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ORIGINAL ARTICLE

Expression of specificity protein 1 and collagen I in primary pterygial tissues

Expressão da proteína de especificidade 1 e colágeno I em tecidos pterigiais primários

Bo Jiang[1], Yong Wang[1], Zi-Cheng Zhu[2], Zhang-You Wu[2]

1. Department of Ophthalmology, Anhui No. 2 Provincial People's Hospital, Hefei, Anhui Province, China.
2. Department of Ophthalmology, Anhui Provincial Hospital, Hefei, Anhui Province, China.

ABSTRACT | Purpose: To determine the expression profiles of the transcription factor specificity protein 1 and collagen I in primary pterygial and normal conjunctival tissues, and to explore the role of specificity protein 1 and collagen I in pterygial development. **Methods:** The pterygial tissues of 20 patients who underwent resection of primary pterygial tissue in our hospital from June 2016 to December 2017 and the conjunctival tissues of 10 patients with enucleation due to trauma were collected. Reverse transcription quantitative-polymerase chain reaction and western blot analyses were used to detect the relative expression levels of specificity protein 1 and type I collagen at the mRNA and protein levels. **Results:** The content of specificity protein 1 and collagen I mRNA and protein was significantly greater in primary pterygial tissue than it was in conjunctival tissue ($p<0.05$). There was a positive correlation between the mRNA and protein levels of specificity protein 1 and collagen I in primary pterygial tissues (protein: $r=1$, $p<0.05$; mRNA: $r=1$, $p<0.05$). **Conclusion:** Specificity protein 1 and collagen I are expressed in normal conjunctival and pterygial tissues, but expression is significantly greater in the latter. Specificity protein 1 and collagen I may be involved in the regulation of the development of primary pterygium.

Keywords: Pterygium; Conjunctiva; Sp1 transcription factor; Collagen type I

RESUMO | Objetivo: Determinar os perfis de expressão do fator de transcrição da proteína de especificidade 1 e do colágeno I em tecidos pterigiais primários e conjuntivais normais, e explorar o papel da proteína de especificidade 1 e colágeno I no desenvolvimento pterigial. **Métodos:** Foram coletados os tecidos pterigiais de 20 pacientes submetidos à ressecção de tecido de pterígio primário em nosso hospital no período de junho de 2016 a dezembro de 2017 e os tecidos conjuntivais de 10 pacientes com enucleação por trauma. A reação em cadeia da polimerase quantitativa de transcriptase reversa e a análise de *Western blot* foram utilizadas para detectar os níveis de expressão relativa da proteína de especificidade 1 e colágeno tipo I nos níveis de mRNA e proteína. **Resultados:** O conteúdo de especificidade da proteína 1 e do mRNA e proteína do colágeno I foi significativamente maior no tecido de pterígio primário do que no tecido conjuntival ($p<0,05$). Houve correlação positiva entre os níveis de mRNAs e proteína de especificidade 1 e colágeno I nos tecidos primários do pterígio (proteínas: $r=1$, $p<0,05$; mRNA: $r=1$, $p<0,05$). **Conclusão:** A proteína de especificidade 1 e do colágeno I é expressa nos tecidos conjuntivais e pterigiais normais, mas a expressão é significativamente maior no segundo. A especificidade da proteína 1 e do colágeno I pode ser envolvida na regulação do desenvolvimento do pterígio primário.

Descritores: Pterígio; Túnica conjuntiva; Fator de transcrição Sp1; Colágeno tipo I

INTRODUCTION

Pterygium is a common disease of the ocular surface that is associated with prolonged exposure to ultraviolet (UV) light and has a wing-shaped appearance that invades the cornea with proliferating tissue on the conjunctiva that often leads to corneal astigmatism, resulting in visual impairment[1-2]. Histological studies have found that pterygium is composed of atrophic conjunctival epithelium, hypertrophic vascularized tissue, and degenerated connective tissue, while ultrastruc-

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Corresponding author: Yong Wang.

Department of Ophthalmology. Anhui NO.2 Provincial People's Hospital

No. 1868 Dangshan Road - Hefei 230001, Anhui Province, China
E-mail: 553797875@qq.com

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tural studies have shown that the prominent pathological changes of pterygium are the proliferation and degeneration of elastic and collagen fibers[3-4]. Although some environmental factors, such as wind-blown sand, infections, smog, and certain chemical substances, are considered to play an important role in the pathogenesis of pterygium, its exact etiology and pathogenesis remain controversial[5].

The proliferation and degeneration of collagen fibers play a huge role in the occurrence and development of pterygium, especially the expression of collagen I[6]. There are three subtypes of transforming growth factor beta (TGF-β): TGF-β1; TGF-β2; and TGF-β3. TGF-β1 is among the most complex growth factors and is expressed in the fibers of the heart, liver, and kidney. Hence, TGF-β1 has received extensive attention in various fields. Some studies have shown that high expression levels of TGF-β1 may play an important role in the development of pterygium[7], while other studies have confirmed that the transcription factor specificity protein 1 (Sp1) is a downstream target of TGF-β1 that plays a role in the synthesis and degradation of collagen I[8,9]. Therefore, the aim of the present study was to examine the expression profiles of Sp1 and collagen I in primary pterygium, and to explore the role and underlying mechanism of Sp1 in the regulation of pterygial development, thereby providing an experimental basis and new ideas for the further study of associated signal transduction pathways.

METHODS

Subjects

The study protocol was approved by the Ethics Committee of Anhui No. 2 Provincial People's Hospital (Hefei, Anhui Province, China) and all patients submitted signed consent forms before participation in the study. The pterygial tissues of 20 patients who underwent primary pterygium resection in our hospital from June 2016 to December 2017, and the conjunctival tissue of 10 patients who had enucleation due to trauma in our hospital were randomly collected. The 20 patients in the pterygium group consisted of eight males and 12 females with a mean age of 62.12 ± 11.51 (range, 43-75) years, while the normal conjunctiva group consisted of six males and four females with a mean age of 62.41 ± 12.24 (range, 49-77) years. All patients had no history of corneal disease, glaucoma, uveitis, diabetes, or hypertension. The isolated pterygial and conjunctival tissues were stored at -80 °C until analysis.

Western blot analysis

About 100 mg of tissue was ground in liquid nitrogen, lysed by the addition of 1 mL of radioimmunoprecipitation assay buffer, and then centrifuged for 10 min. After protein denaturation, a 20-μL aliquot of the supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis in a current of 300 mA (Sp1 membrane, 90 min; collagen I membrane, 20 min). The protein bands were then transferred to a polyvinylidene difluoride membrane that was sealed at room temperature for 2 h. Afterward, the membranes were incubated overnight with antibodies against Sp1 (BS1598; dilution, 1:500; bioWORLD, Dublin, OH, USA) and collagen I (bs-0578R; dilution, 1:300; Bioss, Shanghai, China) at 4 ºC with mild shaking. The next day, the membranes were washed three times with phosphate-buffered saline plus Tween-20 (PBST) for 10 min for each wash and then incubated at room temperature for 2 h with horseradish peroxidase-conjugated secondary antibodies (dilution, 1:20,000). Finally, the membranes were washed three times with PBST for 10 min each wash. Proteins were detected using an ultrasensitive electrochemiluminescence kit and analyzed with Image J software (https://imagej.nih.gov/ij/).

Reverse transcription quantitative-polymerase chain reaction (qRT-PCR)

The tissues were ground in liquid nitrogen and total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), in accordance with the manufacturer's instructions. Total RNA (1 μg) and 10 μ a/L Oligo (dT) were added to 0.2 mL of RNase-free water and heated for 5 min at 65 °C. After the reaction, the tubes were immediately placed on ice for 3 min. Then, the RNA was reverse transcribed into cDNA in a tube containing 4.0 μL of 5× reaction buffer, 2 μL of 10 mmol/L dNTP mix, 1 μL of RibolockTM RNase inhibitor, and 1 μL of Revert Aid TM M-MuLV reverse transcriptase (Thermo Fisher Scientific) at 42 °C for 60 min and 70 °C for 5min, and then were stored at -80 °C until qRT-PCR analysis was done. The qRT-PCR amplification protocol included an initial denaturation step at 95 °C for 2 min followed by 40 cycles at 95 °C for 5 s and 60 °C for 10 s. The $2^{-\Delta\Delta CT}$ method was used to quantify target genes relative to β-actin as an endogenous control. Each reaction was performed in triplicate with use of the primer sequences listed in table 1.

Table 1. Primers for qRT-PCR

Gene	Forward primer	Reverse primer
β-actin	GGGAAATCGTGCGTGACATTAAGG	CAGGAAGGAAGGCTGGAAGAGTG
Collagen I	CACCAATCACCTGCGTACAG	GCAGTTCTTGGTCTCGTCAC
Sp1	ACAACTCAAGCCATCTCCCA	ACTGTTGGTGTCCGGATGAT

qRT-PCR= Quantitative reverse transcriptase polymerase chain reaction.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows software (version 22.0; IBM Corporation, Armonk, NY, USA). The results are expressed as the mean ± standard deviation (SD). Differences were identified using the paired *t*-test and analysis of variance (ANOVA). The relationship in each group between the RNA expression levels of Sp1 and collagen I was evaluated using the Pearson's correlation coefficient and hypothesis testing of correlation coefficients. A probability (p) value <0.05 was considered statistically significant.

RESULTS

Changes in protein expression levels of Sp1 and collagen I

Western blot analysis was used to assess the protein expression levels of Sp1 and collagen I in the pterygial and conjunctiva tissues. As shown in figure 1, protein expression levels of Sp1 and collagen I were significantly greater in the pterygial tissues than they were in the conjunctival tissues (p<0.05).

Changes in Sp1 and collagen I mRNA expression levels

Sp1 and collagen I mRNA expression levels in the pterygial and conjunctiva tissues were quantified by qRT-PCR. As shown in figure 2, mRNA expression levels of Sp1 and collagen I were significantly greater in pterygial tissues than they were in the conjunctival tissues (p<0.05).

Correlation analysis of Sp1 and collagen I expression

In the pterygial tissue, expressions of collagen I and Sp1 were significantly correlated both at the levels of protein (r=1; p=0.00 and <0.05) and mRNA (r=1; p=0.00 and <0.05).

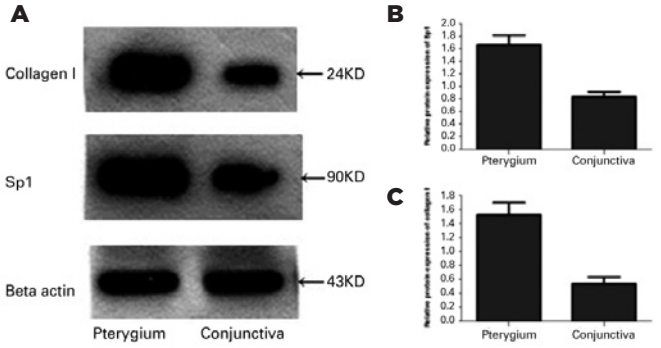

Figure 1. (A) Protein expression of Sp1 and collagen I. This Sp1 and collagen I protein expression was significantly higher in pterygial tissue than it was in conjunctival tissue. (B, C) Relative protein expression levels of Sp1 and collagen I in pterygial and conjunctival tissues. Data are presented as the mean ± standard deviation (SD) (p<0.05).

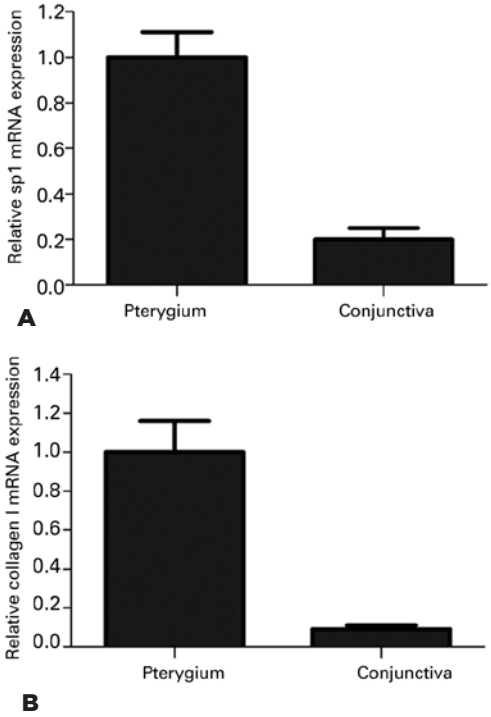

Figure 2. (A) Comparison of changes in mRNA expression profiles of Sp1 in pterygial and conjunctival tissues, p<0.05. (B) Comparison of changes in mRNA expression profiles of collagen I in the pterygial and conjunctival tissues, p<0.05.

DISCUSSION

The basic pathological mechanism of pterygium is keratoconjunctival epithelial cells undergoing epithelial-mesenchymal cell metaplasia, which induces the formation of pterygial epithelial cells and further fibroblast activation, resulting in the gradual formation of pterygium[10]. Pterygium is characterized by the centripetal growth of a group of altered limbal stem cells, followed by squamous metaplasia and goblet cell hyperplasia, with dissolving of the Bowman's membrane, which is accompanied by the activation of a large number of fibroblasts, inflammation of the proliferative stroma, neovascularization, and extracellular matrix (ECM) formation[11]. In pterygial development, local leukemic stem cells gradually change through chronic exposure to UV radiation. Then, progression of the corneal conjunctiva occurs due to limbal barrier dysfunction. In addition to participation in pterygial development, UV radiation also causes the upregulation of multiple inflammatory cytokines, growth factors, and matrix metalloproteinases. These effectors play an important role in inflammation, fibrosis, angiogenesis, and ECM remodeling, which are characteristic of pterygium[12].

TGF-β1 is a very important multifunctional cytokine, which is involved in the regulation of various physiological and pathological processes in the human body, such as cell proliferation, differentiation, ECM synthesis, immunity, inflammation, and apoptosis[13]. TGF-β1 can also inhibit synthesis of proteolytic enzymes in the ECM, thereby inhibiting decomposition of the ECM, resulting in abnormal proliferation and accumulation of collagen and elastin fibers, which leads to the occurrence and development of pterygium[14].

Sp1 is a downstream target of TGF-β1 signaling in many physiological processes[15]. Sp1 belongs to the specificity protein/Kruppel-like factor family of transcription factors, which contains three conservative Cys2/His2 zinc finger DNA-binding sites. Hence, damage to the zinc finger structure not only affects Sp1 binding to DNA, but also its nuclear transfer. In mammalian cells, Sp1 regulates multiple cellular processes, including the cell cycle, growth, proliferation, metabolism, and apoptosis[16-18]. In this study, the content of Sp1 in primary pterygial tissue was significantly higher than the content of Sp1 in conjunctival tissue. Pterygium is rich in collagen fibers, mainly collagen I and III[19,20]. In this study, the content of collagen I in primary pterygial tissue was significantly higher than the content of collagen I in conjunctival tissue, and the mRNA and protein expression levels of Sp1 and collagen I were highly correlated. Therefore, it can be concluded that Sp1 and collagen I may play an important role in pterygial development.

To conclude, the results of the present study have demonstrated that Sp1 is expressed in both human pterygial and conjunctiva tissues, but the expression level is significantly increased in the pterygial tissues. The correlation between the expression levels of Sp1 and collagen I indicates that both expression levels are involved in the regulation of the development of pterygial tissue, thereby providing a new direction for the study of pterygial development so as to improve preventive measures. Our group is planning future experiments to further study the relationships among Sp1, collagen I, and TGF-β1.

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