Transforming Growth Factor-β2 Induces Synthesis and Secretion of Endothelin-1 in Human Trabecular Meshwork Cells

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PURPOSE. Analysis of aqueous humor from patients with primary open-angle glaucoma (POAG) revealed marked increases in the content of endothelin-1 (ET-1) and transforming growth factor-beta (TGF-β). We determined the consequences of TGF-β signaling on ET-1 expression and secretion by human trabecular meshwork (TM) cells.

METHODS. Primary or transformed (NTM5 and GTM3) human TM cells conditioned in serum-free media were incubated in the absence or presence of TGF-β1 or -β2. Relative changes in preproendothelin (ppET)-1 mRNA content and secreted ET-1 peptide were quantified by real-time PCR and ELISA, respectively. In some experiments, TGF-β or ET-1 receptor antagonists, or Rho G-protein inhibitors, were evaluated for effects on TGF-β signaling. Filamentous actin organization was visualized by phalloidin.

RESULTS. Primary or transformed human TM cells cultured in the presence of TGF-β1 or -β2 exhibit a marked (>8-fold) increase in ppET-1 mRNA content compared to vehicle controls. Coincubation with SB-505124, an inhibitor of TGFβRI/ALK-5 signaling, prevented TGF-β–mediated ppET-1 mRNA expression. In contrast, coincubation with ET_A (BQ-123) or ET_B (BQ-788) receptor antagonists had no effect on TGF-β–mediated ppET-1 mRNA expression. TGF-β1 and -β2 each elicited a robust (>7-fold) secretion of ET-1 while enhancing stress fiber organization. Inhibition of Rho signaling attenuated TGF-β–mediated increases in ppET-1 mRNA content, ET-1 secretion, and stress fiber organization.

CONCLUSIONS. TGF-β, signaling through the TGFβRI/ALK-5 receptor, elicits marked increases in ET-1 mRNA content and ET-1 secretion from cultured primary or transformed human TM cells. Elevated levels of TGF-β2 present in AH of POAG patients may elevate intraocular pressure, in part, by eliciting aberrant Rho G-protein dependent cell contraction, and increasing ET-1 synthesis and secretion, in human TM cells. (*Invest Ophthalmol Vis Sci.* 2012;53:5279–5286) DOI:10.1167/iovs.11-9289

Primary open-angle glaucoma (POAG) is one of the most common causes of blindness worldwide, affecting over 2 million individuals 45 years or older in the United States.[1] In POAG patients, irreversible loss of peripheral vision frequently is associated with a pathologic elevation of intraocular pressure (IOP).[2] Clinically, elevated IOP remains a poorly understood hallmark of POAG. In healthy eyes, normal IOP is maintained through a balance between production and outflow of aqueous humor (AH). In adults, the majority (>50%) of AH exits the eye by a conventional outflow pathway involving the trabecular meshwork (TM) at the iridocorneal angle.[3]

TM cells regulate AH outflow facility partly through contraction and relaxation of their actin cytoskeleton. Under pathologic conditions, chronic aberrant contraction of TM cells increases resistance to AH outflow, leading to abnormal and sustained elevation of IOP. The mechanism that promotes harmful chronic aberrant TM cell contraction in POAG remains unknown, but may involve dysregulation of small monomeric Rho G-protein mediated organization of the actin cytoskeleton.[4,5] Interestingly, analysis of AH samples from POAG patients reveal marked increases in the content of endothelin-1 (ET-1),[6–10] a potent vasoconstrictor that elicits Rho G-protein dependent TM cell contraction through ET_A receptor signaling. Under nonpathologic conditions, ET-1 immunoreactivity has been localized to the corneal epithelium, iris, ciliary body, choroid, retinal blood vessels, and the ciliary and optic nerves, but not the TM.[11,12] By comparison, TM cells differentially express high levels of contraction-promoting ET_A receptor, and contain little to no relaxation-promoting ET_B receptor.[12–16] In vitro, experimental application of ET-1 to TM cells promotes activation of the Rho/ROCK pathway, enhances Rho-dependent phosphorylation of myosin light chain, and contraction of the actomyosin cytoskeleton.[15,17–22] Similarly, ET-1 decreases outflow facility through bovine anterior segments in vitro.[18]

An alternative pathologic mechanism that may increase IOP in POAG patients is enhanced synthesis and secretion of extracellular matrix (ECM) components by TM cells.[23–26] Levels of transforming growth factor (TGF)-β2, a cytokine known to promote synthesis and release of ECM components, similarly are increased aberrantly in AH from POAG patients.[27–33] In the healthy anterior chamber, TGF-β2 is expressed in limbal epithelial cells, the ciliary body, and the conjunctival stroma, and largely serves to maintain immune privilege of the area.[34] In glaucomatous eyes, by comparison, chronically elevated TGF-β2 levels are associated with increased synthesis and deposition of ECM components by optic nerve head astrocytes[35–37] and by TM cells.[23,25,36,38,39] Similarly, TGF-β2 significantly enhances outflow resistance through human, monkey, or porcine anterior segments[23,40,41] and is associated with a concomitant increase in ECM accumulation.[41]

Emerging evidence strongly supports a pathologic association between either ET-1 or TGF-β2 with elevated IOP in the

pathogenesis of POAG. Mechanisms responsible for regulating endogenous synthesis and secretion of ET-1 and TGF-β2 within the eye currently remain unknown. A role for TGF-β in promoting transcription and release of ET-1 has been suggested.[42–47] In our study, we determined the effect of TGF-β signaling on expression and secretion of ET-1 by human TM cells.

MATERIALS AND METHODS

Human Trabecular Meshwork Cell Culture

The use of human cadaver material in our study was approved by the Edward Hines Jr. VA and Loyola University Chicago institutional review boards in compliance with the tenets of the Declaration of Helsinki. Fresh cadaver corneoscleral rims were obtained (Illinois Eye Bank, Chicago, IL) at the time of corneal transplant and primary human TM cells were prepared using a collagenase-free procedure as described previously.[3,48,49] Individual TM cell lines were restricted to 3–6 passages. SV40-transformed human TM cell lines derived from patients without or with glaucoma (NTM5 and GTM3, respectively) were a generous gift from Alcon Laboratories (Fort Worth, TX). Primary TM cell cultures were established on Falcon Primaria flasks in Eagle's minimum essential medium (EMEM) containing 2 mM L-glutamine supplemented with 5% adult bovine serum, 10% fetal bovine serum (FBS), 0.1% gentamicin, 1% amphotericin B, and a mixture of essential (Life Technologies, Grand Island, NY) and nonessential (Sigma-Aldrich, St. Louis, MO) amino acids.[3,48] Transformed TM cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM GlutaMAX-I supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). Human TM cell cultures were maintained at 37°C under a humidified atmosphere of 5% CO_2/95% air.

Treatment

Subconfluent primary or transformed human TM cells were cultured for 24 hours in serum-free media before treatment. Recombinant human TGF-β1 or TGF-β2 (Cell Signaling Technology, Danvers, MA) was reconstituted as a stock solution in 4 mM HCl containing 0.1% BSA for 30 minutes at 23°C before use. Serum-starved cultures were treated (24 hours) in the absence (vehicle, 200 nM HCl) or presence (5 ng/mL) of TGF-β1 or TGF-β2 in fresh serum-free media. TM cell viability was routinely determined by Trypan Blue dye exclusion and was consistently >90%. To determine mechanism of action, GTM3 cells were co-treated (24 hours) with TGF-β2 (5 ng/mL) in the absence or presence of SB-505124 (1 μM), a TGF-β type I receptor (TGFβRI)/activin receptor-like kinase 5 (ALK-5) inhibitor; BQ-123 (1 μM), an ET_A antagonist; or BQ-788 (1 μM), an ET_B antagonist. To determine the role of Rho G-proteins, TM cells were pretreated (1 hour) with chemically-activated lovastatin (10 μM)[3,48,50]; GGTI-298 (10 μM, a geranylgeranyl transferase-I inhibitor) or with cell-permeable exoenzyme C3 transferase (10 μg /mL, a selective Rho G-protein subfamily inhibitor; Cytoskeleton, Denver, CO). Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich.

Real Time RT-PCR

Total RNA was extracted from primary or transformed human TM cells using TRIzol reagent, and 5 μg were reverse-transcribed using Super Script III First Strand Synthesis system (Life Technologies) as described previously.[3,48] Human specific ppET-1 or GAPDH cDNA sequences were amplified by real-time PCR on a Mini-Opticon PCR detection system using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Human specific ppET-1 primer sequences were as follows: sense, 5′-TATCAGCAGTTAGTGAGAGG, antisense, 5′-CGAAGGTCTGTCACCAA TGTGC. GAPDH, sense, 5′-TCCCTCAAGATTGTCAGCAA, antisense, 5-AGATCCACAAC-GGATACATT primers were used as a reference

control. Optimized amplification steps of 94°C × 5 minutes followed by 94°C × 45 seconds, annealing at optimized temperatures (58°C for ppET-1, 62°C for GAPDH) for 30 seconds, and 72°C × 60 seconds were used. For each sample, the specificity of the real-time reaction product was determined by melting curve analysis. Reaction efficiencies typically were >90%. Data are expressed as relative fold-changes in mRNA content normalized to GAPDH.

Endothelin-1 ELISA

The content of ET-1 in cell culture media was assessed using a commercially-available ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. The working range for this human-specific ET-1 ELISA kit is 0.39–25 pg/ml. Media from primary or transformed human TM cells cultured in 12-well cell culture plates were harvested, centrifuged ($700g \times 5$ minutes) to remove particulate, and aliquots (75 μL) were added to microtiter wells precoated with a monoclonal antibody against human ET-1. Samples were read at 450 nm with a 540 nm correction, and results expressed as pg of ET-1.

Filamentous Actin Staining

NTM5 or GTM3 cells were cultured on Nunc Lab-Tek II chambered slides overnight (24 hours) in serum-free DMEM and treated × 24 hours without (vehicle, 200 nM HCl) or with 5 ng/mL TGF-β1 or TGF-β2. Some cultures were pretreated (1 hour) with chemically-activated lovastatin (10 μM) or GGTI-298 (10 μM) before TGF-β2 treatment. Treated cells were fixed ×15 minutes at 23°C by immersion in phosphate buffered (pH 7.4) 4% paraformaldehyde. Filamentous actin stress fiber organization was visualized using AlexaFluor488-conjugated phalloidin. Stained slides were mounted using Fluoroshield containing DAPI (Sigma), and visualized by confocal microscopy.

Statistical Analysis

Results are expressed as mean ± SD of duplicate (primary) or triplicate (transformed) cultures, repeated at least one additional time unless otherwise specified. Parametric data were analyzed by Student's t-test or by one-way ANOVA followed by either a Dunnett's or Bonferroni's multiple comparison post-hoc analysis, as indicated. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

TGF-β2 Increases ppET-1 mRNA Content

Transformed human TM cells (NTM5) conditioned overnight in serum-free media and incubated subsequently for 24 hours in the presence of TGF-β2 (5 ng/mL) exhibited a >8-fold increase in ppET-1 mRNA content compared to vehicle-treated controls (Fig. 1A). TM cells derived from a glaucomatous donor (GTM3) exhibited, by comparison, a marked >18-fold increase in ppET-1 mRNA content (Fig. 1B) in response to 5 ng/mL TGF-β2 (24 hours). These responses were not unique to transformed human TM cells, as primary human TM cells cultured from discarded corneoscleral rims exhibited a marked >30-fold increase in ppET-1 mRNA content following TGF-β2 treatment (Fig. 3B). TGF-β1 (5 ng/mL, 24 hours) treatment similarly enhanced ppET-1 mRNA content 10 ± 1-fold ($n = 6$, $P < 0.01$) and 11 ± 2-fold ($n = 6$, $P < 0.01$) in serum-free conditioned NTM5 and GTM3 cells, respectively (data not shown).

The effect of TGF-β2 on relative changes in ppET-1 mRNA content was dose- and time-dependent (Figs. 1C, 1D). Conditioned GTM3 cells responded maximally to a 5 ng/mL × 24 hours dose of TGF-β2 (Fig. 1C), consistent with what commonly is reported in the literature.[39,51–53] GTM3 cells also responded to 5 ng/mL TGF-β2 stimulation in a time-dependent
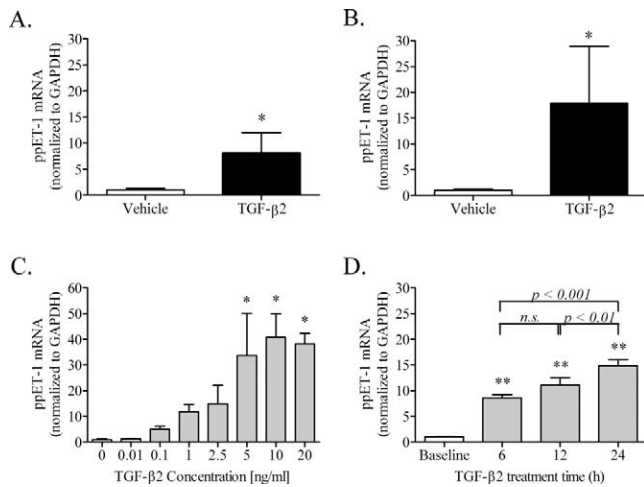
FIGURE 1. TGF-β2 increases prepro (pp) ET-1 mRNA content in human TM cells. (**A**) Normal NTM5 or (**B**) glaucomatous GTM3 cells were conditioned in serum-free DMEM (24 hours) and then incubated in the absence (vehicle, 200 nM HCl) or presence (5 ng/mL) of TGF-β2 in fresh serum-free DMEM for 24 hours. (**C, D**) Conditioned GTM3 cells were cultured in fresh serum-free DMEM (**C**) containing 0–20 ng/mL TGF-β2 as indicated for 24 hours, or (**D**) for 0 (baseline), 6, 12, or 24 hours with 5 ng/mL TGF-β2. Levels of ppET-1 mRNA were quantified by qRT-PCR. Data shown are the GAPDH-normalized fold changes from 2 to 3 separate experiments ($N = 6-9$) each performed in triplicate and expressed as mean ± SD. Statistical significance between groups is shown. (**A, B**) *$P < 0.01$, unpaired Student's t-test. (**C**) *$P < 0.01$ compared to 0 ng/mL, one-way ANOVA with Dunnett's post-hoc analysis. (**D**) *$P < 0.01$ compared to Baseline, one-way ANOVA with Bonferroni's post-hoc analysis.

manner, exhibiting significant and sustained changes in ppET-1 mRNA content at 6–12 hours of incubation (Fig. 1D).

The mechanism by which TGF-β2 elicits marked changes in ppET-1 mRNA content in TM cells was determined partly by the use of selective TGFβRI/ALK-5 and endothelin receptor antagonists (Fig. 2). Conditioned GTM3 cells were co-incubated for 24 hours with TGF-β2 (5 ng/mL) in the absence (vehicle, 200 nM HCl) or presence of SB-505124 (a TGFβRI/ALK-5 antagonist). SB-505124 (1 μM) prevented TGF-β2–mediated increases in ppET-1 mRNA content (Fig. 2A). In contrast, co-incubating conditioned cells with ET_A or ET_B antagonists BQ-123 (1 μM) or BQ-788 (1 μM), respectively, had no effect on TGF-β2 induced changes in ppET-1 mRNA
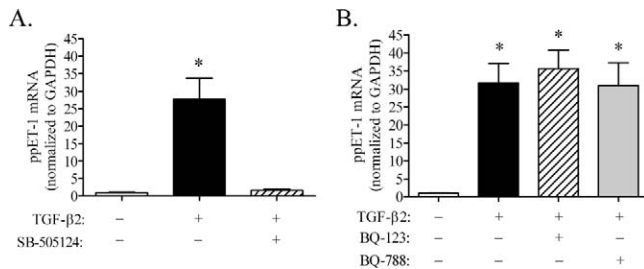

FIGURE 2. Blocking TGFβRI/ALK-5, but not ET_A or ET_B, prevents TGF-β2–mediated increases in ppET-1 mRNA content. GTM3 cells were cultured in serum-free DMEM (24 hours) and subsequently incubated without (vehicle, 200 nM HCl) or with TGF-β2 (5 ng/mL, 24 hours) in the absence or presence of (**A**) SB-505124 (1 μM), or (**B**) BQ-123 (1 μM) or BQ-788 (1 μM) as indicated. Levels of ppET-1 mRNA were quantified by qRT-PCR. Data shown are the GAPDH-normalized fold changes ($N = 3-6$) from 1 to 2 separate experiments each performed in triplicate and expressed as mean ± SD. *$P < 0.01$, one-way ANOVA with Dunnett's post-hoc analysis.
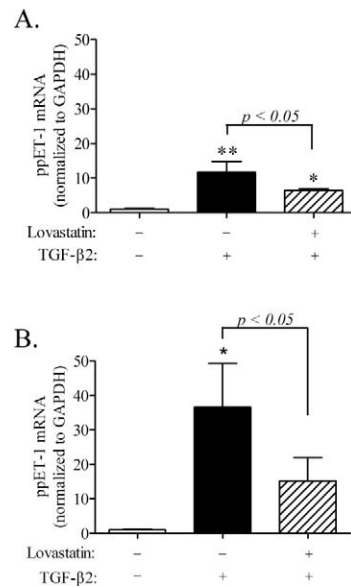

FIGURE 3. Lovastatin attenuates TGF-β2–mediated increases in ppET-1 mRNA content in transformed and primary human TM cells. (**A**) NTM5 or (**B**) primary human TM cells were conditioned in serum-free medium (24 hours) and pretreated × 1 hour without (0.01% ethanol) or with lovastatin (10 μM) as indicated. Pretreated cultures subsequently were incubated in the absence (200 nM HCl) or presence of TGF-β2 (5 ng/mL) as indicated for an additional 24 hours. Levels of ppET-1 mRNA content were quantified by qRT-PCR. Data shown are the GAPDH-normalized fold changes from 1 to 2 separate experiments ($N = 2-6$) performed in (**A**) triplicate or (**B**) duplicate and expressed as mean ± SD. Statistical significance between groups is shown. *$P < 0.05$; **$P < 0.01$ compared to control, one-way ANOVA with Bonferroni's post-hoc analysis.

expression (Fig. 2B). These findings are consistent with previous studies showing that Smad3/AP-1 transcriptional activation of the ET-1 promoter accounts for TGF-β–mediated increases in endothelial cell ppET-1 mRNA levels.[44]

Pretreating TM cells with SIS3, a specific inhibitor of the canonical TGF-β effector Smad3, however, did not reduce TGF-β2 enhanced ppET-1 mRNA expression (data not shown), suggesting the involvement a non-canonical TGF-β signaling pathway. Previously, a role for small monomeric G-proteins in the regulation of ppET-1 expression has been suggested.[54-56] To determine the involvement of small G-protein signaling in TGF-β2–mediated increases in ppET-1 expression, conditioned primary or transformed human TM cells were pretreated (1 hour) with vehicle (0.01% ethanol) or lovastatin (10 μM, an indirect inhibitor of small G-protein signaling) followed by the addition of TGF-β2 (5 ng/mL, 24 hours). Pretreatment with lovastatin attenuated significantly TGF-β2–mediated increases in ppET-1 mRNA content in transformed and primary human TM cells (Fig. 3, see Table). Lovastatin pretreated NTM5 cells exhibited a 45 ± 4% decrease in ppET-1 mRNA content as compared to vehicle controls (Fig. 3A). Importantly, conditioned primary human TM cells pretreated with lovastatin also exhibited a marked attenuation (59 ± 14%) of TGF-β2–induced ppET-1 mRNA content (Fig. 3B). Similarly, TGF-β2–enhanced ppET-1 mRNA content was attenuated significantly following lovastatin pretreatment in GTM3 cells (see Table).

One mechanism by which statins indirectly inhibit small G-protein signaling in TM cells is by limiting the availability of isoprenoid intermediates required for post-translational modification and activation of Rho G-proteins.[3,48,50] Pretreating (1 hour) conditioned GTM3 cells with GGTI-298 (10 μM, an inhibitor of Rho, Rac, Cdc42 G-protein geranylgeranylation)

TABLE.   Percent of TGF-β2 Mediated ppET-1 mRNA Content following Inhibition of Rho G-Protein Signaling

	Percent Content		
	Lovastatin	GGTI-298	C3
Vehicle	100 ± 26%	100 ± 18%	100 ± 2%
Inhibitor	26 ± 5%	13 ± 2%	26 ± 10%
P value	0.0080	0.0012	0.0008

GTM3 cells were conditioned for 24 hours in serum-free DMEM and then pretreated ×1 hour in the absence (vehicle) or presence (inhibitor) of lovastatin (10 μM), GGTI-298 (10 μM), or C3 exoenzyme (10 μg/mL). Pretreated cultures subsequently were incubated in the presence of TGF-β2 (5 ng/mL) for an additional 24 hours. Levels of ppET-1 mRNA content were quantified by qRT-PCR. Data shown are representative GAPDH-normalized fold changes from 2 separate experiments performed in triplicate (*N* = 6), expressed as the mean ± SD percent of vehicle. Statistical significance to respective vehicle control groups is shown (Student's *t*-test).

attenuated significantly TGF-β2–mediated increases in ppET-1 mRNA content (see Table). To target specifically the Rho subfamily (RhoA/B/C) of G-proteins, conditioned GTM3 cells were treated with the ADP ribosyl transferase C3 exoenzyme (10 μg/mL, 1 hour pretreatment). Pretreatment with C3 mimicked lovastatin-dependent attenuation of TGF-β2–mediated increases in ppET-1 mRNA content in GTM3 cells (see Table).

TGF-β2 Increases Mature ET-1 Peptide Secretion

Culture medium collected from TGF-β2 (5 ng/mL, 24 hours) treated TM cells contained increased levels of mature ET-1 peptide (Fig. 4). The content of ET-1 peptide secreted by resting NTM5 (1.7 ± 0.3 pg/mL), resting GTM3 (0.1 ± 0.1 pg/mL), or resting primary human TM (0.05 ± 0.00 pg/mL) cells was at or below the limit of detection. TGF-β2 (5 ng/mL, 24 hours) induced marked, significant increases in secreted ET-1

content in the culture medium from NTM5 cells (Fig. 4A), GTM3 cells (Fig. 4B), and primary human TM cells (Fig. 4C). These changes were not unique in response to TGF-β2, as similar changes in the level of secreted ET-1 (NTM5 24.2 ± 6.1 pg/mL, GTM3 7.4 ± 1.1 pg/mL) were seen in the culture medium following incubation with TGF-β1 (5 ng/mL, 24 hours). TGF-β2–induced changes in the content of secreted ET-1 were dose- and time-dependent (Figs. 4D, 4E). Significant TGF-β2–mediated increases in ET-1 secretion were observed at 1 ng/mL, and were near maximal at 2.5 ng/mL (Fig. 4D). By comparison, accumulation of secreted ET-1 was enhanced significantly by 6 hours, and enhanced further following 24 hours of TGF-β2 treatment (Fig. 4E).

Inhibition of TGFβRI/ALK-5 with SB-505124 (1 μM) completely prevented TGF-β2–mediated increases in secreted ET-1 peptide (Fig. 5A), whereas the endothelin receptor antagonists BQ-123 and BQ-788 (1 μM each) had no effect on the accumulation of extracellular ET-1 peptide by TGF-β2 (Fig. 5B). By comparison, inhibition of Rho G-protein signaling markedly attenuated TGF-β2–mediated increases in secreted ET-1 content (Fig. 6). Conditioned GTM3 cells pretreated with either 10 μM lovastatin (Fig. 6A) or GGTI-298 (Fig. 6B) exhibited marked, significant (>70%) reduction in ET-1 peptide accumulation in the culture medium. By comparison, specific inhibition of RhoA/B/C with exoenzyme C3 transferase (10 μg/mL, 1 hour pretreatment) completely prevented the effect of TGF-β2 on secreted ET-1 peptide content in GTM3 (Fig. 7A) and primary human TM (Fig. 7B) cells.

TGF-β2 Increases Filamentous Actin Stress Fiber Organization

TM cells treated with TGF-β1 or TGF-β2 exhibit enhanced expression of α-smooth muscle actin[51,57,58] as well as organization of F-actin stress fibers.[58,59] Consistent with these reports, conditioned NTM5 or GTM3 cells in our study responded to TGF-β1 (5 ng/mL, 24 hours, data not shown)
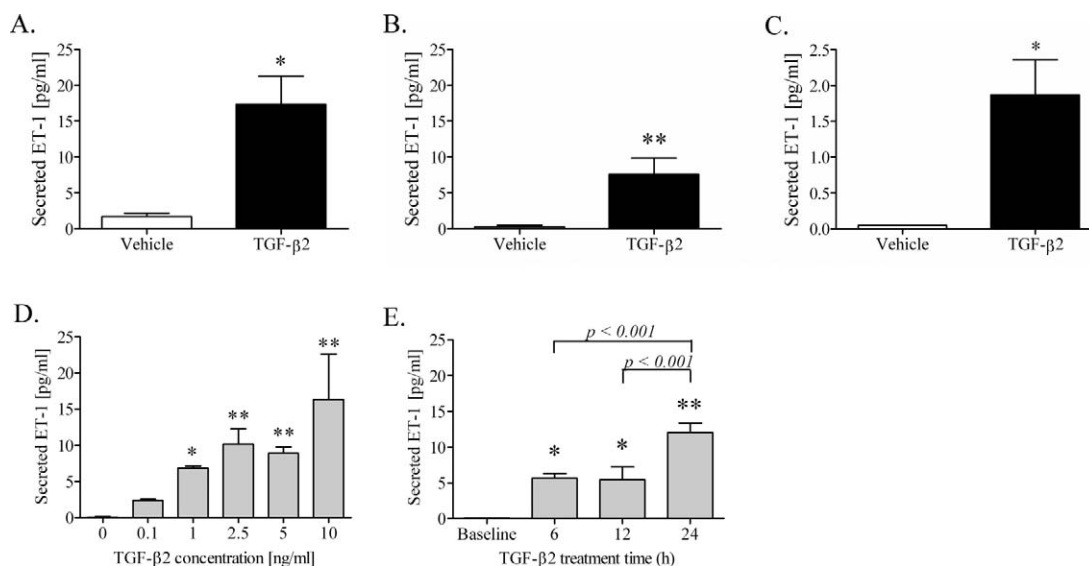

FIGURE 4.   TGF-β2 induces ET-1 secretion in transformed and primary human TM cells. (A) NTM5, (B) GTM3, or (C) primary human TM cells were conditioned in serum-free medium (24 hours) and then incubated × 24 hours in the absence (vehicle, 200 nM HCl) or presence (5 ng/mL) of TGF-β2 in fresh serum-free medium. (D, E) Conditioned GTM3 cells were cultured in fresh serum-free DMEM (D) containing 0–10 ng/mL TGF-β2 for 24 hours, or (E) with 5 ng/mL TGF-β2 for 0 (baseline), 6, 12, or 24 hours. ET-1 content present in culture medium was quantified by ELISA. Data shown are the mean ± SD (*N* = 3–9) from 1 to 3 separate experiments each performed in triplicate. Statistical significance between groups is shown. (A–C) *P < 0.01; **P < 0.0001, Student's *t*-test. (D) *P < 0.05; **P < 0.01 compared to 0 ng/mL, one-way ANOVA with Dunnett's post-hoc analysis. (E) *P < 0.01; **P < 0.001 compared to baseline, one-way ANOVA with Bonferroni's post-hoc analysis.
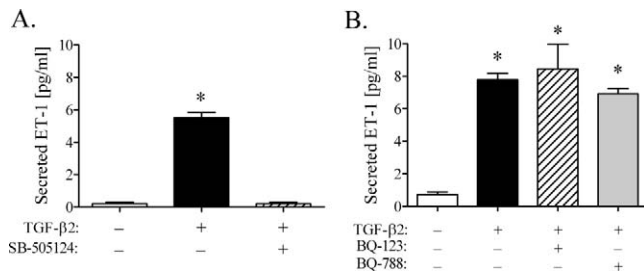
FIGURE 5. Blocking TGFβRI/ALK-5, but not ET_A or ET_B, prevents TGF-β2–mediated increases in secreted ET-1 content. GTM3 cells were conditioned in serum-free DMEM (24 hours) and subsequently incubated without (vehicle, 200 nM HCl) or with TGF-β2 (5 ng/mL, 24 hours) in the absence or presence of (A) SB-505124 (1 μM), or (B) BQ-123 (1 μM) or BQ-788 (1 μM) as indicated. ET-1 content present in culture medium was quantified by ELISA. Data shown are the mean ± SD ($N = 3$) from single experiments performed in triplicate. Statistical significance between groups is shown. *$P < 0.01$, one-way ANOVA with Dunnett's post-hoc analysis.

or TGF-β2 (5 ng/mL, 24 hours) by markedly enhancing F-actin stress fiber organization (Fig. 8). Pretreating (1 hour) conditioned GTM3 cells with lovastatin (10 μM) or with GGTI-298 (10 μM) prevented this effect of TGF-β2 (Fig. 8), suggesting the involvement of small monomeric G-proteins in facilitating TGF-β2–mediated increases in F-actin stress fiber organization.

DISCUSSION

To our knowledge, primary and transformed human TM cells are shown for the first time to respond to TGF-β2 signaling, in
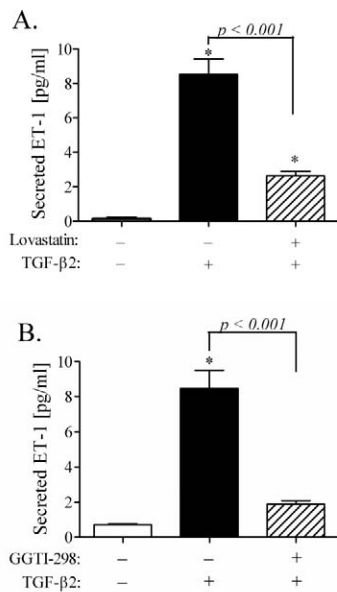

FIGURE 6. Inhibition of geranylgeranylation attenuates TGF-β2–induced ET-1 secretion in transformed human TM cells. GTM3 cells conditioned for 24 hours in serum-free DMEM were pretreated ×1 hour in the absence or presence of (A) lovastatin (10 μM) or (B) GGTI-298 (10 μM) as indicated. Pretreated cultures subsequently were incubated without (200 nM HCl) or with TGF-β2 (5 ng/mL) as indicated for an additional 24 hours. ET-1 content present in culture medium was quantified by ELISA. Data shown are the mean ± SD ($N = 3$–6) from 1 to 2 experiments performed in triplicate. Statistical significance between groups is shown. *$P < 0.001$ compared to control, one-way ANOVA with Bonferroni's post-hoc analysis.
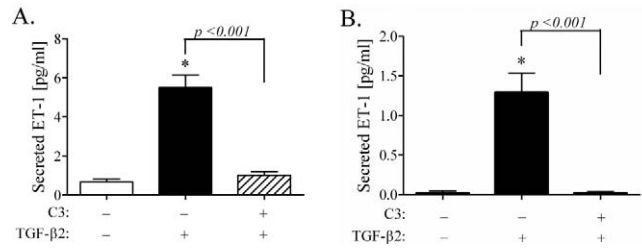
FIGURE 7. Inhibition of Rho G-protein signaling attenuates TGF-β2–mediated increases in secreted ET-1 content. (A) Transformed GTM3 or (B) primary human TM cells were conditioned overnight (24 hours) in serum-free media and then pretreated ×1 hour in the absence or presence of exoenzyme C3 transferase (10 μg/mL) in fresh serum-free medium as indicated. Pretreated cultures subsequently were incubated without (200 nM HCl) or with TGF-β2 (5 ng/mL) as indicated for an additional 24 hours. ET-1 content present in culture medium was quantified by ELISA. Data shown are the mean ± SD ($N = 3$) from single experiments performed in triplicate. Statistical significance between groups is shown. *$P < 0.001$ compared to control, one-way ANOVA with Bonferroni's post-hoc analysis.

vitro, by markedly enhancing the intracellular expression and extracellular content of the vasoconstrictive peptide ET-1. TGF-β2, signaling through the TGFβRI/ALK-5 receptor, elicits marked increases in ET-1 mRNA content and ET-1 peptide secretion from cultured primary and transformed human TM cells. TGF-β2–mediated increases in mature ET-1 secretion involved partly a novel Rho G-protein subfamily mediated non-canonical signaling pathway. The functional consequence of TGF-β2 signaling in human TM cells was underscored by a marked increase in F-actin stress fiber organization that similarly involved the activation of geranylgeranylated small GTP-binding proteins. Here, we propose that elevated levels of TGF-β2 present in AH of POAG patients may elevate IOP partly
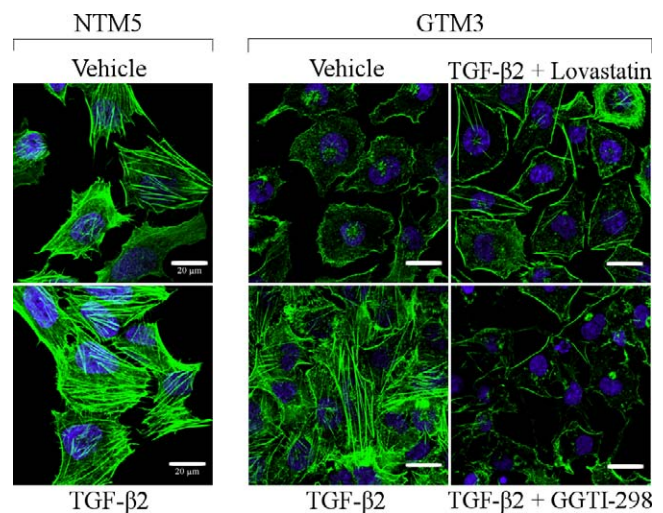
FIGURE 8. TGF-β2 promotes G-protein mediated F-actin stress fiber organization. NTM5 (*left panels*) or GTM3 (*middle, right panels*) cells were incubated overnight (24 hours) in serum-free DMEM and subsequently treated without (vehicle, 200 nM HCl) or with TGF-β2 (5 ng/mL) for an additional 24 hours as indicated. In some experiments, GTM3 cells were pretreated (1 hour) with lovastatin (10 μM) or GGTI-298 (10 μM) before the addition of TGF-β2 as indicated. Data shown are confocal photomicrographs of stained cells representative of 7 to 20 separate cultures. Nuclei are counterstained with DAPI. Bar: 20 μm.

by eliciting Rho G-protein mediated aberrant ET-1 synthesis and secretion, and concurrent actin stress fiber organization, in human TM cells.

Of the three TGF-β isoforms present in mammalian cells, TGF-β2 has been associated most frequently with POAG.[27–33] Elevated levels of TGF-β2 within the AH of POAG patients raise the possibility of a pathologic correlate to ocular hypertension and subsequent retinal injury. A similar thesis recently has been proposed for elevated levels of ET-1 observed in AH of POAG patients.[6–10,15,17–22] Whereas an association between TGF-β2 and POAG has been proposed previously, a causal relationship between TGF-β2 and ET-1 in association with the pathogenesis of POAG has not been addressed previously to our knowledge. In our study, TGF-β1 and TGF-β2 were found to be equally effective at inducing marked increases in ppET-1 mRNA expression and extracellular ET-1 peptide release by primary human TM cells as well as established transformed human TM cell lines. The relative synthesis of ppET-1 and constitutive release of mature ET-1 peptide by resting primary or transformed human TM cells, in comparison, was negligible. These findings are consistent with ET-1 functioning within the anterior chamber as a potentially potent inducible modulator of ocular pressure.

TGF-β induction of ppET-1 expression and ET-1 peptide secretion is not without precedence, and has been established in vascular endothelial cells.[42–47] The mechanism by which TGF-β induces ppET-1 expression and ET-1 peptide release by human TM cells is unclear, but may involve the activation of canonical (Smad) and non-canonical (Rho G-protein, MAPK) signaling pathways.[42,43,45,47,60] A role for TGF-β at enhancing ppET-1 mRNA stability, while not determined in our study, remains unlikely given the inducible nature of this prepropeptide.[61] However, treating GTM3 cells in our study with SIS3, a Smad3 specific inhibitor, did not reduce TGF-β2–induced ppET-1 mRNA expression (data not shown), suggesting the involvement of a non-canonical TGF-β signaling pathway in the expression of this message. Disrupting small G-protein signaling with lovastatin, GGTI-298, or with cell-permeable exoenzyme C3 transferase significantly reduced or completely prevented TGF-β2–induced ppET-1 mRNA expression and ET-1 peptide secretion. These collective findings strongly support a non-canonical role of small monomeric G-proteins, particularly those belonging to the Rho subfamily, in TGF-β2–induced upregulation of ET-1 expression and release by cultured human TM cells.

Consistent with these observations are reports of Rho kinase-dependent enhancement of TGF-β1–induced ppET-1 mRNA expression in hepatic stellate cells[47] and of Rho G-protein activation of activator protein-1 (AP-1) transcriptional activity.[62–64] The ET-1 promoter sequence contains Smad3 and AP-1 responsive elements.[42–44] We propose that in human TM cells, TGF-β2–mediated upregulation of ppET-1 mRNA expression occurs by a mechanism involving non-canonical Rho G-protein–dependent activation of AP-1. The mechanisms that regulate TGF-β2–induced release of mature ET-1 peptide from human TM cells remain unclear, but similarly may involve activation of small G-proteins.

A comparison, as presented within our study, between TM cells derived from healthy (NTM5) and glaucomatous (GTM3) donors revealed some compelling differential responses to TGF-β2 signaling. Compared to NTM5 cells, GTM3 and primary human TM cells responded more robustly to TGF-β2 as evidenced by greater relative increases in induced ppET-1 mRNA content. In contrast, media harvested from NTM5 cells treated with either TGF-β1 or –β2 contained more ET-1 peptide compared to GTM3 cells. Whereas consequences of cellular transformation may influence TM cell responses, our results presented using primary human TM cells indicated that primary human TM cells also respond robustly to TGF-β2 treatment. These findings corroborated our previously reported studies, which demonstrate phenotypic and morphologic similarities between transformed and primary human TM cells.[3,48,50] While interesting, the definitive evaluation of these differential findings awaits the use of primary human TM cells harvested from glaucomatous patients.

Previous studies suggest that TGF-β elevates IOP by primarily promoting changes in ECM synthesis or ECM elasticity.[23] Other studies, however, support a functional role for TGF-β2 at inducing changes to TM cell actin cytoskeletal dynamics.[51,57–59,65] Specifically, TGF-β2 enhances experimental collagen gel contraction by inducing contractile actin stress fiber organization in human and bovine TM cells.[58,59] In NTM5 and GTM3 cell lines, TGF-β2 induced marked increases in actin stress fiber organization, consistent with the role of TGF-β2 at enhancing the contractile tone of TM and increasing resistance to AH outflow. O'Reilly et al. reported a modest increase in the organization of cross-linked actin networks (CLANs) in TGF-β2–treated TM cells.[65] However, NTM5 or GTM3 cell lines used here failed to exhibit recognizable CLAN formation in response to TGF-β signaling. Similar to induction of ppET-1 mRNA, the mechanism by which TGF-β2 induces F-actin stress fiber reorganization in human TM cells may involve the activation of non-canonical Rho G-protein pathways as evidenced by inhibition with lovastatin or GGTI-298.

In conclusion, our study provides the first evidence to our knowledge that TGF-β2 elicits marked increases in ppET-1 mRNA content, as well as mature ET-1 peptide secretion, from primary and transformed human TM cells, partly through a non-canonical Rho G-protein–mediated mechanism. Elevated levels of TGF-β2 present in AH of POAG patients may elevate IOP partly by eliciting Rho G-protein–mediated aberrant ET-1 synthesis and secretion, and concurrent actin stress fiber organization in human TM cells.

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