·Basic Research·

Inhibitory effect of Meloxicam on the cultured fibroblasts from the excised pterygium

Li-Na Huang, Xiao-Ming Yao

Shenzhen Ophthalmic Center, Medical College of Jinan University, Shenzhen Eye Hospital, ShenZhen 518001, Guangdong Province, China

Correspondence to: Li-Na Huang. Shenzhen Ophthalmic Center, Medical College of Jinan University, Shenzhen Eye Hospital, Shenzhen 518001, Guangdong Province, China. pengyunzg@msn.com

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Abstract

• AIM: To investigate the association between cycloxygenase-2 (COX-2) expression and vascular endothelial growth factor (VEGF) intervention as well as the inhibitory effect of Meloxicam on the cultured human pterygium fibroblasts (HPF).

• METHODS: Expression of COX-2 was measured by immunohistochemistry in the cultured HPF from twenty excised pterygium cases. Expression of COX-2 in HPF was measured by Western blot following the treatment of VEGF at different concentrations. In addition, the effect of Meloxicam on proliferation of HPF was studied by adding different concentrations into the cultured HPF plates by Mono-nuclear cell direct cytotoxicity (MTT) reduction assay.

• RESULTS: COX-2 expression was present in the cultured HPF. The level of the expression increased following VEGF treatment. The proliferation of the cultured HPF decreased following the addition of different concentrations of Meloxicam (from 75μ mol/L to 300μ mol/L) and the magnitude of the inhibition was dose-time dependent.

• CONCLUSION: COX-2 levels in the cultured HPF were positively associated with VEGF stimulation and Meloxicam was inhibitory to HPF proliferation.

• KEYWORDS: pterygium; vascular endothelial growth factor; cycloxygenase-2; Meloxicam

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INTRODUCTION

Pterygium is a common ocular surface disease, apparently only observed in human. At present, the pathogenesis of this disease is still not clear. Pterygium treatment depends mainly on the surgical excision coupling with the adjunctive medicine treatment. The therapeutic efficacy of drugs available for this disease is not so encouraging. Meloxicam is one kind of COX-2 inhibitor, commonly used in treatment of acute and chronic inflammation and pain. From the literature, COX-2 has been reported to have association with the development of pterygium [1]. We hypothesize that Meloxicam could be of inhibitory effect on pterygium growth. In this research we observed the interaction of the cultured human pterygium fibroblasts (HPF) from excised pterygium and exogenous vascular endothelial growth factor (VEGF) stimulation as well as Meloxicam treatment.

MATERIALS AND METHODS

Immunohistochemical Analysis Human pterygial tissue samples were obtained from Shenzhen Ophthalmic Center. The samples used in this study were primary pterygium (n=20) and bulbar conjunctiva from strabismus surgery (n=20). The tissue specimens were obtained from 8 men and 12 women with pterygium, with the mean age of 55 (32 to 66 years old). The conjunctivas were obtained from 12 boys and 8 girls who had strabismus surgery. The tissue samples were processed and analyzed according to manufacturer's instruction for COX-2 detection (SABC method). The negative control was processed by replacing primary antibody with PBS. The positive cells were identified by examing the yellowish brown colour or the deep yellowish brown colour of the cytoplasm. The samples were considered COX-2 positive if the COX-2 positive counting≥10%.

Pterygium-derived Cells Culture Pterygial tissues were obtained from 4 different patients and cultured within 6 hours after the surgical excision. Minced pterygial tissues were attached onto the culture dish and incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 100mL/L fetal bovine serum (FBS) at 37℃ in a humidified atmosphere of 50mL/L CO_2 in air. Outgrowth

Table1 Absorbance data (n=4) and cell inhibition values of the effect of Meloxican at different concentration in HPF by MTT

Meloxican (μmol/L)	Absorbance data				Cell inhibition values (%)			
	24h	48h	72h	96h	24h	48h	72h	96h
control group	0.935±0.019	0.949±0.031	0.961±0.027	0.966±0.031				
19	0.931±0.014$^{\Delta}$	0.837±0.020$^{※}$	0.758±0.022$^{※}$	0.659±0.021$^{※}$	0.43	11.80	21.12	31.78
37.5	0.920±0.021$^{\Delta}$	0.803±0.018$^{※}$	0.721±0.012$^{※}$	0.613±0.022$^{※}$	1.60	15.38	24.97	36.54
75	0.908±0.018$^{\Delta}$	0.746±0.019$^{※}$	0.672±0.025$^{※}$	0.499±0.019$^{※}$	2.89	21.39	30.07	48.34
150	0.767±0.029$^{※}$	0.605±0.017$^{※}$	0.492±0.028$^{※}$	0.382±0.024$^{※}$	17.97	36.25	48.8	60.46
300	0.582±0.037$^{※}$	0.449±0.030$^{※}$	0.278±0.036$^{※}$	0.165±0.031$^{※}$	37.75	52.69	71.07	82.92

$^{\Delta}P$>0.05; $^{※}P$<0.001

cells were harvested by using 2.5g/L trypsin solution, and then subcultured. After we compared their growth speed, the cell line with the fastest growth rate was selected and used (passage 4-6) in the remainder of the study.

Western Blotting Confluent HPF in 6-well plates were washed with serum-free media (SFM) and then incubated in SFM for 1 hour before treatment with 1,10,100μg/L of VEGF in SFM or SFM alone for 24 hours. The cells were scraped off the plates, transferred to centrifuge tubes, and centrifuged at 1 000r/min. The medium was removed, and the cells were resuspended in protein extraction buffer (50mmol/L Tris-CL, pH 7.5, 100mL/L glycerol, 5mmol/L magnesium acetate, 0.2mmol/L EDTA, 0.5mmol/L DTT, 1mmol/L PMSF).The cells were lysed by freeze-thawing, and the membrane fraction was separated from the soluble fraction by centrifuging at 14 000r/min for 30 minutes. The soluble fraction was mixed with sample buffer and boiled for 10 minutes. The membrane fraction was resuspended in 2 volumes of protein extraction buffer, and boiled for 10 minutes. Electrophoresis was performed using 100g/L NuPAGE Bis-Tris gel (1.5 hours, 150V, 80mA, 2μg protein per lane). Separated proteins were transferred to nitrocellulose membranes (1.6 hours, 30V). Membranes were incubated in PBS containing 1g/L Tween-20,50g/L dry milk, and 10g/L cold water fish gelatin to block nonspecific IgG binding, followed by treatment with COX-2-specific antibodies for 2 hours. The membranes were then incubated with horseradish peroxides-conjugated goat anti-mouse IgG for 1 hour. The Western blot analysis was developed using Luminal as a substrate. The experiments were performed in triplicate.

Mttreduction Assay Pterygium-derived cells in DMEM containing 100mL/L FBS were seeded on a 96-well chamber at 1×10^4 cells/plate. After 1 day, the medium was aspirated and replaced with fresh medium containing various concentrations of Meloxicam at final concentrations of 19, 37.5, 75, 150 and 300μmol/L. After 24, 48, 72 and 96 hours of incubation at 37℃, the cells reacted with MTT for 4 hours at 37℃, and solved with 0.05g/L DMSO. Each well was measured at 490nm. Results were expressed as the percentage of total cells assuming that the adhesion of cells in control was 100%. The percentage of adhesion was determined using the formula: (A490 nm after being rinsed with PBS/A490 nm no rinse) 100%. The experiments were performed in triplicate.

Statistical Analysis Factor analysis of variance, including analysis of variance (ANOVA) and student-newman-keuls test (SNK-q test) was conducted on each absorbance data by SAS version 8.0 for Windows. P<0.05 was considered statistically significant. Bliss method was conducted on inhibitory concentration 50% (IC50).

RESULTS

Expression of COX-2 in normal conjunctiva and pterygia immunohistochemical analysis was performed to determine the expression and status of COX-2 in normal conjunctiva and pterygia. The normal conjunctiva showed negative immunostaining for COX-2 (Figure 1) .The positive immunostaining for COX-2 in HPF were 14 samples (Figure 2). The positive ratio was 70%. The difference between them was statistically significant (P<0.05).

Western blotting experiments were conducted to determine whether the treatment of HPF with VEGF altered COX-2 protein expression. COX-2 protein expression in HPF was augmented by VEGF (Figure 3).

Mttreduction Assay The effect of Meloxicam on the proliferation of HPF was investigated by MTT reduction assay. Our experiment showed that Meloxicam dramatically decreased the proliferation of fibroblasts. As seen in table 1, the absorbance data of 19, 37.5, 75μmol/L Meloxicam for 24 hours was similar to those in the control group(P>0.05). HPF preincubated with Meloxicam for 48 hours at concentrations ranging from 19.5μmol/L to 300μmol/L significantly reduced the proliferation of HPF compared with the control

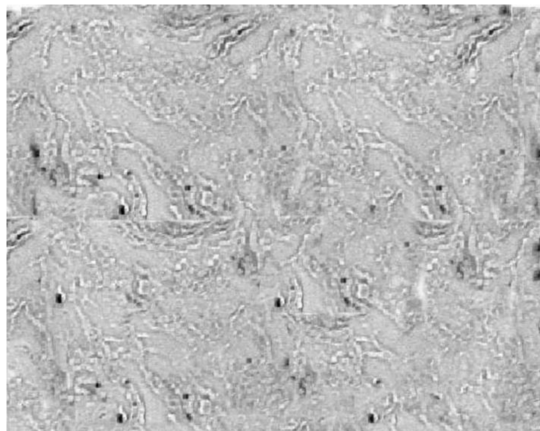

Figure 1 The negative staining in HPE SABC×400

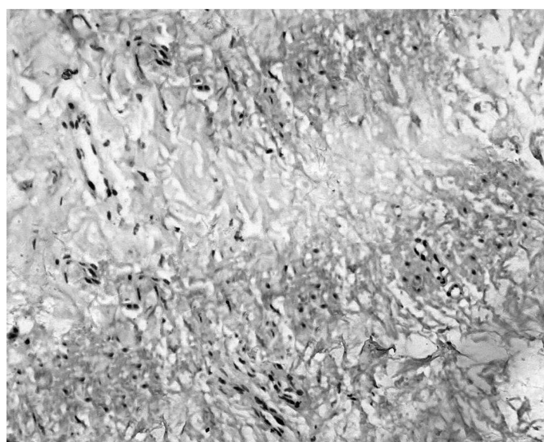

Figure 2 The positive staining in HPE SABC×400

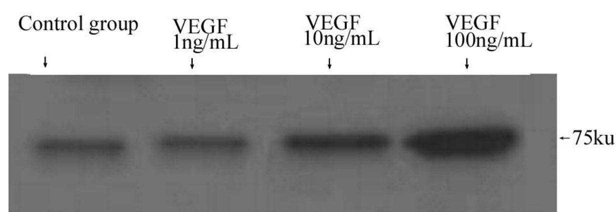

Figure 3 The difference from COX-2 protein expression induced by VEGF at a concentration of 1μg/L to the control group was considered not statistically significant. COX-2 protein was 1.5 times more abundant in the treatment of HPF with VEGF at a concentration of 10μg/L. Similarly, COX-2 was increased by approximately 3-fold at a concentration of 100μg/L. COX-2 protein was showed specifically at the 75ku on the Western blot

group ($P<0.01$) .The IC50 value of Meloxicam for the inhibition of HPF proliferation was 65.8μmol/L, with a confidence interval of 39.56μmol/L to 109.46μmol/L.

DISCUSSION

The pathogenesis of pterygium is characterized by proliferation with neovascularization, an increase in mast cells, and infiltration by small lymphocytes and plasma cells. Many angiogenic and fibrogenic growth factors are over-expressed in pterygium [2]. Apart from the proliferative fibroblast, angiogenesis is also essential for progressive pterygium growth. The new vessels in pterygia not only give the necessary nutritional ingredient to the pterygium growth, but also drive the processes of the fibroblast proliferation by producing growth factors.

The VEGF has well-known roles in angiogenesis. Additionally, several immunohistochemical studies has shown increased VEGF in pterygium epithelium and vascular endothelium where the epithelial staining could be basal or superficial depending on the antibody used [3-6]. The up-regulation of VEGF observed in pterygium could be trigged by the effects of the environmental factor which are those and some inflammation stimulates references here, which lead to the development of pterygium and it's recurrence after surgical treatment.

COX recently has been shown to exist in two forms: a constitutive form (COX-1) and an inducible form (COX-2). COX-2 is induced by proinflammatory stimuli such ascytokines and mitogens [7]. To the best of our knowledge, this is the first report to show COX-2 in pterygium. The high level expression of COX-2 occurs in a wide range of several physiological events and various pathophysiological processes, such as inflammation, damage, reparation, tumor growth and metastasis [8]. Moreover, high levels of COX-2 are detected in activated and proliferating vascular tissues. We have previously shown that secretion of angiogenic factors, such as VEGF and fibroblast growth factor (b-FGF), are modulated by COX-2 overexpression [9]. The mechanistic details of how COX-2 regulates angiogenic growth factor expression are not well understood. *In vitro* studies with cultures of human umbilical vein endothelial cells (HUVEC) treated with VEGF showed that COX-2 protein and mRNA expression were augmented by VEGF [7], on the other hand, Liu *et al*[10] showed that COX-2 expression produced VEGF in HUVEC. Therefore, there is a positive feedback network between COX-2 and VEGF in HUVEC, both of which promote angiogenesis. The present study showed that VEGF enhanced the expression of COX-2 in HPF. Although research on COX-2 inhibitors has focused mainly on inflammation and pain, experimental and epidemiological data suggest that COX-2 inhibitors could be used in the treatment or prevention of a broader range of diseases [11]. Meloxicam inhibits cell proliferation by inhibiting the expression of COX-2 in cells. In the current study, Meloxicam from 75μmol/L to 300μmol/L, demonstrated a

dose and time-dependant inhibitory effect on the proliferation of fibroblasts of pterygium. Meloxicam could be a useful drug to control pterygium growth.

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