Anti-fibrotic effect of rosmarinic acid on inhibition of pterygium epithelial cells

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INTRODUCTION

PTerygium, an uncontrolled proliferation of tissue, excessively migrates centripetally from the bulbar conjunctiva into the cornea. Upon pterygium, a lesion invades the cornea and covers the visual axis, potentially causing an irregular corneal astigmatism and eventually resulting in blindness. Ultraviolet (UV) radiation is a major cause that initiates the formation of pterygium[1]. Overexposure to UV radiation not only impairs limbal stem cells, disrupting a barrier of the interval cornea and conjunctiva, but also mutates the p53 gene of conjunctival epithelial cells, resulting in proliferative conjunctival epithelial cells invading the corneal epithelium[2-3]. Additionally, UV radiation increases the expression of transforming growth factor beta (TGF-β), especially TGF-β1[4]. Increasing TGF-β1 signaling was accompanied by enhanced type I collagen synthesis, a feature of fibrosis[5].

PTerygium is a fibrotic disease on the ocular surface that is characterized by type I collagen deposition[6-8]. Type I collagen, a major component of extracellular matrix (ECM), is critical for contributing several important functions in normal physiology and wound healing in various human organs. However, when the balance of synthesis and degradation of type I collagen is disrupted, excessive production of type I collagen will lead to fibrosis that may alter corneal or conjunctival tissue into opacity and induce vision loss[9-10]. Surgical removal of the lesion is a principle and useful treatment for pterygium, but the postoperative recurrence is up to 88%, which is extremely high[11]. Although a chemotherapeutic agent is used to efficiently decrease the recurrence of pterygium, serious complications cause other major problems after inhibition of recurrent pterygium[12-13]. Since increasing ECM production is contributed to recurrent pterygium, exploring more potent medication with an anti-fibrotic effect is necessary[14-15]. Increasing evidence has indicated that a natural compound has many biological abilities to ameliorate the disease both in vivo and in vitro, and includes anti-tumor[16], anti-angiogenesis[17], anti-inflammatory[18], and anti-fibrosis[19] effects.

Abstract

● AIM: To investigate the anti-fibrosis effect of rosmarinic acid (RA) in pterygium epithelial cells (PECs) to determine if RA is a potent agent for treating pterygium.
● METHODS: The PECs (1×10⁴ cells/mL) were treated with 100 μmol/L of RA for 1, 3 and 6h. After RA treatment, the cell viability was determined by staining with acridine orange/DAPI and analysis via a NucleoCounter NC-3000. The protein expression levels of type I collagen, transforming growth factor beta-1 (TGF-β1), TGF-β type II receptor (TGF-βRII), p-Smad1/5, p-Smad2, p-Smad3, and Smad4 of the cell lysates were measured by Western blot analysis.
● RESULTS: The cell viability of PECs was significantly decreased after RA treatment (P<0.01). As the result, RA reduced the protein expression of type I collagen and TGF-β1 of PECs. Additionally, RA also inhibited TGF-β1/Smad signaling by decreasing the protein expressions of TGF-βRII, p-Smad1/5, p-Smad2, p-Smad3, and Smad4.
● CONCLUSION: This study demonstrate that RA could inhibit fibrosis of PECs by down-regulating type I collagen expression and TGF-β1/Smad signaling. Therefore, RA is a potent therapeutic agent for the treatment of pterygium.
● KEYWORDS: fibrosis; pterygium; rosmarinic acid; transforming growth factor beta-1; type I collagen

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Rosmarinic acid inhibits pterygium cells

Rosmarinic acid (RA), an ester of hydrocinnamic acid and 3,4-dihydroxyphenyllactic acid, is a natural phenolic compound that is derived from plants of the Lamiaceae family, such as Rosmarinus officinalis and Salvia officinalis[20]. Medicinal values of RA, including anti-inflammatory[21], anti-angiogenesis[22], anti-oxidant[23], anti-tumor[24], and anti-photodamage[25], have been reported. Several studies have also reported the effect of RA against fibrosis both in vivo and in vitro. In a carbon tetrachloride (CCL4)-induced rat liver fibrosis model, RA ameliorated the histopathological morphology, reduced the fibrosis grade, and decreased TGF-β1 and connective transforming growth factor (CTGF) expression in the fibrotic liver. Additionally, RA could inhibit hepatic stellate cell (HSC) proliferation and decreased TGF-β1, CTGF and α-smooth muscle actin (α-SMA) expression in HSCs[26]. Moreover, RA alleviated cardiac fibrosis in a fructose-fed rat model by significantly reducing the expression of TGF-β1, α-SMA, and collagen[27].

Our previous study reported that RA inhibited PECs through induction of apoptotic cell death; however, the effect of RA to inhibit fibrosis in pterygium epithelial cells (PECs) remains unclear. Therefore, the aim of this study is to investigate the anti-fibrotic effect of RA in PECs to evaluate if RA has the potential to inhibit pterygium recurrence.

MATERIALS AND METHODS

Reagents RA, Trypsin-EDTA solution, and protease inhibitor cocktails were obtained from Sigma-Aldrich (St. Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) buffer was purchased from Bio Basic (Markham, ON, Canada). Anti-β-actin, anti-TGF-β1, anti-TGF-β type II receptor (TGF-βRII), anti-type-I-collagen, and anti-pan-Cytokeratin Alexa Fluor® 488 antibodies were purchased from Santa Cruz Technology (Santa Cruz, CA, USA), and anti-Smad1, anti-Smad2, anti-Smad3, anti-Smad4, anti-p-Smad1/5, anti-p-Smad2, and anti-p-Smad3 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). HyClone™ Dulbecco’s Modified Eagle’s medium (DMEM)/high glucose and HyClone™ Dulbecco’s phosphate buffered saline (PBS) were purchased from GE HealthCare Life Sciences (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Life Technologies (Danvers, MA, USA).

Cell Culture The cell line of PECs was a gift from Prof. Ya-Wen Cheng (Taipei Medical University, Taiwan, China)[28]. The cell type of the PECs was confirmed by pan-Cytokeratin after we received it (data not shown). PECs were maintained in DMEM/high glucose with 10% FBS in a humidified incubator at 37°C in an atmosphere of 5% CO2. Culture medium was replaced with fresh medium every two to three days. Only PECs that underwent less than ten passages were used in our study.

Cell Viability The PECs (5×10⁴ cells/mL) were seeded onto a 3.5 mm dish and incubated overnight. According to the condition that we have used in our previous experiment, 100 μmol/L of RA is an efficient and safe concentration[28]. Thus, PECs were treated with 100 μmol/L of RA for 1, 3 and 6h. After treatment, PECs were washed twice with PBS and detached by reaction of trypsin-EDTA. The viability of harvested PECs was measured by using a NucleoCounter NC-3000 (Copenhagen, Denmark), according to the manufacturer’s instructions.

Western Blot Analysis The PECs were treated with 100 μmol/L of RA for 1, 3 and 6h, and cell lysates were harvested using RIPA buffer containing 1% protease inhibitor cocktail. The protein concentrations of cell lysates were determined by the Bradford protein assay. The cell lysates were separated by 10% polyacrylamide gel and transferred onto PVDF. After incubating the membrane with blocking buffer (5% nonfat milk in PBST buffer) for 1h at 4°C, it was performed with primary antibodies, followed by adding horseradish peroxidase-conjugated secondary antibodies. The immunocomplexes were visualized using the ImageJ analysis system.

Statistical Analysis The results were expressed as the mean±standard deviation (SD), and statistical significance was determined by one-way analysis of variance (ANOVA), followed by a post hoc Tukey’s test using SPSS (version 20.0) software (SPSS Inc.). Statistically significant differences in values were considered when P<0.05.

RESULTS

Effect of Rosmarinic Acid on Cell Viability in Pterygium Epithelial Cells To determine the effect of RA on cell viability in PECs, the cells were treated with 100 μmol/L of RA for 1, 3 and 6h and stained with acridine orange/DAPI to analyze cell viability using a NucleoCounter NC-3000. In Figure 1, RA significantly reduced the cell viability of PECs (P<0.01) compared to the cells without RA treatment. The results showed that RA remarkably inhibited the viability of PECs.

Effect of Rosmarinic Acid on Type I Collagen Expression in Pterygium Epithelial Cells Type I collagen deposition is a notable feature of fibrosis in various organs. Although type I collagen is the main component of the cornea and conjunctiva, excessive accumulation of type I collagen induces fibrotic diseases on the ocular surface. A previous study indicated that a higher level of type I collagen was observed in patients with pterygium[29]. To examine the anti-fibrotic effect of RA in PECs, Western blot analysis was used to detect the protein expression of type I collagen after RA treatment. In Figure 2, type I collagen protein expression of PECs decreased after RA treatment, and the result had a significant difference at 6h (P<0.05).
Effect of Rosmarinic Acid on Transforming Growth Factor Beta-1 Signaling in Pterygium Epithelial Cells

In the early stage of TGF-β signaling transduction, TGF-β binds to TGF-βRII[30], TGF-β, a pro-fibrotic protein[31], has three isoforms, including TGF-β1, TGF-β2, and TGF-β3[32]. Excessive expression of TGF-β1 was detected in patients with pterygium[6]. To identify the effect of RA on pro-fibrotic protein and its receptor, we examined TGF-β1 and TGF-βRII protein expressions by Western blot analysis. In Figure 3, the result showed that RA decreased TGF-β1 and TGF-βRII protein expression in PECs.

Effect of Rosmarinic Acid on Smad Pathway in Pterygium Epithelial Cells

TGF-βRII results in phosphorylation of receptor-regulated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5); then, the phosphorylated R-Smads complexes with the common mediator Smad (Co-Smad: Smad4) and regulates collagen synthesis[30]. To further elucidate the underlying mechanism of RA against fibrosis in PECs, we examined the associated protein expression of the Smad pathway by Western blot analysis. After normalizing to β-actin, the ratios of p-Smad1/5 protein and total Smad1 (Figure 4A), p-Smad2 protein and total Smad2 (Figure 4B), and p-Smad3 and total Smad3 (Figure 4C) decreased after RA treatment. In addition, RA also decreased the protein expression of Smad4 (Figure 4D). Therefore, our results confirmed that RA down-regulated the TGF-β1/Smad signaling of PECs.

DISCUSSION

Pterygium is a benign tumor that is characterized by hyperproliferation, overexpression of anti-apoptosis, and ECM deposition. Currently, the primary therapy for pterygium is surgical excision; however, postoperative recurrence is a common complication. Recurrent pterygium is difficult to remove surgically because the recurrent lesion is more aggressive and rapidly progressive and may be larger and thicker compared to primary pterygium[35]. Since increasing ECM production after surgery is contributed to recurrent pterygium[15], mitomycin C, a chemotherapeutic agent with an anti-fibrotic effect, has been used to inhibit recurrent pterygium in clinical practice. Although mitomycin C can effectively decrease the recurrence rate of pterygium, the complications of mitomycin C severely impair normal ocular tissues, resulting in scleral or corneal melting, ulceration, and perforation[12-13]. Therefore, it is necessary to explore more efficient therapies to inhibit the recurrence of pterygium.

RA, a natural compound found primarily in plants from the Lamiaceae family, has shown numerous biological activities,
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![Image](image-url)

Figure 4 RA down-regulated the protein expression of p-Smad1/5, p-Smad2, p-Smad3, and Smad4 in PECs PECs were treated with 100 μmol/L RA for 1, 3 and 6h. After RA treatment, cell lysates were extracted to assess p-Smad1/5 and Smad1 (A), p-Smad2 and Smad2 (B), p-Smad3 and Smad3 (C), and Smad4 (D) expressions. Forty micrograms of protein were loaded on a 10% SDS-polyacrylamide gel and evaluated by Western blot analysis. *p<0.05 compared with 0h (untreated group). All data are expressed as the mean±SD (n=3).

such as anti-UV ability, as well as free radical scavenging and anti-oxidant activities. RA was reported to decrease radical oxygen species (ROS) production and lipid peroxidation levels in a H2O2-induced astrocytes injury model. Additionally, it could ameliorate UV-B radiation damage in keratinocytes by increasing NF-E2-related factor 2 transcription to enhance anti-oxidant activities. RA was reported to decrease radical generation such as anti-UV ability, as well as free radical scavenging and anti-oxidant activities. RA was reported to decrease radical oxygen species (ROS) production and lipid peroxidation levels in a H2O2-induced astrocytes injury model. Additionally, it could ameliorate UV-B radiation damage in keratinocytes by increasing NF-E2-related factor 2 transcription to enhance anti-oxidant activities. RA was reported to decrease radical generation.

However, RA has been reported to induce tumor cell death by suppression of ROS generation. Thus, PECs were killed because their normal physiological functions could not work. Since RA showed the remarkable effect on inhibiting PECs, we continued to use RA for further research.

Fibrosis plays a crucial role in modulating recurrent pterygium. TGF-β1/Smad signaling is the most important pathway among all of the fibrotic process transductions. The TGF-β signal is transduced through TGF-βRI and TGF-βRII. The binding of TGF-β and TGF-β receptors allows TGF-βRII to phosphorylate TGF-βRI and activates R-Smads (Smad1, Smad2, Smad3, Smad5), then, the phosphorylated R-Smads complexes with the common mediator Smad (Smad4) and regulates ECM proteins synthesis. Previous studies indicated that RA ameliorated fibrosis in the liver, as well as in renal and cardiac organs. Therefore, we investigated the anti-fibrotic effect of RA in PECs in this study. The results demonstrated that RA decreased type I collagen expression and TGF-β1 expression and down-regulated TGF-β1/Smad signaling.

Type I collagen deposition is an indicator of fibrotic diseases; increasing type I collagen has been detected in active fibrosis in the liver, as well as in renal and cardiac organs. Type I collagen deposition is also widely observed in tumors; it is a critical protein in tumor progression, promoting tumor cell proliferation, migration, invasion, and metastasis. Many studies have indicated that pterygium had a higher expression of type I collagen compared to normal conjunctival. Additionally, mitomycin C was reported to effectively inhibit recurrent pterygium through decreasing proliferation and type I collagen synthesis. In our results, RA significantly decreased cell viability (Figure 1) and protein expression of type I collagen (Figure 2) in PECs, which showed that RA is a promising agent to inhibit fibrotic pterygium.

UV radiation, an inducer of primary pterygium, has also been reported to be associated with recurrent pterygium. Sekelj et al. indicated that the incidence of recurrent pterygium was significantly higher in patients who were exposed longer to UV radiation. However, the underlying mechanism of UV radiation-induced recurrence of pterygium is still unclear. Because UV radiation increases pro-fibrotic protein and TGF-β1 expression, and fibrosis is contributed to pterygium recurrence, we further investigated the effect of RA in TGF-β1 and TGF-β1 associated fibrotic signaling. TGF-β1 is a member of TGF-β ligands that are a superfamily of cytokines with multi-functionality, including the regulation of numerous signaling pathways and cell processes, such as proliferation, migration, angiogenesis, differentiation and ECM synthesis. Overexpression of TGF-β1 induces fibrosis in various organs via activation of Smad and non-Smad signaling, which results in ECM synthesis. Additionally, overexpression of TGF-β1 has also been found in diverse...
tumor types and TGF-β1 stimulation enhances metastasis in tumor cells by increasing type I collagen deposition. A previous study indicated that excessive expression of TGF-β1 is detected in pterygium tissue and contributes to progression in pterygium. Our results revealed that RA inhibited the expression of TGF-β1 protein of PECs (Figure 3A), showing that RA could decrease type I collagen production by decreasing TGF-β1 signaling.

TGF-β1 signaling is involved in the progression of numerous diseases. Induction of TGF-β1/Smad signaling induces fibrotic diseases by increasing ECM production. Additionally, up-regulating the expression of phosphorylated Smad proteins increases tumor cell adhesion, migration and invasion. Previous studies reported that down-regulating TGF-β1/Smad signaling contributes to ameliorating the diseases. In a CCl₄-induced rat liver fibrosis model, 18α-glycyrrhizin inhibited fibrosis by suppressing TGF-β1, p-Smad2, p-Smad3 and type I collagen expression. Additionally, oxymatrine, a natural product, inhibited cell migration in colorectal carcinoma cells by reducing the expression of TGF-β1, p-Smad2, Smad4, and ECM proteins. Thus, targeting TGF-β1/Smad signaling is a potential way to explore novel therapeutic agents. To the best of our knowledge, no study to date has been published that investigated TGF-β1/Smad signaling in pterygium. Since TGF-β1/Smad signaling is indicated to be a common pathway that regulates the activation of fibrosis in corneal and conjunctival cells, we investigated the effect of RA in TGF-β1/Smad signaling in PECs. Our results showed that RA decreased the protein expression of TGF-βRII (Figure 3B), p-Smad1/5, p-Smad2, p-Smad3, and Smad4 of PECs (Figure 4). Although Smad1 and Smad5 were members of R-Smads, they were categorized in bone morphogenetic protein signaling, instead of TGF-β signaling. However, a recent report indicated that p-Smad1/5 could be activated via TGF-β1 stimulation in various epithelial cells. Thus, we also evaluated p-Smad1/5 expression in this study.

Primary pterygium can be easily removed by surgery; a high rate of recurrence is the main postoperative complication. Currently, mitomycin C is still the most efficient agent for inhibition of recurrent pterygium; it inhibits the recurrence by decreasing the fibrosis. However, the usage of mitomycin C induces severe complications in normal ocular tissue. RA has been reported to have an anti-fibrosis effect in various organs. Despite this, our study is the first to demonstrate the anti-fibrotic effect of RA by down-regulating TGF-β1/Smad signaling. To our knowledge, this study is the first to demonstrate an anti-fibrotic effect of RA in PECs. In this study, RA decreased cell viability of PECs, and ameliorated the fibrosis of PECs by decreasing type I collagen production and down-regulating TGF-β1/Smad signaling, which showed the excellent effect on inhibiting fibrosis in PECs (Figure 5). Therefore, RA is a potent candidate for treating pterygium. In future research, we will evaluate the effects of RA that is topically applied via eye-drops on both primary pterygium and recurrent pterygium in animal models to determine the possibility of RA in clinical application.

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