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Elevated IGFBP3 levels in diabetic tears: a negative regulator of IGF-1 signaling in the corneal epithelium

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Abstract

Purpose—To determine the ratio of IGFBP3:IGF-1 in normal and diabetic human tears, and in telomerase-immortalized human corneal epithelial cells (hTCEpi) cultured under elevated glucose conditions and to correlate these changes with total and phosphorylated levels of IGF-1R.

Methods—Tear samples were collected noninvasively from diabetic subjects and non-diabetic controls; corneal sensitivity was assessed using a Cochet-Bonnet Aesthesiometer. Conditioned media were collected following culture of hTCEpi cells in normal (5 mM) and elevated (25 mM) glucose conditions; mannitol was used as an osmotic control. IGFBP3, IGF-1, and phosphorylated IGF-1R levels were assessed by ELISA. IGFBP3 and IGF-1R mRNA were assessed by real time polymerase chain reaction (PCR). Total and phosphorylated IGF-1R expression in whole cell lysates was assessed by western blot.

Results—There was a 2.8-fold increase in IGFBP3 in diabetic tears compared to non-diabetic controls ($P=0.006$); IGF-1 levels were not significantly altered. No difference in corneal sensitivity was detected between groups. The concentration of IGFBP3 in tears was independent of IGF-1. Consistent with human tear measurements in vivo, IGFBP3 secretion was increased 2.2 fold ($P<0.001$) following culture of hTCEpi cells under elevated glucose conditions in vitro. Treatment with glucose and the mannitol control reduced IGFBP3 mRNA ($P<0.001$). Total IGF-1R levels were unchanged. The increase in the IGFBP3:IGF-1 ratio detected in diabetic tears compared to normal controls blocked phosphorylation of the IGF-1R by IGF-1 ($P<0.001$) when tested in vitro.

Conclusions—Taken together, these in vivo and confirmatory in vitro findings suggest that the observed increase in IGFBP3 found in human tears may attenuate IGF-1R signaling in the diabetic cornea. A long-term increase in IGFBP3 may contribute to epithelial compromise and the pathogenesis of ocular surface complications reported in diabetes.

Keywords

Diabetes; cornea; epithelium; tears; IGFBP3; IGF-1; IGF-1R

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INTRODUCTION

Diabetic keratopathy can result in substantial and sometimes permanent visual loss secondary to chronic erosions, scarring, and infectious corneal ulceration. Reported to occur in more than 70% of patients with diabetes, diabetic keratopathy includes a large spectrum of conditions, ranging from superficial punctate keratitis to recurrent corneal erosions both after surgery and *de novo*, as well as corneal neuropathy.^{1–4} Alterations in the epithelium of the diabetic cornea that contribute to the disease phenotype include impaired wound healing resulting from delayed cellular migration and reduced cellular adhesion and the concomitant loss of hemidesmosomes, which function to anchor the epithelium to the underlying basement membrane.^{5–7} Accompanied by alterations in the basement membrane itself, these collective changes result in epithelial fragility and disruption of the normal epithelial barrier.^{5, 8–12}

Insulin-like growth factor binding protein 3, IGFBP3, is an N-linked glycosylated, phosphorylated, secretory protein with known antiproliferative and pro-apoptotic functions.¹³ IGFBP3 belongs to a family of high affinity insulin-like growth factor (IGF) binding proteins, which function to sequester extracellular IGF-1, preventing IGF-1 activation of the insulin-like growth factor receptor, IGF-1R.¹⁴ The IGF-1R is a glycosylated, transmembrane receptor tyrosine kinase that has vital roles in development and normal tissue maintenance.¹⁵ Both IGFBP3 and IGF-1R have been previously identified in the corneal epithelium and in cultured corneal epithelial cells^{16, 17}; however, the functional significance of this localization in mediating homeostatic renewal is unknown. In addition to mediating IGF-1R signaling, IGFBP3 has also been shown to regulate insulin resistance^{18, 19} and apoptosis^{20–24} in a variety of cell types via IGF-1 independent pathways. IGFBP3 has also been identified as a hypoxia-responsive protein^{25, 26} with the potential to regulate angiogenesis.^{27–30}

Changes in tear production and composition have been associated with diabetes, including elevated glucose levels,³¹ an increase in advanced glycation end product modified proteins,³² and a reduction in reflex tearing.³³ In this study, we investigated the expression levels of IGFBP3 and IGF-1 in human tears of normal and diabetic patients *in vivo* and following *in vitro* culture of telomerase-immortalized human corneal epithelial cells in elevated glucose. We further investigated IGF-1R expression in normal and high glucose conditions and phosphorylation status when stimulated at various IGFBP3:IGF-1 ratios. Significantly, we show for the first time that IGFBP3 is present in human tears and that it is increased in the tears of patients with diabetes. Moreover, secreted IGFBP3 is similarly increased following *in vitro* culture in high glucose. The increase in the IGFBP3:IGF-1 ratio is associated with a reduction in phosphorylated IGF-1R. Taken together, these findings suggest that prolonged elevated IGFBP3 expression levels in human tears of diabetics may contribute to the pathogenesis of the high incidence of corneal complications through the attenuation of normal IGF-1R-mediated signaling by IGF-1.

METHODS

Study Population

Thirty-three patients, 18 diabetics and 15 non-diabetic normal controls, who met the inclusion criteria signed an informed consent and were enrolled in this study between June and August 2011. Inclusion criteria included no recent history of contact lens wear; no use of topical medications including artificial tears; non-smoking; no use of systemic hormones, anti-histamines, or anti-depressants; or any previous history of ocular surgery. Diabetic patients were recruited from the Internal Medicine Diabetic Clinic at UT Southwestern Medical Center. A diagnosis of diabetes in the patient's medical chart was required for

admittance to the study. Normal, non-diabetic patients were recruited from within the Department of Ophthalmology, UT Southwestern Medical Center. Pertinent medical history was obtained following consent. Current glycosylated hemoglobin levels were obtained from medical records. All procedures were approved by the Institutional Review Board at UT Southwestern Medical Center and were conducted in accordance with the tenets of the Declaration of Helsinki ethical principles for medical research involving human subjects.

Tear Collection

Each subject underwent one visit at which 10 μ l of basal tears were collected sequentially from the inferior meniscus of the right, then left, eye using a 10 μ l glass microcapillary tube (Drummond, Fisher Scientific, Houston, TX). Samples from right and left eyes were not pooled but were used to test levels of IGF-1 and IGFBP3, respectively. Care was taken to minimize reflex tearing. Tear samples were eluted into 1.5 ml Eppendorf tubes and immediately placed on ice. All samples were collected between 8 a.m. and noon to control for diurnal fluctuations. Tear samples were stored at -80°C until use. All tear samples were obtained by a single trained investigator.

Corneal Sensitivity

Corneal sensitivity was measured in the right eye after tear collection using a Cochet-Bonnet Aesthesiometer (C-BA, Luneau, Paris, France). The C-BA was mounted to a slit lamp base using a standard lab clamp that allowed for movement in the x, y, z directions. A 0.08 mm diameter nylon filament applanated the inferior cornea approximately 2 mm above the inferior limbus. Measurements were initiated with the filament fully extended to 6.0 mm. Filament length was systematically reduced in 0.5 mm increments until the patient correctly reported 2 or more of the four stimulus presentations. False presentations were included to control for incorrect patient responses. All measurements were obtained by a single trained investigator.

Cell Culture

Human telomerase-immortalized corneal epithelial cells (**hTCEpi**) were initially isolated and thereafter routinely maintained in serum-free keratinocyte growth media (KGM-2, Clonetics-BioWhittaker) containing 5 mM glucose, 0.15 mM calcium and supplemented with 0.4% bovine pituitary extract, 0.1% human epidermal growth factor, 0.1% insulin, 0.1% hydrocortisone, 0.1% transferrin, 0.1% epinephrine and 0.1% gentamicin sulfate amphotericin B, as previously described.³⁴ Cells were subcultured on T75 tissue culture flasks (Falcon Labware; BD Biosciences, Bedford, MA), incubated at 37°C in 5% CO_2 , and passaged every 5 days. For high glucose conditions, cells were cultured in serum-free keratinocyte basal media without supplements for three days on 6-well polystyrene tissue culture plates (Corning, Lowell, MA). Media contained an additional 20 mM glucose (Sigma, St. Louis, MO) or 20 mM mannitol (Sigma, St. Louis, MO) as an osmotic control. Media was changed at day 2. Conditioned media samples were collected at day 2 and day 3 and pooled for later analysis. For all cell culture experiments, assays were performed in triplicate and repeated three times independently.

ELISA

All samples collected from right and left eyes were used for IGF-1 and IGFBP3 analysis, respectively. Samples were diluted 1:5 in assay buffer. Conditioned media samples were concentrated using iCON protein concentrators (Pierce, Rockford, IL). To detect phosphorylated IGF-1R, 3×10^5 cultured cells were starved in basal media for 24 hours and then stimulated by 25 ng/ml of recombinant human IGF-1 (BioVision Inc, Mountain View, California) with or without recombinant human IGFBP-3 (BioVision Inc, Mountain View,

California) for 15 minutes at IGFBP3:IGF-1 ratios that simulated the ratios detected in normal and diabetic human tears. Whole cell lysates were harvested directly in the culture dish using RIPA buffer containing Halt™ protease and phosphatase inhibitor single-use cocktail (Thermo Fisher, Rockford, IL) on ice for 5 minutes. Total protein concentration was measured using a Bradford Assay. IGFBP3, IGF-1, and phosphorylated IGF-1R were detected by ELISA assay (IGFBP3 and IGF-1, R&D Systems, Minneapolis, MN; phosphorylated IGF-1R, Cell Signaling, Danvers, MA). Media and whole cell lysate samples were assayed in triplicate. ELISA results of media and whole cell lysate samples were further normalized to total protein concentration. Each experiment was repeated three times.

Western Blot

Whole cell lysates were harvested in RIPA buffer as described above. To assess IGF-1R activation, 3×10^5 cultured cells were starved in basal media for 24 hours and then stimulated by 100 ng/ml of recombinant human IGF-1 with or without recombinant human IGFBP-3 for 15 minutes at IGFBP3:IGF-1 ratios identical to those utilized for the ELISA assays. Supernatant of the lysates were boiled for 5 minutes in 2× SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 0.01% bromophenol blue, 2% β-mercaptoethanol), resolved on a TGX™ precast polyacrylamide gel (Bio-rad, Hercules, CA), and subsequently transferred to an immobilon-P PVDF membrane (Millipore, Temecula, CA). Membranes were blocked in 5% non-fat milk for 30 minutes at room temperature and blotted using an antibody against IGF-1R, pIGF-1R, or β-actin overnight at 4°C. Following a 1-hour incubation with a peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), membranes were visualized using ECL Plus Detection Reagents (Amersham Biosciences, Piscataway, NJ) and imaged on a Typhoon Variable Mode Imager.

Real-Time RT-Polymerase Chain Reaction (PCR)

Total RNA from hTCEpi cells was extracted with the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The synthesis of cDNA was performed by reverse transcription of 1 ug of total RNA using the QuantiTect RT Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The expression of β-actin, IGF-1R, and IGFBP-3 was quantified on an iCycler iQ real-time detection system (Bio-rad, Hercules, CA) using the QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. For each sample, 100 ng of cDNA was subjected to 40 cycles of real-time RT-PCR in a total volume of 25 μl containing 1 μM of each primer sets. Non-template controls were performed in parallel. Acquisition of fluorescence signals was monitored on an iCycler and data analysis was performed with the iCycler iQ real-time detection system software (Bio-rad, Hercules, CA). IGF-1R and IGFBP-3 expression in samples were normalized to β-actin mRNA expression using the ΔCt method. The primers used in PCR were obtained from QuantiTect Primer Assay (Qiagen, Valencia, CA) as follows: β-actin probe (Cat. QT00095431); IGF-1R probe (Cat. QT00005831); IGFBP-3 probe (Cat. QT00072737). All samples were assayed in triplicate and mean values were calculated. Each experiment was repeated three times.

Statistical Analysis

Statistical analysis was performed using SigmaStat 3.1 (Systat Software, Inc., San Jose, CA). For comparisons of differences in age and in protein levels between diabetic and non-diabetic tears, a student's t-test was used to determine which groups were significantly different. For statistical analysis, the diabetic population consisted of patients with either type 1 or type 2 diabetes. To compare differences in gender between diabetic and non-diabetic groups, a chi-square test was used. To evaluate a correlation between IGFBP3 and

IGF-1 levels present in human tears, a Pearson Product Moment correlation was used. For comparisons in protein levels between conditioned media and cell lysates, a one-way ANOVA was used with an appropriate post-hoc multiple comparison test. Statistical significance was set at $P < 0.05$.

RESULTS

Identification of IGFBP3 and IGF-1 in Human Tears

Patient demographics are reported in Table 1. There was no difference in age between populations ($P = 0.559$); however, due to limited availability of patients who met the inclusion criteria, there was a difference in gender distribution between groups ($P < 0.001$). The mean concentration of IGFBP3 in normal non-diabetic human tears was 6.1 ng/ml; IGF-1 levels were detected at approximately 1.2 ng/ml. In diabetic tears, IGFBP3 was increased 2.8-fold compared to the level in non-diabetic tears (Figure 1A, $p = 0.006$). When stratified by disease type, patients with type 2 diabetes had significantly elevated levels of IGFBP3 in tears compared to non-diabetic controls ($P = 0.016$, data not shown). IGFBP3 was similarly increased in tears of patients with type 1 diabetes; however, this increase was not significantly different from controls ($P > 0.05$). There was a trend toward a reduction in IGF-1 levels in diabetic tears, which was not statistically significant (Figure 1B, $P = 0.096$). When stratified by type, both type 1 and type 2 showed a slight reduction in IGF-1 in diabetic tears compared to non-diabetic controls, which was not significant ($P = 0.072$, data not shown). Changes in IGFBP3 levels were independent of IGF-1 (Figure 1C, $p = 0.383$). Based upon the UTSW clinical laboratory, HbA1c values of less than 5.8% are considered normal and 7.0% are considered to be good diabetic control. HbA1c within the diabetic group (mean of 7.2%) demonstrated moderately well-controlled diabetics, but in whom the disease was still active. There was no correlation between IGFBP3 in diabetic tears and HbA1c ($r = 0.383$, $P = 0.186$). No differences in corneal sensitivity were detected between groups (Figure 2, $P = 0.247$).

IGFBP3 and IGF-1R Expression and Activation in hTCEpi Cells

After 3 days in culture, 5.6 ng/mg of IGFBP3 was detected in conditioned media under basal conditions (Figure 3A). In the presence of elevated glucose, there was a 2.2-fold increase in IGFBP3 compared to the normal glucose control (Figure 3A, $P < 0.001$). There was no increase in the mannitol control. Western blot for total IGF-1R in whole cell lysates showed that IGF-1R levels were unchanged in the presence of elevated glucose (Figure 3B). Real time PCR demonstrated a significant decrease in IGFBP3 mRNA in cells treated with glucose and the mannitol control compared to non-treated cells (Figure 3C, $P < 0.001$); there was no change in IGF-1R mRNA between test groups (Figure 3D, $p = 0.491$). In non-hyperglycemic cells, when stimulated with IGF-1, there was a significant increase in IGF-1R phosphorylation compared to the non-stimulated control (Figure 4, $P < 0.001$). A similar increase in receptor phosphorylation was evident when cells were treated with IGFBP3 and IGF-1 at a ratio that paralleled that seen in the normal tear fluid ($P < 0.001$). Treatment with IGFBP3 and IGF-1 at the ratio present in diabetic tears completely attenuated IGF-1R phosphorylation to non-stimulated levels. A schematic illustrating the relationship between IGFBP3, IGF-1 and IGF-1R activation is presented in Figure 5.

DISCUSSION

This study reports the first identification of IGFBP3 in human tears and demonstrates a three-fold increase in IGFBP3 in diabetic tears compared to non-diabetic controls. When stratified by disease type, IGFBP3 was significantly increased in type 2 diabetics. While type 1 diabetics showed a two-fold increase in tear IGFBP3 levels, this increase was not

significant. This could be due to the small number of type 1 diabetics recruited in the study (n=6) or it may be the result of confounding effects from insulin treatment. Importantly, the change in IGFBP3 identified in this study occurred in the absence of a compensatory increase in IGF-1 tear levels. While IGFBP3 is a multifunctional protein, the primary role of extracellular IGFBP3 is to sequester IGF-1 and regulate subsequent IGF-1:IGF-1R interactions.¹⁴ It has been reported that extracellular IGFBP3 binds IGF-1 with a greater affinity than IGF-1 binding to the IGF-1R.³⁵ Thus, an alteration in the IGFBP3:IGF-1 ratio through an overall increase in IGFBP3 results in a net reduction in extracellular bioavailable IGF-1. The impact of reduced free IGF-1 coupled with static IGF-1R levels was demonstrated in this study through the reduction in the ability of IGF-1 to stimulate IGF-1R phosphorylation in corneal epithelial cells. Specifically, when tested in vitro at ratios that were detected in normal human tears, IGFBP3 was not able to inhibit activation of the receptor by IGF-1; however, IGFBP3 completely abrogated IGF-1 activation of the IGF-1R when tested at a ratio replicating that found in diabetic tears.

A second potential mechanism by which an increase in IGFBP3 may contribute to ocular surface alterations is through non-IGF-1R pathways. IGFBP3 has well-established roles in mediating insulin resistance^{18, 19, 36} and apoptosis,^{20–24} and, more recently, it has also been identified as a regulator of oxidative damage, which is currently regarded as a central mechanism that underlies the induction of diabetic complications.^{37, 38} Caspase-mediated apoptotic pathways are implicated in the pathogenesis of diabetic microvascular changes,³⁹ and IGFBP3 has been reported to potentiate high glucose-induced damage in specific cell types within the kidney.²¹ This includes proximal tubular epithelial cells, mesangial cells and podocytes, where IGFBP3 has been shown to potentiate oxidative stress and stimulate high glucose-mediated apoptosis.^{21, 40, 41}

Both the attenuation of IGF-1R signaling via IGFBP3 sequestration of IGF-1, as well as IGFBP3-mediated signaling pathways independent of the IGF-1R, have the potential to disrupt regulatory mechanisms essential for normal epithelial maintenance and renewal. Moreover, in the normal corneal epithelium, the IGF-1R has been shown to localize specifically to E-cadherin complexes at sites of cell-to-cell contact.⁴² The significance of the IGF-1R:E-cadherin complex is unknown; however, it likely involves modulation of IGF-1R signaling. The possible impact of elevated IGFBP3 in human tears and subsequent abrogation of IGF-1R activation on IGF-1R:E-cadherin signaling may explain in part the disruption in cellular adhesion that is commonly reported in the diabetic cornea.

Of more importance than the absolute tear concentration in this study is the fold increase in IGFBP3 in human tears, which was similarly found in conditioned media samples collected from cultured corneal epithelial cells challenged with high glucose conditions. Growth factors and other proteins present in the tear fluid have been reported to be derived from the lacrimal gland, epithelial cells, and blood proteins.^{43, 44} While the source(s) of IGFBP3 in the tears has not yet been identified, these data suggest that secretion by corneal epithelial cells in response to hyperglycemia may account, at least in part, for the presence of IGFBP3 in human tear fluid and support the use of the hTCEpi cell line as an in vitro model to study these regulatory interactions.

In contrast to protein, IGFBP3 mRNA was significantly reduced following treatment with glucose and the mannitol control compared to non-treated cells. The simultaneous reduction in IGFBP3 mRNA by both sugars suggests that IGFBP3 is transcriptionally down-regulated following osmotic stress in corneal epithelial cells. The specific increase in extracellular IGFBP3 detected following treatment in high glucose suggests that hyperglycemia regulates post-transcriptional or post-translational modification of IGFBP3 that may regulate stability and function. Serum IGFBP3 has been previously identified to undergo non-enzymatic

glycosylation^{45, 46}; consistent with the hypothesis that IGFBP3 is post-translationally modified in diabetic tears in response to hyperglycemia, an increase in total glycosylated proteins in diabetic tears has been reported.³² Further biochemical studies are necessary to investigate the potential for hyperglycemic-induced post-translation modification of IGFBP3 and the corresponding impact of these changes on IGFBP3 function.

The absence of any detectable difference in corneal sensitivity using the C-BA in the current study is not altogether unsurprising. While the C-BA has been reported to be a viable methodology for obtaining cornea touch thresholds in diabetic eye disease and has been shown to correlate with severity of neuropathy,⁴⁷ other studies have suggested that within the normal population, the C-BA lacks the ability to detect early subtle changes in overall sensitivity or identify true thresholds due to the limited testing range.⁴⁸ The mean HbA1c levels found in the diabetic study group and the absence of any reported treatment for diabetes-related corneal or retinal complications suggests that these patients are in an earlier stage of the disease; this is supported by the absence of any significant neuropathy. Data from one patient were excluded, however, due to a past medical history for surgical treatment of severe diabetic retinopathy. The excluded patient, a 51-year-old Hispanic male with type 2 diabetes, presented with an HbA1c of 8.5% and a corneal sensitivity of 0.5 mm. Interestingly, his tested IGFBP3 level was 54 ng/ml, which was outside the standard testing range of the assay. This represents a 9-fold increase in IGFBP3 over normal tear values and supports the need for further studies to evaluate the effects of prolonged increased tear IGFBP3 levels in patients at various stages in the disease process.

The collective findings from this study suggest that the expression of IGF-binding proteins in tears may modulate IGF-1R signaling in the diabetic cornea. The primary limitations of this study include a relatively small sample size of type 1 and type 2 diabetics and the absence of sex-matched controls due to the stringent inclusion criteria. In addition, dry eye is commonly reported in diabetics and was not included as a test parameter in this study.⁴⁹ Although patients who were undergoing treatment for dry eye were excluded, a clinical assessment to confirm the absence of any dry eye symptoms in study subjects was not performed. Thus, an expanded investigation will be needed to reconfirm and extend the current findings. Such investigations would include a comprehensive anterior segment examination to assess ocular surface integrity and tear production and function for potential confounding effects from asymptomatic dry eye conditions, as well as correlation between serum and tear glucose levels in patients with both mild and severe disease.

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Figure 1.

IGFBP3 and IGF-1 expression in human tears. A. IGFBP3 demonstrated a 2.8-fold increase in expression in diabetic tears compared to normal controls (* $p=0.006$, Mann-Whitney Rank Sum test). B. IGF-1 was detected at low levels in normal patients and showed a trend toward reduced levels in diabetic tears ($P=0.096$, Mann-Whitney Rank Sum test). Data represented as mean $\pm$ se; $n=18$ diabetic subjects, 15 non-diabetic controls. C. IGFBP3 levels plotted as a function of IGF-1 demonstrated that the concentration of IGFBP3 in tears was independent of IGF-1 ($P=0.383$, Pearson Product Moment Correlation).

Figure 2.

Corneal sensitivity measurements. Corneal sensitivity measured in the inferior peripheral cornea following tear collection using a Cochet-Bonnet Aesthesiometer. Sensitivity measurements are expressed as mean $\pm$ se. No difference was detected between diabetics and non-diabetic controls ($P=0.247$, Mann-Whitney Rank Sum test).

Figure 3.

IGFBP3 and IGF-1R expression in hTCEpi cells. A. IGFBP3 levels in conditioned media were increased by 2.2-fold under high glucose (25 mM glucose) culture conditions compared to normal glucose levels (5 mM glucose, $P < 0.001$, One-way ANOVA, Holm-Sidak post hoc multiple comparison test, $n=3$). Cells cultured in 20 mM mannitol + 5 mM glucose control failed to alter IGFBP3 secretion compared to the control. Data represented as mean $\pm$ se. Asterisks indicative of $P < 0.001$. Graph representative of 3 repeated experiments. B. Western blot for total IGF-1R in hTCEpi cells cultured under normal glucose (5 mM), high glucose (25 mM), and the mannitol control (5 mM glucose, 20 mM mannitol). There was no detectable difference in total IGF-1R in any test condition. Beta-

actin was used as a loading control. Blot representative of 3 repeated experiments. C and D. Real-time RT-PCR for IGFBP3 (C) and IGF-1R (D) in hTCEpi cells cultured under normal glucose (5 mM), high glucose (25 mM), and the mannitol control (5 mM glucose, 20 mM mannitol). There was no significant difference in IGF-1R mRNA level in any test condition ($P=0.491$). IGFBP3 mRNA levels were decreased under high glucose and the mannitol control culture conditions compared to normal glucose levels (5 mM glucose, $P<0.001$, One-way ANOVA, Holm-Sidak post hoc multiple comparison test, $n=3$). Data represented as mean $\pm$ se. Asterisks indicative of $P<0.001$. Graphs representative of 3 repeated experiments.

A

B

Figure 4.

Phosphorylation of the IGF-1R. A. Stimulation of hTCEpi cells with 25 ng/ml IGF-1, 25 ng/ml IGF-1 + 125 ng/ml IGFBP3 (5× IGFBP3:IGF-1 ratio), or 25 ng/ml IGF-1 + 365 ng/ml IGFBP3 (14.6× IGFBP3:IGF-1 ratio). Both IGF-1 alone and IGF-1 with IGFBP3 at a 5× ratio increased phosphorylation of the IGF-1R compared to the non-stimulated control ($P < 0.001$, One-way ANOVA, Holm-Sidak post hoc multiple comparison test, $n = 3$). Treatment with IGF-1 and IGFBP3 at a 14.6× ratio completely blocked phosphorylation of the IGF-1R. Data represented as mean $\pm$ se. Asterisks indicative of $P < 0.001$. Data representative of three repeated experiments. B. Western blot for phosphorylated IGF-1R in hTCEpi cells under the stimulation with 100 ng/ml IGF-1, 100 ng/ml IGF-1 + 500 ng/ml

IGFBP3 (5×), or 100 ng/ml IGF-1 + 1.46 µg/ml IGFBP3 (14.6×). Both IGF-1 alone and IGF-1 with IGFBP3 at a 5× ratio increased phosphorylation of the IGF-1R compared to the non-stimulated control. Treatment with IGF-1 and IGFBP3 at a 14.6× ratio blocked phosphorylation of the IGF-1R. Total IGF-1R was used as a loading control. Blot representative of 3 repeated experiments.

Figure 5.

Schematic illustrating extracellular modulation of IGF-1R signaling. A. In normal tears, in the presence of low levels of IGFBP3, free IGF-1 can bind and activate the IGF-1R. B. In diabetic tears, an increase in IGFBP3 binds free IGF-1, preventing activation and phosphorylation of the IGF-1R.

Table 1

Patient demographics

	Diabetics	Non-diabetics
<i>Number of participants</i>	18 (6 type 1; 12 type 2)	15
<i>Age (years)</i>		
mean $\pm$ sd	48.8 $\pm$ 8.5	46.9 $\pm$ 10.6
range	31–62	25–63
<i>Gender</i>		
male	11 (61%)	5 (33%)
female	7 (39%)	10 (67%)
<i>Race</i> [*]		
Asian	0	4 (27%)
African American	3 (17%)	5 (33%)
Caucasian	12 (67%)	4 (27%)
Hispanic	3 (17%)	2 (13%)
<i>Duration of disease (years)</i>		
mean $\pm$ sd	14.8 $\pm$ 9.7	NA
range	1 – 31	
<i>HbA1c</i> ^{**}		
mean $\pm$ sd	7.2% $\pm$ 1.2	Data not available
<i>Retinopathy</i>	None diagnosed in medical record	None diagnosed in medical record

* Sum of mean values greater than 100% due to rounding.

** Normal HbA1c level at the Aston Ambulatory Care Center Laboratory, UT Southwestern Medical Center is considered $\leq 5.8\% \pm 1.5$.