TGFB1-Induced Extracellular Expression of TGFB1p and Inhibition of TGFB1p Expression by RNA Interference in a Human Corneal Epithelial Cell Line

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PURPOSE. To report the increased production of extracellular transforming growth factor β-induced protein (TGFB1p) by human corneal epithelial cells (HCECs) after induction by TGFB1 and the inhibition of TGFB1p production in induced and noninduced HCECs by RNA interference (RNAi).

METHODS. HCECs were cultured in serum-free medium and treated with 0 or 10 ng/mL TGFB1 over a period of 72 hours. Commercially available siRNAs targeting TGFB1 mRNA were mixed with a transfection reagent and used to reverse transfet TGFB1-induced and noninduced HCECs. Extracellular and intracellular concentrations of TGFB1p were measured by ELISA and Western blot analysis, respectively, and TGFB1 RNA was assayed using semiquantitative RT-PCR.

RESULTS. HCECs constitutively express TGFB1p, and treatment with TGFB1 results in up to a fourfold increase in the amount of extracellular TGFB1p. Four commercially available siRNAs targeting TGFB1 mRNA produced a >70% decrease in extracellular TGFB1p within 48 hours after transfection of noninduced HCECs but a <25% decrease in extracellular TGFB1p by 48 hours after transfection of TGFB1-induced HCECs. The suppression of extracellular TGFB1p production correlated with a decrease in intracellular TGFB1p production and TGFB1 mRNA expression after transfection.

CONCLUSIONS. Extracellular TGFB1p expression by HCECs is increased several fold after exposure to TGFB1. Both HCEC-constitutive and HCEC-induced TGFB1p production can be inhibited with RNA interference, though the effect was greater and lasted longer for constitutive than induced TGFB1p production. Given that the corneal deposits in the TGFB1 dystrophies consist of TGFB1p derived from HCECs, RNAi represents a potential means to inhibit primary dystrophic deposit formation and recurrence after surgical intervention. (Invest Ophthalmol Vis Sci. 2011;52:757–763) DOI:10.1167/iovs.10-5362

O f the 35,000 to 40,000 corneal transplants performed in the United States annually, approximately 15% to 25% are performed for management of a corneal dystrophy.1–4 The genetic basis of two-thirds of the approximately 30 corneal dystrophies has been elucidated, with five of the most common dystrophies associated with dominant mutations in the transforming growth factor β-induced gene (TGFB1; MIM 601692).5 Mutations in TGFB1 result in the deposition of dysfunctional TGFB1p in the corneal stroma in the form of discrete or confluent dystrophic deposits.6–7 If the dystrophic corneal deposits are superficially located, painful recurrent epithelial erosions may develop. Management of corneal stromal opacification or recurrent corneal erosions is typically achieved with laser phototherapeutic keratotomy (PTK), lamellar keratoplasty, or penetrating keratoplasty (PK). Although PTK is an effective technique to remove superficial dystrophic corneal deposits, it is not effective in a large percentage of patients with TGFB1 dystrophies caused by the presence of visually significant deposits in the mid and posterior stroma. Additionally, PTK is associated with several potential complications such as induced corneal scarring, irregular astigmatism, and hyperopia. Penetrating and lamellar keratoplasty are also associated with a multitude of potential intraoperative and postoperative complications, including recurrence of the dystrophic deposits in the transplanted cornea, and thus are reserved for patients in whom more conservative therapies have failed.

In patients with TGFB1 corneal dystrophies, the dystrophic deposits typically recur after both PTK and PK.8–10 In four published series documenting the recurrence of the TGFB1 dystrophies after PK, the percentage of patients who experienced recurrence of the dystrophic deposits in the transplanted cornea was approximately 43% for granular corneal dystrophy (GCD), 48% to 60% for lattice corneal dystrophy (LCD), and 88% to 100% for corneal dystrophy of Bowman layer type I (CBD I) and CBD II10–15 The median time to recurrence is highly variable but was estimated to be approximately 2 years for CBD I and II and 8 years for LCD.15 The incidence and rate of recurrence of the TGFB1 corneal dystrophies after PTK vary widely, likely secondary to the differences in the number of patients, lengths of follow-up, and definitions of recurrence in the various reports. If the data from each of the reports are combined, recurrent deposits developed in approximately 52% of patients with CBD I and II, 25% of patients with LCD, 38% of patients with GCD, and 100% of patients with combined granular lattice corneal dystrophy (CGLCD) after corneal transplantation.8,9,13–16 Mean time to recurrence was shortest in patients who were homozygous for the mutation associated with CGLCD (9.5 months) and in patients with CBD I and II (26 months).

The need for surgical management of the dystrophic corneal deposits in most patients with TGFB1 dystrophies has led to interest in nonsurgical means to prevent the development or the recurrence of the dystrophic corneal deposits, which may be associated with significant visual morbidity in affected patients. Immunohistochemical analysis of the corneal buttons removed from affected patients at the time of PK has shown that the deposits consist of mutated TGFB1p.17–20 Interestingly, dystrophic deposits are limited to the corneas of affected persons, as confirmed by clinical and autopsy studies.21 TGFB1p is
constitutively produced by human corneal epithelial cells (HCECs), although the amount produced may be significantly increased in HCECs and in corneal stromal keratocytes in response to injury or surgery, as has been reported after LASIK procedures.

We investigated the usefulness of RNA interference (RNAi) to inhibit or impede the formation of visually significant dystrophic corneal deposits in patients with TGFBI dystrophies and as a means to prevent the recurrence of visually significant dystrophic corneal deposits after PTK and corneal transplantation. Evidence to support the usefulness of this approach comes from previous in vitro and in vivo studies using RNAi to inhibit pathologic processes involving the cornea. This therapeutic strategy has been shown to significantly suppress the expression of vascular endothelial growth factor (VEGF) induced by hypoxia in HCECs and to inhibit injury-induced murine corneal neovascularization in vivo. RNAi has also been used in vitro (cultured human corneal fibroblasts) and in vivo murine models of ocular inflammation to suppress the activity of TGF-β.

In addition, RNAi has been shown to effectively decrease the expression of TGFBIp in an experimentally transformed cell line (HEK 293) transfected with plasmids expressing TGFBIp and TGFBIp-specific shRNA-generating plasmids. We report the efficacy of RNAi in decreasing TGFBIp expression in both TGFBI-induced and noninduced HCECs, which are the primary source of the dystrophic TGFBIp in persons with a TGFBI dystrophy.

**Materials and Methods**

**Cell Line and Cultivation**

An HCEC line (CRL-11515) was obtained from American Tissue Culture Collection (Manassas, VA) and cultured in serum-free medium (Keratinocyte-SFM; Invitrogen, Carlsbad, CA) supplemented with 5 ng/mL human recombinant endothelial growth factor (Invitrogen), 0.05 mg/mL ovine pituitary extract (Invitrogen), 0.005 mg/mL insulin, 500 ng/mL hydrocortisone (both from Sigma-Aldrich, St. Louis, MO), and 1× penicillin-streptomycin mix (Invitrogen). All flasks and plates for experiments were coated with the following cocktail for 2 hours at 37°C: 0.01 mg/mL fibronectin (Fisher Scientific, Pittsburgh, PA), 0.01 mg bovine serum albumin (Sigma-Aldrich), and 0.03 mg/mL type I collagen (Sigma-Aldrich) prepared in sterile phosphate-buffered saline. Cells were cultured at 37°C in the presence of 5% CO₂.

**TGFBI1-Mediated Induction of TGFBIp**

To stimulate the production of TGFBI, TGFBI (Millipore, Billerica, MA) was dissolved in 10 mM citrate buffer at pH 3.5 and added to keratinocyte-SFM. The number of viable HCECs between a duplicate set of siRNA-treated and untreated samples. The HCECs were trypsinized, washed once with phosphate-buffered saline (PBS), and resuspended in PBS containing 0.2% Trypan blue and counted using a hemocytometer. A second method of identifying siRNA-mediated cytotoxicity assayed intracellular glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) enzyme levels for differences between siRNA-treated and untreated HCECs. GAPDH enzyme levels were measured in HCECs collected 48 hours after transfection using a GAPDH assay kit (KDalet; Ambion) according to the manufacturer’s instructions.

**Determination of Cytotoxicity of siRNAs**

To identify and quantify possible cytotoxic effects of the siRNA treatment, Trypan blue dye (Sigma-Aldrich) exclusion was used to count the number of viable HCECs between a duplicate set of siRNA-treated and untreated samples. The HCECs were trypsinized, washed once with phosphate-buffered saline (PBS), and resuspended in PBS containing 0.2% Trypan blue and counted using a hemocytometer. A second method of identifying siRNA-mediated cytotoxicity assayed intracellular glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) enzyme levels for differences between siRNA-treated and untreated HCECs. GAPDH enzyme levels were measured in HCECs collected 48 hours after transfection using a GAPDH assay kit (KDalet; Ambion) according to the manufacturer’s instructions.

**Determination of Efficacy of siRNAs**

**Targeting TGFBI**

Transfection of HCECs and Measurement of Extracellular TGFBI Protein in Noninduced HCECs. Each of the four predesigned siRNAs that provided the greatest suppression of extracellular TGFBI protein expression in the initial experiment was added to 50 μL keratinocyte-SFM to obtain five different final concentrations (15 nM, 30 nM, 50 nM, 75 nM, and 100 nM). These dilutions were then mixed with 50 μL transfection reagent (siPORT NeoFX; Ambion)/keratinocyte-SFM in triplicate in precoated 24-well plates (Becton Dickinson, Franklin Lakes, NJ) in quadruplicate. The cultures were incubated at 37°C in a 5% CO₂ environment. The medium was replaced after 24 hours with medium that did not contain either an siRNA or the transfection reagent. The efficacy of each predesigned siRNA in decreasing the amount of extracellular TGFBI protein was determined at 24, 48, and 72 hours after transfection. The four siRNAs with the highest average knockdown percentage were then selected for use in subsequent experiments.

**Testing and Selection of Predesigned siRNAs**

To identify the most effective predesigned siRNAs, we performed an initial experiment using seven commercially available siRNAs targeting various portions of the coding region (siRNAs 138708, 138709, s14068, s14069, s14070, and 8327; Ambion, Austin, TX) for the 3' UTR (siRNA 8518; Ambion) of TGFBI mRNA. Transfection was performed by the reverse transfection approach in which the siRNA and transfection reagent (siPORT NeoFX; Ambion) were mixed with keratinocyte-SFM and added to the plates, after which the HCECs were added. Each siRNA, obtained as 5 nmol dried-down RNA, was dissolved in 1 mL nuclease-free water. To achieve a final concentration of 30 nM in a 500-μL medium, 1.5 μL of each predesigned siRNA was then added to 50 μL keratinocyte-SFM and mixed with an equal volume of medium containing 1.5 μL transfection reagent (siPORT NeoFX; Ambion). The mixes were allowed to form complexes for 10 minutes at room temperature. HCECs used in all experiments were between passages 45 and 50 and had been actively growing for at least three passages. Next, 4 × 10⁶ HCECs in 400 μL medium were added to 100 μL transfection reagent (siPORT NeoFX; Ambion) complexes in precoated 24-well plates (Becton Dickinson, Franklin Lakes, NJ) in quadruplicate. The cultures were incubated at 37°C in a 5% CO₂ environment. The medium was replaced after 24 hours with medium that did not contain either an siRNA or the transfection reagent. The efficacy of each predesigned siRNA in decreasing the amount of extracellular TGFBI protein was determined at 24, 48, and 72 hours after transfection. The four siRNAs with the highest average knockdown percentage were then selected for use in subsequent experiments.
Transfection of HCECs and Measurement of Extracellular TGFBI Protein in TGFB1-Induced HCECs. Transfection of induced HCECs was performed in the same manner as described for noninduced HCECs, with the inclusion of 10 ng/mL TGFBI in the medium. Only the optimal concentration of each siRNA, as determined in the previous experiment, was used.

Measurement of Intracellular TGFBI Protein in Noninduced HCECs. Transfection of HCECs was performed using the same reverse transfection technique described previously. Each experiment was performed using the four predesigned siRNAs that provided the greatest suppression of extracellular TGFBI protein expression in the initial experiment at the five different concentrations (15 nM, 30 nM, 50 nM, 75 nM, and 100 nM). The efficacy of each of the four siRNAs in decreasing the amount of intracellular TGFBI protein was determined 24 and 48 hours after transfection. Protein samples were extracted from the HCECs using RIPA lysis and extraction buffer containing Halt protease inhibitor cocktail (Pierce, Rockford, IL). After denaturation in Laemmli sample buffer (Bio-Rad, Richmond, CA), the samples (6 μg as determined by the microBCA kit; Pierce) were loaded onto 8% to 16% polyacrylamide gradient gels (ReadyGels; Bio-Rad) and electrophoresed at 200 V for 45 minutes. Also loaded in one lane as an external standard was 1 ng pure TGFBI (Millipore). The gels were then transferred to polyvinylidene difluoride membranes at 100 V for 1 hour at 4°C. The membranes were probed using reagent transport (SNAP i.d. Protein Detection System; Millipore), 0.4 μg/mL biotinylated goat–human βG-H5 antibody (R&D Systems), and 1:500 dilution of streptavidin-HRP (R&D Systems). Chemiluminescence was used to detect bound antibody using extended-duration substrate (Super Signal West Dura; Pierce) and film (CL-Xposure; Pierce). GAPDH quantification in the HCEC lysate served as an internal control. To confirm that an equivalent amount of total protein was loaded in each lane, each blot was stripped and probed with a 1:6000 dilution of anti–GAPDH antibody (4 μg/mL; Ambion) using a similar chemiluminescent detection method.

Measurement of TGFB1 mRNA in Noninduced HCECs. Two-step qRT-PCR was performed to monitor TGFB1 mRNA levels in HCECs using GAPDH mRNA levels as the reference. Total RNA was isolated from HCECs using a purification kit (GenElute Mammalian Total RNA Miniprep Kit; Sigma-Aldrich) according to the manufacturer’s instructions. Extracted mRNA was first converted to cDNA (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). The cDNA was then used in the RT-PCR experiments (SYBR Fast qPCR Kit; Kapa Biosystems, Woburn, MA). Exonic primers (TGFB1 forward, 5′-AGATCGAGGACACCTTGTGAG-3′; TGFB1 reverse, 5′-TTGTTCAGCAGGTCCTCAG-3′; GAPDH forward, 5′-CTGGGCAGTGCTCATCCTG-3′; GAPDH reverse, 5′-GCCATGCAAGTTGCTCC-3′) used to amplify TGFB1 and GAPDH cDNA were designed to selectively amplify cDNA rather than genomic DNA. The percentage knockdown of TGFB1 mRNA was determined using the ΔΔCT method, in which the percentage knockdown = 100 × 2−ΔΔCT where ΔΔCT = (Ct for TGFB1 experimental sample − Ct for GAPDH experimental sample) − (Ct for TGFB1 control sample − Ct for GAPDH control).33

Positive and Negative Controls. Positive and negative controls were used to determine the degree and specificity of the effect of each siRNA on the production of extracellular TGFBI. The measurement of intracellular GAPDH before transfection of HCECs with a siRNA complementary to GAPDH mRNA (AM4631; Ambion) served as a positive control (data not shown). The measurement of extracellular TGFBI was performed as well using several negative controls: after transfection of HCECs with a siRNA that does not target any human genes (AM4636; Ambion) (scrambled control); after transfection of HCECs with the transfection reagent but without the addition of an siRNA (no siRNA control); and using HCECs that were not exposed to either the transfection reagent or an siRNA (untreated control).
RESULTS

TGFB1-Mediated Induction of TGFBIp

Cultured HCECs constitutively expressed TGFBIp, which was transported out of the cells into the surrounding medium, and were measured by ELISA 24 hours after plating. The amount of TGFBIp produced by HCECs increased in an exponential fashion, with extracellular levels more than doubling every 24 hours for the first 3 days (Fig. 1). Exposure of the cells to TGFB1 in the media resulted in an increased expression of TGFBIp at 24, 48, and 72 hours after the initial exposure, with levels increased approximately fourfold at 24 hours and twofold at 48 hours compared with TGFBIp expression by the uninduced cells (Fig. 1).

Testing and Selection of Predesigned siRNAs

Transfection of noninduced cells with each of the predesigned siRNAs targeting TGFBI mRNA at 30 nM concentration resulted in decreased expression of extracellular TGFBIp at all measured time points for all seven of the siRNAs tested (Fig. 2). The greatest knockdown effect was obtained with siRNA 8518, which demonstrated the suppression of extracellular TGFBIp levels by 73%, 74%, and 68% at 24, 48, and 72 hours after transfection, respectively. Three other siRNAs—138709, s14068, and s14070—produced knockdown of >58% at each time point and gave an average knockdown across the three time points of >50%. Only one of the other three siRNAs produced knockdown of >58% at any time point (64% knockdown with siRNA 8327 at 24 hours). A reduction in the extracellular TGFBIp level was also seen in cells exposed to the nontargeted (scrambled) siRNA when compared with the no siRNA control, though the reduction was much less than that seen with the targeted siRNAs.

Determination of Cytotoxicity of siRNAs

Viable cell counts and intracellular GAPDH enzyme levels measured in both treated and untreated cells showed no evidence of cytotoxicity by any of the siRNAs (data not shown).

Determination of Efficacy of siRNAs Targeting TGFBI

Measurement of Extracellular TGFBI Protein in Noninduced HCECs. HCECs transfected with siRNAs 138709, s14068, s14070, and 8518 targeting TGFBI mRNA produced less extracellular TGFBIp at each measured time point compared with the no siRNA control. Determination of the mean knockdown percentage for each siRNA at each of the five concentrations tested revealed that siRNA 138709 was the most effective siRNA tested, achieving the greatest average knockdown at each concentration, with maximal average knockdown of 76% at 75 nM and the greatest knockdown at any time point (85% at 75 nM 48 hours after transfection) (Fig. 3). For three of the four siRNAs tested, maximal knockdown of extracellular TGFBI protein was observed 48 hours after transfection of HCECs, whereas for siRNA 8518, maximal knockdown was observed 24 hours after transfection (Fig. 3). The optimal concentration of each siRNA, defined as the concentration that gave >5% additional knockdown when compared with the next lowest concentration of the siRNA, was determined to be 30 nM for siRNAs 138709 and s14068, 50 nM for siRNA s14070, and 75 nM for siRNA 8518.

Measurement of Extracellular TGFBI Protein in TGFB1-Induced HCECs. Transfection of TGFB1-induced cells with siRNAs 138709, s14068, s14070, and 8518 reduced the expres-
Inhibition of TGFBIp Expression with RNAi

We report the successful inhibition of extracellular TGFBIp production by HCECs using RNA interference, a process wherein intracellular double-stranded RNA (dsRNA) elicits the selective elimination of mRNA with sequence homology to the dsRNA. We have demonstrated >70% knockdown of extracellular TGFBIp production using four commercially available siRNAs at either 24, 48, or 72 hours after transfection of HCECs and have correlated this decrease with a decrease in the intracellular TGFBIp and TGFBI mRNA. At three of the five concentrations tested (15 nM, 30 nM, and 50 nM), the knockdown values obtained with siRNA 8518 were lower than the other three siRNAs (12%–19% lower than the values obtained with siRNA 138709 at these concentrations). The fact that this siRNA targets the 3′UTR while the other three siRNAs against which it was tested target exonic regions of TGFBI is suggestive that the most effective means of inhibiting expression of the TGFBI transcript is by targeting the coding region. We also noted that the scrambled siRNA used in these experiments produced a modest reduction in TGFBIp production by HCECs, 43% less than the average knockdown seen with each of the four siRNAs targeting TGFBI. Multiple mechanisms have been proposed to explain the nonspecific inhibitory effects that a scrambled siRNA control may have on expression of the target gene. Activation of the host cell’s microRNA pathway by the introduced foreign siRNA and partial sequence homology between the scrambled siRNA and a portion of the target gene’s mRNA allowing binding of the siRNA are just two such proposed mechanisms.

The study design we used to transfect HCECs with nonselective siRNAs limited our ability to speculate on the potential clinical usefulness of topical siRNA therapy targeting the TGFBI dystrophies. Although the HCECs in this study were exposed to the siRNAs targeting TGFBI for 24 hours before replacing the medium, in the clinical setting, a topically applied siRNA would likely remain in contact with the ocular surface for a shorter period. The length of exposure of a cell to a transfection reagent does affect the amount of knockdown of the targeted gene transcript, with gene silencing in cell cultures exposed to a transfection reagent for 4 hours found to be 30% less than that in cell cultures exposed to an siRNA in a medium that was not changed (Applied Biosystems TechNotes volume 8518 at 75 nM resulted in a reduction of expression of TGFBI mRNA by ≥60% at 24, 48, and 72 hours after transfection for each of the siRNAs except siRNA s14070 at 24 hours (Fig. 6). Maximum knockdown of expression at 24 hours (66.5%) was observed with siRNA s14068 and at 48 hours (78%) and 72 hours (81%) with siRNA 8518 (Fig. 6).

**DISCUSSION**

FIGURE 6. TGFBI mRNA expression by HCECs is reduced after transfection with siRNAs targeting TGFBI mRNA. Data shown reflect the mean reduction in TGFBI mRNA expression in HCECs after exposure to the optimal concentration of each siRNA compared with the TGFBI mRNA expression in HCECs exposed to the transfection reagent but not to an siRNA (no siRNA control).
12:1; www.ambion.com/techlib/trn/121/9.html; accessed July 2, 2010). Just how long HCECs will be exposed to a transfection reagent contained in a topical preparation containing a vehicle that allows for prolonged retention on the ocular surface is unknown. Additionally, because we transfected only a single HCEC line and because we did not measure the degree of knockdown beyond 72 hours, the generalizability of the results we report to HCECs from many different persons and the duration of the knockdown effect are not known. Finally, whether nonsense suppression of the TGFBI transcript, both wild-type and mutant, may lead to a compensatory increase in the production of both wild-type and mutant TGFBIp by HCECs and keratocytes, negating the effect of RNA interference, will have to be determined by monitoring intracellular and extracellular TGFBIp production for a much longer period than the 72 hours used in this study.

The pathogenic corneal deposits that characterize the TGFBI dystrophies represent a gain of function, or dominant negative effect, with the dysfunctional protein product, leading to loss of vision and painful corneal erosions. In patients reported to be homozygous for mutations in TGFBI, in whom both copies of the TGFBI gene are mutated, dystrophic deposits appear earlier in life and are more dense than in patients who are heterozygous for these mutations.36–37 However, the dystrophic deposits are the only cause of corneal opacification in these patients because the stroma between, posterior, and peripheral to these deposits remains clear. Therefore, it can be inferred that the expression of wild-type TGFBI may not be necessary for the maintenance of corneal clarity and that a loss of function of the TGFBI transcript would not be expected to lead to a loss of corneal clarity. Thus, although the roles that TGFBIp plays in the corneal stroma have yet to be defined, we are not aware of evidence that indicates inhibition of the expression of both wild-type and mutant TGFBIp would necessitate the restoration of expression of the wild-type TGFBIp or that an allele-specific siRNA that targets only the mutant form of the TGFBI mRNA would be necessary to avoid inhibition of the wild-type TGFBI transcript.

Our results indicate that RNAi appears to be more effective in inhibiting constitutively expressed TGFBIp than TGFBI-induced TGFBIp expression by HCECs. The potential clinical implications of these results are that a topically applied RNAi targeting the TGFBI transcript is likely to be more effective in inhibiting or impeding dystrophic TGFBIp deposition that occurs primarily or secondarily after corneal transplantation and LASIK procedures, which are not associated with significant increases of TGFBI expression.38–41 In contrast, topically applied RNAi targeting the TGFBI transcript is expected to be less effective after procedures that are associated with an increased expression of TGFBI by stromal keratocytes, such as photorefractive keratectomy/LASEK and phototherapeutic kerectomy, given the TGFB1-induced expression of TGFBIp by HCECs and keratocytes, negating the effect of RNA interference, will have to be determined by monitoring intracellular and extracellular TGFBIp production for a much longer period than the 72 hours used in this study.

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References


