HGF Signaling Impacts Severity of *Pseudomonas aeruginosa* Keratitis

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Submitted: December 10, 2013
Accepted: March 1, 2014

Citation: Jiang X, McClellan SA, Barrett R, Foldenauer M, Hazlett LD. HGF signaling impacts severity of *Pseudomonas aeruginosa* keratitis. *Invest Ophthalmol Vis Sci*. 2014;55:2180–2190. DOI:10.1167/iovs.13-13743

Purpose. To determine whether rapamycin altered corneal growth factor levels to impact severity of *Pseudomonas aeruginosa* keratitis.

Methods. BALB/c mice were injected intraperitoneally with rapamycin or PBS and infected with *P. aeruginosa*. Corneas were harvested and mRNA levels of growth factors (EGF, HGF, FGF-7/KGF), receptors (EGFR, c-met, FGFR-2), and signaling molecules (PI3K, Akt, S6K1, and IGF-1R) tested. ELISA determined HGF/c-met, IGF-1, and Substance P (SP) protein levels. Corneal application of recombinant (r)HGF was assessed by clinical score, photography with a slit lamp, real-time RT-PCR (mRNA for mT0R, IL-10, IL-12, IL-18, PI3KCα, Akt), and ELISA (total and phosphorylated [p]c-met); rIGF-1 effects also were tested by ELISA. In vitro, RAW cells and peritoneal macrophages were stimulated with LPS ± rHGF ± c-met inhibitor (CI) and mTOR mRNA levels tested.

Results. Rapamycin disparately regulated infected corneal mRNA levels of EGF/EGFR and FGF-7/FGFR-2, but HGF/c-met mRNA levels both increased. ELISA confirmed elevated HGF protein. Rapamycin did not change PI3KCα or Akt signaling molecule expression, downregulated S6K1, but upregulated IGF-1R mRNA levels; IGF-1 and SP proteins also were upregulated. After infection, topical rHGF versus PBS increased mRNA levels of IL-12p40, IL-18, PI3KCα, and Akt; mTOR and IL-10 mRNA were downregulated; rIGF-1 increased HGF protein. In vitro, rHGF and LPS lowered RAW cell and macrophage mTOR levels; CI addition restored them.

Conclusions. Collectively, these data provide evidence that enhanced corneal HGF levels increase signaling through the c-met receptor, decrease mTOR levels, and enhance proinflammatory cytokines, while decreasing anti-inflammatory cytokines, and that HGF signaling is central to disease outcome.

Keywords: bacteria, keratitis, HGF, c-met, IGF-1R, mice

Bacterial keratitis is associated with contact lens wear and has significant medical and financial impact, with approximately 25,000 to 30,000 cases occurring annually in the United States.[1] A gram-negative bacterium, *Pseudomonas aeruginosa*, is one of the most common keratitis-inducing microbial isolates.[2] Among numerous virulence factors, it produces endotoxin (i.e., lipopolysaccharide [LPS]) that elicits an acute inflammatory response in the cornea that can contribute to eradication of the bacterium, but unless precisely regulated, adverse events, such as stromal destruction and loss of vision, are common outcomes.

The innate immune response in the cornea has received considerable attention, with experimental infection studies focusing on infiltration of leukocytes (e.g., polymorphonuclear leukocytes [PMNs], macrophages, dendritic cells),[3] production of pro- and anti-inflammatory cytokines,[3] and the interplay of cellular apoptosis versus necrosis.[4] Studies have provided consistent evidence that a key regulatory molecule associated with better disease outcome in murine models of *P. aeruginosa* keratitis is the anti-inflammatory cytokine IL-10.[5,6]

Transcription of the IL-10 gene is partially under the control of the central regulator of protein synthesis and cell growth kinase: mammalian target of rapamycin (mTOR).[7–9] mTOR is a downstream effector of the PI3K/Akt pathway that is used by a variety of ligands, including nutrients, cellular stress, and growth factors.[10] Previous work by our laboratory provided evidence that in the infected cornea, IL-10 balances proinflammatory cytokines, such as IFN-γ, in BALB/c (Th2-responsive, -resistant) mice, leading to better disease outcome than in the Th1/Th17-responsive B6 susceptible mouse in which corneal perforation is common.[11–13] Moreover, we recently determined that, in BALB/c mice, inhibition of mTOR by the macrolide antibiotic, rapamycin, diminishes levels of IL-10, leading to a cascade of events, including upregulation of proinflammatory cytokines, such as IL-12 and IL-23, that worsen bacterial keratitis.[14]

Another effect of rapamycin treatment is increased transcription of preprotachykinin-A, the precursor of active Substance P (SP).[14] In this regard, our laboratory has characterized the role of SP, a proinflammatory neuropeptide, in both B6 and BALB/c mouse models of disease.[6,15] In a recent study, SP regulation of growth factors was examined after infection,[16] as others have reported that they are essential in tissue repair and have healing properties when administered exogenously in noninfectious wound or trauma models.[17,18] That study[16] revealed that SP injection had a localized effect and increased growth factors, such as hepatocyte growth factor (HGF), in both the normal and infected cornea. Additionally,

Table. Nucleotide Sequences of Mouse Primers for Real-Time RT-PCR

Gene	GenBank No.	Primer Sequence, 5′–3′	Size, bp
β-Actin	NM_007393.3	F - GAT TAC TGC TCT GGC TCC TAG C R - GAC TCA TCG TAC TCC TGC TTG C	147
Akt	NM_001165894.1 NM_009652.3	F - AGA AGA GAC GAT GGA CTT CCG R - TCA AAC TCG TTC ATG GTC ACA C	111
c-met	NM_008591.2	F - GTG AAC ATG AAG TAT CAG CTC CC R - TGT AGT TTG TGG CTC CGA GAT	100
EGF	NM_010113.3	F - ACG GTT TGC CTC TTT TCC TT R - GTT CCA AGC GTT CCT GAG AG	130
EGFR	NM_007912.4 NM_207655.2	F - GTG GAG GGA CAT CGT CCA AA R - ATT GGG ACA GCT TGG ATC ACA T	100
FGF-7	NM_008008.4	F - AAC AGC TAC AAC ATC ATG GAA ATC AG R - AAT CAG TTC TTT GAA GTT GCA ATC CT	153
FGFR2	NM_010207.2	F - GGT ACT TCA TGG TGA ATG TCA CA R - CTC TGG TTG CTC CTG TTC TCA	103
HGF	NM_010427.4	F - ACT TCT GCC GGT CCT GTT G R - GGG ATG GCG ACA TGA AGC A	66
IL-10	NM_010548.2	F - TGC TAA CCG ACT CCT TAA TGC AGG AC R - CCT TGA TTT CTG GGC CAT GCT TCT C	126
IL-12p40	NM_008352.2	F - GGT CAC ACT GGA CCA AAG GGA CTA TG R - ATT CTG CTG CCG TGC TTC CAA C	121
IL-18	NM_008360.1	F - GCC ATG TCA GAA GAC TCT TGC GTC R - GTA CAG TGA AGT CGG CCA AAG TTG TC	122
mTOR	NM_020009.2	F - ACC GGC ACA CAT TTG AAG AAG R - CTC GTT GAG GAT CAG CAA GG	110
PI3KCα	NM_008839.2	F - ATC ATG CAA ATC CAG TGC AA R - CAG CTG TCC GTC ATC TTT CA	219
S6K1	NM_001114334.1	F - GGG GCT ATG GAA AGG TTT TTC A R - CGT GTC CTT AGC ATT CCT CAC T	113
IGF-1R	NM_010513.2	F - GTG GGG GCT CGT GTT TCT C R - GAT CAC CGT GCA GTT TTC CA	127

this effect was overwhelmed by a proinflammatory cytokine response, leading to increased stromal destruction, higher bacterial plate counts, and a decrease in M2 arginase-producing cells (critical to disease resolution through production of anti-inflammatory mediators, such as IL-10). These data, based on experimental modeling, led to the conclusion that treatment with SP to hasten wound closure was contraindicated clinically in the presence of infection.[16]

In the current work, we examined the effects of rapamycin inhibition of mTOR on expression levels of growth factors, their receptors, and signaling molecules in the *P. aeruginosa*–infected cornea. Data provide evidence that of the growth factors and receptors tested, only HGF/c-met were similarly elevated. Furthermore, rapamycin downregulated mRNA for S6K1, an important downstream signaling molecule of mTOR, and upregulated insulin-like growth factor-1 receptor (IGF-1R), and protein for both IGF-1 and SP; and rIGF-1 injection also was sufficient to upregulate HGF protein. Topical rHGF treatment provided further confirmatory evidence that enhancement of HGF levels in infected cornea resulted in increased signaling through the c-met receptor to decrease mTOR levels, enhance proinflammatory cytokines and decrease anti-inflammatory cytokines, and that HGF signaling is central to disease outcome.

Materials and Methods

Infection of Mice

Eight-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized with ether, placed beneath a stereoscopic microscope (Stereomaster; Fisher Scientific, Asheville, NC, USA), and the cornea of the left eye wounded (three 1-mm incisions) using a sterile $25^{5/8}$-gauge needle. A 5-μL aliquot containing 1×10^6 CFU/μL of *P. aeruginosa,* strain 19660 (American Type Culture Collection, ATCC, Manassas, VA, USA), prepared as described before,[5] was topically applied. Eyes were examined at 1 day postinfection (p.i.) and/or at times described below, to ensure mice were infected and to monitor disease. Animals were treated humanely and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal Response to Infection

Disease was graded using an established scale[19]: 0, clear or slight opacity, partially or fully covering the pupil; +1, slight opacity, covering the anterior segment; +2, dense opacity, partially or fully covering the pupil; +3, dense opacity, covering the anterior segment; and +4, corneal perforation. A clinical score was recorded for each mouse after infection (1, 3, and 5 days p.i.) for statistical comparison of disease severity.

Rapamycin Treatment

Rapamycin (LC Laboratories, Woburn, MA, USA) was prepared to a concentration of 20 μg/μL in 100% ethanol and stored at −20°C. Before intraperitoneal (IP) injection, the rapamycin in ethanol was diluted in sterile PBS. BALB/c mice ($n = 5$/group/time/assay) were anesthetized with ether and IP injected with 100 μL rapamycin (3.0 mg/kg)[7,14] or sterile PBS (Mediatech, Manassas, VA, USA) on the day before infection (day = −1) and each day through 5 days p.i.

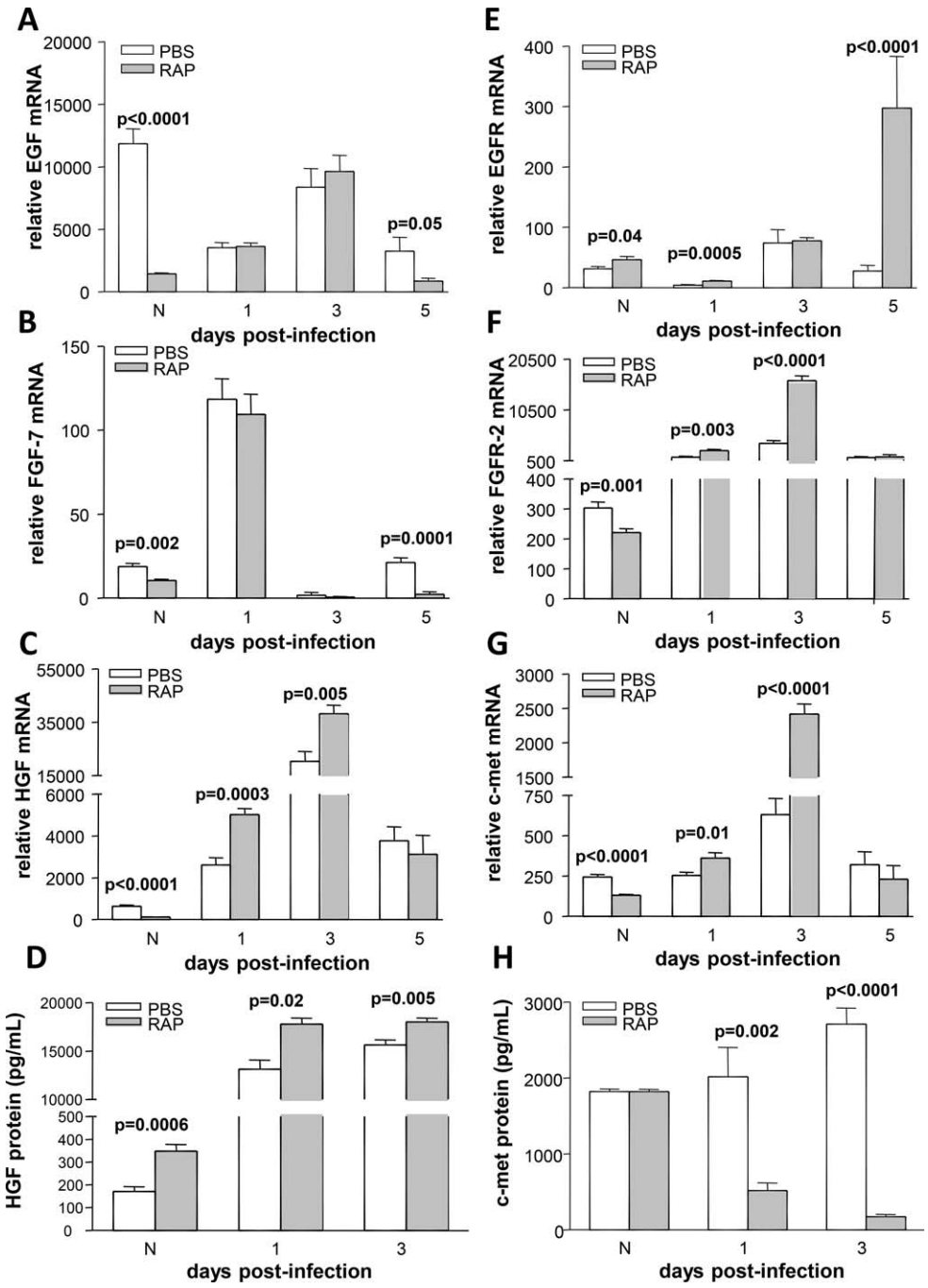

Figure 1. Effects of rapamycin treatment on growth factor and growth factor receptor mRNA. After rapamycin treatment, EGF mRNA levels (**A**) were significantly downregulated in the normal cornea and at 5 days p.i. In contrast, EGFR mRNA (**E**) was significantly upregulated in the rapamycin-treated normal cornea and at 1 and 5 days p.i. Rapamycin versus PBS treatment significantly decreased FGF-7 mRNA (**B**) in the normal cornea and at 5 days p.i. Although FGFR-2 mRNA (**F**) was significantly decreased in the normal cornea, elevated levels were detected at 1 and 3 days p.i. HGF mRNA (**C**) and its receptor, c-met (**G**), were significantly downregulated after rapamycin treatment in the normal cornea, but both were elevated significantly at 1 and 3 days p.i. Rapamycin treatment significantly increased HGF protein levels (**G**) in both the normal cornea and at 1 and 3 days p.i. Protein levels of total c-met (**H**), the HGF receptor, were decreased at 1 and 3 days p.i. and not different in the normal cornea.

rHGF Treatment

rHGF (R&D Systems, Minneapolis, MN, USA) was reconstituted in PBS to a final concentration of 40 μg/mL.[20] After infection, 5 μL of the growth factor (0.2 μg/mL) was applied topically to the cornea of BALB/c mice on the day of infection, twice on 1 day p.i. and once daily through 4 days p.i. Controls received 5 μL PBS similarly. Corneas were harvested at 1 and 5 days p.i. for real-time RT-PCR or ELISA assays for c-met and phosphorylated c-met (p-c-met).

rIGF-1 Treatment

Recombinant protein (R&D Systems) (1 μg/5 μL) or PBS (5 μL) was given subconjunctivally the day before infection. On days 1 and 3 p.i., an additional 1 μg (100 μL) was injected IP;

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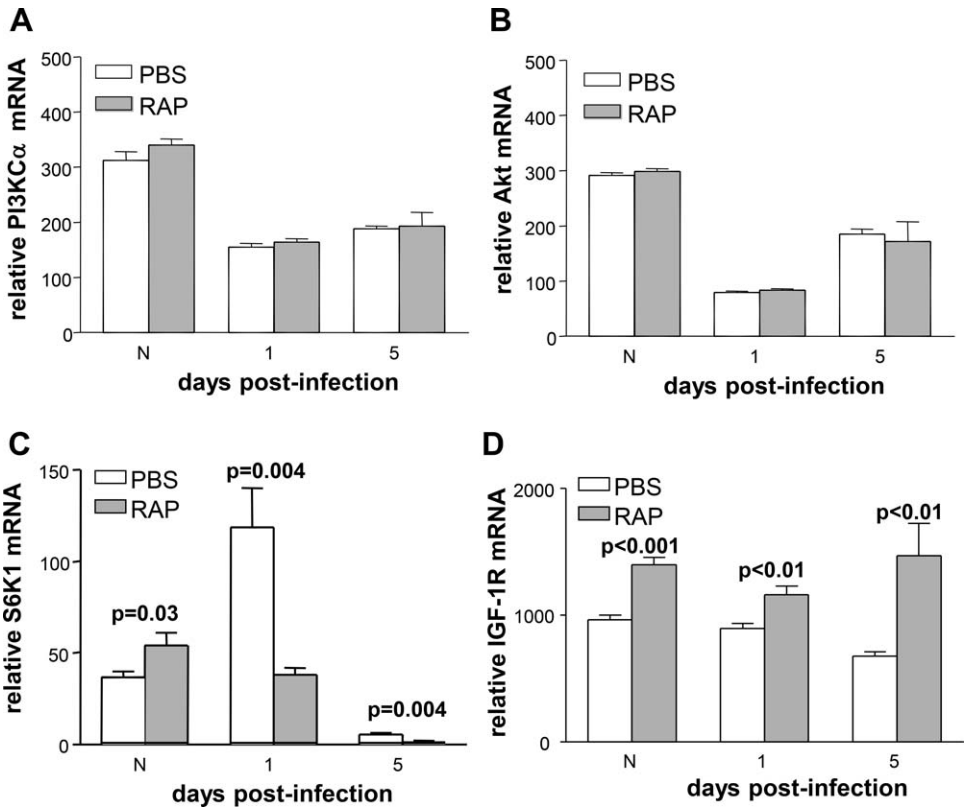

Figure 2. The effect of rapamycin on components of the c-met-mTOR pathway. Rapamycin had no effect on PI3KCα or Akt mRNA levels (**A**, **B**). In contrast, rapamycin significantly increased S6K1 (**C**) mRNA levels in the normal cornea, but decreased levels significantly at 1 and 5 days p.i. IGF-1R mRNA levels were significantly elevated in normal cornea and at 1 and 5 days p.i. after rapamycin treatment (**D**).

controls received 100 μL PBS. Normal and infected corneas were harvested at 3 and 5 days p.i., and assayed by ELISA for HGF and SP protein.

Real-Time RT-PCR

After animals were killed, normal (uninfected) and infected corneas from rapamycin- and PBS-treated mice were removed at 1, 3, and 5 days p.i. (n = 5/treatment/time). Similarly, corneas were harvested at 5 days p.i. from rHGF- and PBS-treated mice (n = 5/treatment). Total corneal RNA was extracted (RNA STAT-60; Tel-Test, Friendswood, TX, USA) per the manufacturer's instructions and used to produce a cDNA template for PCR reaction. After spectrophotometric quantification (260 nM), 1 μg of each RNA sample was reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase. The 20-μL reaction mixture contained 10 U RNasin, 500 μg oligo(dT) primers, 10 mM deoxyribonucleotide triphosphates (dNTPs), 100 mM dithiothreitol (DTT), and M-MLV reaction buffer (all from Invitrogen, Carlsbad, CA, USA). cDNA products were diluted 1:25 with diethylpyrocarbonate-treated water and a 2-μL cDNA aliquot was used for real-time RT-PCR (20 μL total reaction volume). mRNA levels of epidermal growth factor (EGF), EGF receptor (EGFR), FGF-7, FGFR-2, HGF, c-met, PI3KCα, Akt, S6K1, IGF-1R, mTOR, IL-10, IL-12p40, and IL-18 were tested by real-time RT-PCR (MyiQ Single Color Real-Time PCR Detection System; Bio-Rad, Hercules, CA, USA). Real-Time SYBR Green/Fluorescein PCR Master Mix (Bio-Rad) was used for the PCR reaction with primer concentrations of 5 μM. Optimal conditions for PCR amplification of cDNA were established using routine methods.[21] The fold differences in gene expression were calculated after normalization with β-actin. The primer pair sequences used for real-time RT-PCR are shown in the Table.

ELISA

All individual corneas taken for ELISA analysis were homogenized in 500 μL PBS with 0.1% Tween 20 with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and centrifuged at 12,000*g* for 5 minutes. After rapamycin or PBS treatment, normal (uninfected) and infected samples were harvested and prepared as described above at 1 and 3 days p.i. A 100-μL aliquot of each supernatant was assayed in duplicate for HGF or c-met protein according to the manufacturer's instructions (R&D Systems). Sensitivity for the assays was determined by comparison with a standard curve generated from known concentrations of the target protein. In a similar experiment (rapamycin or PBS treatment) normal and infected corneas were harvested at 1, 3, and 5 days p.i., and 50 μL of each sample was assayed in duplicate for IGF-1 using a Quantikine ELISA kit (R&D Systems) with a sensitivity of less than 3.5 pg/mL. SP protein was similarly tested using a High-Sensitivity Substance P EIA kit (Bachem, San Carlos, CA, USA) per the manufacturer's instructions. Sensitivity of the SP assay was less than 39 pg/mL. Corneas from rIGF-1– (or PBS)-injected mice were tested at 3 and 5 days p.i. for HGF and SP protein, as described above. After rHGF (or PBS) injection, corneas were harvested at 1 and 5 days p.i. and tested for total (R&D Systems) and p-c-met protein (Cell Signaling, Danvers, MA, USA).

RAW 264.7 Cell Culture and Real-Time RT-PCR

Immortalized RAW 264.7 macrophage-like cells were purchased from the ATCC and grown to confluency. Cells were

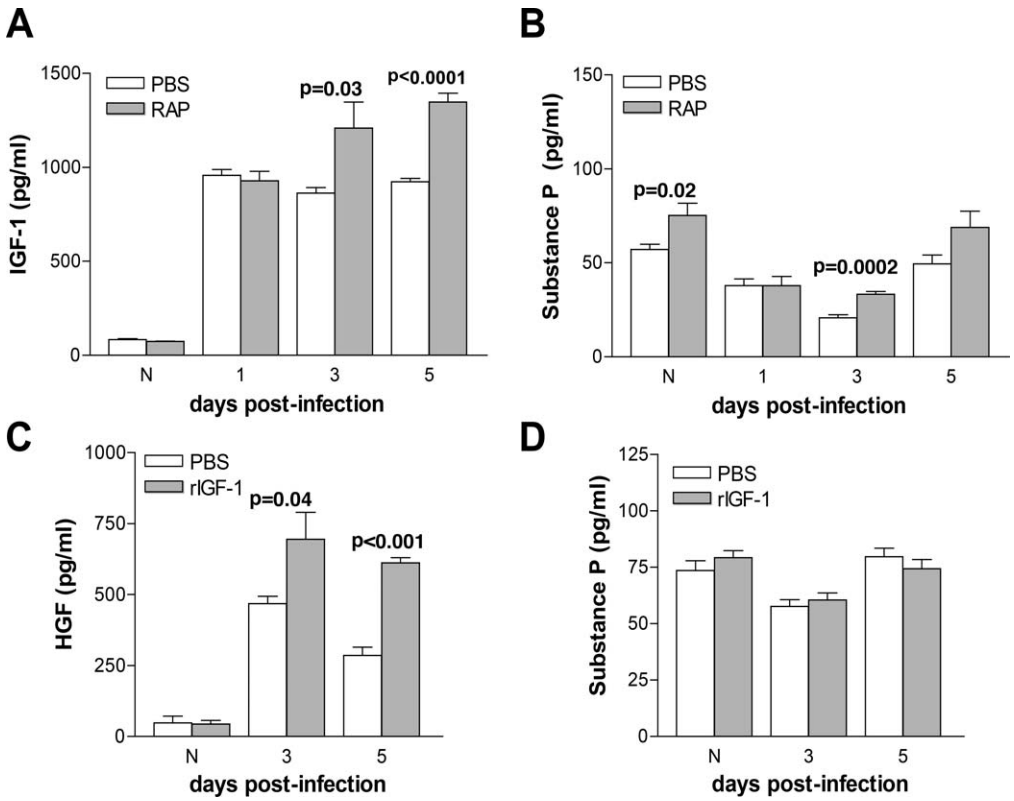

Figure 3. The effect of rapamycin or rIGF-1 treatment on IGF-1 and SP. Rapamycin had no effect on IGF-1 protein levels in normal cornea or at 1 day p.i.; however, protein levels were significantly upregulated at 3 and 5 days p.i. (**A**). Rapamycin significantly increased SP protein (**B**) levels in the normal cornea, and at 3 days p.i., but there were no differences between groups at 1 or 5 days p.i. rIGF-1 treatment (**C**) had no effect on HGF protein levels in normal cornea, but significantly elevated HGF at 3 and 5 days p.i. over controls. rIGF-1 had no significant effect on SP protein levels at any time tested (**D**).

harvested and centrifuged at 208*g* for 10 minutes. The pellet was resuspended in 10 mL Dulbecco's modified Eagle's medium and a 10-μL aliquot stained with Trypan Blue dye (1:1; Invitrogen) to determine cell viability (≥87.3%) using a hemacytometer (Bright-Line; American Optical, Buffalo, NY, USA). Cells were seeded in 6-well plates at a density of 3×10^6 cells per well ($n = 3$ wells/group). After incubation for 1 hour to allow cells to adhere, media containing nonadherent cells was removed, fresh media added, and the remaining adherent cells stimulated as follows: 1 μg/mL LPS (L9143 from *P. aeruginosa*, serotype 10; Sigma-Aldrich, St. Louis, MO, USA) ± 0.2 μg/mL rHGF; c-met inhibitor (CI) (SU11274; Sigma-Aldrich); a combination of LPS, rHGF, and CI; or media alone (in a total volume of 1 mL per well). Cells were incubated for 18 hours at 37°C. Afterward, cells and media were removed from the wells and centrifuged for 5 minutes at 5100*g*. Pellets were reconstituted in RNA STAT-60 (Tel-Test) and processed for mRNA as described above.

Elicited Peritoneal Macrophage Analysis

BALB/c mice ($n = 10$) were injected IP with 1 mL 3% Brewer's thioglycollate medium (Becton-Dickinson, Franklin Lakes, NJ, USA). Seven days later, mice were killed and macrophages harvested.[22] Cells were prepared as above and seeded in 12-well plates at a density of 3×10^6 cells per well ($n = 3$ wells/group, repeated once) and incubated at 37°C for 1 hour to allow cells to adhere. Elicited macrophages were treated, processed, and assayed using real-time RT-PCR as described above.

Statistical Analysis

The difference in clinical score between two groups at each time was tested by the Mann-Whitney *U* test. An unpaired, two-tailed Student's *t*-test was used to determine the statistical significance of the real-time RT-PCR, and ELISA data and a one-way ANOVA was used to compare mTOR expression in RAW cells and elicited macrophages. Data were considered significant at $P \leq 0.05$. All experiments were repeated once to ensure reproducibility and data from a representative experiment are shown as mean ± SEM.

Results

Real-Time RT-PCR Analysis of Growth Factors and Their Receptors After Rapamycin Treatment

Figure 1 (A–H) depicts growth factor (Figs. 1A–C) and growth factor receptor (Figs. 1E–G) mRNA levels in the cornea after rapamycin versus PBS treatment. EGF mRNA levels (Fig. 1A) were decreased in the normal, uninfected cornea and at 5 days p.i. ($P < 0.0001$ and $P = 0.05$, respectively) after treatment. However, there was no difference between groups at 1 or 3 days p.i. FGF-7 mRNA expression (Fig. 1B) was significantly decreased in the normal cornea and at 5 days p.i. ($P = 0.002$, $P = 0.0001$, respectively), with no difference between groups at 1 or 3 days p.i. HGF mRNA expression (Fig. 1C) was significantly reduced in the normal cornea ($P < 0.0001$), but at 1 and 3 days p.i., levels were increased significantly ($P = 0.0003$, $P = 0.005$, respectively), when compared with PBS

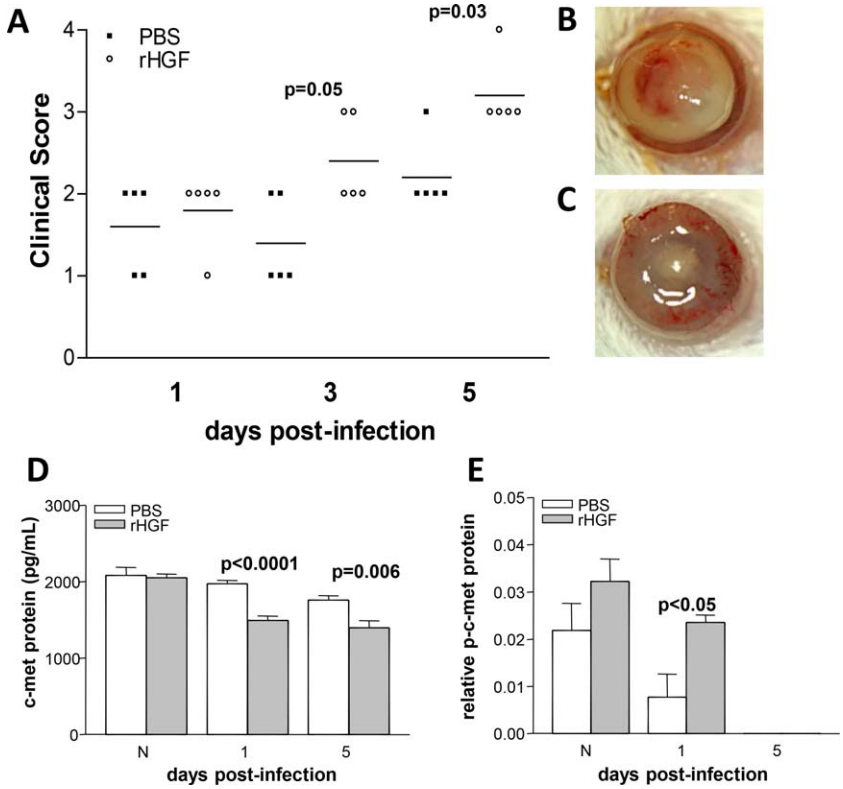

FIGURE 4. rHGF treatment in vivo. Clinical scores (**A**) were significantly increased at 3 and 5 days p.i. in rHGF-treated versus PBS-treated mice. Photographs with a slit lamp confirmed that the cornea of rHGF-treated (**B**) versus PBS-treated (**C**) mice had increased opacity and corneal thinning. rHGF treatment had no effect on total c-met protein levels (**D**) in normal cornea, but decreased levels significantly at 1 and 5 days p.i. p-c-met protein (**E**) was increased after rHGF treatment in the normal cornea, but was not significantly elevated until 1 day p.i.; levels were below assay detectability in both groups at 5 days p.i.

controls; no difference between groups was seen at 5 days p.i. For the receptors, EGFR mRNA levels (Fig. 1E) were significantly increased in the rapamycin- versus PBS-treated group in the normal cornea and 1 and 5 days p.i. ($P = 0.04$, $P = 0.0005$, $P < 0.0001$, respectively), but no difference between groups was detected at 3 days p.i. FGFR-2 mRNA levels (Fig. 1F) were significantly decreased in the normal cornea ($P = 0.001$), but increased at 1 and 3 days p.i. ($P = 0.003$, $P < 0.0001$, respectively) after rapamycin treatment, with no difference between groups at 5 days p.i. After rapamycin treatment, c-met mRNA levels (Fig. 1G) were significantly decreased in normal cornea ($P < 0.0001$), but significantly increased at 1 and 3 days p.i. ($P = 0.01$, $P < 0.0001$, respectively). No difference between groups was observed at 5 days p.i.

ELISA Analysis of HGF and c-met

Because treatment with rapamycin elevated only HGF and c-met levels similarly when compared with the other growth factors and their receptors, an ELISA assay was used to confirm the effect at the protein level (Figs. 1D, 1H). Rapamycin treatment significantly increased levels of HGF, in the normal cornea and at 1 and 3 days p.i. (Fig. 1D; $P = 0.0006$, $P = 0.02$, $P = 0.005$, respectively). To test further involvement of the relevant signaling pathway, c-met total protein levels were tested at 1 and 3 days p.i. Rapamycin treatment decreased total protein levels of c-met at 1 and 3 days p.i. when compared with controls (Fig. 1H; $P = 0.002$, $P < 0.0001$, respectively).

Rapamycin Affects Signaling Molecules

Signaling between c-met and mTOR is canonically known to require PI3K and Akt.[10] Rapamycin had no direct effect on either PI3KCα or Akt mRNA levels in the normal, uninfected, cornea, or at 1 or 5 days p.i. (Figs. 2A, 2B). However, it significantly decreased S6K1 (the downstream effector of mTOR) mRNA levels at 1 and 5 days p.i. compared with PBS (Fig. 2C; $P = 0.004$, for both). In contrast, S6K1 mRNA was significantly increased in the normal, uninfected cornea ($P = 0.03$). S6K1 also regulates IRS-1, an associated protein of IGF-1R, and has been implicated in participating in cross talk with c-met, providing a potential link between the pathways.[23] IGF-1R mRNA levels were upregulated in normal cornea and at 1 and 5 days p.i. (Fig. 2D; $P < 0.001$ and $P < 0.01$ for both 1 and 5 days p.i.).

ELISA analysis of IGF-1 and SP

Because mRNA for IGF-1R was elevated, ELISA analysis was used to test whether protein levels of IGF-1 also were upregulated by rapamycin treatment. No differences were detected in the normal cornea or at 1 day p.i. between groups, but at 3 and 5 days p.i., IGF-1 protein was significantly increased (Fig. 3A; $P = 0.03$ and $P < 0.0001$, respectively). Because rapamycin treatment also has been shown before to upregulate mRNA for cebpb,[14] a precursor of SP that elevates HGF levels,[16] SP protein also was tested. Rapamycin treatment elevated SP in normal cornea and at 3 days p.i. (Fig. 3B; $P = 0.02$ and $P = 0.0002$, respectively) with no difference between groups at 1 or 5 days p.i. IGF-1 also has been shown to elevate

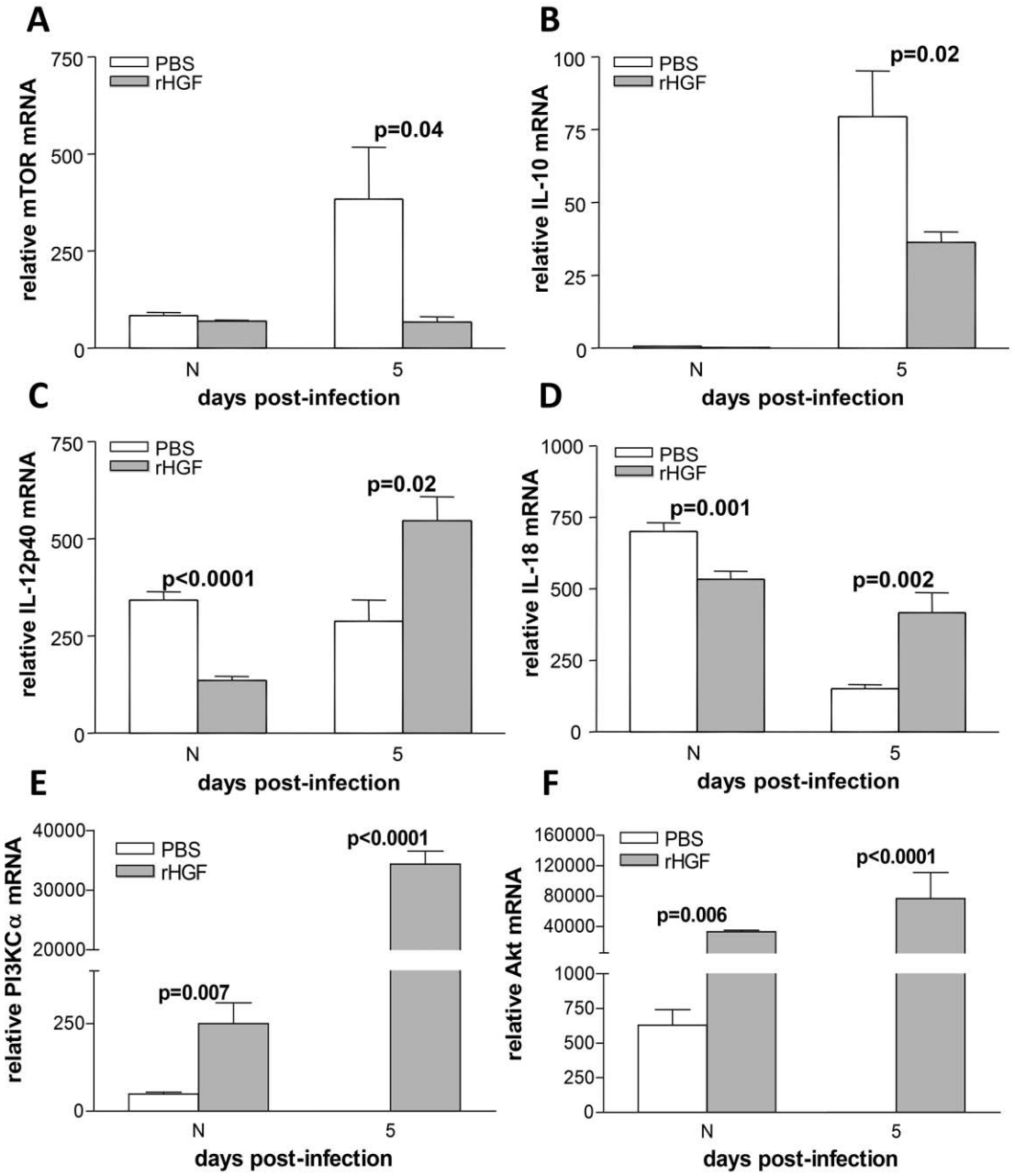

Figure 5. rHGF versus PBS treatment on mTOR, cytokines, and signaling molecules. rHGF treatment decreased mTOR mRNA (**A**) and IL-10 mRNA levels (**B**) significantly only at 5 days p.i., with no differences in the normal cornea. In contrast, proinflammatory cytokines IL-12p40 (**C**) and IL-18 (**D**) mRNA were significantly decreased in the normal, uninfected, cornea and significantly increased at 5 days p.i. Signaling molecules PI3KCα (**E**) and Akt (**F**) mRNA levels were increased significantly in normal cornea and at 5 days p.i after rHGF treatment.

HGF in other systems[24] and associate with SP.[25] Thus, mice were injected with rIGF-1 and tested to determine if this resulted in elevated HGF and/or SP protein levels. Treated mice showed increased HGF at 3 and 5 days p.i. ($P = 0.04$, $P < 0.001$, respectively) (Fig. 3C), but no differences were detected between groups for SP (Fig. 3D) at any time tested.

Disease Response After rHGF Treatment

To further determine the significance of elevated HGF after infection and rapamycin treatment to disease outcome, animals were infected and treated with rHGF. Clinical scores showed that rHGF treatment increased disease severity at 3 and 5 days p.i. (Fig. 4A; $P = 0.05$, $P = 0.03$, respectively) when compared with PBS-treated controls with no significant difference at 1 day p.i. Photographs of representative eyes from both groups (Figs. 4B, 4C) taken with a slit lamp at 5 days p.i. confirmed that rHGF treatment increased corneal opacity (Fig. 4B) when compared with the cornea of PBS-treated mice, which exhibited slight central opacity (Fig. 4C).

ELISA Analysis of c-met After rHGF Treatment

ELISA was used to confirm protein levels of c-met (total and p-c-met) after rHGF treatment, and the data are shown in Figures 4D, 4E. No significant difference was seen in the normal, uninfected, cornea between the rHGF-treated and PBS control–treated groups. However, c-met protein was significantly

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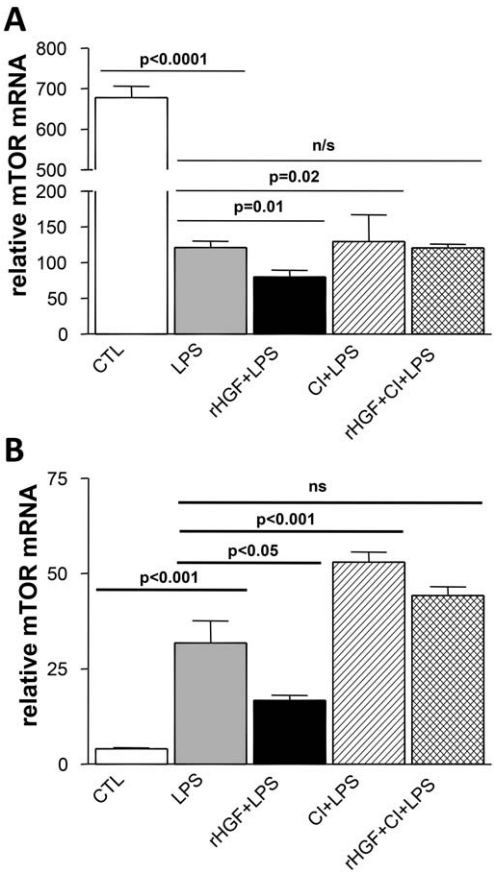

Figure 6. mTOR mRNA levels in vitro. (**A**) In immortalized RAW 264.7 cells, high levels of mTOR are detected in the media-only control. Stimulation with LPS significantly decreased mTOR levels and the addition of rHGF+LPS significantly decreased mTOR levels further. A c-met inhibitor (CI) significantly increased mTOR levels versus LPS alone, whereas a cocktail of rHGF+CI+LPS had no effect versus LPS alone. (**B**) In nonimmortalized elicited peritoneal macrophages, mTOR levels are low in the media-only control. LPS significantly increased mTOR levels versus control. rHGF+LPS treatment significantly decreased mTOR versus LPS stimulation alone, whereas treatment with CI+LPS significantly increased mTOR levels versus LPS alone. rHGF+CI+LPS treatment had no effect.

decreased at 1 and 5 days p.i. after rHGF treatment (Fig. 4D; $P < 0.0001$, $P = 0.006$, respectively). In contrast, p-c-met levels (Fig. 4E) were elevated in both the normal cornea and at 1 day p.i. (latter significantly, $P < 0.05$) with no difference (and low detectability) in either treatment group at 5 days p.i.

Real-Time RT-PCR of mTOR, Inflammatory Cytokines, and Signaling Molecules After rHGF Treatment

The next series of studies tested the effect of rHGF on mTOR, and both pro- (IL-12p40, IL-18) and anti-inflammatory (IL-10) cytokines (Fig. 5), as well as signaling molecules downstream of c-met following infection. rHGF versus PBS treatment significantly decreased mTOR mRNA (Fig. 5A) at 5 days p.i. ($P = 0.04$), with no significant difference in the normal, uninfected, cornea between groups. Likewise, IL-10 (Fig. 4B) was significantly decreased at 5 days p.i. ($P = 0.02$) with no significant difference in the normal cornea between groups. In contrast, proinflammatory cytokines, IL-12p40 (Fig. 5C) and IL-18 (Fig. 5D), were significantly increased at 5 days p.i. ($P = 0.02$ and $P = 0.002$, respectively), but levels for both cytokines were decreased in the normal, uninfected, cornea ($P < 0.0001$, $P = 0.001$). In contrast, rHGF treatment significantly increased both PI3KCα (Fig. 5E; $P = 0.007$, $P < 0.0001$), and Akt (Fig. 5F; $P = 0.006$, $P < 0.0001$) mRNA expression in the normal cornea and at 5 days p.i.

Real-Time RT-PCR of mTOR in RAW Cells and Elicited Macrophages

RT-PCR was used to confirm the effects on mTOR and c-met signaling after rHGF treatment and infection seen in vivo using an in vitro approach. rHGF treatment of LPS stimulated RAW cells (Fig. 6A), and elicited macrophages (Fig. 6B) provided evidence that mRNA for mTOR was detected in control-treated (media only) immortalized RAW cells. After LPS treatment, mTOR levels decreased when compared with media control (Fig. 6A; $P < 0.0001$). After rHGF+LPS treatment, mTOR levels were decreased compared with LPS alone ($P = 0.01$); rHGF alone had no inhibitory effect compared with control levels (data not shown). However, in cells treated with CI+LPS, mTOR levels were increased compared with LPS alone ($P = 0.02$). Finally, a cocktail of rHGF, CI, and LPS restored mTOR levels to that of LPS stimulation alone. In elicited macrophages (nonimmortalized cells), the effects of the individual treatments were similar to those in RAW cells, with the exception that mTOR levels were lower in the media-only control and increased with LPS treatment (Fig. 6B; $P < 0.001$). rHGF+LPS treatment decreased levels of mTOR compared with LPS alone ($P < 0.05$). Likewise, treatment with CI+LPS significantly increased mTOR levels compared with LPS alone ($P < 0.001$), and the combination of rHGF, CI, and LPS restored macrophage mTOR levels to that of LPS stimulation alone.

Discussion

Growth factors facilitate intercellular communication to enhance cell proliferation and migration,[26–29] essential to restoring tissue functionality after injury.[30–32] HGF/scatter factor is a potent growth factor in the cornea,[33] where its inactive form is produced by stromal fibroblasts[34] and, after activation, it acts primarily on the c-met receptor present on epithelial cells.[35] After wounding or injury, the availability of HGF and c-met in the cornea increases dramatically.[30] Several groups have shown that if not properly regulated, HGF can be deleterious during tissue restoration in the eye after injury. Moreover, HGF is upregulated in the human tear film after cataract surgery and penetrating keratoplasty.[36] Carrington and Boulton[34] determined that HGF delays corneal reepithelialization, resulting in the formation of aberrant epithelia (through its "scattering" effects) and mass differentiation of keratocytes into myofibroblasts. Also, c-met is expressed in the retinal pigmented epithelium of patients with proliferative vitreoretinopathy (PVR)[37] with concurrent HGF elevation in the vitreous,[38] suggesting c-met/HGF involvement in PVR pathogenesis.

Other tissues with epithelial layers (lung,[39] pancreas,[40] among others) experience elevated HGF during a variety of pathological conditions. Giebeler et al.[41] demonstrated that HGF and inflammatory cytokines (IL-6, TNF-α) are upregulated during cholestatic liver injury. In addition, this group provided evidence that c-met–dependent signaling leads to TLR4-dependent pathway activation. In this regard, our laboratory has determined that regulation of TLR4 is essential for balanced inflammatory cytokine production in the cornea in response to *P. aeruginosa* infection.[42] Dysregulation of this pathway leads

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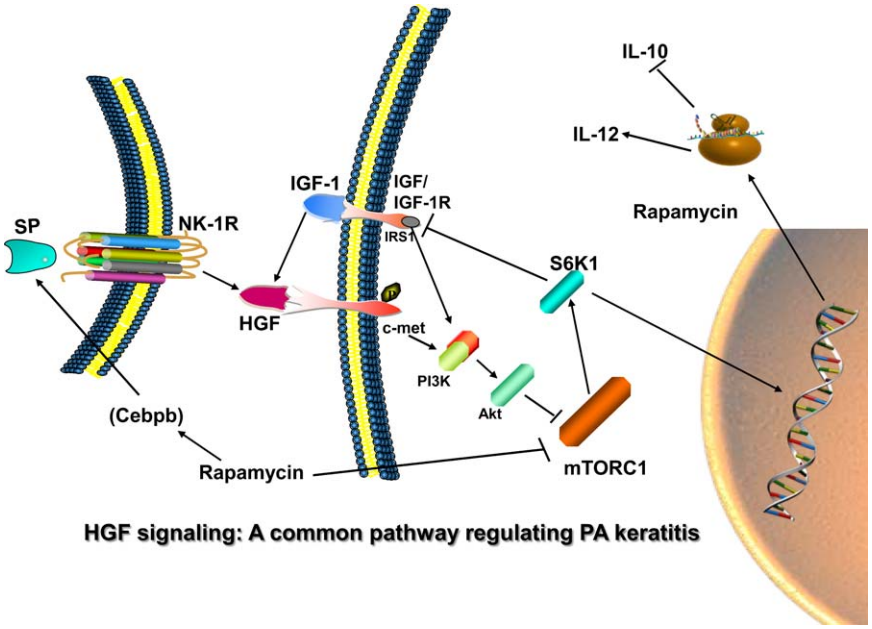

Figure 7. Proposed signaling through mTOR involving SP, HGF/c-met, its downstream effects, and a feedback loop through S6K1, IGF-1R, and IGF-1 signaling.

to worsened disease, potentially resulting in corneal perforation.[3]

Additionally, associations have been established between HGF and common bacterial strains. Both *Listeria monocytogenes*[43,44] and *Helicobacter pylori*[45,46] use c-met directly to induce their uptake into host cells. In periodontal disease, *P. gingivalis* LPS has been shown to enhance HGF production by human gingival fibroblasts.[47] This study implicated HGF and c-met in both the destructive and reparative phases of periodontal disease pathogenesis, as it is localized on both osteoclasts and osteoblasts. *Escherichia coli* LPS has been shown to stimulate the TLR4 pathway with concurrent HGF upregulation in septic patients with obstructive jaundice,[48] and in vitro kidney cell studies determined that HGF increased the invasiveness and cytotoxicity of *P. aeruginosa*.[49] In the cornea, however, little is known about the relationship between *P. aeruginosa* and HGF/c-met. The current work has provided evidence that HGF/c-met is upstream of the mTOR signaling pathway that directs the regulation of inflammatory cytokine production during *P. aeruginosa* infection, as depicted diagrammatically (Fig. 7).

In this regard, recently, this laboratory demonstrated that blocking mTOR signaling with rapamycin disrupted the balance between pro- (IL-12p40, IL-23) and anti-inflammatory (IL-10) cytokines in the cornea and increased bacterial plate count.[14] Additionally, overexpression of inducible nitric oxide synthase and matrix metalloproteinases was observed, as was impeded bactericidal function of corneal PMN.[50] Unexpectedly, this study[14] also showed that depressed mTOR signaling led to an increase in levels of cebpb mRNA. The cebpb acts to increase expression of the preprotachykinin-A gene that gives rise to the neuropeptide SP.[51] In this regard, SP treatment worsens disease in the *P. aeruginosa*–infected cornea, while concurrently elevating HGF levels.[16] Furthermore, SP treatment has been associated with elevated levels of proinflammatory cytokines (IFN-γ, IL-18, and TNF-α), along with decreased anti-inflammatory (IL-10) cytokines and elevated bacterial plate count. This cytokine imbalance after SP treatment, promotes susceptibility in the dominant Th2 responder, BALB/c (resistant) mouse, and furthermore, outcome is similar to the effects of rapamycin treatment[14] (as depicted in the diagram in Fig. 7). The current study provided evidence that rapamycin treatment also raised levels of not only SP, but also IGF-1 protein, downstream of mTOR signaling, and furthermore, rIGF-1–treated mice showed upregulation of HGF protein (as depicted in the diagram in Fig.7), providing evidence for an autologous loop effect in which levels of HGF protein appear critical to disease responsiveness in keratitis.

The effects of SP on corneal growth factors, and the similarity between rapamycin and SP treatment on the inflammatory response in the cornea, led to further examination of the effects of rapamycin on the growth factors. After infection, only HGF was elevated similarly at both the mRNA and protein levels. To confirm the importance in disease pathogenesis of HGF and its putative signaling through c-met, rHGF was applied topically to the infected cornea. Paralleling effects observed with either SP or rapamycin treatment, rHGF exacerbated *P. aeruginosa*–induced keratitis, lowered mTOR levels, and disrupted the pro- and anti-inflammatory cytokine balance. Other studies have shown that rhHGF treatment had deleterious effects in patients with liver disease that led to renal hypertrophy, glomerular hyaline droplet deposition, and tubule/mesangial expansion with associated albuminuria and proteinuria.[52]

Likewise, in vitro studies demonstrated that exogenous administration of rHGF (+LPS) decreased mTOR levels in macrophage-like RAW 264.7, and macrophages elicited from the peritoneal cavity of BALB/c mice. These cells have already been shown to be critical to regulation of PMN and inflammatory cytokine production in the BALB/c cornea after *P. aeruginosa* infection.[5] Furthermore, previous work by our laboratory identified the macrophage as a source of growth factors, including HGF in the cornea.[16] Next, in vitro administration of a c-met inhibitor (SU11274) in cultured macrophage-like and elicited cells (+LPS) resulted in restoration of mTOR levels seen after LPS treatment alone. This inhibitor has been characterized as proapoptotic (possibly through regulation of mitogenic mTOR),[53] whereas rHGF has been shown to be antiapoptotic and able to prevent Fas-induced cell death.[54] Early apoptosis is protective against

infection and tissue damage. However, after SP or rapamycin treatment, apoptosis is delayed in the resistant BALB/c mouse.[4,14] However, whether HGF/c-met plays a role in the regulation of apoptosis in the infected cornea is yet unknown, but appears likely.

Last, we examined PI3K and Akt for their putative role in HGF/c-met–mTOR signaling after rHGF treatment. rHGF elevated mRNA levels of both molecules, whereas rapamycin had no effect, perhaps because both molecules are downstream of HGF and upstream of mTOR.[10] However, rapamycin treatment downregulated mRNA levels of S6K1. In this regard, others have shown that S6K1, an important downstream effector of mTOR, when downregulated, upregulates signaling through IGF-1R, which can lead to increased levels of HGF,[55] consistent with the data presented herein. IGF1-R also has been shown to work cooperatively with c-met,[23] which would provide another link between mTOR inhibition and HGF/c-met signaling.

Collectively, the data, together with past studies, suggest an important regulatory role for HGF (depicted diagrammatically in Fig. 7), whose levels appear critical to c-met signaling, downstream mTOR regulation (promoting or inhibiting), and disease outcome. HGF signaling through c-met, if not balanced, results in enhanced *P. aeruginosa* keratitis, whether that balance is manipulated by rHGF application, SP treatment, or rapamycin inhibition of mTOR.

Acknowledgments

Supported by National Institutes of Health Grants R01EY002986, R01EY016058, and P30EY004068.

Disclosure: **X. Jiang**, None; **S.A. McClellan**, None; **R. Barrett**, None; **M. Foldenauer**, None; **L.D. Hazlett**, None

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