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All-trans retinoic acid stimulates the secretion of TGF-β2 via the phospholipase C but not the adenylyl cyclase signaling pathway in retinal pigment epithelium cells

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Abstract

Background: By investigating that (i) all-trans retinoic acid (ATRA) affects human retinal pigment epithelium (RPE) in expressing and secreting transforming growth factor (TGF)-β2 and (ii) U73122 (phospholipase C inhibitor) and SQ22536 (adenylyl cyclase inhibitor) regulate the ATRA-induced secretion of TGF-β2 in human RPE, we sought to interpret the signaling pathway of ATRA in promoting the development of myopia.

Methods: The RPE cell line (D407) was treated with (i) ATRA (10 μM), (ii) U73122 (5–40 μM) and ATRA (10 μM), or (iii) SQ22536 (5–40 μM) and ATRA (10 μM). The control group was no-treated. After stimulated at 2, 4, 8, 16, 24, and 48 h, The expression and secretion of TGF-β2 was detected.

Results: TGF-β2 in the cytoplasm was time-dependent increased by ATRA ($p < 0.001$). A time-dependent increase in the TGF-β2 protein of the supernatant was induced by ATRA ($p < 0.001$). U73122 (in the range of 5 to 40 μM) could suppress the secretion of TGF-β2 induced by ATRA ($p < 0.001$), and 40 μM U73122 could completely inhibit the up-regulated effect of 10 μM ATRA. However, SQ22536 (in the range of 5 to 40 μM) had no impact on the secretion of TGF-β2 induced by ATRA ($p > 0.05$).

Conclusions: In RPE cells, ATRA stimulates the secretion of TGF-β2 via the phospholipase C signaling pathway but not the adenylyl cyclase signaling pathway. U73122 may inhibit the promotion of ATRA in the development of myopia.

Keywords: Transforming growth factor-β2, U73122, SQ22536, Retinal pigment epithelium cells, All-trans retinoic acid

Background

Retinoic acid (RA) is a biological activity meditator with many functions including proliferation, cell differentiation and apoptosis [1, 2]. It has been reported that RA may be a retinal signal involved in inhibiting the remodeling of scleral extracellular matrix, resulting in slower ocular growth. Yuko Sekoa found that retinal RA increased within 5 days after form-deprivation in the chicken eyes. McFadden SA found that retinal RA accelerates the elongation of the eyes and increases in the myopic eyes of guinea pig. And conversely, it becomes lower in the inhibited elongation eyes

[3–8]. Previous studies have reported that all-trans retinoic acid (ATRA) can not only up-regulate the expression of tight junction-associated protein in the retinal pigment epithelium (RPE)-choroid complex and also promotes the epithelial barrier function of the RPE monolayer during the development of myopia [9, 10]. All of these results indicate that RPE plays an important role in the retinal RA signal controlling the scleral remodeling of myopia. It was reported that several cytokines synthesized and secreted by RPE cells have been involved in the progression of myopia [11–13]. Of these factors, it has been proved that transforming growth factor (TGF)-β2 affects the production of collagen and proliferation of scleral fibroblast cells and control the development of myopia [14]. TGFβ is both a critical mediator of collagen, MMP-2, and proteoglycan production in the extracellular matrix. It implies that TGFβ may have

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potential influence in controlling scleral remodeling, which induces myopia development [15–20]. According to these studies, we infer that retinal RA regulates the TGF-β2 level of RPE in the development of myopia, which controls the scleral remodeling. However, the mechanism by which ATRA could be transported from the choroid to the sclera has not been clarified. In the present study, we sought the effect of ATRA on the expression and secretion of human RPE in vitro and inhibited the signaling pathways of RPE with U73122 (phospholipase C and A2 inhibitor) and SQ22536 (adenylyl cyclase inhibitor) in order to survey which signaling pathway inhibitor can suppress the changes of TGF-β2 induced by ATRA in RPE.

Methods
Materials and reagents
In this study, human RPE cell line (D407) was purchased from the Retinal Cell Biology Laboratory, University of South Carolina (Columbia, South Carolina, USA). All-trans retinoic acid (ATRA), U73122, SQ22536 and DMSO were purchased from Sigma-Aldrich (Poole, UK).

The human TGF-β2 ELISA kit was purchased from Bender MedSystems (Austria). Antibodies of TGF-β2 and β-actin were purchased from Santa Cruz (Santa Cruz, CA, USA). Complete protease inhibitor cocktail tablets were obtained from Roche Diagnostics (Roche, Switzerland). The protein marker was purchased from NEB (New England Biolabs, China), and the ECL western blot kit was purchased from Pierce (Lockford, IL, USA). All-trans retinoic acid (ATRA), U73122 and SQ22536 were dissolved in DMSO for subsequent coculture with cells.

Cell culture and treatment
D407 cells, cultured as previously described [21]. There were 4 groups, Group A: Cells were treated with 10 μM ATRA for 2, 4, 8, 16, 24 and 48 h; Group B: Cells were treated with U73122 (B_1, 5 μM; B_2, 10 μM; B_3, 20 μM; B_4, 40 μM) for 30 min, then treated with 10 μM ATRA for 24 h; Group C: Cells were treated with SQ22536 (C_1, 5 μM; C_2, 10 μM; C_3, 20 μM; C_4, 40 μM) for 30 min, then treated with 10 μM ATRA for 24 h; Group D: Cells
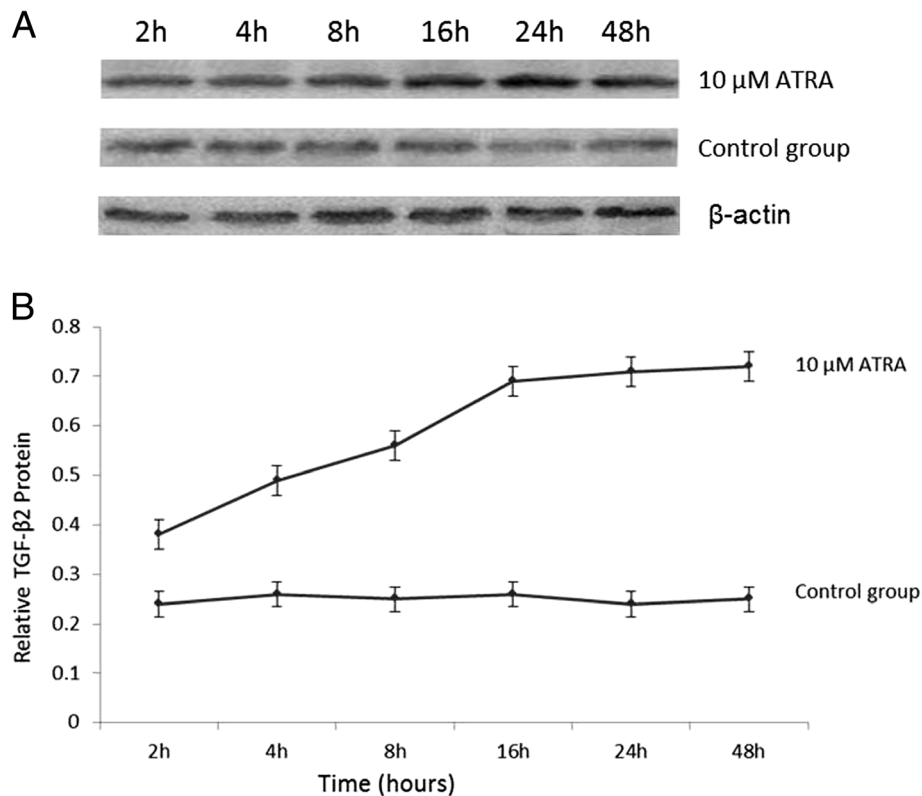

Fig. 1 ATRA stimulated the expression of TGF-β2 mRNA in D407 cells. D407 cells were treated with 10 μM ATRA for 2, 4, 8, 16, 24, and 48 h, and the expression of TGF-β2 protein was detected by western blot analysis. **a** The electrophoretogram of TGF-β2 protein in the 10 μM ATRA-treated and control groups for 2, 4, 8, 16, 24, and 48 h. It was found that 10 μM ATRA stimulated the expression of TGF-β2 protein in a time-dependent manner. **b** After treatment with ATRA for 2 h, the level of TGF-β2 protein in D407 cells was increased significantly compared with that of the control group ($p < 0.001$) and peaked at 16 h. However, there were no statistically significant differences in the level of TGF-β2 protein at 16 h, 24 h and 48 h ($p > 0.05$; $n = 6$ per treatment)
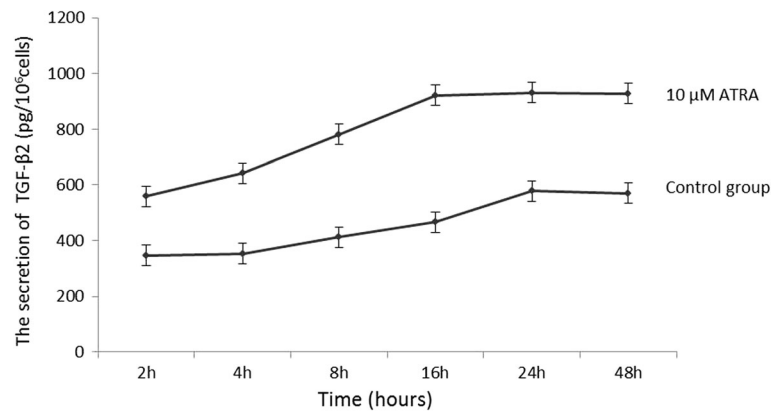
Fig. 2 Treatment with 10 μM ATRA stimulated the secretion of TGF-β_2 protein in the supernatants of D407 cells. TGF-β_2 protein in the conditioned media was measured by ELISA and normalized to cell counts (1×10^6). The concentration of secreted TGF-β_2 in the control group increased at 8 h and peaked at 24 h, and there was no statistically significant difference between 24 h and 48 h. After treatment with 10 μM ATRA for 2 h, the concentration of secreted TGF-β_2 of the ATRA-treat group increased ($p < 0.001$) and peaked at 16 h. However, there was no statistically significant difference the concentrations of secreted TGF-β_2 in the 10 μM ATRA-treated group at 16 h, 24 h and 48 h ($p > 0.05$; $n = 3$ per treatment). The effects of U73122 on the ATRA-induced secretion of TGF-β2 in D407 Cells . Cells were pretreated with U73122 (5 μM, 10 μM, 20 μM and 40 μM) for 30 min, followed by exposure to ATRA (10 μM) for 24 h. After treatment with 5–40 40 μM U73122 + 10 μM ATRA, the concentrations of secreted TGF-β2 in the supernatants were significantly lower than those of the ATRA-treated group ($p < 0.01$). The concentration of secreted TGF-β2 decreased with the increase of U73122. When the concentration of U73122 reached 40 μM, the concentration of secreted TGF-β2 was not significantly different from that of the control group ($p > 0.05$) (Fig. 3). The results indicated that the secretion of TGF-β_2 induced by ATRA is inhibited by U73122 (5–40 μM) in D407 cells. This suppressive effect of U73122 was enhanced with increasing concentrations, and the effect of 10 μM ATRA was completely inhibited by 40 μM U73122

treated only with equivalent volume of DMSO served as blank controls.

Western blot analysis and ELISA
Western blot assay and ELISA were performed according to our previous study [21].

Data and statistical analysis
SPSS software 18.0 (IBM Corp, NY, USA) was used to perform the statistical analysis in the present study. Data are presented as the mean ± SD. Student's t-test (SNK) and One-way ANOVA were used to determine the level of significance; $p < 0.05$ was defined as statistically significant. All in vitro cell experiments were repeated three times.
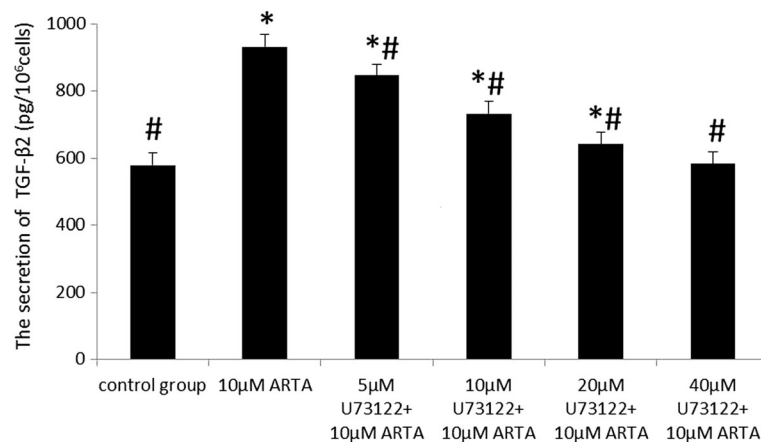

Fig. 3 U73122 suppressed the quantity of TGF-β_2 protein induced by ATRA in the supernatants of D407 cells. TGF-β_2 protein levels in conditioned media were measured by ELISA and normalized to cell counts (1×10^6). The secretion of TGF-β_2 induced by ATRA was inhibited by U73122 (5–40 μM) in D407 cells. The suppressive effect of U73122 was enhanced with increasing concentrations of U73122, and the effect of 10 μM ATRA was completely inhibited by 40 μM U73122. Data are represented as the mean ± SEM. Statistical comparisons were performed by one-way ANOVA. *Significance versus control group ($p < 0.001$; $n = 3$ per treatment). #Significance versus ATRA-treated group (10 μM) ($p < 0.001$; n = 3 per treatment). The effects of SQ22536 on the ATRA-induced secretion of TGF-β2 in D407 Cells. Cells were pretreated with SQ22536 (5 μM, 10 μM, 20 μM and 40 μM) for 30 min, followed by exposure to ATRA (10 μM) for 16 h. There was no significant difference between the secretion of TGF-β_2 in the 5–40 μM SQ22536 + ATRA-treated group and ATRA-treated group ($p > 0.05$) (Fig. 4). These results indicate that SQ22536 (5–40 μM) had no suppressive effects on the secretion of TGF-β_2 induced by ATRA in D407 cells

Results

ATRA stimulates the expression and secretion of TGF-β_2 in D407 cells

To identify whether ATRA can stimulate D407 cells in expressing and secreting TGF-β_2, we examined the concentration of TGF-β_2 in the cytoplasm, and the level of TGF-β2 secreted in the supernatant in the ATRA treatment group and control group at 2, 4, 8, 16, 24 and 48 h. In the control group, the concentration of TGF-β_2 in the cytoplasm did not change in the whole time period ($p > 0.05$) (Fig. 1). The level of TGF-β_2 of the control group in the supernatant increased at 8 h and peaked at 24 h (Fig. 2) At each time point, the expression and secretion of TGF-β_2 in the ATRA treated group were significantly higher than in the control group ($p < 0.001$). The TGF-β_2 level in the cytoplasm and supernatant increased at 2 h and peaked at 16 h. However, there were no significant differences between the concentrations of TGF-β_2 and the levels of secreted TGF-β_2 at 16 h, 24 h and 48 h. ($p > 0.05$) (Figs. 1, 2). These results proved that ATRA could induce the significant up-regulation of TGF-β_2 in both cytoplasm and supernatant in a time-dependent manner.

The effects of U73122 on the ATRA-induced secretion of TGF-β2 in D407 cells

Cells were pretreated with U73122 (5 μM, 10 μM, 20 μM and 40 μM) for 30 min, then exposure to ATRA (10 μM) for 24 h. After treatment with (5-40 μM) U73122 + 10 μM ATRA, the concentrations of secreted TGF-β2 in the supernatants were significantly lower than those of the ATRA-treated group ($p < 0.01$). The concentration of secreted TGF-β2 decreased with the increase of U73122. When the concentration of U73122 reached 40 μM, the concentration of secreted TGF-β2 was not significantly different from that of the control group ($p > 0.05$) (Fig. 3).

The results indicated that the secretion of TGF-β_2 induced by ATRA is inhibited by U73122 (5–40 μM) in D407 cells. This suppressive effect of U73122 was enhanced with increasing concentrations, and the effect of 10 μM ATRA was completely inhibited by 40 μM U73122.

The effects of SQ22536 on the ATRA-induced secretion of TGF-β2 in D407 cells

Cells were pretreated with SQ22536 (5 μM, 10 μM, 20 μM and 40 μM) for 30 min, followed by exposure to ATRA (10 μM) for 16 h. There was no significant difference between the secretion of TGF-β_2 in the (5–40 μM) SQ22536 + ATRA-treated group and ATRA-treated group ($p > 0.05$) (Fig. 4). These results indicate that SQ22536 (5-40 μM) had no suppressive effects on the secretion of TGF-β_2 induced by ATRA in D407 cells.

Discussion

The metabolism of Vitamin A and its derivative retinoid happen in the retina. As a product of Vitamin A metabolism, RA is a critical factor of maintaining the normal functions of the eye. It has been reported that the structure and functions of RPE cells is regulated by RA [22]. RA causes growth arrest of RPE cells and induces hexagonal changes in their shapes, which plays a crucial role in maintaining the RPE's monolayer structure [23]. In addition, RA was reported to up-regulate TSP-1 and enhance the concentration of TGF-β1 in RPE cells [24]. In our research, we attempted to investigate the effect of RA on TGF-β2, which is closely related to myopia. We found that ATRA could up-regulate the expression of TGF-β2 in the RPE cells and increase the secretion of TGF-β2 time-dependently. In the mice, guinea pig and mouse, TGF-β2 was proved to be expressed in the retina, RPE-choroid complex and sclera [25, 26]. In a
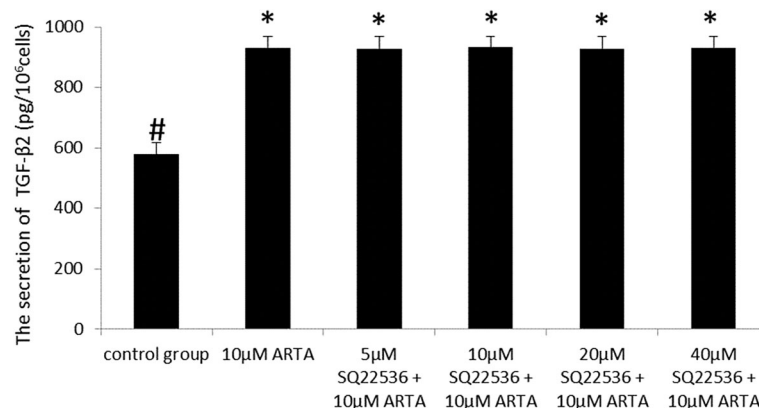

Fig. 4 SQ22536 did not suppress the quantity of TGF-β_2 protein induced by ATRA in the supernatants of D407 cells. TGF-β_2 protein levels in conditioned media were measured by ELISA and normalized to cell counts (1×10^6). There was no significant difference in the secretion of TGF-β_2 of the ATRA-treated group and SQ22536 (5–40 μM) + ATRA-treated group ($p > 0.05$). Data are represented as the mean ± SEM. Statistical comparisons were performed with one-way ANOVA. *Significance versus control group ($p < 0.001$; $n = 3$ per treatment). #Significance versus ATRA-treat group (10 μM) ($p < 0.001$; $n = 3$ per treatment)

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recent study, the TGF-β2 concentration of aqueous in highly myopic patients was significantly up-regulated, and was positively related to axial length of myopic patients.. According to these results, we inferred that increased RA in the retina of patients with myopia might induce the up-regulation of TGF-β2 in the RPE, which leads to the chondrogenic change in the sclera during the development of myopia. Nonetheless, the signaling pathway by which RA regulates TGF-β2 in RPE was not clear until now.

In the RPE, RA is bound to the retinoic X receptor (RXR) and retinoic acid receptor (RAR) to regulate gene transcription. There are two signaling pathways in RPE: (i) phospholipase C (PLC), calcium(Ca^{2+}) channels, and protein kinase C (PKC), and (ii) adenylyl cyclase, calcium (Ca^{2+}) channels, and cyclic AMP (cAMP)-dependent protein kinase A (PKA) [27]. In our study, we used two inhibitors (U73122 and SQ22536) to inhibit the signaling pathways. As the inhibitor of phospholipase C and A2, U73122 can reduce the free cytosolic $Ca2^+$ via inhibiting the hydrolysis of PPI (phosphatidylinositol) to IP3 (inositol triphosphate). It also inhibits the activation of G-protein phospholipase C coupling while remaining unaffected by the production of cAMP [28]. SQ22536 is a cell-permeable adenylyl cyclase inhibitor. SQ22536 reduces the activity of adenylate cyclase, when the concentrations of GTP increases with Mg^{++} and Mn^{++}, SQ22536 can inhibit that the activating effect of Mg^{++} and Mn^{++} on adenylate cyclase in the presence of catecholamines, [29]. We found that after treatment with U73122 + ATRA, the concentration of secreted TGF-β2 was significantly lower than that of the ATRA-treated group. When the concentration of U73122 reached to 40 μM, the concentration of secreted TGF-β2 was not significantly different from that of the control group. These results indicated that ATRA stimulated the secretion of TGF-$β_2$ via the PKC signaling pathway in RPE. On the other hand, we found that in the SQ22536-treated groups, there was no significant difference between the secretion of TGF-$β_2$ in the SQ22536 + ATRA-treated group and the ATRA-treated group, which indicated that the adenylyl cyclase signaling pathway was not involved in the effect of the stimulation of ATRA on the secretion of TGF-$β_2$. We concluded that retinal RA might bind to the RA receptors of RPE and activate the PKC signaling pathway, stimulating the secretion of TGF-$β_2$ in the RPE and inducing scleral remodeling in the myopia.

In further studies, our group will inject ATRA and U73122 into the sub-retina of mice, observe the effects of the suppression of U73122 on the changes in RPE induced by ATRA, and determine whether the growth of eyeball induced by ATRA can be suppressed by U73122. We will attempt to clarify the mechanism behind ATRA's control of myopia.

Conclusions

In RPE cells, ATRA stimulates the secretion of TGF-$β_2$ via the phospholipase C signaling pathway but not the adenylyl cyclase signaling pathway. U73122 may inhibit the secretion of TGF-β2 induced by the stimulation of ATRA, which slow down the scleral remodeling in the myopia.

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