opacification, and significant vision loss. Patients suffering corneal damage would greatly benefit from safe and effective pharmaco-therapeutic agents that enhance the healing of the cornea.

We hypothesize that HGH promotes corneal wound healing and that it will do so via inducing local IGF-1 expression. To this day, very few peer-reviewed publications have explored the possible effect of HGH on corneal wound healing *in vivo*, with the exception of a preliminary study showing rHGH to promote corneal wound healing in a rabbit cornea debridement model [11].

To understand the molecular and cellular mechanism of this potential beneficial effect of HGH in corneal wound healing, we previously performed cell culture studies and discovered that HGH activates STAT-5 signaling and promotes human corneal epithelial cell migration but not proliferation *in vitro* [12]. We also determined that the migratory effect requires an intact communication between corneal epithelia and fibroblasts, and is not mediated by IGF-1. Given these data *in vitro*, we embarked on testing the hypothesis that HGH will promote corneal wound healing possibly via inducing IGF-1 *in vivo*.

Materials and Methods

Preparation of rHGH eye drop

rHGH (National Hormone and Peptide Program, Torrance, CA, USA) was dissolved in sterile phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) to prepare 10 μM stocks, which were stored at -20-degree Celsius. rHGH stocks were further diluted in 0.5% carboxymethylcellulose sodium (CMC, vehicle) eye drops (Allergan, Irvine, CA, USA) to 10 nM and 6.7 μM respectively on the day of corneal wounding. Diluted eye drops were stored at 4-degree Celsius for use in the study.

Animal treatment

Three-month old C57BL/6J male mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Schepens Eye Research Institute Animal Care and Use Committee.

Mice were anesthetized with an intraperitoneal injection of a Ketamine (120 mg/kg) /Xylazine (20 mg/kg) mixture. One drop of 0.5% Proparacaine eye drop (Bausch and Lomb Inc., Tampa, FL, USA) and one drop of 0.5% phenylephrine hydrochloride (Altaire Pharmaceuticals, New York, NY, USA) were applied once respectively with an interval of 5 minutes. A 1.5 mm diameter epithelial wound was made in both eyes by demarcating an area on the central cornea with a trephine and removing the epithelium within the circle with a rotating burr (Alger brushII, Alger equipment Co., Lago Vista, TX, USA) This procedure left an intact basement membrane [13]. A pain-relieving medication was provided by subcutaneously injecting Meloxicam (5-10 mg/kg, Norbrook Laboratories Limited, Newry, North Ireland, UK) immediately after the surgery and 24 hours later.

Corneal fluorescein staining was performed by placing 1µl of 0.125% sodium fluorescein dissolved in sterile saline on the ocular surface of both eyes. Corneal defects were evaluated by a slit lamp biomicroscopy (Topcon SL-D4, Tokyo, Japan), photographed and recorded. 5µl 10nM or 6.7 µM rHGH eye drop (in separate experiments) was applied to the right eye and 5 µl vehicle was administrated as a control treatment to the left eye. 5 minutes after rHGH instillation, neomycin/polymyxin B sulfates/bacitracin zinc ophthalmic ointment (Putney Inc., Portland, ME, USA) was applied. Corneal fluorescein imaging was taken at various time points post debridement, and was quantified with Image J (downloadable at (http://rsb.info.nih.gov/ij/download.html). rHGH eye drop and antibiotic ointment were administrated three times a day throughout the study period.

Cell culture

Immortalized human corneal epithelial cells (iHCECs), a gift from Dr. James Jester (University of California Irvine), were cultured in keratinocyte serum-free medium (KSFM; Thermo-Fisher Scientific, Grand Island, NY, USA) supplemented with 5 ng/ml epidermal growth factor (EGF; Thermo-Fisher Scientific) and 50 µg/ml bovine pituitary extract (BPE; Thermo-Fisher Scientific), as previously described [12]. Primary human corneal fibroblasts (HCFs), a gift from Dr. James Zieske (Schepens Eye Research Institute, Boston) were grown in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F12; Mediatech, Inc., Manassas, VA, USA) containing 10% fetal bovine serum (FBS; Thermo-Fisher Scientific). Co-culture of iHCECs with HCFs were maintained in DMEM/F12 containing 10% FBS for 5 days similarly described previously [12]. All these cells were then treated with or without 10 nM rHGH for 3 days.

SDS-PAGE and immunoblotting

Whole cell lysates were used for Western Blot analyses, and human recombinant IGF-1 (National Hormone and Peptide Program) was used as a positive control for Western Blotting. Briefly, cells were lysed in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA) supplemented with 1% protease inhibitor cocktail, 200 µM sodium orthovanadate, and 5% β-mercaptoethanol (all from Sigma-Aldrich, St. Louis, MO, USA), followed by denaturation at 95^{0}C for 10 minutes. Lysates were run on 4-20% Tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with Tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, probed with antibody against IGF-1 (1:1000; R and D Systems, Minneapolis, MN, USA) or β-actin (1:10000, Cell Signaling Technology) as a loading control, and incubated with the required horseradish peroxidase-conjugate secondary antibody (1:5000, Cell Signaling Technology). Visualization of proteins was accomplished using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

Statistical Analyses

Student's t-test was performed within each time point using commercially available software (Prism 5; GraphPad Software, Inc).

Results and Discussion

Results

To determine the effect of rHGH on corneal wound healing, we established a mouse model of corneal epithelium debridement *in vivo*, treated mice with two different doses of rHGH in separate experiments, and evaluated their corneal re-epithelialization (Figure 1). We found that rHGH of both doses were well-tolerated in these mice without adverse reactions. As shown in figure 1D, an average of 97.1% and 20.3% CEDs remained in control eyes 6h and 24h post debridement respectively. A more detailed result showed an average of 97.2%, 94.2%, 79.5%, 52.0%, 27.7% and 9.0% CEDs in control eyes at 4h, 8h, 12h, 18h, 24h and 48h post debridement respectively (Figure 1E). There was no significant difference in epithelial wound healing rate in HGH treated vs control eyes at any of the time points tested. Complete re-epithelialization was observed between 24 and 48 hours in both conditions.

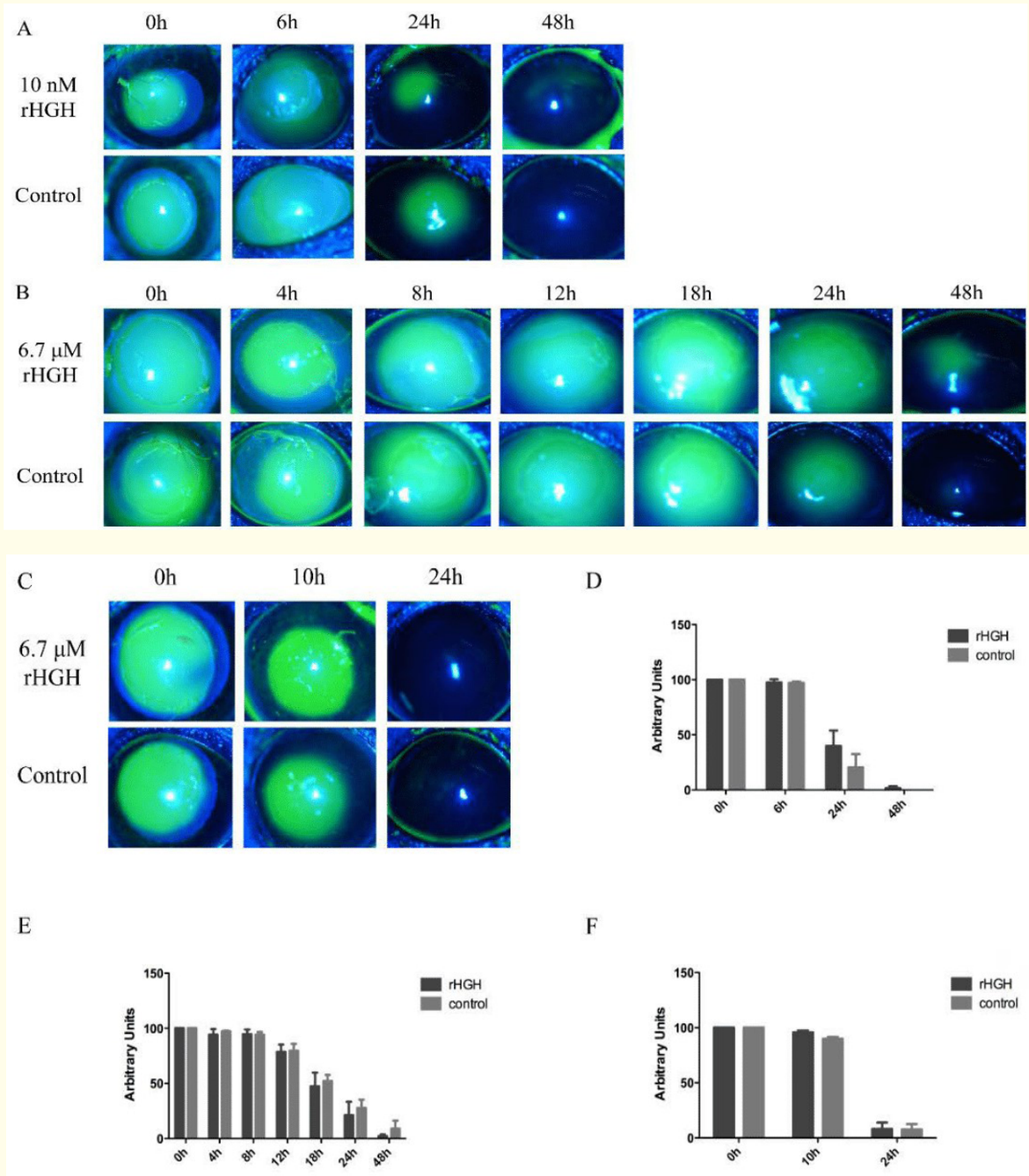

Figure 1: *Corneal fluorescein staining and quantitation of epithelial defect area following debridement in mice. A, B and C show corneal fluorescein staining following epithelial debridement treated with physiological (10 nM) and pharmacological dose (6.7 µM) rHGH at various time points in mice. D, E and F are the graphic representation of the defect areas. Each mouse had both corneas debrided, with GH treatment in one eye and vehicle control in the other eye. No significant difference was found between HGH treated and control eyes at any time point. N = 3 mice were used for each experiment.*

To test whether rHGH plays a role in corneal wound healing via IGF-1, iHCECs and HCFs were seeded separately or co-cultured in the presence or absence of rHGH. As demonstrated in figure 2, iHCECs and HCFs did not produce detectable levels of IGF-1 with or without rHGH treatment *in vitro*, while a rIGF-1 positive control band was present.

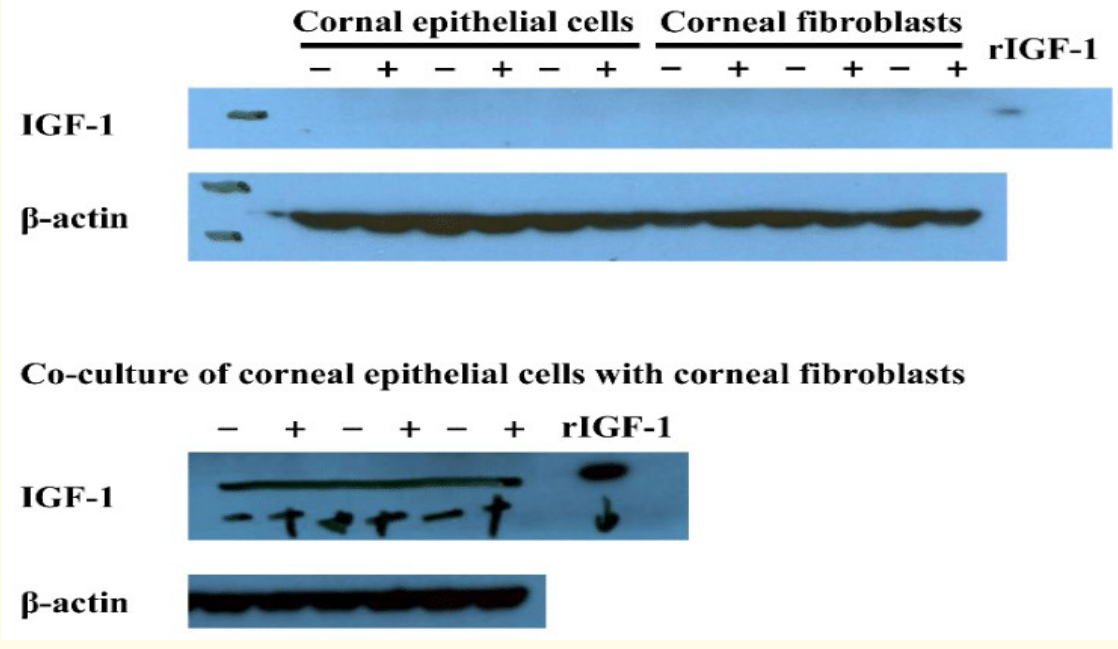

Figure 2: *Immortalized human corneal epithelial cells (passage 67, cultured in KSFM/EGF/BPE medium) and primary human corneal fibroblasts (passage 4, cultured in DMEM/F12+ 10% FBS medium) were seeded separately or co-cultured for 5 days, and then treated with or without rHGH for 3 days. Whole cell lysates were used for Western Blot analyses, and rIGF-1 was used as a positive control for Western Blotting. While a rIGF-1 positive control band was present, no evidence of IGF-1 was detected in corneal epithelial cells and/or fibroblasts with or without growth hormone treatment. Experiments were performed in triplicates. One representative result of 3 total experiments is shown.*

Discussion

Corneal epithelial defects are a common ocular disorder, which may be due to trauma, ocular surface disease, systemic disease or surgical intervention. Mild CEDs usually undergo quick spontaneous recovery without complications. However, corneal wound healing may be retarded by dry eye, lid malfunction, inflammation, infection, and/or damage to corneal nerves, resulting in persistent CEDs. Persistent CEDs may lead to corneal ulceration, scarring, opacification, neovascularization, and, ultimately, visual compromise and loss of vision [14].

During an acute injury, corneal epithelium responds rapidly to many growth factors released into the injury sites. These growth factors, including epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF), come from corneal epithelium and stromal fibroblasts [15,16] and the tear film [17], facilitating corneal re-epithelialization in a relatively short time. It is not known whether GH or IGF-1 are released in a similar manner like other growth factors when a corneal injury occurs. This may be a subject of future study.

Corneal wound healing is a complex processes involving epithelial proliferation and migration and interaction of the epithelium and stroma [18]. We expected rHGH to promote corneal wound healing in the mouse model based on previous studies using rabbits and co-culture of epithelia and fibroblasts *in vitro*, the latter of which shows that rHGH facilitates migration of iHCECs [12]. However, the differences in corneal epithelial wound healing rate between HGH treated eyes and control eyes were not significant at any of the time points tested in the current study.

Production of IGF-1 is stimulated by GH in several target tissues, including the liver, muscle and bone, and the GH/IGF-1 axis acts on these tissues to promote growth and regulate metabolism. A role of GH/IGF-1 on the cornea is not known. We did not detect IGF-1 with or without HGH treatment in HCECs or HCFs or co-cultured cells. This indicates that under the current cell culture system, GH does not stimulate IGF-1 expression, unlike in some of the other tissues.

Conclusion

In summary, we have shown that HGH is well tolerated on the ocular surface in mice, but under the conditions tested, we did not find a significant effect of HGH on corneal epithelial healing, and it appeared that HGH did not stimulate IGF-1 production in human corneal cell lines *in vitro*. Dose- and time-dependent studies in females are needed before ruling out a positive effect of HGH on corneal wound healing *in vivo*. Further, the current study tested normal corneal epithelial wound healing, which is a fast process. It is possible that GH may help in cases where a persistent corneal epithelial wound defect happens, which is much slower and often recurrent. This also needs to be studied in future.

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Conflict of Interest

There are no conflicts of interest with regard to this manuscript.

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