Inhibition of TGF-β2-induced migration and epithelial-mesenchymal transition in ARPE-19 by sulforaphane

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Abstract

• AIM: To investigate the effects of sulforaphane (SFN) on transforming growth factor (TGF)-β2 stimulated migration and epithelial-mesenchymal transition (EMT) in ARPE-19 cells.  
• METHODS: ARPE-19 cells were cultured in the presence or absence of SFN or TGF-β2. SFN toxicity was assessed by performing a lactate dehydrogenase assay (LDH) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays, and cell migration was evaluated by Transwell migration assay. Actin stress fiber formation in ARPE-19 cells was determined using immunofluorescence analysis. Immunoblotting analysis was used to determine fibronectin and α-smooth muscle actin expressions along with the degree of Smad and Akt phosphorylation.  
• RESULTS: SFN inhibited ARPE-19 migration. Additionally, SFN attenuated TGF-β2-induced appearance of actin stress fibers as well as fibronectin and α-smooth muscle actin expressions in these cells. SFN also hindered the TGF-β2-stimulated phosphorylation of Smad2, Smad3, and Akt. SFN showed no cytotoxicity towards ARPE-19 cells.  
• CONCLUSION: SFN inhibits TGF-β2-stimulated migration and EMT in ARPE-19 cells, probably by preventing the establishment of actin stress fibers and Akt and Smad2/3 signaling.

• KEYWORDS: transforming growth factor-β2; epithelial-mesenchymal transition; sulforaphane; ARPE-19

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INTRODUCTION

Successful repair of rhegmatogenous retinal detachment, penetrating globe trauma, as well as several other ocular diseases are primarily hindered by proliferative vitreoretinopathy (PVR)¹-². PVR is characterized by membrane growth on both the posterior hyaloid and detached retina³. PVR may result in blindness without appropriate treatment; the occurrence of PVR is approximately 5%-10% among cases of retinal detachment based on epidemiological evidence⁴. At present, surgical repair is the most widely recommended treatment for PVR. However, the benefits of surgery are limited⁵, with 45%-85% anatomic success rates and 26%-67% technical success rates following PVR detachment surgery¹. Pharmacological intervention before or after surgery may improve surgical success. Anti-inflammatory, anti-growth factor, anti-proliferative, and anti-neoplastic agents have been introduced to treat PVR based on the results of studies using animal models⁶. However, these drugs show several side effects in humans⁷. Thus, newer approaches for preventing the progression of PVR are needed.

Retinal pigment epithelium (RPE) cells are essential for the development of PVR and form monolayers of cells with pigmented microvilli within the choroid and neural retina⁸. When the blood-retina barrier is damaged, RPE cells begin to proliferate, disrupting epithelial morphology and resulting in their migration into the vitreous humor through the neuroretina fissure, thus triggering the production of extracellular matrix (ECM)⁹. RPE proceed to transform into fibroblasts or myofibroblasts, during which α-smooth muscle protein (α-SMA), glial fibrillary acidic protein, and vimentin are expressed⁸-⁹. This progression is known as epithelial-
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mesenchymal transition (EMT). Among all fibrogenic cytokines, transforming growth factor (TGF)-β2 represents the strongest and has been closely associated to the development of several malignancies and fibrotic diseases[10][12]. This protein stimulates the transformation of myofibroblast-like cells, raises α-SMA levels, and upregulates fibrogenic production in cultured human RPE cells[19], all of which are essential for the progression of EMT[11] and PVR[14]. Additionally, patients with PVR overexpress TGF-β2 in the vitreous humor[4,15]. The Smad pathway is the principal signaling transmitter for TGF-β and regulates cell proliferation, and is inherently fundamental in the development of fibrogenic responses and RPE cells[16-17]. Sulforaphane (SFN), an organosulfur compound, is found in several cruciferous plants such as cabbage, brussel sprouts and broccoli[19]. SFN exerts anti-oxidative and anti-fibrotic effects on several fibrotic diseases[19-22]. Recently, increasing evidence revealed that SFN could reverse EMT and fibrosis via TGF-β/Smad signaling[23-25].

The inhibitive effect of SFN on EMT and TGF-β2 suggests that it can be used as a treatment for PVR. However, no studies have evaluated this potential treatment, and the effects of SFN on ocular fibrosis diseases remain unclear. Therefore, we examined the efficacy of SFN on TGF-β2-stimulated migration and EMT in ARPE-19 cells.

**MATERIALS AND METHODS**

**Materials** TGF-β2 was procured from R&D Systems (Minneapolis, MN, USA). Promega (Madison, WI, USA) provided the cytotoxicity assay kit (CytoTox 96® Non-Radioactive and CellTiter96®Aqueous One Solution Cell Proliferation Assay). Penicillin-streptomycin, fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were bought from Invitrogen (Carlsbad, CA, USA). The cell culture dishes and Transwell plates were bought from Corning, Inc. (Corning, NY, USA). Rhodamine phalloidin was purchased from Cytoskeleton (Denver, CO, USA). The 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) was from MP Biomedicals (Irvine, CA, USA). Crystal violet staining solution was obtained from Beyotime (Shanghai, China). Antibodies against Akt, phosphorylated Akt, Smad3, phosphorylated Smad3, Smad2, and phosphorylated Smad2 were bought from Cell Signaling Technology (Danvers, MA, USA), while Proteintech (Chicago, IL, USA) provided the antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Abcam (Cambridge, UK) provided fibronectin. SFN and antibodies to α-SMA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase and all secondary antibodies used in immunoblotting analysis were obtained from Beyotime (Shanghai, China). All reagents and media utilized for cell culture in the current investigation contained minimal amounts of endotoxins.

**Cell Culture** The RPE cell line ARPE19 (ATCC® CRL-2302™) was procured from the American Type Culture Collection (Manassas, VA, USA). ARPE-19 cells were maintained in DMEM containing streptomycin and penicillin and 10% FBS in a 5% CO₂ and 37°C incubator in 100-mm dishes. All culture media was replenished once every two to three days. All subsequent experiments utilized cells from passage 19 onwards.

**Cell Viability Assays** Cell viability was detected using the lactate dehydrogenase (LDH) and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays. To quantify LDH activity in the supernatants of the ARPE-19 cell culture, the cells were cultured at 2×10⁴ cells/24-well plate for 72h in 10% FBS-DMEM. The cells were deprived of serum for 24h prior to inoculation with increasing doses of SFN (0, 0.3, 1, 3, and 10 μmol/L in serum-free medium) for an additional 24h. Next, the cells were simultaneously exposed to both 5 ng/mL TGF-β2 in serum-free medium and the same SFN doses for 3d. The supernatants and positive control (50 μL) were re-plated on a 96-well plat. Reconstituted substrate mix (50 μL; Promega; Madison, USA) was added, and the plate was placed in an incubator in a darkened room for 30min at room temperature. Stop solution (50 μL) was then added to each well. A microplate reader (Bio-Rad; USA), allowed for evaluation of the OD value at a wavelength of 490 nm.

For MTS assay, the ARPE-19 cells were cultured in 96-well plates (5×10⁴ cells/well). Then, cells were treated with TGF-β2 and different concentrations of SFN for 72h. Next, 20 μL for MTS assay solution was added to the 96-well plate for 1-4h. A microplate reader (Bio-Rad; USA) was allowed for evaluation of the OD value at a wavelength of 490 nm.

**Cell Migration Assay** Transwell plates were used to study cell migration. ARPE-19 cells were grown in 60-mm dishes (4×10⁵ cells/dish) in 10% FBS-DMEM for 1d before being serum-starved for 24h. After pretreatment with 10 μmol/L SFN for 24h, the cells were incubated with either SFN (10 μmol/L), TGF-β2 (5 ng/mL) or a combination of both for 48h. The ARPE-19 cells were placed in DMEM deprived of serum in the top chamber at 2.5×10⁴ cells per well. The lower section contained DMEM with 10% FBS. The system and cells were allowed to culture for 24h at the end of this period, a cotton swab was used to separate non-migrated cells on the upper side of the filter (upper chamber interior). Migrated cells placed in the bottom filter (upper chamber external) were dried and immobilized with 4% paraformaldehyde and crystal violet-stained for 24h. Five photos were taken of each well (up, down, left, right, middle), three of which were used for cell counting using Photoshop (ZEISS Axiovert 40; Oberkochen, Germany).
Immunofluorescence Staining Immuno-fluorescence staining was used to detect the appearance of actin stress fibers in ARPE-19 cells. All cells were incubated in 60-mm dishes (2×10^5 cells/dish, four slides per container) containing 10% FBS-DMEM for 1d and then deprived of serum for 24h. After pretreatment using 10 μmol/L SFN for 24h, the cells were exposed to either SFN (10 μmol/L), TGF-β2 (5 ng/mL) or a combination of both for 48h. Cells were then fixed for 20min at room temperature with 4% paraformaldehyde before they were exposed for 15min to 0.5% Triton at room temperature. Next, a blocking buffer supplemented with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was used to immerse the cells at room temperature for 30min. Actin stress fibers were labeled at room temperature with rhodamine-phalloidin (100 nmol/L in 1% BSA in PBS) for 30min. Nuclei were stained with DAPI (100 nmol/L in 1% BSA in PBS) for 10min at room temperature. A fluorescence microscope (ZEISS Axio Scope A1) was then used to observe cells.

Immunoblot Analysis For immunoblot assessment of α-SMA and fibronectin, 60-mm dishes (5×10^5 cells/dish) were used to incubate ARPE-19 cells with 10% FBS-DMEM for 1d. After serum starving the cells for 24h, they were pretreated with SFN (0, 0.3, 1, 3, and 10 μmol/L) for another 24h. Following this, ARPE-19 cells were exposed with TGF-β2 (5 ng/mL) and SFN (0, 0.3, 1, 3, and 10 μmol/L) for an additional 72h. To assess the total and phosphorylated forms of Smad 2, Smad 3 and Akt, 60-mm dishes were used to house ARPE-19 cells (5×10^5 cells/dish) containing 0.5% FBS-DMEM for 1d, and then starved and pretreated with SFN (10 μmol/L) for 1d. ARPE-19 cells were stimulated with TGF-β2 (5 ng/mL) and SFN (10 μmol/L) for 0, 1, 2, 6, 12, and 24h. Proteins were extracted at specific time points. The cells were collected, and proteins were extracted. The protein products were first electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide 10% gel prior to transferring them onto to polyvinylidene fluoride membranes. A blocking solution [0.1% Tween-20, 5% dried non-fat milk and 20 mmol/L Tris-HCl (pH 7.4)] was exposed to the samples for an hour at room temperature and incubated with primary antibodies at a 1:1000 dilution in blocking solution at 4°C overnight. The membranes were rinsed, then subjected to a 1h incubation with horseradish peroxidase-conjugated secondary antibodies at 1:3000 dilution at room temperature. They were then exposed to electrochemiluminescence reagents and films. The intensity of immunoreactive bands was assessed using Tanon software (Shanghai, China).

Statistical Analysis Quantitative data are shown as mean± standard deviation. Intergroup variation was assessed using one-way analysis of variance and then by least significant difference comparison. P<0.05 indicated statistical significance. All experiments were conducted independently thrice. The SPSS version 24.0 software (SPSS, Inc., Chicago, IL, USA) was used to carry out all statistical analyses.

RESULTS Increasing Concentrations of SFN Imparted No Cytotoxicity Towards ARPE-19 Cells ARPE-19 cells were exposed to TGF-β2 (5 ng/mL) and SFN (0, 0.3, 1, 3, and 10 μmol/L) for 72h to determine the potential cytotoxic effects of SFN. After this period, the culture media was assessed for LDH activity. SFN at concentrations of 0.3, 1, 3, and 10 μmol/L imparted no significant effect on LDH release when co-cultured with 5 ng/mL of TGF-β2 (P>0.05; Figure 1A), suggesting that SFN had no cytotoxic effect on ARPE-19 cells. The result of MTS assay showed that the cell viability was not affected by SFN (Figure 1B).

SFN Inhibits Cell Migration Stimulated by TGF-β2 To explore the impact of SFN on ARPE-19 cell migration, Transwell assays were performed. The crystal violet staining results for migrated cells on the lower filter (upper chamber external) showed that compared with the control group, significantly fewer cells had migrated in the SFN (10 μmol/L) group (P<0.05). In contrast, the TGF-β2 (5 ng/mL) group showed significantly increased cell migration in stark contrast to the control group (P<0.05). Additionally, co-treatment with TGF-β2 (5 ng/mL) and SFN (10 μmol/L) induced substantially lower cell migration than that those stimulated with TGF-β2
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Figure 2 SFN inhibits TGF-β2-triggered cell migration  A: ARPE-19 cells were grown with the indicated TGF-β2 and SFN concentrations for 48h and then placed in the top well of a Transwell plate at 2.5×10^4 cells per well in serum-free DMEM media. The lower section contained DMEM medium with 10% FBS. The outer surface of the lower filter in the upper chamber contained migrated cells, which were labeled with crystal violet. Five images of each well were acquired (up, down, left, right, middle), and three images were chosen for cell counting by Photoshop. The quantity of migrated cells is depicted in terms of mean±standard deviation; B: Representative images of migrated cells in each group after crystal violet staining. *P<0.05 in contrast to cells incubated without the addition of TGF-β2,  #P<0.05 in contrast to cells treated with TGF-β2 only. All experiments were repeated three times independently. Scale bar, 200 μm.

Figure 3 SFN attenuates TGF-β2-stimulated actin stress fiber formation  ARPE-19 cells were exposed to both TGF-β2 (5 ng/mL) and SFN (10 μmol/L) alone or in combination for 48h. After cell fixation with 4% PFA and permeabilizing with 0.5% Triton, rhodamine-phalloidin was used to stain actin, and DAPI was used to stain the nuclei. An inverted fluorescence microscope was used to visualize the cells. A: Control; B: TGF-β2; C: TGF-β2 + SFN. Scale bar, 100 μm.

Figure 4 SFN decreases α-SMA levels upregulated by TGF-β2 in ARPE-19 Cells  SFN decreased ARPE-19 cell migration induced by TGF-β2 (Figure 2A), indicating that SFN decreased ARPE-19 cell migration induced by TGF-β2 (Figure 2A). Representative crystal violet staining photos of migration cells in each group are shown in Figure 2B.

SFN Attenuates Actin Stress Fiber Formation Stimulated by TGF-β2  To examine the impact of SFN on actin stress fiber formation, ARPE-19 cells were subjected to a 48h exposure period to either TGF-β2 alone or in combination with SFN. In contrast to the untreated control groups (Figure 3A), TGF-β2 clearly increased actin stress fiber formation and induced spreading of ARPE-19 cells (Figure 3B). However, SFN hindered actin stress fiber formation and cell spreading, which were stimulated by TGF-β2 in ARPE-19 cells (Figure 3C).

SFN Decreased α-SMA Levels Upregulated by TGF-β2 in ARPE-19 Cells  As EMT is critical in PVR, we measured ARPE-19 cell levels of α-SMA as a marker of myofibroblasts. After stimulation with TGF-β2 alone or in combination with SFN for 72h, α-SMA levels were noted to be significantly raised when TGF-β2 was present in the culture system (P<0.05). In contrast, this stimulation was considerably prevented in a concentration-dependent manner by the addition of SFN (Figure 4).

SFN Suppresses Fibronectin Expressions Upregulated by TGF-β2 in ARPE-19 Cells  Fibronectin is a protein biomarker of fibrosis, which was detected by Western blotting after treatment with TGF-β2 with or without SFN. TGF-β2 substantially increased fibronectin expression compared to that of the controls. In contrast, SFN inhibited the upregulation of fibronectin stimulated by TGF-β2 in a concentration-dependent manner (Figure 5).

SFN Inhibits Smad Pathway Activation by TGF-β2  As Smad family proteins have a crucial function in transmitting TGF-β signals between the cell surface receptors and the nucleus, we detected levels of phosphorylated Smad2 and Smad3. TGF-β2 (5 ng/mL) upregulated expressions of both p-Smad2 and p-Smad3 in a manner dependent on time. Conversely, SFN reversed TGF-β2-triggered phosphorylation of Smad2 and Smad3 in a time-dependent manner, demonstrating that SFN inhibited Smad pathway activation by TGF-β2 (Figure 6).
SFN Restrains the p-Akt Pathway Activated by TGF-β2

To investigate the mechanisms by which SFN attenuates TGF-β2-triggered upregulation of ARPE-19 cell ECM molecules, Western blotting was used to determine the influence of SFN on components of the cell signaling pathway. Detection of total or phosphorylated Akt with antibodies showed that TGF-β2 (5 ng/mL) upregulated p-Akt expressions in a time-dependent manner. However, ARPE-19 cells which were exposed to both TGF-β2 (5 ng/mL) and SFN (10 μmol/L) demonstrated notably suppressed Akt phosphorylation in a time-dependent manner, suggesting that SFN inhibited the p-Akt pathway activated by TGF-β2 (Figure 7).

DISCUSSION

RPE cells, which form a monolayer of closely connected pigmented cells, constitute most of the cells involved in PVR. Generally, RPE cells remain stationary in order to maintain their distinctive morphology and functions [14]. When the blood-retinal barrier is damaged, RPE cells dedifferentiate, migrate, grow, and finally develop into myofibroblasts, thereby causing PVR [14,26-27]. PVR severely damages vision. Although the clinical treatment of PVR has seen several improvements, neither surgical treatment nor medication is able to achieve satisfactory results, making this condition an important clinical problem.

Numerous studies have shown that EMT is essential for PVR progression. Recent studies have reported that natural plant compounds such as SFN might suppress the progression of EMT [21]. However, no relevant studies have explored...
Tumors and PVR. Therefore, SFN has anti-fibrotic activity by attenuating TGF-β signaling. In addition to detecting the influence of SFN on cell migration and levels of EMT markers, we evaluated the possible pathways involved in these mechanisms. Previous evidence suggested that pathways both independent and dependent on Smad, including the PI3K/Akt pathways, can be activated, or inhibited by TGF-β2 in various cell types and under different stimuli. In the Smad-dependent pathway, downstream signaling is mainly mediated by Smad2 and Smad3. Smad proteins are critical for intracellular signaling, which are considered to be an important clinical therapeutic target in managing ocular fibrotic diseases. A Smad4 complex is formed upon the union on phosphorylated Smad2 and Smad3. This complex undergoes nuclear translocation where it goes on to activate or suppress gene transcription. By directly phosphorylating Smad2/3, TGF-β is able to trigger EMT. Smad3 is a crucial component for post-retinal detachment dedifferentiation of the RPE, therefore, EMT may be hindered by blocking the Smad3 pathway in mice. With regards to PI3K/Akt pathways, Akt functions as a serine/threonine kinase that is upregulated in breast and gastric cancers. It participates in pathways related to cell differentiation, apoptosis inhibition, and rearrangements of the actin cytoskeleton. Wang et al. reported that in endothelial cells, TGF-β1 can upregulate α-SMA and vimentin levels and increase the phosphorylation of Smad2/3 and Akt. TGF-β1 has been noted to induce EMT in tumor cell lines through PI3K/Akt. Additionally, blocking the Akt signaling was reported to reduce α-SMA expression, migration, and ECM synthesis in conjunctival fibroblasts. In the present study, SFN inhibited Smad2/3 and Akt phosphorylation that were triggered by TGF-β2, thereby indicating that SFN inhibits TGF-β2-induced EMT through Smad pathways and Akt pathways in ARPE-19 cells.

In summary, our results support an inhibitory role for SFN in cell migration and EMT progression via TGF-β2 signaling in ARPE-19 cells. The findings support the notion that SFN may be able to function as a preventive and therapeutic agent in fibrotic diseases, particularly in fibrotic fundus disease. However, the association between SFN and PVR or the mechanism of this interaction is beyond the scope of this study. The current investigation demonstrates that SFN suppressed TGF-β2-upregulated EMT biomarkers, such as α-SMA and fibronectin in a concentration-dependent manner. Furthermore, SFN inhibited both Akt and Smad pathways which were activated by TGF-β2 in a time-dependent manner, suggesting that SFN exerts antifibrotic effects on ARPE-19 cells.
Conflicts of Interest: Huang YB, None; Liu PP, None; Zheng H, None; Yang XX, None; Yang CC, None; Liu Y, None; Liu Y, None.

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