

Triamcinolone acetonide modulates TGF- β 2-induced angiogenic and tissue-remodeling effects in cultured human retinal pigment epithelial cells

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Abstract. Transforming growth factor- β 2 (TGF- β 2) has been implicated in the pathogenesis of proliferative vitreoretinopathy (PVR) and proliferative diabetic retinopathy (PDR), due to its ability to stimulate the overproduction of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), and remodeling of the extracellular matrix (ECM). Although intravitreal triamcinolone acetonide (TA) is clinically useful in the treatment of PVR and PDR, its molecular mechanism has yet to be fully elucidated. The present study investigated whether TA treatment altered TGF- β 2-driven biological effects on the behavior of cultured human retinal pigment epithelial (RPE) cells, in order to determine which signaling pathway may be essential for the pharmacological action of TA. The R-50 human RPE cell line was treated with TA in the presence of TGF- β 2, followed by analyses of cell viability and contraction using cell viability and collagen gel contrac-

tion assays. VEGF mRNA expression and protein production were measured using reverse transcription-quantitative PCR and ELISA, respectively. The phosphorylation status of signaling mediators and the protein expression of type I collagen (COL1A1), α -smooth muscle actin (α -SMA), and ECM-remodeling enzymes, including MMP-2 and MMP-9, were analyzed using western blotting. The gelatinolytic activity of MMPs was detected using gelatin zymography. TA treatment exhibited no prominent cytotoxicity but markedly antagonized TGF- β 2-induced cytostatic effects on RPE cell viability and TGF- β 2-enhanced contractility in collagen gels. In the context of TGF- β 2-related signaling, TA significantly attenuated TGF- β 2-elicited Smad2, extracellular-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) phosphorylation. Moreover, TA markedly mitigated TGF- β 2-induced VEGF upregulation through ablation of p38 signaling activity. TA also partially attenuated TGF- β 2-elicited expression of COL1A1, α -SMA, MMP-2, and MMP-9, but only suppressed TGF- β 2-induced MMP-9 gelatinolytic activity. Mechanistically, the MEK/ERK signaling pathway may have a critical role in the TGF- β 2-induced upregulation of COL1A1, α -SMA and MMP-9. In conclusion, TA may be considered a useful therapeutic agent for treating TGF- β 2-associated intra-ocular angiogenesis and tissue remodeling, the underlying mechanism of which may involve the ERK and p38 MAPK signaling pathways.

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Introduction

Retinal pigment epithelial (RPE) cells form a monolayer between the retina and choriocapillaris. In response to changes in the microenvironment, RPE cells may be activated to produce autocrine/paracrine growth factors, which can affect not only their microenvironment but also adjacent compartments, such as the choroid plexus and retinal neurons (1-3).

Neovascularization in diabetic retinopathy and other retinal vasculopathies has long been known to be associated with retinal capillary nonperfusion and retinal hypoxia (4,5). Notably, retinal hypoxia has been experimentally evidenced in pig and monkey retinas following laser vein occlusion (6). A previous study indicated that angiogenic factors, including vascular endothelial cell growth factor (VEGF), are detectable in vitreous fluid and epiretinal membrane samples derived from patients with proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR) (7). Therefore, these factors may be involved in the pathogenesis of proliferative membranes; however, their pathogenic mechanisms have yet to be fully understood.

Transforming growth factor- β (TGF- β) is a multifunctional cytokine that regulates RPE cell proliferation (8,9), differentiation (10), migration (11,12) and extracellular matrix (ECM) production (12,13). Vitreous TGF- β 2 levels have been reported to be increased in patients with PDR and other vitreoretinal disorders, and this increase has been shown to be correlated with the severity of fibrous proliferation (14). Oxidative stress can induce the synthesis of TGF- β 2 and increase ECM gene expression in cultured RPE cells; this cellular response may contribute to the deposition of ECM in the aging macula (15). There is growing evidence that RPE cells are able to synthesize TGF- β 2 (16,17) and the release of TGF- β 2 may regulate VEGF production in human RPE cells (18-20). Moreover, TGF- β 2 has been reported to activate RPE cell transformation by upregulating the expression of α -smooth muscle actin (α -SMA) (21). The main signaling response induced by TGF- β is mediated via Smad proteins; however, evidence has suggested that TGF- β is capable of signaling independently of Smads. While TGF- β has been found to activate non-Smad signaling pathways, including p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase and extracellular-regulated kinase (ERK), the specific pathway involved appears to vary depending on the cell type and tissue function (22,23).

Since the breakdown of the blood-retinal barrier (BRB) is symptomatic of several sight-threatening diseases (24), intravitreally injected triamcinolone acetonide (TA), a corticosteroid agent, has long been proposed for the stabilization of the inner BRB and to prevent macular edema in retinopathies (25-27). Thus, it is considered a promising treatment modality for a variety of proliferative, edematous, neovascular and inflammatory ocular disorders (28). Since the upregulation of VEGF is known to be associated with the presence of diabetic macular edema and PDR (29), the pharmacological mechanisms of TA treatment include modulation of intercellular adhesion molecule-1 expression and cellular permeability in endothelial cells (25), reduction of VEGF production in RPE cells under oxidative stress (30), and VEGF receptor-associated signaling (31). These findings suggest that TA may exhibit an anti-angiogenic effect against choroidal neovascularization. In addition, TA has been reported to abrogate the regulatory loop between VEGF and matrix metalloproteinase (MMP)-9 in RPE cells (32), and its intravitreal injection was shown to improve wound healing in mice with laser retinal photocoagulation (33). However, the pharmacological action of TA on TGF- β 2 signaling in RPE cells and the corresponding cellular behaviors have yet to be fully defined. Therefore, the present study aimed to investigate whether TA may modulate

TGF- β 2-mediated angiogenic and tissue-remodeling effects in cultured human RPE cells.

Materials and methods

Chemicals and reagents. TA (cat. no. T6501) was purchased from MilliporeSigma and maintained in methanol (MeOH) at 10 mM. Recombinant human TGF- β 2 (cat. no. 302-B2) was purchased from R&D Systems, Inc. Oligonucleotide primers for PCR analyses were synthesized by Genomics BioSci & Tech Co., Ltd. Selective kinase inhibitors of p38 MAPK (SB203580; cat. no. S8307), MEK1/MEK2 (PD98059; cat. no. P215), the selective inhibitor for TGF- β type I receptor (SB431542; cat. no. S4317) and the mouse monoclonal antibody against β -actin (cat. no. MAB1501) were purchased from MilliporeSigma. Rabbit monoclonal antibodies against total Smad2 (cat. no. 5339s), phosphorylated (p)-Smad2 (Ser465/467; cat. no. 3108s), total ERK1/2 (cat. no. 4695s), p-ERK1/2 (Thr202/185 and Tyr204/187; cat. no. 9101s), total p38 MAPK (cat. no. 8690s) and p-p38 MAPK (Thr180/Tyr182; cat. no. 4631) were purchased from Cell Signaling Technology, Inc.

Cell culture and drug treatment. A spontaneously immortalized human RPE R-50 cell line established from a donor eye with informed consent was provided by Professor D.N. Hu (New York Eye and Ear Infirmary of Mount Sinai, New York, USA), according to previously described methods (34,35). Briefly, R-50 cells were grown in Ham's F-12 nutrient medium supplemented with 10% FBS (both HyClone; Cytiva), 4 mM glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin and 250 ng/ml amphotericin (MP Biomedicals, Inc.) in a humidified atmosphere containing 5% CO₂ at 37°C.

R-50 cells were dissociated by incubating with 0.25% trypsin at 37°C for 2 min and seeded on either dishes or 96-well plates at an appropriate cell density of 2x10⁴ cells/cm² growth area, and were incubated overnight until they attached. The cells were treated with TA (0.1-100 μ M), TGF- β 2 (10 ng/ml) or with the different kinase inhibitors (10 μ M) at 37°C. The specimens collected after a 6-h incubation were used for gene expression study, the cells collected after a 24-h incubation were used for the cell viability assay and protein expression analyses, and the supernatants collected (obtained by centrifugation at 1,1000 x g; 5 min; 4°C) after 48 h exposure were used for ELISAs. Cells cultured in medium without supplementation were used as negative controls, and those incubated with 0.1% MeOH or 0.1% DMSO were used as solvent controls for TA or kinase inhibition experiments. The morphology of the treated cells was examined using a phase-contrast light microscope (Axio Vert.A1; Carl Zeiss AG) and documented using a digital imaging system (AxioVision; v4.8.2.0; Carl Zeiss AG).

Cell viability assay. A commercial cell viability assay kit (CellTiter™ Aqueous One Solution; cat. no. G3582; Promega Corporation) using MTS reagent as the chromogenic substrate was used to determine the effect of TA on RPE cell viability in the presence or absence of TGF- β 2. The group treated with 0.1% MeOH was used as a solvent control equivalent to TA treatment at 10 μ M. The assay was performed according to the manufacturer's protocol, as described previously (36).

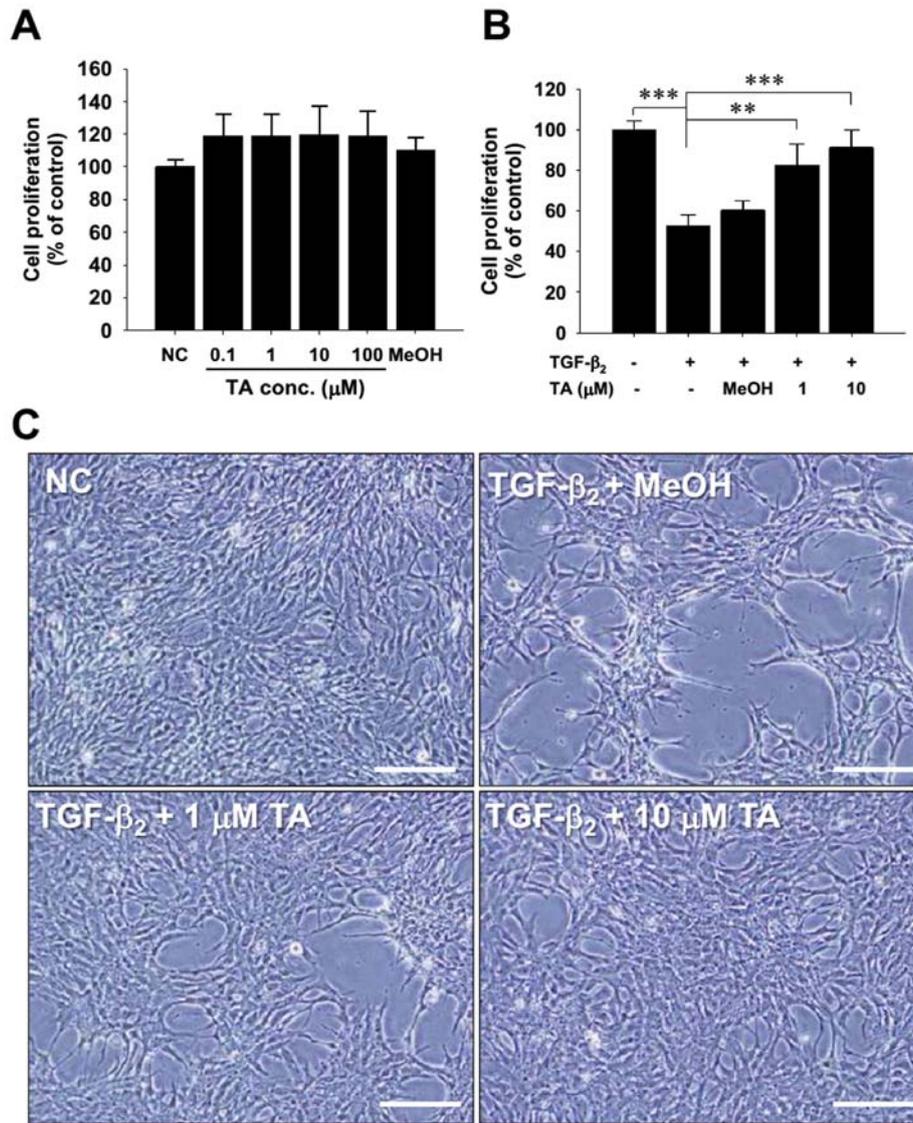


Figure 1. TA antagonizes the TGF- β_2 -induced cytostatic effect on cultured human RPE cells. (A) Effect of TA on viability of RPE cells. Human R-50 cells were treated with TA at the indicated doses or 0.1% MeOH as a solvent control for 24 h. Cell viability was measured using an MTS-based cell viability assay. (B) TA suppressed TGF- β_2 -induced cytostatic effect. After 2 h of serum starvation, R-50 cells were treated with 10 ng/ml TGF- β_2 in the presence of TA or 0.1% MeOH for 24 h. Cell viability data in triplicate are presented as the mean \pm SD. ** $P < 0.01$, *** $P < 0.001$. (C) Representative microphotographs of R-50 cells treated with 10 ng/ml TGF- β_2 in the presence of TA or 0.1% MeOH. Scale bar, 100 μm . RPE, retinal pigment epithelial; TA, triamcinolone acetonide; MeOH, methanol; TGF- β_2 , transforming growth factor- β_2 ; NC, negative control.

The end-point optical density was measured using a microplate reader (SunriseTM; Tecan Group, Ltd.). The relative cell viability was normalized to that of the negative control (without drug treatment).

Rat tail tendon collagen preparation and 3-dimensional (3D) collagen gel contraction assay. Rat tails were collected from adult male Sprague-Dawley rats under the approval and supervision of the Institutional Animal Care and Use Committee at E-Da Hospital (approval no. IACUC-102016; Kaohsiung, Taiwan). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) (37). The rat tails (n=5) were excised from the sacrificed animals of a previous study (38), in which deep anesthesia was induced and maintained with 2.5 and 2% isoflurane, respectively, and supplemental oxygen was administered using a calibrated vaporizer. Animal death

was caused by collagenase solution perfusion and subsequent removal of the liver for hepatocyte isolation. The extraction of collagen from rat tail tendons and 3D collagen gel contraction assays were performed as previously described (39). Briefly, 1.5×10^5 R-50 cells were repopulated in a 3D lattice collagen gel by rapidly mixing 1 ml culture medium containing 20 mM HEPES, 0.5 ml cell suspension containing freshly trypsinized RPE cells, and 2.5 mg rat tail collagen solution in a 35-mm dish. After complete gelling by incubating for 5 min in a CO_2 incubator at 37°C , the gels were detached and allowed to float in dishes. The gel area was directly measured at 24-h intervals. Data are expressed as the percentage of the area compared to the original size of the gel measured at 0 h.

Western blotting. Total cellular protein extracts from RPE cells were obtained by lysing the cells in ice-cold RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.25%

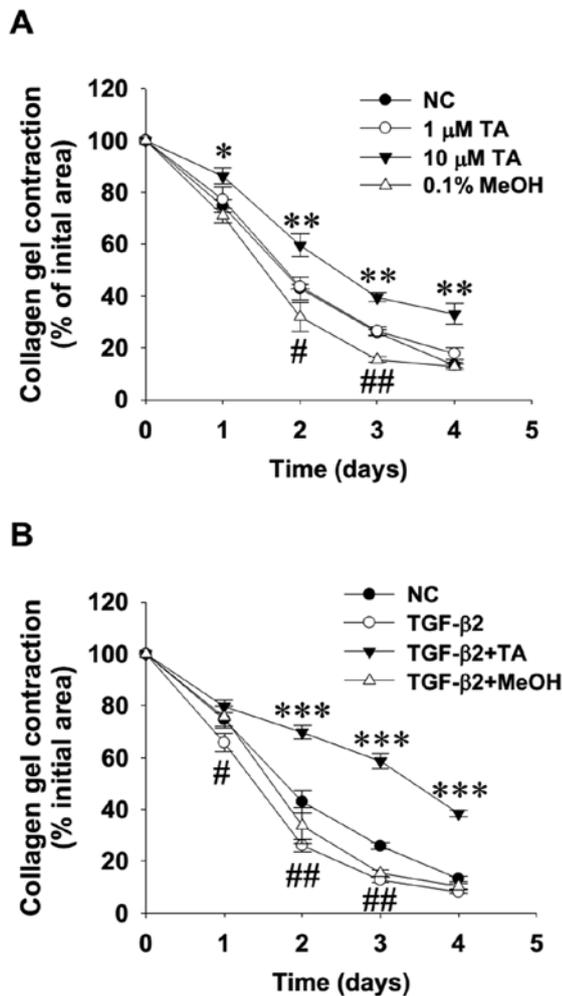


Figure 2. TA suppresses TGF- β 2-enhanced human RPE cell contractility in a 3D-collagen gel. (A) Effect of TA on human RPE cell-mediated collagen gel contraction. R-50 RPE cells (1.5×10^5) were re-populated into each collagen gel and were treated with TA at the indicated dose or with 0.1% MeOH as a solvent control. (B) Suppressive effect of TA on TGF- β 2-enhanced RPE cell-mediated collagen gel contraction. The diameters of collagen gels were measured at 24-h intervals and the time-course profiles of gel contraction were plotted. Data are presented as the mean \pm SD of triplicate results from three independent experiments. # $P < 0.05$, ## $P < 0.01$ vs. NC; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. MeOH or vs. TGF- β 2 + MeOH groups. RPE, retinal pigment epithelial; TA, triamcinolone acetonide; MeOH, methanol; TGF- β 2, transforming growth factor- β 2; NC, negative control.

sodium deoxycholate and 0.1% SDS in 50 mM Tris pH 7.4) in the presence of a protease inhibitor cocktail (Roche Applied Science) and phosphatase inhibitors (1 mM sodium fluoride and 1 mM sodium orthovanadate). Total protein was quantified using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) and 40 μ g total protein per lane was separated by SDS-PAGE, using 8% acrylamide gels under reducing conditions. After electrophoresis and electrotransfer onto a PVDF membrane, the blots were blocked in 5% skim milk in PBS-0.1% Tween-20 (PBST) at room temperature for 1 h, followed by incubation with one of the following antibodies at 4°C overnight: Anti- β -actin (1:10,000), anti-Smad2 (1:1,000), anti-p-Smad2 (1:1,000), anti-ERK1/2 (1:1,000), anti-p-ERK1/2 (1:1,000), anti-p38 MAPK (1:1,000), anti-p-p38 MAPK (1:1,000), anti-COL1A1 (1:1,000), anti- α -SMA (1:1,000), anti-MMP-2 (1:1,000) or anti-MMP-9 (1:1,000). After washing with

PBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000; cat. no. 7074; Cell Signaling Technology, Inc) or goat anti-mouse IgG (1:10,000; cat. no. 115-035-003; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody at room temperature for 1 h. The immunoreactive signals were visualized using an enhanced chemiluminescence detection system (MilliporeSigma), documented on a digital imaging system and densitometrically analyzed using ImageJ software (1.48v; National Institutes of Health). Relative protein expression levels were presented as ratios of the densities between target proteins and actin or compared to their total protein levels, and further normalized to that of the untreated negative control. The normalized density data were expressed as the mean \pm standard deviation (SD) from three independent experiments.

Total RNA extraction, semi-quantitative reverse transcription (RT)-PCR and RT-quantitative PCR (RT-qPCR). RNA extraction, RT and semi-quantitative multiplex PCR detection of VEGF isoform gene transcripts were performed as previously described (36). Briefly, total RNA was extracted from 1×10^6 R50 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was generated using avian myeloblastosis virus reverse transcriptase (cat. no. M5108; Promega Corporation) and oligo-dT primers (cat. no. C1101; Promega Corporation) in the presence of a ribonuclease inhibitor (RNasin; cat. no. N2111; Promega Corporation). For the multiplex amplification of VEGF gene transcripts, the following primers were synthesized: VEGF sense, 5'-CGA AGTGGTGAAGTTCATGGATG-3' and anti-sense, 5'-ATATCCATCACACTGGCGGCCGC-3'; and β -actin sense, 5'-GCCAAGGTCATCCATGACAAC-3' and anti-sense, 5'-GTCCACCACCCTGTTGCTGTA-3'. PCR was performed according to a previously described protocol (36). The PCR products were titrated to establish standard curves for documenting linearity and permitting semi-quantitative analysis using a densitometrical analysis system (UVITEC Imaging system; UVITEC). The relative gene expression levels were expressed as the ratio of densities between the PCR products and β -actin. For RT-qPCR detection of the VEGF₁₆₅ transcript levels, the RNA extraction and RT reaction were performed as described previously for semi-quantitative PCR. The cDNA was used for qPCR analysis on a real-time PCR machine (Eco Real-Time PCR system; Illumina, Inc.) according to a previously described protocol (40). Briefly, VEGF₁₆₅ mRNA was amplified using a SYBR Green PCR Master Mix kit (Thermo Fisher Scientific, Inc.) and specific primers for detecting VEGF₁₆₅ (forward, 5'-TTGCCTTGCTGCTCTACCTC-3' and reverse, 5'-AAATGCTTTCTCCGCTCTGA-3') and the housekeeping β -actin gene (forward, 5'-TCCTGTGGCATCCACGAAACT-3' and reverse, 5'-GAAGCATTGCGGTGGACGAT-3'). The following thermocycling conditions were used for the qPCR reaction: Initial denaturation at 95°C for 10 min; followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec and 72°C for 30 sec. After amplification, a final melting curve protocol was used to confirm the specificity of PCR products. Data were analyzed using the $2^{-\Delta\Delta C_t}$ method (41) and normalized to β -actin expression levels.

ELISA detection of VEGF. Cultured R-50 cells were treated with 10 ng/ml TGF- β 2 at 37°C in the presence of either TA (1 or 10 μ M), kinase inhibitors (10 μ M) or equivalent solvent

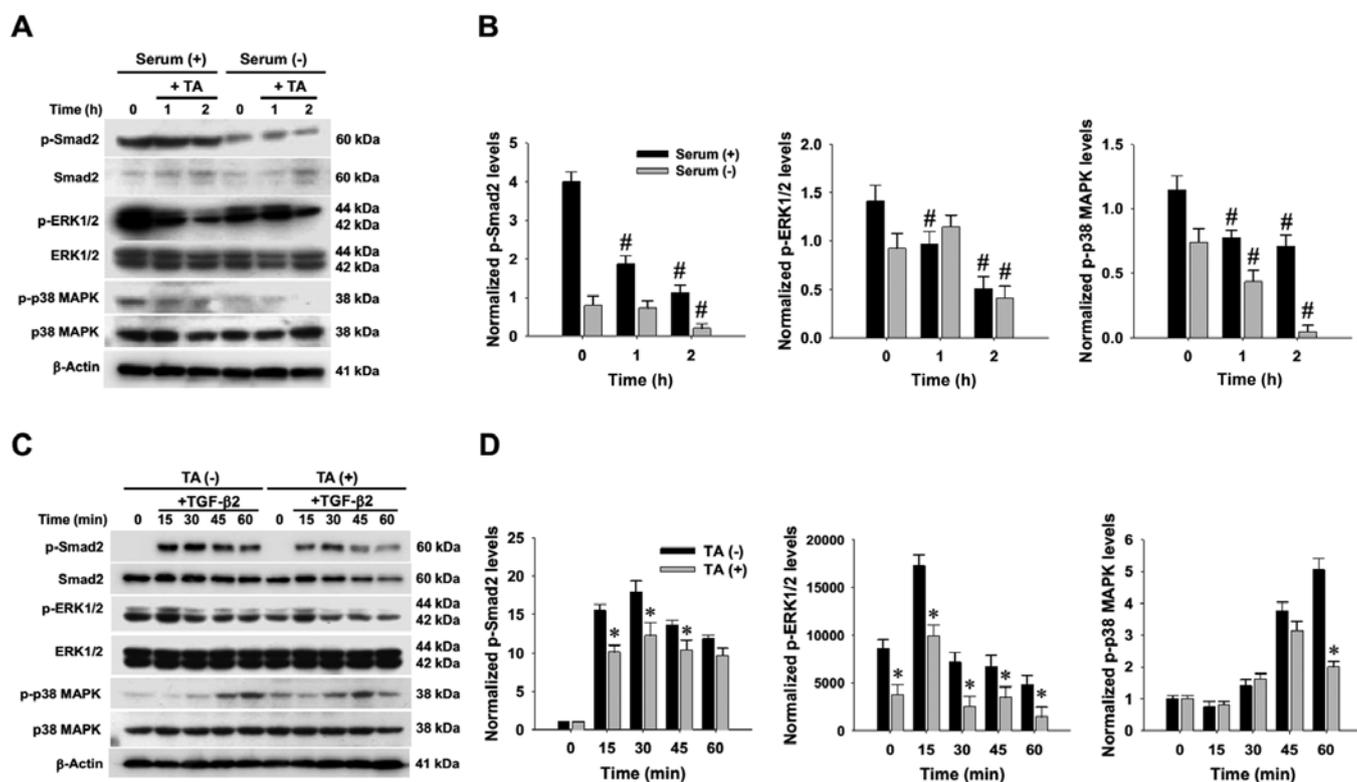


Figure 3. Effects of TA on constitutive and TGF- β 2-stimulated signaling activities in cultured human RPE cells. (A) Human R-50 cells were treated with TA at 10 μ M under serum-supplemented or -starved conditions for the indicated periods. Total proteins were subjected to western blot analysis of p-Smad2, p-ERK1/2 and p-p38 MAPK. (B) Densitometric analysis of (A). (C) R-50 RPE cells received 2 h of serum starvation, followed by treatment with 10 ng/ml TGF- β 2 in the presence or absence of 10 μ M TA. (D) Densitometric analysis of (C). Data are presented as the mean \pm SD from three independent experiments. [#]P<0.05 vs. respective 0 h group; ^{*}P<0.05 vs. respective TA (-) group. RPE, retinal pigment epithelial; TA, triamcinolone acetonide; TGF- β 2, transforming growth factor- β 2; p, phosphorylated; ERK1/2, extracellular-regulated kinase 1/2; MAPK, mitogen-activated protein kinase.

control (0.1% MeOH for TA or 0.1% DMSO for kinase inhibitor treatments). The supernatants were collected after 48 h of incubation by centrifugation at 1,000 \times g for 5 min at 4°C. Soluble VEGF release from RPE cells was assessed using a commercially available ELISA kit (cat. no. DVE00; R&D Systems, Inc.) according to the manufacturer's instructions.

Gelatin zymography. To determine the level of gelatinolytic activities of MMP-2 and MMP-9 in the conditioned media from the drug-treated cells, non-reducing SDS-PAGE and gelatin zymography were performed as previously described (42). Briefly, 10 μ l conditioned medium was mixed with SDS-PAGE sample buffer without reducing agent and boiling. After electrophoresis via an 8% polyacrylamide gel containing 2 mg/ml gelatin (Sigma-Aldrich; Merck KGaA), the gel was soaked in 50 mM Tris-HCl buffer (pH 7.4) containing 0.25% Triton X-100 for 30 min at room temperature, followed by an incubation with a digestion buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl₂, 2 μ M ZnSO₄, 0.01% Brij-35 and 5 mM phenylmethylsulfonyl fluoride) at 37°C overnight. After washing with distilled water, the gelatin-digested gels were stained with 0.25% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid at room temperature for 2 h, and destained with 10% methanol and 10% acetic acid at room temperature for 20 min. The gelatinolytic activities shown as clear bands in the gels were determined by analyzing the band densities using ImageJ software.

Statistical analysis. All data are expressed as the mean \pm SD from three independent experiments. Statistical analysis was performed using JMP Version 5.1.2 statistical software (SAS Institute, Inc.). Unpaired Student's t-test or one-way ANOVA and Bonferroni post hoc test were used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference among the groups tested.

Results

TA antagonizes the cytostatic effect of TGF- β 2 on RPE cells. Preservatives (such as MeOH) in commercial TA solution, but not TA itself, were previously revealed to be cytotoxic to RPE cells (43). To confirm that TA did not have a cytotoxic effect on cultured human RPE cells, various concentrations of TA (0-100 μ M) were used to evaluate cell viability using the MTS cell viability assay. After 24 h of treatment, no alteration in cell morphology was observed (data not shown) and cell growth was not affected by treatment with TA or MeOH, a solvent control (Fig. 1A). These results demonstrated that TA treatment exhibited no cytotoxicity on cultured human RPE cells *in vitro*.

TGF- β has long been known to induce arrest of RPE cell proliferation (44). Thus, the present study further examined whether TA at doses not affecting RPE cell proliferation had the ability to modulate TGF- β 2-induced growth arrest. TA was added to cultured cells in the presence of 10 ng/ml

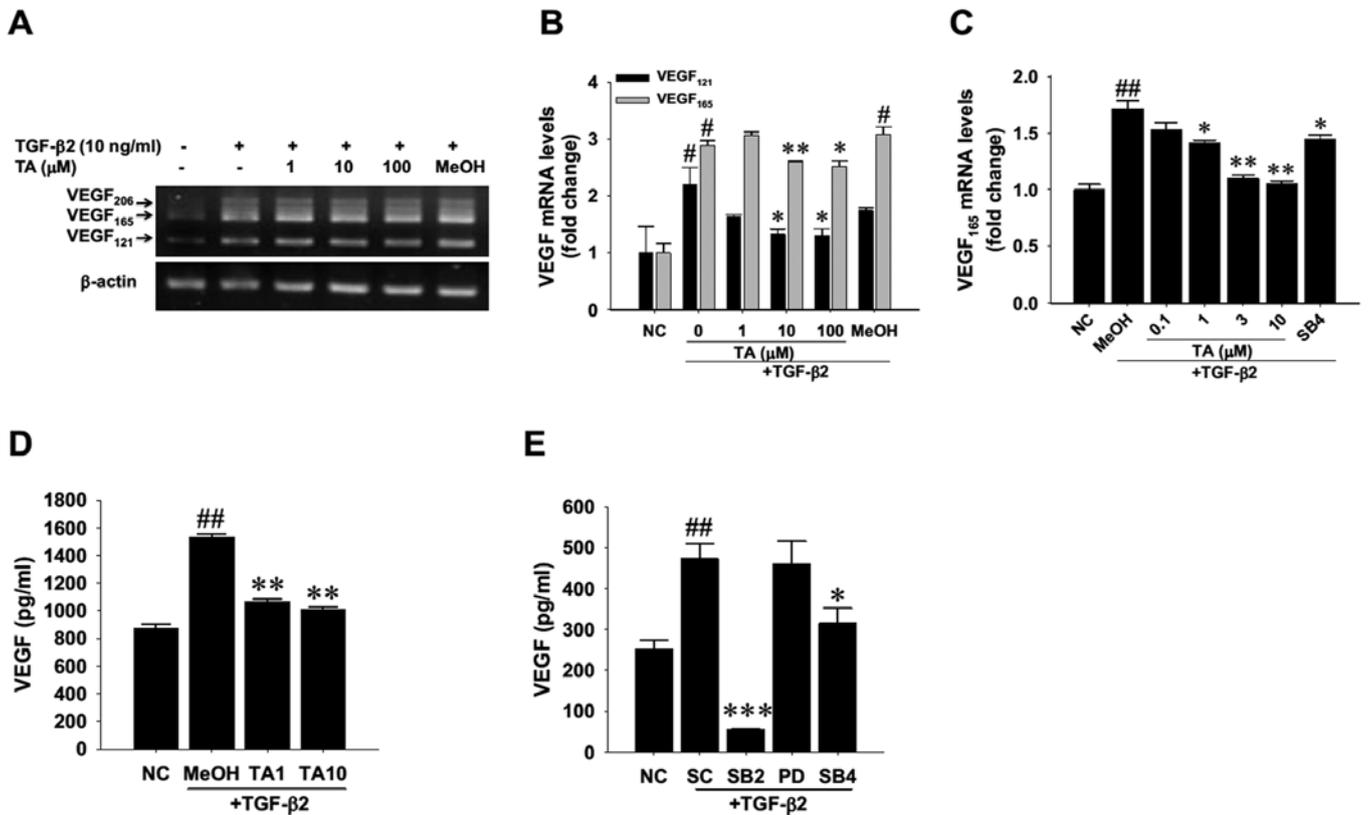


Figure 4. Suppressive effect of TA on TGF-β2-induced de novo synthesis of VEGF in cultured human retinal pigment epithelial cells. (A) Human R-50 cells were treated with TGF-β2 for 6 h in the presence of TA at the indicated doses or 0.1% MeOH as a SC. Total RNA was extracted and a multiplex reverse transcription-PCR was performed to simultaneously detect three isoforms of VEGF transcripts. (A) Representative gel showing VEGF isoform PCR products. (B) Densitometric analysis of the two major VEGF₁₂₁ and VEGF₁₆₅ transcripts. The density values of genes were normalized to those of the respective internal control, β-actin. Data are presented as the mean ± SD. (C) Reverse transcription-quantitative PCR analysis of the mRNA expression levels of VEGF₁₆₅. TGF-β receptor blocker SB4 at 10 μM was used to block receptor kinase activity. (D) ELISA of R-50 cells receiving 10 ng/ml TGF-β2 for 48 h in the presence of 1 μM (TA1) or 10 μM (TA10) TA. (E) ELISA of R-50 cells receiving 10 ng/ml TGF-β2 for 24 h in the presence of p38 mitogen-activated protein kinase inhibitor SB2, MEK inhibitor PD, TGF-β receptor blocker SB4 at 10 μM or equivalent DMSO as a SC. Data are presented as the mean ± SD from three independent experiments. #P<0.05, ##P<0.01 vs. NC; *P<0.05, **P<0.01, ***P<0.001 vs. MeOH or vs. DMSO SC. TA, triamcinolone acetonide; TGF-β2, transforming growth factor-β2; VEGF, vascular endothelial growth factor; NC, negative control; SC, solvent control; SB4, SB431542; SB2, SB203580; PD, PD98059.

TGF-β2 and MTS viability assay was performed to evaluate the viability of RPE cells. The results indicated that TA significantly attenuated TGF-β2-induced RPE cell growth arrest (Fig. 1B). Microscopic morphological observations supported the conclusion that the lower confluence of TGF-β2-treated RPE cells was reversed by TA treatment (Fig. 1C). These findings strongly suggested that TA may pharmacologically antagonize the biological effects of TGF-β2.

TA suppresses TGF-β2-enhanced RPE cell contractility. TGF-β2 is known to upregulate α-SMA expression in RPE cells (21), and has also been shown to stimulate RPE cell-mediated collagen gel contraction (45). Therefore, the present study examined the direct effect and antagonism of TA on RPE cell contractility using a 3D collagen gel contraction assay. The results of direct gel-size measurement at 24-h intervals indicated that the addition of TA at 10 μM in RPE cell-populated gels significantly delayed gel contraction, in clear contrast to the enhanced contractility in solvent control cells treated with MeOH (Fig. 2A; representative images not shown due to a lack of documentation). Moreover, the addition of TA at 10 μM markedly suppressed the TGF-β2-induced increase in RPE cell contractility (Fig. 2B), suggesting that

TA is able to suppress RPE cell contraction, most likely by interfering with TGF-β2-driven signaling.

TA mitigates TGF-β2-induced Smad2, ERK1/2 and p38 MAPK hyperphosphorylation. To characterize the pharmacological effect of TA on the signaling machinery in RPE cells under serum-supplemented and -deprived conditions, R-50 cells were grown in media with or without serum supplementation, and then further treated with TA at 10 μM for different durations up to 2 h. Western blotting and densitometry indicated that, under serum-deprived conditions, TA exposure markedly reduced not only phosphorylation of Smad2, but also that of non-Smad signaling mediators, including ERK1/2 and p38 MAPK (Fig. 3A and B). To determine whether TA attenuated the signaling response of RPE cells to TGF-β2 stimulation, serum-starved R-50 cells were stimulated with TGF-β2 in the presence or absence of TA. The results clearly confirmed that TA mitigated the TGF-β2-induced hyperphosphorylation of Smad2, ERK1/2 and p38 MAPK proteins (Fig. 3C and D).

TA suppresses TGF-β2-induced VEGF expression in RPE cells. Since TGF-β signaling is of critical importance for

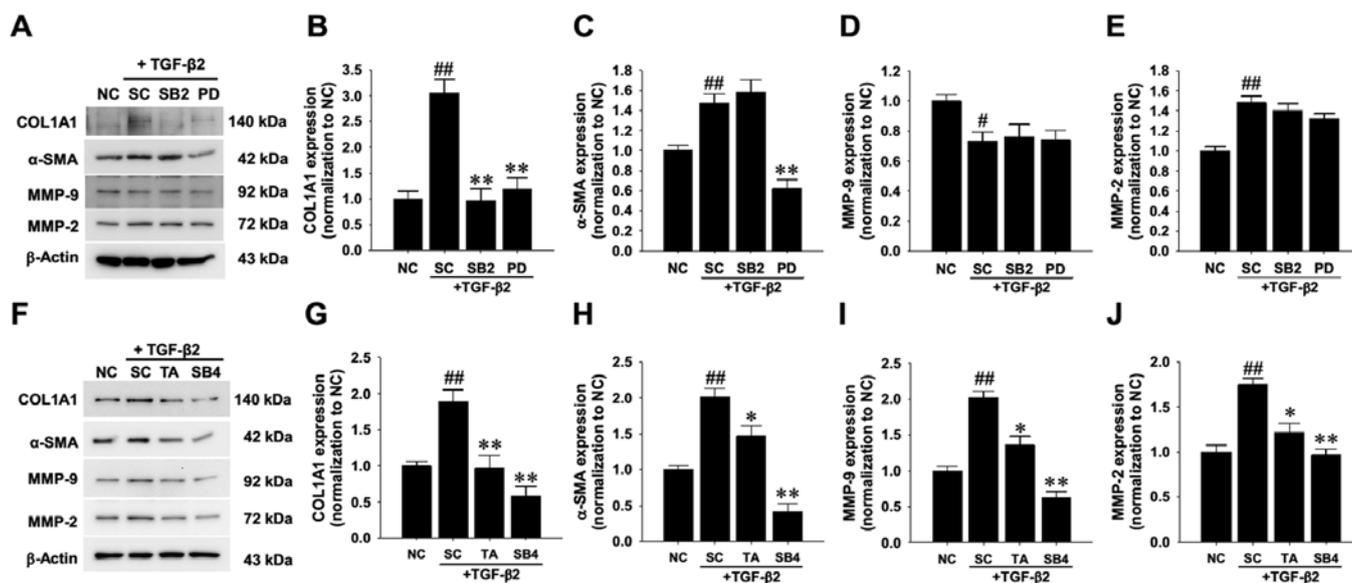


Figure 5. Effect of TA treatment on TGF- β 2-driven ECM remodeling in cultured human retinal pigment epithelial cells. (A) Western blot analysis of signaling pathway proteins involved in TGF- β 2-driven ECM remodeling. Human R-50 cells were treated with 10 ng/ml TGF- β 2 for 24 h in the presence of either 10 μ M p38 mitogen-activated protein kinase inhibitor SB2, MEK inhibitor PD or equivalent DMSO as a SC. Bands were densitometrically semi-quantified and the relative expression levels of (B) COL1A1, (C) α -SMA, (D) MMP-9 and (E) MMP-2 were normalized to internal actin expression levels. (F) R-50 cells were treated with 10 ng/ml TGF- β 2 for 48 h in the presence of 10 μ M TA, TGF- β receptor blocker SB4 or 0.1% methanol as a SC. The relative expression levels of (G) COL1A1, (H) α -SMA, (I) MMP-9 and (J) MMP-2 were densitometrically semi-quantified and normalized to β -actin. Fold changes are expressed as the mean \pm SD from three independent experiments. * P <0.05, ** P <0.01 vs. NC; ## P <0.01 vs. SC. TA, triamcinolone acetate; TGF- β 2, transforming growth factor- β 2; α -SMA, α -smooth muscle actin; MMP, matrix metalloproteinase; NC, negative control; SC, solvent control; SB4, SB431542; SB2, SB203580; PD, PD98059; COL1A1, collagen type I α 1 chain.

normal vascular development and physiology, and since aberrant TGF- β signaling forms the basis for several disorders due to neovascularization (46), the present study aimed to determine whether TGF- β 2 upregulated VEGF production in cultured RPE cells and whether TA modulated the TGF- β 2-induced upregulation of VEGF. The measurement of VEGF isoform transcripts by multiplex PCR indicated that TGF- β 2 at 10 ng/ml significantly increased the expression levels of both VEGF₁₂₁ and VEGF₁₆₅ transcripts in R50 cells. By contrast, this upregulation was significantly reduced by TA treatment in a dose-dependent manner (Fig. 4A and B). In parallel, RT-qPCR analysis demonstrated that TA exerted a dose-dependent suppression on the mRNA expression levels of VEGF₁₆₅ in TGF- β 2-stimulated cells, and that, compared with the TA treatment group, a less obvious suppression of VEGF₁₆₅ was observed in the cells pretreated with SB431542, an inhibitor of TGF- β 2 receptor kinase that abolishes Smad2 phosphorylation (Fig. 4C). Consistent with the changes detected in mRNA expression levels, TGF- β 2 markedly increased the release of soluble VEGF₁₆₅ peptides from R-50 cells after 48-h treatment, and the increase in VEGF peptide release was significantly alleviated by TA treatment (Fig. 4D). As TGF- β 2-induced p38 MAPK signaling activation has been demonstrated to regulate VEGF expression in RPE cells and critically contributes to its pro-angiogenic effect (19), we did not monitor the phosphorylation status of signaling mediators due to kinetic fluctuations after long-term exposure. Consistent with the previous observation, treatment of R-50 cells with kinase inhibitors demonstrated that blockade of p38 MAPK activity by SB203580 not only significantly suppressed TGF- β 2-induced VEGF expression, but also

reduced constitutive VEGF expression. Moreover, abolishment of Smad2 phosphorylation by SB431542 markedly attenuated TGF- β 2-induced VEGF overproduction (Fig. 4E). These findings suggested that both TGF- β 2 canonical and non-canonical signaling pathways may be essential for VEGF expression in RPE cells.

TA modulates TGF- β 2-induced tissue-remodeling effects on RPE cells. Given that TGF- β 2 induced a tissue-remodeling effect on RPE cells (2), the present study explored which signaling pathway participated in this remodeling effect. After R-50 RPE cells were treated with 10 ng/ml TGF- β 2 for 48 h with or without the addition of selective kinase inhibitors, the protein lysates were subjected to western blotting (Fig. 5A). The results consistently revealed that TGF- β 2 treatment significantly upregulated the protein expression levels of COL1A1, α -SMA and MMP-2 in the RPE cells. Treatment with kinase inhibitors demonstrated that the activities of both the MEK/ERK and p38 MAPK pathways were involved in TGF- β 2-induced upregulation of COL1A1 (Fig. 5B), whereas only the MEK/ERK pathway appeared to be essential for TGF- β 2-induced α -SMA upregulation (Fig. 5C). Notably, neither pathway was involved in MMP-9 and MMP-2 protein expression in cultured RPE cells (Fig. 5D and E). Western blotting and densitometric analysis were also used to determine whether TA interfered with this remodeling effect; the results revealed that TA exposure significantly abrogated the TGF- β 2-induced upregulation of COL1A1 (Fig. 5F and G), α -SMA (Fig. 5H), MMP-9 (Fig. 5I) and MMP-2 (Fig. 5J) in R-50 cells. A discrepancy in the TGF- β 2-modulated cellular MMP-9 abundance is likely due to the fact that the solvent

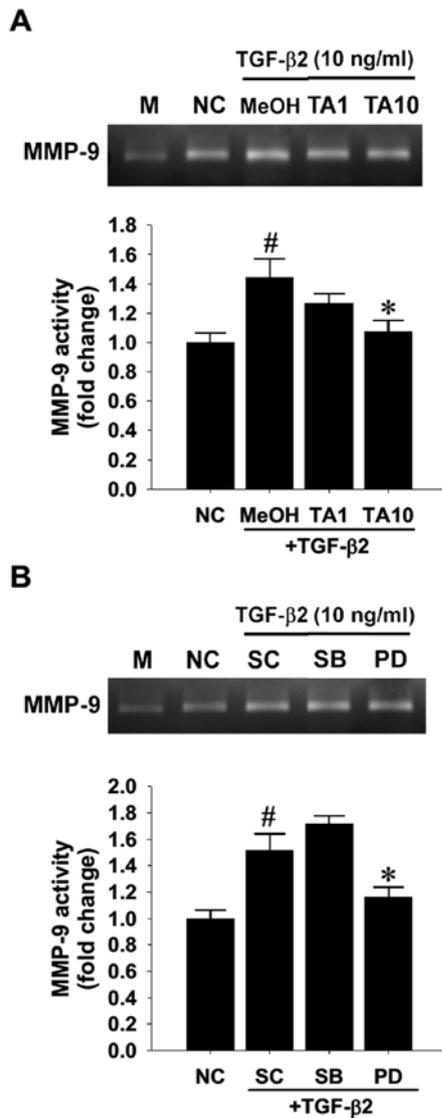


Figure 6. Effect of TA treatment on TGF-β2-driven MMP-9 gelatinolytic activity in cultured human retinal pigment epithelial cells. (A) Human R-50 cells were treated with TGF-β2 for 48 h in the presence of 1 μM (TA1) or 10 μM (TA10) TA or 0.1% MeOH as a SC. The supernatants were collected and subjected to gelatin zymography detection of MMP-9 activity. Bands were densitometrically semi-quantified and the scanned densities were subtracted from the background levels in the M group. (B) R50 cells were treated with TGF-β2 for 24 h in the presence of either 10 μM p38 mitogen-activated protein kinase inhibitor SB, MEK inhibitor PD or equivalent DMSO as a SC. Fold changes presented as the mean ± SD were obtained from three independent experiments. [#]P<0.05 vs. NC; ^{*}P<0.05 vs. MeOH or SC. TA, triamcinolone acetonide; TGF-β2, transforming growth factor-β2; MMP, matrix metalloproteinase; NC, negative control; SC, solvent control; M, medium; MeOH, methanol; SB, SB203580; PD, PD98059.

DMSO used for kinase inhibitor treatment might exhibit an unexpected blunting effect (Fig. 5D). Notably, TGF-β2 receptor kinase blockade significantly suppressed the protein expression levels of these remodeling mediators.

To confirm the modulatory effect of TA, gelatin zymography was used to detect the gelatinolytic activities of both MMPs. The results revealed that, despite no detectable MMP-2 gelatinolytic activity in the conditioned media (data not shown), TA at a higher concentration (such as 10 μM) significantly attenuated the TGF-β2-induced increase in MMP-9 gelatinolytic activity

(Fig. 6A). Furthermore, pretreatment with PD98059 markedly reduced the TGF-β2-induced MMP-9 activation (Fig. 6B), suggesting the involvement of the MEK1/MEK2 pathway in TGF-β2-induced MMP-9 upregulation.

Discussion

The present study demonstrated that the ERK and p38 MAPK signaling pathways may have a crucial role in the therapeutic mechanism of TA for the treatment of TGF-β2-associated intraocular angiogenesis and tissue remodeling, which are known to be associated with retinal disorders, such as PVR and PDR. TA was shown to markedly antagonize TGF-β2-induced RPE cell growth arrest and contractility, without exhibiting prominent cytotoxicity. In the context of TGF-β2-induced activation, TA significantly mitigated TGF-β2-elicited Smad2, ERK1/2, and p38 MAPK hyperphosphorylation, whereas inhibition of TGF-β receptor- and p38 MAPK-mediated signaling activities attenuated TGF-β2-induced upregulation of VEGF mRNA and peptide release. Furthermore, TGF-β2-induced ERK1/2 and p38 MAPK activation was involved in COL1A1 overproduction, while only the ERK1/2 signaling pathway was observed to participate in the α-SMA upregulation induced by TGF-β2. Similar to the blunting effects of Smad2 inhibition, TA effectively mitigated TGF-β2-induced upregulation of COL1A1, α-SMA, MMP-2 and MMP-9 expression and that of MMP-9 gelatinolytic activity in R-50 RPE cells. Taken together, TA treatment may prevent pathogenic signaling activation that contributes to angiogenesis and tissue remodeling.

It is well known that the sequential process of fibroblast-like transformation and differentiation of RPE cells is a pathological feature in the genesis of PVR and associated vitreoretinal diseases (47). The contraction of fibrotic and scarring membranes may lead to retinal detachment, increasing the risk of vision deterioration or blindness (48). TGF-β2 is considered a key component in the development of abnormal fibrosis in the pathological environment. Various studies have confirmed TGF-β2 as a dominant factor in the development of age-related macular degeneration and macular fibrosis or atrophy (49,50). The present results demonstrated that TA not only enhanced cell viability in TGF-β2-treated RPE cells, but also suppressed contractility in collagen gels. Furthermore, TA altered the TGF-β2-driven ECM remodeling process in cultured RPE cells, as the protein expression levels of COL1A1, α-SMA, MMP-2 and MMP-9 were revealed to be partially attenuated. Clinically, these results indicated that TA may cause substantial changes in the composition, structure and mechanics of fibrotic vitreoretinal conditions, such as PVR and PDR.

Although TGF-β2 is widely considered a major constituent of the fibrotic transformation process (50), to the best of our knowledge, the specificity of the effects of TA on TGF-β2-driven molecular behaviors remain elusive. Interleukin-2 interacts with TGF-β2, which may lead to the excessive expression of ECM proteins, further aggravating the fibrosis process (49). In the present study, TA was revealed to significantly attenuate TGF-β2-elicited Smad2, ERK1/2 and p38 MAPK phosphorylation, effectively blocking the signaling cascade. According to the present results, PD98059 may prevent TGF-β2-induced COL1A1 and α-SMA upregulation, while SB203580 also inhibit the increase in COL1A1

expression, suggesting the involvement of both the ERK and p38 MAPK signaling pathways.

VEGF is known to be a potent vasopermeability factor, which is involved in the development of diabetic macular edema and PDR (51). The development of diabetic macular edema is multifactorial, and intravitreal injections of VEGF inhibitors and steroids may help to reduce central retinal thickness and therefore improve visual acuity (52). Treatment with VEGF inhibitors requires multiple repeated injections, especially in the first and second years of therapy (53). Compared with the currently available anti-VEGF agents, steroids are advantageous because of their high potency and selectivity, along with a lower release rate, providing a high enough intraocular concentration to achieve therapeutic efficacy in the macular region (54). In a study of pseudophakic eyes at baseline, visual acuity improvement was observed in the combined TA and prompt or deferred laser photocoagulation group compared with in the VEGF-antagonizing ranibizumab group (55,56). At present, TA is used for the treatment of various neovascular and inflammatory ocular disorders with the risk of intraocular pressure elevation, cataract formation and infection (52). The present study confirmed that TA blocked the increase in VEGF transcription caused by TGF- β 2 and that TA attenuated the TGF- β 2-stimulated p38 activation. These results indicated that the attenuated effect was associated with the ablation of p38 signaling activity.

In conclusion, the findings of the present study may have important and novel clinical implications. The results revealed that the pharmacological inhibition via the use of TA may have a role in the inhibition of RPE cell transformation and differentiation through contractility and ECM regulation, suppressing the associated fibrosis in vitreoretinal diseases. In addition, it was hypothesized that both the ERK and p38 MAPK pathways may be applied as strategic targets in utilizing TA to prevent retinal diseases associated with angiogenesis and tissue remodeling.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WCW and YHK obtained the funds, conceived the study and supervised all experiments. CCH, YCC and YHK confirm the authenticity of all the raw data. CCH and YCC interpreted the data and drafted the manuscript. YTH, PHC and MCH performed the experiments. YTH and PHC performed all

statistical analyses. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Rat tails were collected from adult male Sprague-Dawley rats under the approval and supervision of the Institutional Animal Care and Use Committee at E-Da Hospital (approval no. IACUC-102016; Kaohsiung, Taiwan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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