p38 MAPK Mediates the Expression of Type I Collagen Induced by TGF-β2 in Human Retinal Pigment Epithelial Cells ARPE-19

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Purpose. Transforming growth factor (TGF)-β has been implicated as the key mediator of proliferative vitreoretinopathy, but the cellular mechanisms by which TGF-β induces extracellular matrix protein (ECM) synthesis are not fully understood. The current study was conducted to examine whether the mitogen-activated protein kinase (MAPK) pathway is involved in TGF-β2–induced collagen expression in retinal pigment epithelial cells.

Methods. Human retinal pigment epithelial cells ARPE-19 were cultured and stimulated with various concentrations of TGF-β2. The type I collagen gene (COL1A1, COL1A2) expression induced by TGF-β2 was evaluated by real-time RT-PCR. Synthesis of type I collagen was evaluated by the concentration of the C-terminal propeptide of type I (PICP) in the medium. The activation of MAPK pathways by TGF-β2 was assessed by immunoblot with anti-phospho-p38 and anti-phospho-extracellular signal-regulated kinase (ERK)1/2 antibodies. The role of MAPK was assessed using biochemical inhibitors. To examine the transcriptional activities of COL1A1 and COL1A2, luciferase reporter assays were also performed.

Results. mRNA expression of COL1A1 and COL1A2 and type I collagen synthesis were activated by TGF-β2. Both ERK and p38 MAPK pathways were also activated by TGF-β2. The biochemical blockade of p38 MAPK activation, but not ERK activation, inhibited TGF-β2–induced type I collagen mRNA expression and type I collagen synthesis. Moreover, blockade of the p38 MAPK pathway inhibited the increase in both COL1A1 and COL1A2 promoter activities when induced by TGF-β2.

Conclusions. TGF-β2 activates p38 MAPK and p38 MAPK plays a role in relaying the TGF-β2 signal to type I collagen production in the retinal pigment epithelium. (*Invest Ophthalmol Vis Sci.* 2004;45:2431–2437) DOI:10.1167/iovs.03-1276

Proliferative vitreoretinopathy (PVR) is a major cause for failure of rhegmatogenous retinal detachment surgery.[1,2] It is characterized by cellular proliferation and membrane formation within the vitreous cavity and on the surface of the retina.[3,4] When the blood–retinal barrier is breached, serum components and inflammatory cells enter the vitreous cavity and subretinal space, exposing the retinal pigment epithelial (RPE) cells to a variety of cytokines. This exposure leads to activation of the RPE cells and subsequent separation from the established monolayer. Activated RPE cells proliferate and migrate through retinal tears to form pathologic membranes on both surfaces of the neural retina.[3] These PVR membranes are composed of RPE cells, retinal glial cells, fibroblasts, and macrophages, as well as a host of accumulated extracellular matrix proteins (ECM).[5–7]

Type I collagen, the major component of the ECM in PVR membranes,[8] is a heterotrimer composed of coordinately expressed two α1 chains and one α2 chain. They are encoded by distinct genes, COL1A1 and COL1A2, respectively, and their expression is modulated by various cytokines.[9]

Transforming growth factor (TGF)-β is a member of a superfamily of multifunctional cytokines which function during development, wound repair, and other pathologic processes. It is a potent inducer of ECM protein synthesis and accumulation and has been implicated as the key mediator of various fibrogenous diseases, including PVR.[10] Evidence for high concentrations of TGF-β in vitreous aspirates from patients diagnosed with PVR has highlighted its importance in PVR.[11] Of note, immunologic assays showed that most TGF-β in the vitreous aspirates was from the TGF-β2 isoform, not TGF-β1.[11,12] However, to our knowledge, there are no reports describing the effect of TGF-β2 on the expression of type I collagen mRNA in the RPE. Furthermore, despite many data demonstrating the effects of TGF-β on ECM induction in glomerulonephritis, liver cirrhosis, and lung fibrosis,[13] the cellular mechanisms by which TGF-β exerts its effects have not been fully elucidated. Recent evidence has indicated that TGF-β transduces signals through two different pathways, Smad, and mitogen activated protein kinase (MAPK), through extracellular signal regulated kinase (ERK) and p38 MAPK.[14–16]

This investigation was undertaken to determine whether TGF-β2 activates specific MAPK signaling pathways in a cultured human retinal pigment epithelial cell line, ARPE-19, and whether this MAPK activation mediates TGF-β2–induced promoter activity, mRNA expression, and protein synthesis of type I collagen.

Materials and Methods

Reagents

Recombinant human TGF-β2 was purchased from R&D Systems (Minneapolis, MN). SB203580 and PD98059 were purchased from Calbiochem (San Diego, CA). Antibodies were purchased from the following vendors: anti-phospho-p38 MAPK (Thy180/Tyr182) and anti-phospho-Smad2/3 (Ser465/Ser467) rabbit polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). Anti-p38 MAPK, anti-phospho-ERK1/2 (Tyr204), and anti-ERK1/2 rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Smad2/3 rabbit polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY).

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Cell Culture and Stimulation by TGF-β2

Cells of the human retinal pigment epithelial line ARPE-19 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). ARPE-19 is an immortalized, nontransfected cell line that spontaneously arose from cultures of human RPE.[17] Cells were maintained in 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's and Ham's F12 medium (DMEM/F12; ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sanko Junyaku, Tokyo, Japan) and antibiotics (100 U/mL penicillin G and 100 mg/mL streptomycin sulfate) in a humidified incubator at 37°C in 5% CO_2. Cells at passage numbers 13 to 18 were used in the present studies.

When cultures achieved confluence, the medium was removed and replaced with serum-free DMEM/F12. After 24 hours of serum starvation, various concentrations of TGF-β2 were added to the medium, and the cultures were incubated for another 24 hours for RNA isolation or 48 hours for protein analysis. In the experiments using the MEK-1 inhibitor, PD98059, and the p38 MAPK inhibitor, SB203580, we preincubated cells for 30 minutes before treatment with or without exogenous TGF-β2.

RNA Isolation and cDNA Synthesis

Total RNA was prepared using a minikit (RNeasy; Qiagen, Valencia, CA). Cells in lysis buffer containing 1% β-mercaptoethanol were passed through a separation column (QIAShredder; Qiagen), and total RNA was obtained according to the supplier's protocol. cDNA was produced with a kit (Omniscript RT; Qiagen), using random hexamers. To confirm TGF-β2–treated ARPE-19 cells continued to express RPE65 mRNA, cDNA was amplified (HotStarTaq DNA Polymerase; Qiagen). The specific primers used for testing the presence of RPE65 mRNA (NM_000329) are as follows: 5′-TTCTGAGTGTGGTGGTGAGC-3′ (sense) and 5′-GGCCTGTCTCACAGAGGAAG-3′ (antisense).

The thermal cycling conditions included 1 cycle at 95°C for 15 minutes, 30 cycles at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. The PCR product was confirmed by sequence analysis using a sequencer (model 310; Applied Biosystems, Foster City, CA).

Real-Time PCR

Real-time PCR was performed using a sequence-detection system (Prism TM 7700; Applied Biosystems). This system is based on the ability of the 5′ nuclease activity of *Taq* polymerase to cleave a dual-labeled fluorogenic hybridization probe during DNA chain extension. The probe is labeled with a reporter fluorescent dye, FAM, at the 5′ end and a quencher fluorescent dye, TAMRA, at the 3′ end. During the extension phase of PCR, the nucleolytic activity of the DNA polymerase cleaves the hybridization probe and releases the reporter dye from the probe with a concomitant increase in reporter fluorescence. The sequence-specific primers and probe mixtures for human COL1A1, human COL1A2, and human GAPDH were from predeveloped assays (Assays-on-Demand; Applied Biosystems). The thermal cycling conditions included 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, 60°C for 1 minute. The relative mRNA expression levels of COL1A1 and COL1A2 were normalized against that of the GAPDH gene from the same RNA preparations, using a comparative threshold cycle method.[18]

Measurement of the C-Terminal Propeptide of Type I Collagen

Cells were placed in six-well dishes, incubated in medium containing 10% FBS and grown to confluence. The medium was changed to serum-free DMEM/F12 for 24 hours, followed by incubation in the presence or absence of various concentrations of TGF-β2, with or without PD98059 or SB203580 in the medium containing 200 μM of L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries, Osaka, Japan).[19] After 48 hours, 20 μL of the conditioned medium was used in subsequent assays.

The concentration of PICP in the medium was measured with a PICP enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's method (TaKaRa Biochemicals Co., Osaka, Japan).

Preparation of Cell Lysates and Western Blot Analysis

Cells grown to confluence were cultured for 24 hours with serum-free medium and then treated with 10 μg/mL of TGF-β2 for different periods. The cells were washed twice with ice-cold phosphate-buffered saline (PBS), lysed on ice in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Na-deoxycholate) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin). Cell extracts were then centrifuged at 15,000*g* for 10 minutes at 4°C, supernatants were collected, and protein content was determined using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL). Fresh cell extracts were prepared in sample buffer (0.125 M Tris-HCl [pH 6.8]) 4% SDS, 20% glycerol, 0.002% bromophenol blue, and 10% 2-mercaptoethanol). Samples were resolved by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Equal loading and appropriate transfer of each lane was confirmed by staining the PVDF membrane with the ponceau S solution (Sigma-Aldrich, St. Louis, MO). After they were blocked with 5% skim milk or 5% BSA in Tris-buffered saline containing 0.1% Tween-20 for 1 hours at room temperature, membranes were incubated with anti-ERK2 antibody (C-14), anti-phospho-ERK antibody (E-4), anti-p38 MAPK antibody, anti-phosph-p38 MAPK antibody, anti-Smad2/3 antibody, or anti-phospho-Smad2/3 antibody, for 1 hour at room temperature or overnight at 4°C, followed by incubation with the horseradish peroxidase (HRP)–conjugated anti-rabbit IgG for 1 hour at room temperature. The signals were enhanced using a chemiluminescence system (ECL plus; Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to x-ray film.

Plasmid Constructs

For generating the COL1A2-luciferase (COL1A2/Luc) construct in the pGL3 vector (Promega, Madison, WI), a 436-bp fragment containing the sequences from −378 to +58 of the human COL1A2 promoter was amplified by PCR from a COL1A2/Luc plasmid,[20–23] which was kindly provided by Yutaka Inagaki (Tokai University, Kanagawa, Japan). The primers, which are *Sac*I site-linked sense and *Xho*I site-linked antisense primers specific for the promoter, are as follows: 5′-G*GAGCTC*A-GATCTGCAAATTCTGCC-3′ (sense); 5′-T*CTCGAG*GCATGCAGTCGTG-GCCAG-3′ (antisense). The *Sac*I and *Xho*I sites are italic. The PCR product was cloned into a vector (pGEM-T Easy; Promega), followed by digestion with *Sac*I and *Xho*I and subcloned into the *Sac*I/*Xho*I sites of the pGL3-Basic vector.

Similarly, for generating the COL1A1/Luc, a 560 bp-fragment containing the TGF-β response elements of the human COL1A1 gene[24] from −341 to +119 was amplified by PCR from genomic DNA using *Kpn*I site-linked sense and *Xho*I site-linked antisense. The primers are as follows: 5′-C*GGTACC*CAGTTCCACTTCTTCTAG-3′ (sense), 5′-T*CTC-GAG*GTCTAGACCCTAGACATG-3′ (antisense). The *Kpn*I and *Xho*I sites are italic. The PCR product was finally cloned into the pGL3 basic vector. The creation of the mutated Smad binding site (Smad-mut/Luc) was generated by site-directed mutagenesis using the COL1A2/Luc plasmid as a template. The primers used in the PCR amplification were as follows: 5′-G*GAATTC*ACGAGTCAGAGTTTCCCC (sense), 5′-G*GAAT-TC*CATACCTCCGCCCTCCGC (antisense).

The *Eco*RI site for the mutated Smad binding site construct is italic. The PCR product was digested with *Eco*RI, followed by self-ligation. This plasmid was digested with *Sac* I and *Hin*dIII and recloned into the *Sac* I/*Hin*dIII sites of the pGL3-basic vector. All constructs were confirmed by sequence analysis using the sequencer (model 310; Applied Biosystems).

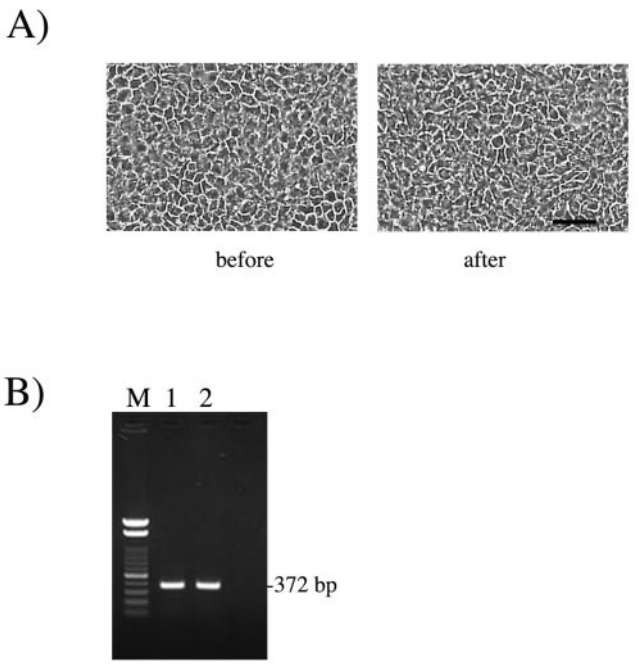

FIGURE 1. (**A**) Phase-contrast photomicrographs of confluent cultures of ARPE-19 cells before and after treatment of TGF-β2 (10 ng/mL) for 24 hours. (**B**) RT-PCR analysis of RPE65 mRNA expression in ARPE-19 cells before and after treatment of TGF-β2 (10 ng/mL) for 24 hours. *Lane M*: 100-bp ladder; *lane 1*: before treatment of TGF-β2; and *lane 2*: after treatment of TGF-β2. Magnification, ×100. Bar, 50 μm.

Transient Transfection and Luciferase Assays

Transcriptional activities of the COL1A1 and COL1A2 gene were determined by transient transfections into ARPE-19 cells. A kit (EndoFree Plasmid Maxi Kit; Qiagen) was used to purify the plasmids. For transient expression reporter assays, ARPE-19 cells were then transfected (Effectene Transfection Reagent; Qiagen). Briefly, cells were seeded the day before transfection in six-well plates. Cells at 60% to 80% confluence were transfected according to the manufacturer's recommendations. Six hours later, cells were stimulated with or without TGF-β2 (10 ng/mL) in the presence or absence of PD98059 or SB203580 for an additional 24 hours. Cells were lysed and assayed using the dual-luciferase reporter assay system (Promega). Luciferase activities were analyzed using a luminometer (Lumat LB.9507; EG and G, Berthold, Germany). A construct containing the renilla luciferase (pRL-TK; Promega) was cotransfected together with COL1A1/Luc or COL1A2/Luc or Smad-mut/Luc and used as a control for transfection efficiency. The values were expressed relative to the activities in nontreated cells.

Statistical Analysis

The values represented the mean ± SD of multiple independent tests and Student's *t*-test was used to evaluate the statistical differences between groups, and a $P < 0.01$ was considered to be significant.

RESULTS

Stimulation of Type I Collagen mRNA Expression and Collagen Synthesis in ARPE-19 Cells by TGF-β2

ARPE-19 cells retained the characteristic features of native RPE. The cells exhibited defined cell borders, a cobblestone appearance, and expressed RPE 65 mRNA before and after TGF-β2 treatment (Fig. 1). The cells expressed a basal level of type I collagen genes, but, when treated with exogenous TGF-β2, type I collagen mRNA expression increased dramatically in a dose-dependent fashion (Fig. 2A). To examine whether the increase of collagen mRNA expression was followed by an increase in collagen protein synthesis, we measured the concentration of PICP in the medium. Similarly, exogenous TGF-β2 increased type I collagen protein levels in the culture media of ARPE-19 in a dose-dependent fashion (Fig. 2B).

Activation of p38 MAPK and ERK 1/2 by TGF-β2 in ARPE-19 Cells

Some of the biological effects of TGF-β1 are mediated by activation of the MAPK signaling pathway in certain cell types.[25–27] We first examined the levels of p38 MAPK and ERK1/2 proteins expression in ARPE-19 cells treated with exogenous TGFβ2 (10 ng/mL) by Western analyses, using anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK1/2, and anti-phospho-ERK1/2 antibodies. The anti-phospho-p38 MAPK and

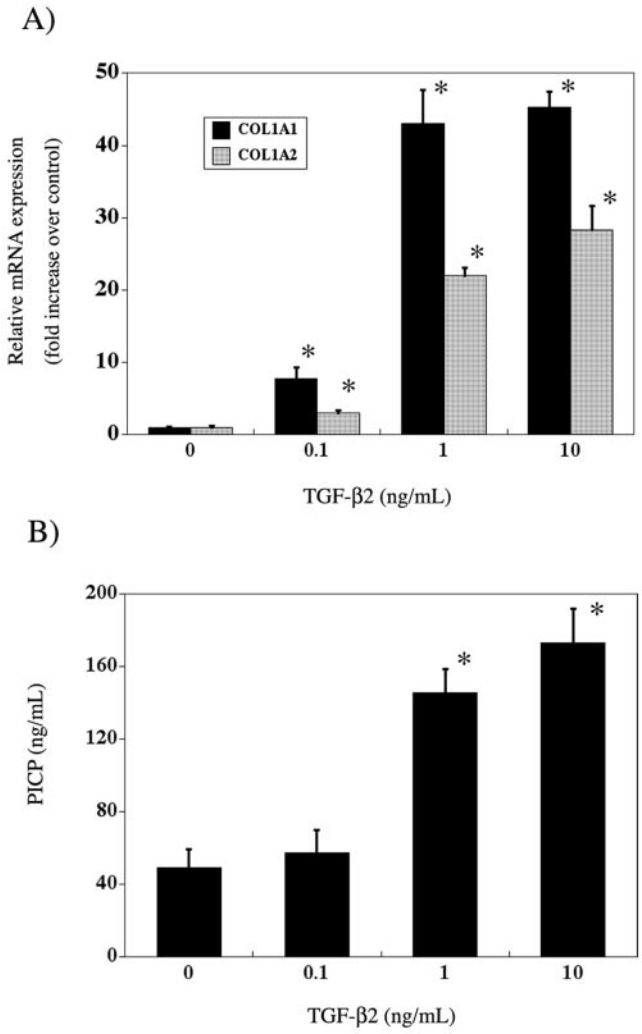

FIGURE 2. (**A**) Analysis of type I collagen mRNA expression using real-time RT-PCR. ARPE-19 cells were treated with various amounts of TGF-β2 for 24 hours. The relative levels of mRNA were normalized against that of GAPDH from the same cDNA preparation. Data represent the mean ± SD of results in six independent experiments. (**B**) The concentration of PICP in the culture media was measured by ELISA. Quiescent cells were treated with various amounts of TGF-β2 for 48 hours. Data represent the mean ± SD of results in four independent experiments. $^*P < 0.01$, compared with no stimulation with TGF-β2.

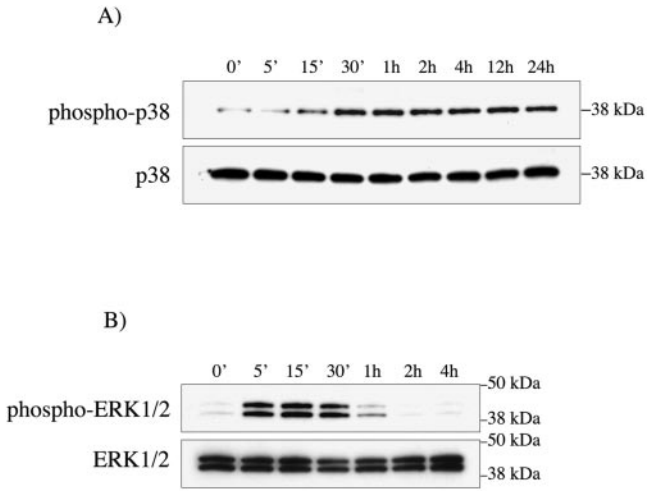

FIGURE 3. Activation of MAPK by TGF-β2 in ARPE-19 cells. Lysates from cells incubated in the presence of exogenous TGF-β2 (10 ng/mL) for the indicated times were subjected to Western blot analysis using various antibodies. (**A**) Bands corresponding to the phosphorylated form of p38 MAPK (*top*) and total p38 MAPK (*bottom*) were detected. (**B**) Bands corresponding to the phosphorylated form of ERK1/2 (*top*) and total ERK1/2 (*bottom*) were detected. In each lane, two bands corresponded to ERK1 (*top*) and ERK2 (*bottom*).

anti-phospho-ERK1/2 antibodies detected specifically the phosphorylated forms of p38 MAPK and ERK1/2, respectively, whereas, the anti-p38 MAPK and anti-ERK1/2 antibodies detected total p38 MAPK or ERK1/2 proteins, respectively (Fig. 3). Exogenous TGF-β2 increased the phosphorylation of p38 MAPK within 30 minutes after stimulation, and its activation persisted for 24 hours (Fig. 3A). In contrast, ERK1/2 phosphorylation increased as early as 5 minutes after stimulation with TGF-β2 and persisted for as long as 60 minutes (Fig. 3B). Preincubation of ARPE-19 cells with the p38 inhibitor SB203580 or ERK1/2 route inhibitor PD98059 blocked TGF-β2–induced p38 or ERK1/2 activation at 10 μM concentration, respectively (Fig. 4).

Effects of SB203580 or PD98059 on the Type I Collagen mRNA Expression and Collagen Synthesis by TGF-β2

Given that TGF-β2 induced increases in both type I collagen genes and MAPK phosphorylation, we posed the question of whether the MAPK pathway is involved in mediating TGF-β2–induced COL1A1 and COL1A2 gene expression. Preincubation of ARPE-19 with SB203580, a specific inhibitor of p38 MAPK, efficiently prevented approximately two thirds of the TGF-β2–induced COL1A1 and COL1A2 mRNA expression (Fig. 5A). In addition, SB203580 inhibited approximately one half of the TGF-β2–induced collagen synthesis (Fig. 5B). In contrast, PD98059, a specific inhibitor of MEK1, which prevents the activation of ERK1/2 pathway, failed to inhibit TGF-β2–induced type I collagen mRNA expression and protein synthesis (Fig. 5). Both inhibitor treatments had very little effect on the basal levels of type I collagen mRNA expression and protein synthesis.

Effects of SB203580 or PD98059 on the Activities of the COL1A1/Luc and COL1A2/Luc Promoter Induced by TGF-β2

The COL1A2 promoter is a widely used experimental model system to study transcriptional regulation of the type I collagen gene at the basal level and in response to exogenous stimuli. The effect of SB203580 or PD98059 on TGF-β2 induction of the COL1A2/Luc promoter was investigated in ARPE-19 (Fig. 6). Treatment with SB203580 significantly reduced TGF-β2 induction. Similar to the COL1A2/Luc promoter, SB203580 also reduced TGF-β2 induction of the COL1A1/Luc promoter activity. PD98059, however, failed to reduce TGF-β2 induction of both promoters. The inhibitor treatment had very little effect on the basal promoter activities of COL1A1/Luc and COL1A2/Luc.

Effects of SB203580 or PD98059 on the Activity of the Smad-mut/Luc Promoter and on the Phosphorylation of Smad2/3 Induced by TGF-β2

TGF-β signals from the cell surface to the nucleus are transduced by Smad2, -3, and -4, and phosphorylation of Smad-2/3 is crucial for this downstream signaling cascade.[28,29] Studies have shown that Smad proteins mediate TGF-β signaling and stimulate COL1A2 mRNA expression.[30–33] Therefore, we evaluated the role of Smads on regulating the COL1A2/Luc promoter when induced by TGF-β2 using the mutated Smad binding site construct (Smad-mut/Luc) in ARPE-19 cells. As predicted, TGF-β2 induction of Smad-mut/Luc promoter activity was significantly diminished, compared with the activity of COL1A2/Luc. Moreover, the treatment with SB203580 reduced TGF-β2 induction of Smad-mut/Luc promoter activity, but treatment with PD98059 had no effect (Fig. 7A). Furthermore, we examined the involvement of two inhibitors in regulating the Smad cascade. Cell extracts from ARPE-19 cells, pretreated with either SB203580 or PD98059 for 30 minutes before administration of TGF-β2 (10 ng/mL), were analyzed by Western blot analysis for phosphorylated Smad2/3 (top) and total Smad2/3 (bottom) using an anti-phospho-Smad2/3 antibody and an anti-Smad2/3 antibody, respectively (Fig. 7B). The ~58-kDa Smad2 and ~50-kDa Smad3 proteins were present in both

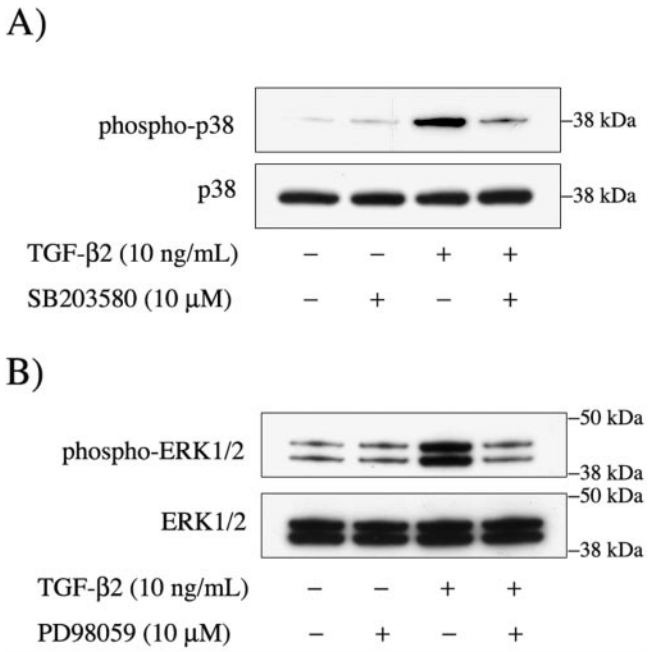

FIGURE 4. Effects of MAPK inhibitors on TGF-β2–induced phosphorylation. (**A**) SB203580 inhibited TGF-β2–induced p38 activation. Cell lysates were harvested after incubation with or without TGF-β2 for 60 minutes in the presence or absence of SB203580. (**B**) PD98059 inhibited TGF-β2–induced ERK1/2 activation. Cell lysates were harvested after incubation with or without TGFβ2 for 30 minutes in the presence or absence of PD98059.

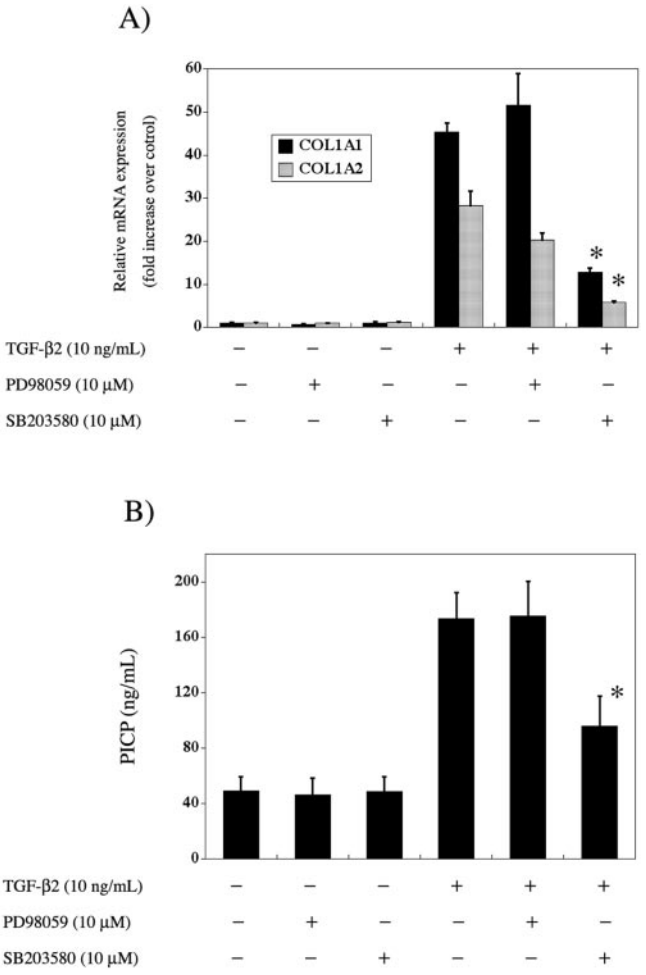

FIGURE 5. Effects of MAPK inhibitors on TGF-β2–induced COL1A1 and COL1A2 mRNA expression (**A**) and type I collagen synthesis (**B**) in ARPE19 cells. (**A**) Analysis of COL1A1 and COL1A2 mRNA expression using real-time RT-PCR. Quiescent cells were pretreated with or without SB203580 (10 μM) or PD98059 (10 μM) and incubated in the presence or absence of TGF-β2 (10 ng/mL) for 24 hours. The relative levels of mRNA were normalized against that of GAPDH from the same cDNA preparation. Data are the mean ± SD of results in six independent experiments. (**B**) Concentration of PICP in the culture media was measured by ELISA. Cells were treated the same as in (**A**) for 48 hours. Data represent the mean ± SD of results in four independent experiments. $^*P < 0.01$, compared with stimulation with TGF-β2 and without both inhibitors.

control and TGF-β2–treated cells, and an increase in phosphorylated Smad2/3 was noticed after TGF-β2 treatment. Thus, Smad2/3 phosphorylation in ARPE-19 cells is increased after TGF-β2 treatment, indicating that these cells can also respond to the ligand through a Smad-dependent pathway. However, SB203580 and PD98059 had no effect on TGF-β2–induced phosphorylation levels of Smad2/3 in ARPE-19 cells.

DISCUSSION

In this study, we examined the role of MAPK signaling cascades in mediating the effects of TGF-β2 on collagen synthesis in the human retinal pigment epithelial cell line, ARPE-19. Exogenous TGF-β2 increased the expression of collagen type I at the transcriptional level, leading to an increase in the synthesis of the protein. Treatment of ARPE-19 cells with exogenous TGF-β2 resulted in p38 MAPK and ERK 1/2 phosphorylation within 5 to 30 minutes. The activation of p38 MAPK continued for at least 24 hours. To examine the relationship of MAPK phosphorylation and collagen synthesis, we used chemical inhibitors of the MAPK pathway on type I collagen expression. Blockade of the ERK 1/2 pathway by PD98059, a specific inhibitor of MEK1 that prevents downstream activation of ERK 1/2, did not prevent induction of COL1A1 and COL1A2 mRNA or type I collagen synthesis by TGF-β2 in the ARPE-19 cells. In contrast, the presence of SB203580, a specific inhibitor of the p38 MAPK pathway, significantly reduced promoter activity, mRNA, and protein expression after induction by exogenous TGF-β2. These data show that p38 MAPK could be involved in TGF-β2–induced collagen synthesis in ARPE-19 cells.

Smads are a family of proteins that operate downstream of various members of the TGF-β superfamily.[34] Smad2 and -3 are downstream effectors of the TGF-β signaling pathway. On ligand binding, they are phosphorylated by the TGF-β type I receptor kinase and translocated to the nucleus in a complex with Smad4.[35] This complex may either bind directly to the promoters of its target genes, or associate with other transcription factors to induce gene transcription.[36] A conserved Smad3/4 DNA binding site, GTCTAGAC,[37] is present within the COL1A2 promoter.[22] Indeed, Smad-mut/Luc promoter activity was significantly diminished when induced by TGF-β2. Therefore, Smads such as p38 MAPK contribute to collagen gene expression in the presence of TGF-β2 in ARPE-19 cells. Over the last few years, cross-talk between the MAPK and Smad signaling pathways in response to TGF-β stimulation have been suggested.[15,38–42] Therefore, we chose to examine the effect of SB203580 and PD98059 on the activity of the Smad-mut/Luc promoter and on the phosphorylation of Smad2/3 induced by TGF-β2. Treatment with SB203580 reduced TGF-β2 induction of the Smad-mut/Luc promoter activity; however, SB203580 and PD98059 had no significant effect on the TGF-β2–induced phosphorylation of Smad2/3 in ARPE-19 cells. These data suggest that p38 MAPK mediates the expression of type I collagen induced by TGF-β2 in ARPE-19, which is independent of the Smad pathway. Similar data have been observed in dermal fibroblasts,[16,43] hepatic stellate cells[44] and mesangial cells,[45] all of which play important roles in fibrogenic diseases.

In ARPE-19 cells, blockage of the ERK1/2 pathway did not significantly prevent induction of collagen gene expression by

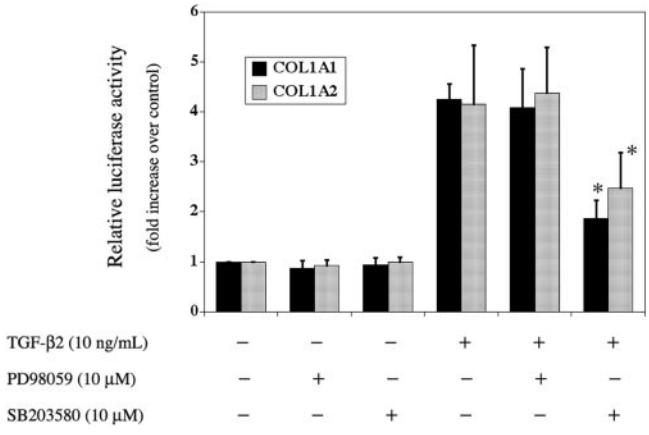

FIGURE 6. Effects of MAPK inhibitors on TGF-β2–induced COL1A1 and COL1A2 promoter activity in ARPE19 cells. Transfected cells were pretreated with or without SB203580 (10 μM) or PD98059 (10 μM) and incubated in the presence or absence of TGF-β2 (10 ng/mL) for 24 hours. Results are expressed as multiples of increase compared with untreated control cells. Data represent the mean ± SD of results five independent experiments. $^*P < 0.01$, compared with the condition of stimulation with TGF-β2 and without both inhibitors.

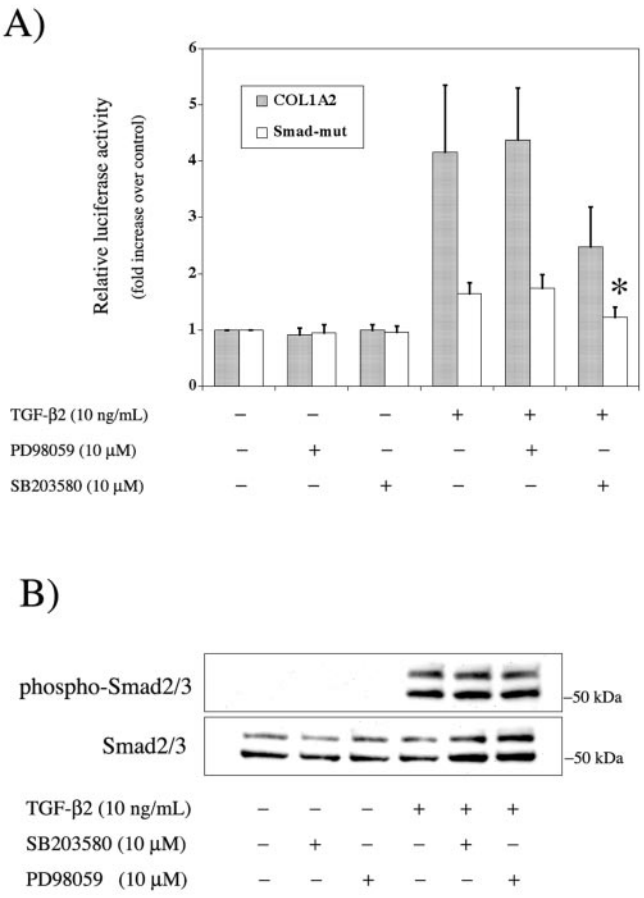

FIGURE 7. Effects of MAPK inhibitors on the TGF-β2–induced Smad-mut/Luc promoter activity (**A**) and phosphorylation of Smad2/3 (**B**) in ARPE-19 cells. (**A**) Transfected cells were pretreated with or without SB203580 (10 μM) or PD98059 (10 μM) and incubated in the presence or absence of TGF-β2 (10 ng/mL) for 24 hours. Results are expressed as multiples of increase compared with the untreated control. Values represent the mean ± SD of five independent experiments. $^*P < 0.01$, compared with stimulation with TGF-β2 and without both inhibitors in the Smad-mut/Luc. (**B**) Quiescent cells were pretreated with or without SB203580 (10 μM) or PD98059 (10 μM) and incubated in the presence or absence of TGF-β2 (10 ng/mL) for 30 minutes. Bands corresponding to the phosphorylated form of Smad2/3 (*top*) and total Smad2/3 (*bottom*) were detected.

TGF-β2. In mesangial cells,[46] ERK1/2 activity is essential for collagen synthesis on induction by TGF-β1, but in dermal fibroblasts,[47] activation of ERK1/2 inhibits type I collagen expression. It seems that MAPK activity could vary, depending on different cell types, culture conditions, and ambient levels of other cytokines.[23]

With respect to the role of MAPK in retinal diseases, such as PVR, ERK1/2 are phosphorylated in RPE cells within 15 minutes and remain phosphorylated for several days after retinal detachment (RD).[48] Thus, ERK1 and -2 play key roles in the control of RPE cell proliferation and migration.[49,50] In pathologic conditions, RPE cells are exposed to a variety of stimuli, including TGF-β, that may induce cell death, in which JNK1 and p38 MAPK induce RPE cell death.[51] In addition, the inflammatory process, due to breakdown of the blood–retinal barrier, causes activation of leukocytes and their subsequent infiltration into the choroid, retina, and vitreous. Bian et al.[52] demonstrated that RPE cells secrete IL-8 and MCP-1 when in coculture with monocytes through activation of p38 MAPK and ERK1/2 signaling pathways. These studies suggest that MAPK signaling pathways play important roles in various processes of ocular disease. In our study, we have demonstrated that p38 MAPK signaling pathway mediates TGF-β2 induced type I collagen synthesis in RPE cells. The MAPK pathway may be a new therapeutic target for treating many ocular diseases, especially PVR.

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