Increased Expression of Gelatinase (MMP-2 and MMP-9) in Pterygia and Pterygium Fibroblasts with Disease Progression and Activation of Protein Kinase C

Shun-Fa Yang,[1,2] *Ching-Yang Lin,*[3] *Pei-Yu Yang,*[4] *Shih-Chun Chao,*[3] *Yi-Zhen Ye,*[4] *and Dan-Ning Hu*[4,5]

Purpose. To study the expression of matrix metalloprotease (MMP)-2 and MMP-9 mRNA and activities in various stages of surgically excised pterygium specimens and cultured pterygium fibroblasts and to study the effects of activation of protein kinase C (PKC) on the expression of these MMPs in pterygium fibroblasts.

Methods. MMP-2 and MMP-9 mRNA expression and activities in 15 pterygium tissues and cultured pterygium fibroblasts were measured by RT-PCR and zymography. Five normal conjunctiva specimens and fibroblasts were tested as the controls. Changes of expression of MMP-2 and MMP-9 of fibroblasts after the simulation of a standard PKC activator, 2-*O*-tetradecanoylphorbol-13-acetate (TPA), were studied.

Results. MMP-2 and MMP-9 expression in pterygium tissues and fibroblasts was greater than those of normal tissues and fibroblasts and was closely relevant to the progression of pterygium. In early-stage pterygium tissues and cultured fibroblasts, MMP-9 was not expressed, activated MMP-2 could not be detected, and only a small amount of latent MMP-2 was present. In advanced-stage pterygium (pterygium head passed the papillary region), MMP-9 was expressed; activated MMP-2 and a large amount of latent MMP-2 could be detected in pterygium tissues and fibroblasts. TPA stimulated the expression of MMP-2 and MMP-9 by pterygium fibroblasts isolated from early-stage specimens in a dose-dependent manner.

Conclusions. MMP-2 and MMP-9 expression by pterygium fibroblasts is significantly increased after the progression of pterygium. Activation of the PKC signaling pathway, aside from other previously reported signaling pathways, may play a role in the development and progression of pterygium. (*Invest Ophthalmol Vis Sci.* 2009;50:4588–4596) DOI:10.1167/iovs.08-3147

From the [1]Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan; [2]Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan; [3]Department of Ophthalmology and [4]Cell Culture Laboratory, Department of Medical Research, Show Chwan Memorial Hospital, Changhua, Taiwan; and [5]Tissue Culture Center, New York Eye and Ear Infirmary, New York, New York.

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Corresponding author: Dan-Ning Hu, Tissue Culture Center, New York Eye and Ear Infirmary, 310 East 14th Street, New York, NY 10003; hu2095@yahoo.com.

Pterygium is a common ocular surface disease that can cause vision loss. Surgical excision is the standard treatment of pterygium, but the recurrence rate is high. Epidemiologic studies indicate that the occurrence of pterygium is related to ultraviolet (UV) radiation.[1–6]

Pterygium is a fibrovascular tissue extending from the bulbar conjunctiva onto the cornea. Recent studies indicate that pterygium originates from transformed limbal epithelial cells and fibroblasts.[7–11] Compared with normal fibroblasts, pterygium fibroblasts grow more quickly in a medium containing a low concentration of serum and can grow in a semisolid agar, indicating that these cells represent tumorlike transformed cells.[10]

Matrix metalloproteinases (MMPs) are a family of enzymes with more than 20 members, most of which degrade extracellular matrix (ECM). They play an important role in various physiological and pathologic processes, such as tissue remolding, healing, angiogenesis, and tumor invasion.[12–16] It has been reported that the expression of several types of MMPs is increased in pterygium.[7–9,17–23] Increased MMPs dissolve the Bowman's layer and cause angiogenesis and the invasion of pterygium onto the cornea. Increased expression of various MMPs is present in epithelial cells and fibroblasts in the pterygium.[7–9,17–23] UV radiation and proinflammatory cytokines increase the expression of MMPs in epithelial cells and fibroblasts.[7,9,17,21–23]

MMPs can be divided into five subgroups based on substrate preference: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10), membrane-associated MMPs (MT-1-MMP, MT2-MMP), and others (e.g., MMP-12, MMP-19, MMP-20).[12] Gelatinases can be divided into gelatinase-A (MMP-2) and gelatinase-B (MMP-9). These MMPs are secreted as latent precursors and can be activated by limited proteolysis. Substrates of gelatinases include gelatin, elastin, laminin, and various collagens.[13,24–27]

It has been documented that the expression of MMP-1 and MMP-3 is increased in pterygium tissues and that the secretion of these MMPs is increased in pterygium epithelial cells and fibroblasts.[7–9,17,19,21,23] Results of studies regarding the expression of MMP-2 and MMP-9 in pterygium and their fibroblasts are conflicting.[7–9,18,19,21,23] Some authors found an increase of these MMPs in pterygium and its fibroblasts, but others did not.[7–9,18,19,21,23] The present study investigates the MMP-2 and MMP-9 mRNA and activity levels in pterygium tissues collected from 15 patients undergoing pterygium removal and receiving cultured fibroblasts isolated from 15 pterygium specimens. Five normal conjunctiva specimens and fibroblasts were tested as controls.

Most previous studies on the changes of MMPs in pterygia compared MMP expression in surgically excised pterygia with that in normal controls. To our best knowledge, no reports have been published of the relationship between MMP level and the extent or stage of pterygium. Therefore, we studied the relationship between MMP-2 and MMP-9 expression in

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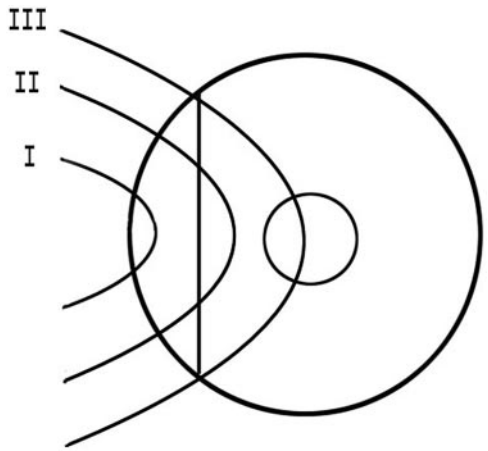

FIGURE 1. Schematic picture shows the different stages of pterygium. Stage I: head of the pterygium did not reach the midline between the limbus and pupillary margin. Stage II: head of the pterygium passed the midline but did not reach the pupil. Stage III: head of the pterygium passed the pupillary margin.

pterygium tissue and stage of pterygium. The production of MMP-2 and MMP-9 in cultured fibroblasts isolated from pterygia at different stages was also studied.

Among the factors modulating the production of MMP by ocular surface cells, the effects of UV radiation and cytokines have been studied.[1,8,9,17,22,23] Di Girolamo et al.[17] reported that UV radiation induced the expression of MMP in pterygium epithelial cells. They found that UV radiation induced increased production of MMPs in pterygium epithelium cells and that this effect was mediated through the MAPK (ERK1/2) signal pathway.[17] The activation of MAPK caused by UV radiation is PKC dependent.[28–30] Therefore, we tested the effect of 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, a standard PKC stimulator) on the production of MMPs by fibroblasts to explore the involvement of the PKC pathway in the modulation of the expression of MMPs in pterygium.

MATERIALS AND METHODS

Pterygium specimens were obtained from patients undergoing pterygium removal after they signed informed consent. The present study was reviewed and approved by the Institutional Review Board of Show Chwan Memorial Hospital and was conducted in accordance with the tenets of the Declaration of Helsinki.

The external eye of each patient was photographed before surgery. Pterygia were classified into three stages by the surgeon based on the extent of the pterygium (Fig. 1), as follows: stage I, the head of pterygium did not reach the midline between the limbus and the pupillary margin; stage II, the head of pterygium passed the midline but did not reach the pupil; stage II, the head of the pterygium passed the pupillary margin. Stages I and II represent pterygium in the relatively early stage, and stage III represents pterygium in the advanced stage.

Pterygium specimens were collected from 15 pterygium patients (seven men, eight women; age, 62.20 ± 11.18 years [mean ± SD]). All these pterygia were progressive in nature. The ages of patients with stages I, II, and III pterygium were 63.60 ± 11.08, 60.4 ± 13.59, and 62.50 ± 11.08 years, respectively. The gender distribution (male/female) was 2:3, 3:2, and 2:3 in stages I, II, and III patients, respectively. Normal conjunctival tissues were obtained from five patients (three women, two men) aged 63.67 ± 7.09 years.

Excised tissues were divided laterally into two symmetrical pieces of identical size. One piece (for measurement of tissue MMP mRNA and activity) was frozen and stored at −70°C until processing. Another piece was used for the isolation and cultivation of pterygium fibroblasts.

Isolation and Cultivation of Pterygium Fibroblasts

Fibroblasts were obtained from 15 pterygium specimens and five normal conjunctiva specimens (one cell line from each specimen) with the use of explant methods reported by Li et al.[8] Briefly, the head of a fresh pterygium specimen or a normal conjunctiva was cut into small pieces (1–2 mm in diameter) under a stereomicroscope, washed in Hanks solution, and placed in a culture dish. Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (FBS) and gentamicin (50 μg/mL) was added to cover the explants (all from Gibco, Carlsbad, CA). The culture dish was put in a CO_2-regulated incubator in humidified 95% air/5% CO_2 atmosphere overnight. Culture medium (identical to the first medium, but the concentration of FBS was reduced to 10%) was added after the explants had adhered. The culture medium was replaced three times a week after the appearance of an outgrowth of cells from the explants. After primary cultures became confluent, cultured fibroblasts were detached from the dish with 0.05% trypsin/0.01% EDTA solution (Gibco) and were passaged for subcultures with a 1:3 split. In some cultures, epithelial cells also migrated from the explants in the early stage. However, epithelial cells rapidly became terminally differentiated and lost their viability in medium with high levels of serum and Ca^{2+}. Furthermore, during subculture, the fibroblasts detached from the well more easily than did the epithelial cells. After detachment of the fibroblasts was nearly complete, serum was added to stop the effect of the enzyme so that the epithelial cells remained in the well and could not be passed to the subculture. Therefore, only fibroblasts were present in the subcultures.

Immunocytochemical study was performed to identify cell types, if necessary. Epithelial cells contained cytokeratin antigens, but fibroblasts did not.[31]

For the MMP experiment, fibroblasts in the second or third subculture were seeded into a 35-mm dish and were cultured with DMEM with 10% FBS (DMEM-FBS). The culture was washed with PBS after near confluence and was cultured with DMEM without FBS for 48 hours. Conditioned medium was collected and centrifuged. The supernatant was stored at −70°C until zymography analysis of MMP activity. Fibroblasts were trypsinized, counted, and stored at −70°C until measurement of mRNA of MMPs. Fifteen fibroblast cell lines from 15 pterygium specimens and five fibroblast cell lines from five normal conjunctiva tissues were tested.

Effect of TPA on MMP Expression by Pterygium Fibroblasts

Cultured fibroblasts isolated from early-stage pterygium specimens were seeded into 24-well plates at a density of 2×10^4cells/well. Cells were cultured with DMEM-FBS for 48 hours and were washed with PBS and then cultured with DMEM without FBS. TPA (Sigma-Aldrich, St. Louis, MO) at different concentrations (0, 1, 10, and 100 ng/mL) was added and cultured. Two cell lines were tested. After culturing for 24 hours, the conditioned culture medium was collected, and cells were trypsinized and stored at −70°C for the zymography and RT-PCR studies, respectively.

Tissue Lysis of Pterygium Tissue

For lysate preparation, pterygium or normal conjunctiva tissue was washed with PBS twice and treated with 0.3 mL cold cell lysis buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO). The protein concentration of lysates was determined by Bradford assay (Bio-Rad, Hercules, CA).[32]

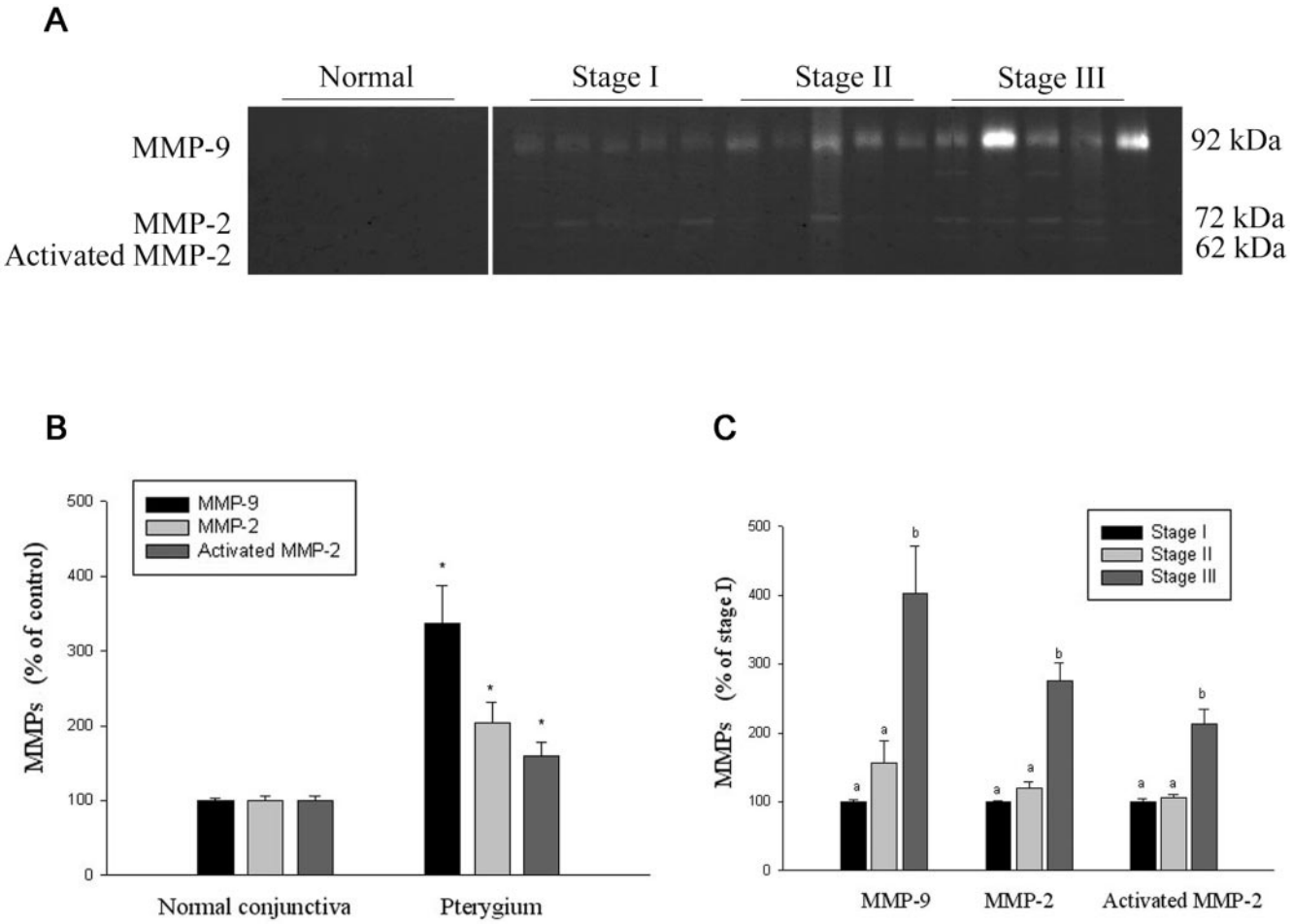

FIGURE 2. MMP-2 and MMP-9 activities in five normal conjunctiva and 15 pterygium tissues at various stages measured by zymography. (**A**) Zymography (MMP-2 activity: 72 kDa; activated MMP-2 activity: 62 kDa; MMP-9 activity: 92 kDa). (**B**) Comparison of MMP-2 activity level between normal conjunctiva and pterygium tissues. *Significant difference ($P < 0.05$) between normal conjunctiva and pterygium. (**C**) Levels of MMP-2 activity (latent and activated) and MMP-9 in pterygium tissues at various stages. Data are given as mean ± SD ($n = 5$). Different letters on MMP levels indicate significant difference ($P < 0.05$).

Measurement of MMP-2 and MMP-9 Activity Levels by Zymography

The activity of MMP-2 and MMP-9 in the conditioned medium and tissue lysates was measured by gelatin zymography protease assays, as previously described by us.[33] Briefly, cultured media or lysates of tissues were prepared with SDS sample buffer without boiling or reduction and then were subjected to 0.1% gelatin/8% SDS-PAGE electrophoresis. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in a reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01% NaN_3) at 37°C for 12 hours. Then the gel was stained with Coomassie brilliant blue (R-250; Sigma). Relative photographic densities of MMP-2 and MMP-9 were measured by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000; Alpha Innotech Corporation, San Leandro, CA).[33] Optical density readings obtained were expressed as percentages of the controls. Levels from normal specimens and stage I specimens were used as the control in the comparison between the normal and the pterygium specimens and in the comparison between the different stages of pterygium, respectively.

RNA Preparation and RT-PCR

Total RNA was isolated from tissues and cultured fibroblasts with reagent (Trizol; Life Technologies, Grand Island, NY) according to the manufacturer's instructions. For reverse transcription, first-strand cDNA synthesis was performed with random primers (hexamers; Promega, Madison, WI) and 100 U Moloney murine leukemia virus reverse transcriptase (Promega), carried out at 42°C for 60 minutes, and terminated at 90°C for 10 minutes. To detect MMP-2 and MMP-9 mRNA, the cDNA (5 μL) was amplified by PCR with the following primers: MMP-9 (400 bp), 5-CAACATCACCTATTGGATCC-3 (sense) and 5-CTGTAGAGTCTCTCGCT-3 (antisense); MMP-2 (474 bp), 5-GGCCCTGTCACTCCTGAGAT-3 (sense) and 5-GGCATCCAGGTTATCGGGGAT-3 (antisense); glyceraldehyde-3-phosphate dehydrogenase (304 bp), 5-CGGAGTCAACGGATISSUESG-GTCGTAT-3 (sense) and 5-AGCCTTCTCCATGGTTGGTGAAGAC-3 (antisense).[34,35] Samples were subjected to 25 cycles, each involving denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 2 minutes, with a final extension phase of 10 minutes performed on a programmable thermal controller. PCR products were subjected to agarose gel electrophoresis, stained by ethidium bromide, and read by a densitometer (AlphaImager 2000; AlphaInnotech Corporation), and the data obtained were analyzed with the method described previously in the study of zymography.

Statistical Analysis

Statistical significance of differences throughout this study was calculated by ANOVA one-way test in comparing data from more than two groups and Student's *t*-test in comparing data between two groups.

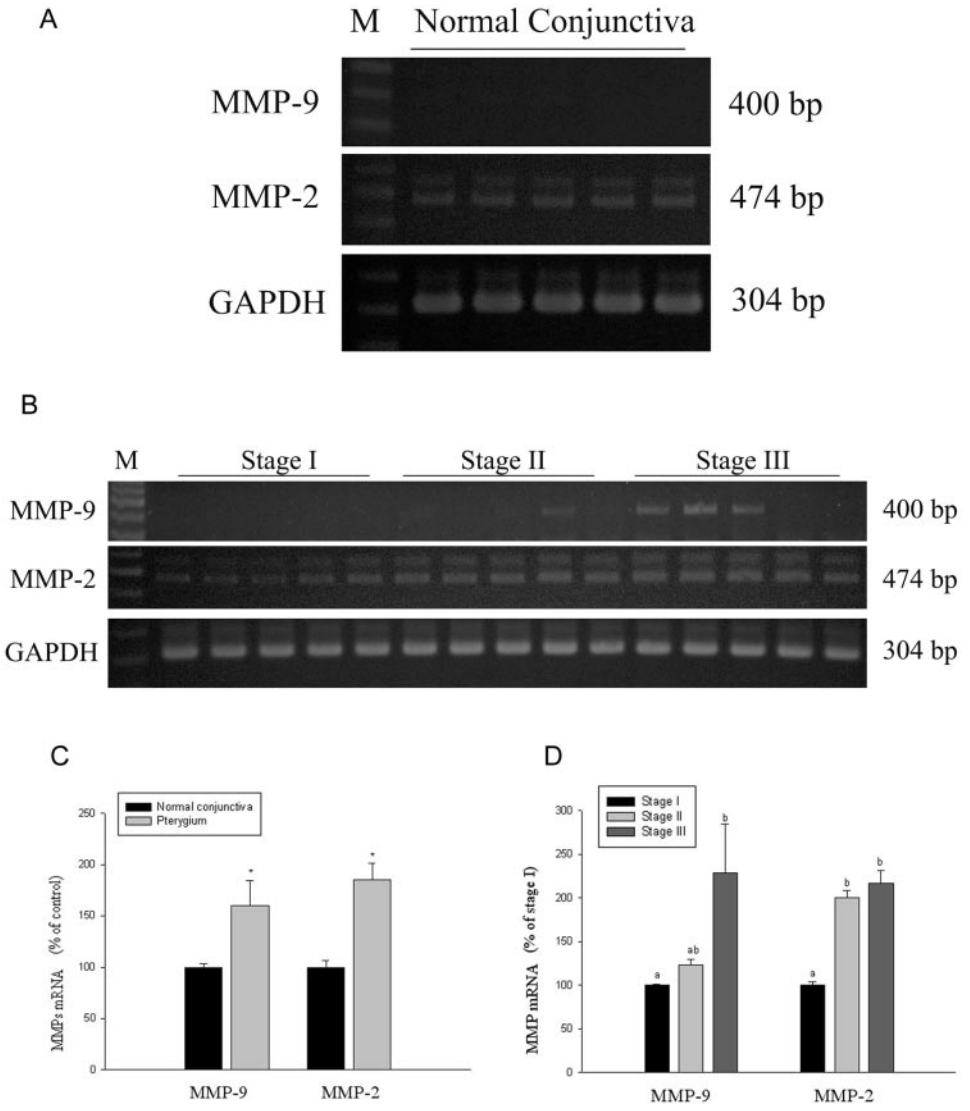

FIGURE 3. MMP-2 and MMP-9 mRNA in normal conjunctiva and pterygium tissue at various stages measured by RT-PCR. (**A**) RT-PCR of normal conjunctiva tissues (MMP-2 mRNA at 474 bp and MMP-9 mRNA at 400 bp). (**B**) RT-PCR of pterygium tissue. (**C**) Comparison of MMP-2 and MMP-9 mRNA levels between normal conjunctiva and pterygium tissues. *Significant difference ($P < 0.05$) between normal conjunctiva and pterygium. (**D**) MMP-2 and MMP-9 mRNA levels in pterygium tissues at various stages. Data are given as mean ± SD ($n = 5$). Different letters on MMP levels indicate significant difference ($P < 0.05$).

Data were analyzed using statistical software (SPSS Inc., Chicago, IL). A difference at $P < 0.05$ was considered to be statistically significant.

RESULTS

MMP-2 and MMP-9 Activities in Tissues

MMP-2 and MMP-9 activities in five normal conjunctiva and 15 pterygium tissues (five tissues of each stage) were measured by gelatin zymography. MMP-2 (latent and activated) and MMP-9 levels in pterygium were significantly greater than those in normal conjunctiva tissues (Figs. 2A, 2B). Latent MMP-2 was present in pterygium tissues of all stages (Fig. 2A). Pterygium tissues at stage III had significantly higher latent MMP-2 levels than those at stages I and II (Figs. 2A, 2C; $P < 0.05$), whereas the difference between stage I and stage II was not statistically significant (Figs. 2A, 2C, $P > 0.05$). Activated MMP-2 was only present in stage III tissues. The difference in activated MMP-2 level between stage III tissues and stage I or II tissues was statistically significant ($P < 0.05$; Figs. 2A, 2C). MMP-9 activity was present in stage III tissues but was either negligibly present or completely absent in stages II and I (Figs 2A, 2C). The difference in MMP-9 level between stage III tissues and stage II tissues was statistically significant ($P < 0.05$; Fig. 2C).

MMP-2 and MMP-9 mRNA Levels in Tissues

MMP-2 and MMP-9 mRNA levels in pterygium were significantly greater than those of normal conjunctiva tissues (Figs. 3A–C). In pterygium specimens, MMP-2 was expressed at various stages (Fig. 3B). Expression of MMP-2 in stages III and II specimens was significantly greater than that of stage I specimens (Figs. 3B, 3D; $P < 0.05$), whereas the difference between stage III and stage II specimens was not statistically significant (Fig. 3D; $P > 0.05$). MMP-9 was expressed in stage III tissues and not expressed or only negligibly expressed in stages I and II tissues (Figs. 3B, 3D). The differences in MMP-9 mRNA level between stage III and II tissues and between stage I and II tissues were statistically significant ($P < 0.05$; Fig. 3D).

MMP-2 and MMP-9 Activities in Conditioned Media from Cultured Fibroblasts

MMP-2 (latent and activated) and MMP-9 activity levels in conditioned media from pterygium fibroblasts were significantly greater than those from normal conjunctival fibroblasts (Figs. 4A–C). In pterygium specimens, latent MMP-2 was present in the conditioned media from fibroblasts of specimens at all stages (Fig. 4B). Latent MMP-2 activity in conditioned

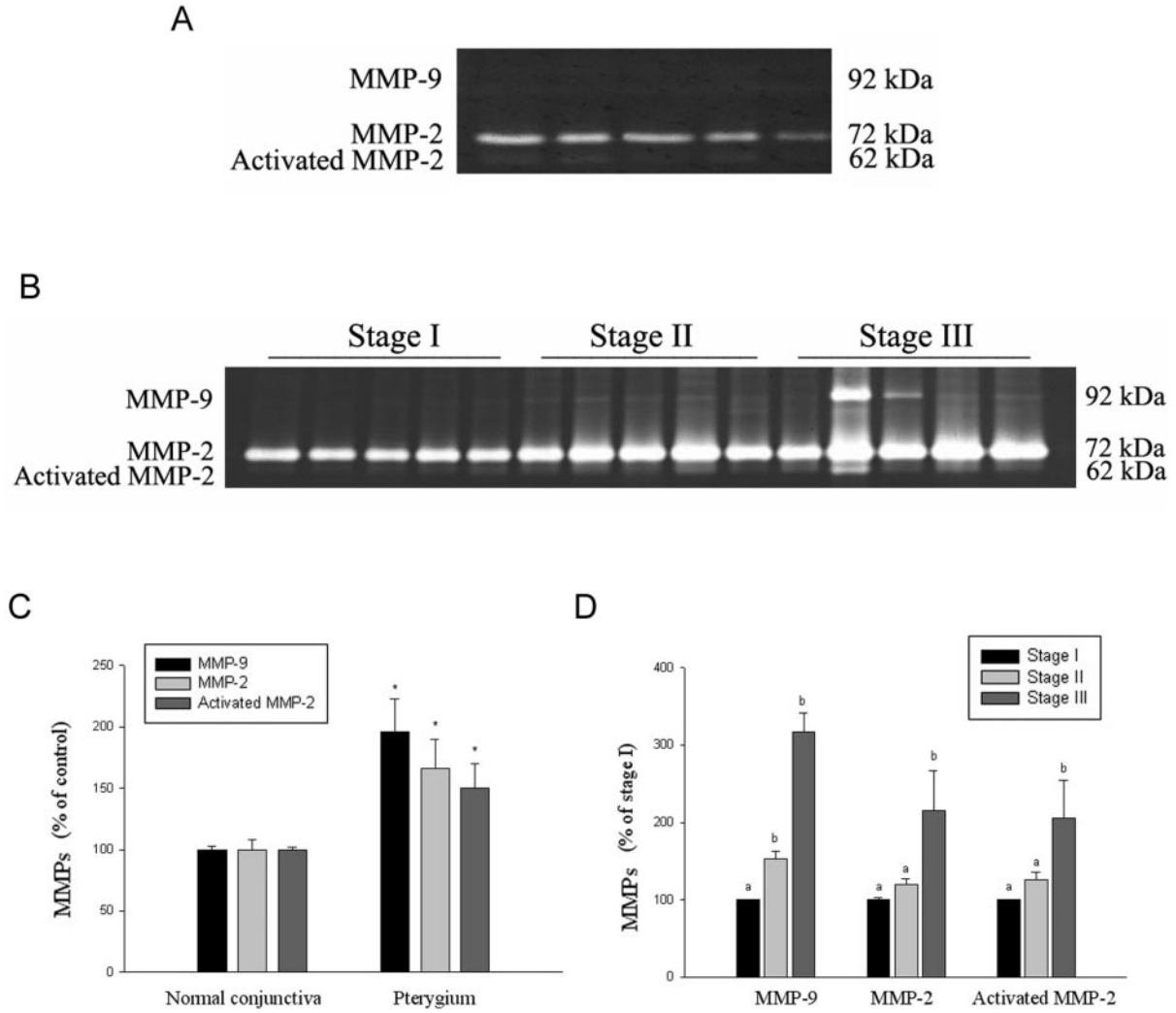

FIGURE 4. MMP-2 and MMP-9 activities in the conditioned medium of normal conjunctival and pterygium fibroblasts of various stages measured by zymography. Fifteen fibroblast cell lines were isolated from 15 pterygia at various stages (five pterygia in each stage of three stages). Five normal fibroblast cell lines were isolated from five normal conjunctival specimens. Cells were cultured in serum-free culture medium for 48 hours. The conditioned medium was collected, and the activity of MMPs was measured by zymography. (**A**) Zymography of medium from normal fibroblasts (MMP-2 activity, 72 kDa; activated MMP-2, 62 kDa; MMP-9, 92 kDa). (**B**) Zymography of medium from pterygium fibroblasts at various stages. (**C**) Comparison of levels of MMP-2 (latent and activated) and MMP-9 activity in conditioned media from normal and pterygium fibroblasts. *Significant difference ($P < 0.05$) between the normal and pterygium fibroblasts. (**D**) Levels of MMP-2 (latent and activated) and MMP-9 activities in conditioned media of fibroblasts from various stages of pterygium. Data are given as mean ± SD ($n = 5$). Different letters on MMP levels indicate significant difference ($P < 0.05$).

medium from fibroblasts of stage III specimens was significantly greater ($P < 0.05$) than that in the medium from fibroblasts of stages I and II specimens (Fig. 4D). Latent MMP-2 level in conditioned medium from fibroblasts of stage II specimens was also significantly greater than that from stage I ($P < 0.05$; Fig. 4D). Activated MMP-2 was present in the conditioned culture medium from fibroblasts of stage III specimens but could not be detected or was only present in a negligible amount in medium from fibroblasts of stages I and II specimens (Figs. 4A, 4D). MMP-9 presented in conditioned medium only from fibroblasts of stage III specimens. The difference in MMP-9 mRNA level between stage III tissues and stage II tissues was statistically significant ($P < 0.05$; Fig. 4D).

MMP-2 and MMP-9 mRNA Levels in Cultured Pterygium Fibroblasts

MMP-2 and MMP-9 mRNA levels in conditioned media from pterygium fibroblasts were significantly greater than those from normal conjunctival fibroblasts (Figs. 5A–C; $P < 0.05$). In pterygium specimens, the difference in mRNA levels of MMP-2 was significant in fibroblasts between stage I and stage II and between stage II and stage III (Figs. 5B, 5D). MMP-9 was expressed in stage III fibroblasts, and not expressed and negligibly expressed in fibroblasts of stages I and II specimens, respectively. The difference in MMP-9 mRNA level between stage III tissues and stage II tissues was statistically significant ($P < 0.05$; Figs. 5B, 5D).

Effects of TPA on MMP-2 and MMP-9 Activities of Pterygium Fibroblasts

TPA showed significantly stimulating effects on the production of MMP-2 (both activated and latent) and MMP-9 by cultured fibroblasts, as shown by gelatin zymography (Fig. 6A). MMP-9 and activated MMP-2 levels in the cultured media from fibroblasts cultured with TPA at 10 to 100 ng/mL were significantly

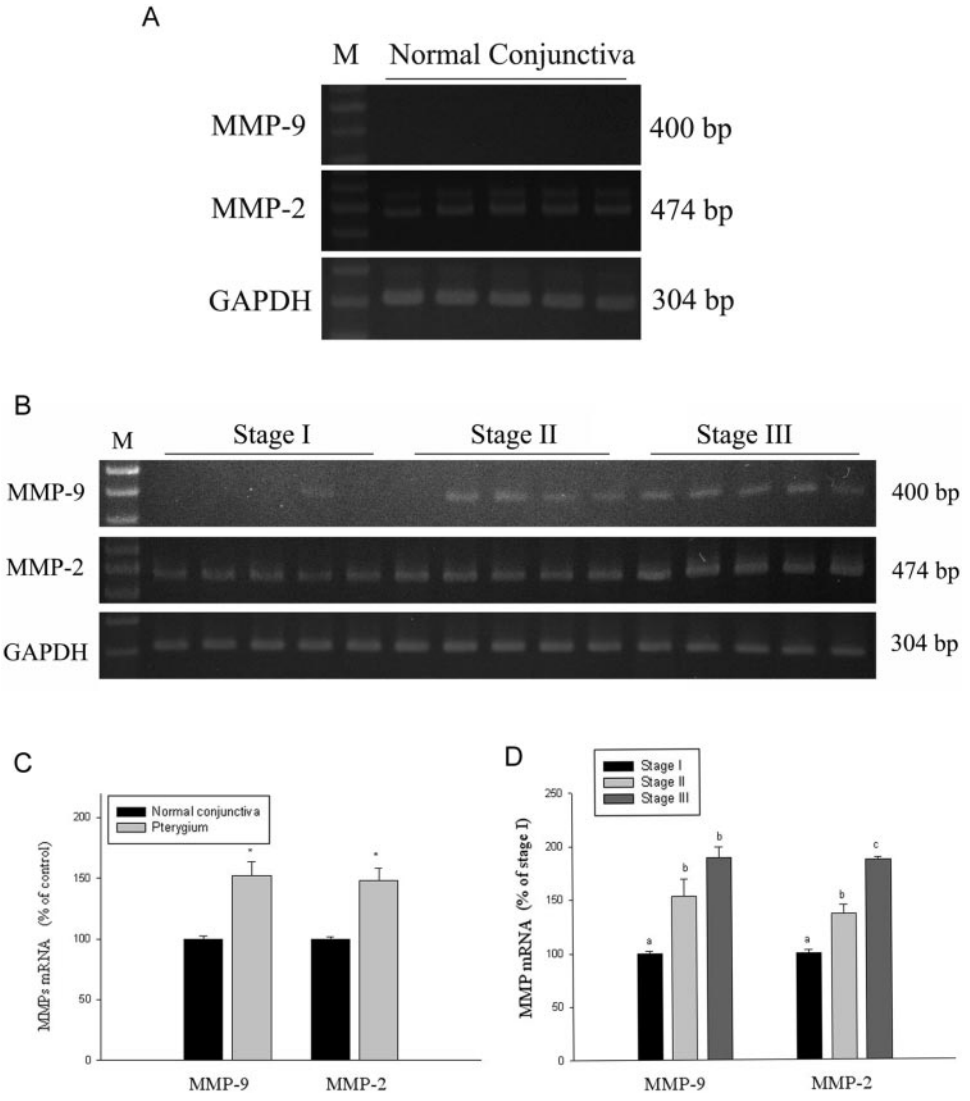

FIGURE 5. MMP-2 and MMP-9 mRNA in five normal conjunctival fibroblast cell lines and 15 pterygium fibroblast cell lines from various stages of pterygium measured by RT-PCR. (**A**) RT-PCR of normal fibroblasts (MMP-2 mRNA at 474 bp; MMP-9 mRNA at 400 bp). (**B**) RT-PCR of pterygium fibroblasts from various stages of pterygium. (**C**) Comparison of MMP-2 and MMP-9 mRNA levels between normal conjunctiva and pterygium fibroblasts. *Significant difference ($P < 0.05$) between the normal conjunctiva and pterygium. (**D**) MMP-2 and MMP-9 mRNA levels in fibroblast from various stages of pterygium. Data are given as mean ± SD ($n = 5$). Different letters on MMP levels indicate significant difference ($P < 0.05$).

greater than those from cells cultured with 1 ng/mL TPA or without TPA (Fig. 6B; $P < 0.05$). MMP-2 levels in the conditioned media from fibroblasts cultured with TPA at 1 to 100 ng/mL was significantly greater than those from cells cultured without TPA ($P < 0.05$). Furthermore, TPA significantly increased the expression of MMP-2 and MMP-9 mRNA of cultured fibroblasts in a dose-dependent manner (Figs. 7A, 7B; $P < 0.05$).

DISCUSSION

MMP-2 (gelatinase A, 72-kDa type IV collagenase) and MMP-9 (gelatinase B, 92-kDa type IV collagenase) are two members of the MMP family that belong to the subgroup of gelatinase. Gelatinases play an important role in the final degradation of fibrillar collagens after they have first been cleaved by collagenases (e.g., MMP-1).[13] In addition, MMP-2 and MMP-9 can degrade fibronectin, laminin, elastin, and various collagens (types I, II, and V).[13,24,25] Furthermore, MMP-2 has been reported to proteolytically activate MMP-9 and MMP-3, which has been demonstrated to play an important role in the occurrence of pterygium.[14,26,27]

MMP-2 and MMP-9 are secreted in a latent precursor and can be activated in vivo by proteinases such as plasmin, trypsin, and stromelysin.[14–16] MMP-2 and MMP-9 also can be activated during zymography by SDS. Therefore, MMP-2 and MMP-9, as well as their latent form and activated form, can be distinguished by their molecular size.

In previous reports, results on the expression of MMP-2 and MMP-9 in pterygium and their fibroblasts have been conflicting.[7–9,18,19,21,23] MMP-2 is constitutively expressed by normal fibroblasts.[36] No difference of MMP-2 mRNA and protein could be detected between pterygium and normal fibroblasts in two reports.[8,9] However, in immunocytochemical studies, MMP-2 was detected in pterygium fibroblasts but not in normal fibroblasts.[7,19] In three reports using zymography to detect the activity of MMP-2, conflicting results were obtained.[8,9,18]

MMPs in pterygium tissues are released by various cell components of the pterygium, such as epithelial cells, fibroblasts, and inflammatory cells.[7,18,19] In the present study, the expression of MMP-2 and MMP-9 was first tested in pterygium tissues. Then fibroblasts were isolated from pterygium, and the expression of these MMPs was measured in fibroblasts to study the role of fibroblasts in the release of MMPs to the pterygium and adjacent tissues.

In the present study, activated MMP-2 could not be detected in normal conjunctival tissues/fibroblasts and stage I pterygium tissue/fibroblasts. Activity and mRNA levels of MMP-2 in ptery-

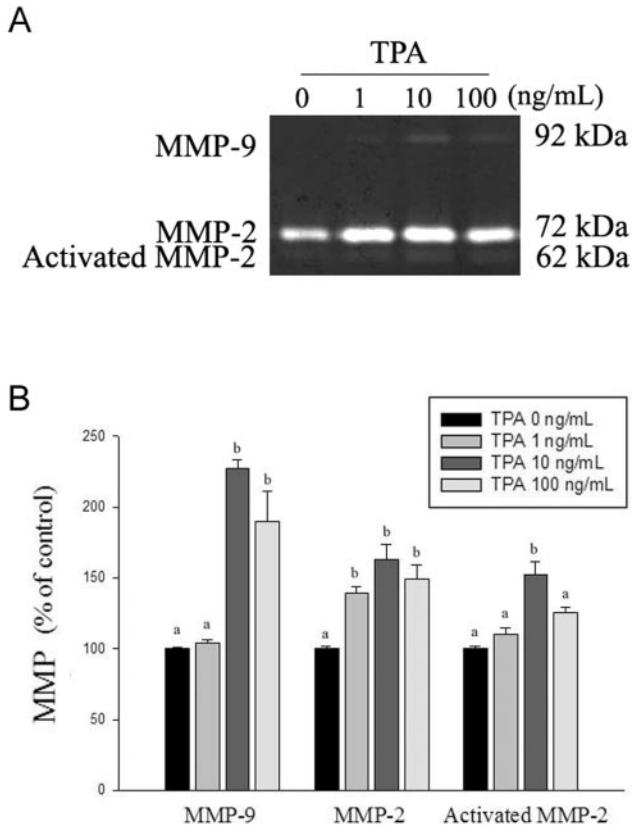

FIGURE 6. Effect of TPA on the activities of MMP-2 and MMP-9 by pterygium fibroblasts from early stage pterygium. TPA at various concentrations (0 to 100 ng/mL) was added into the culture medium of cultured pterygium fibroblasts. After culturing for 24 hours, the conditioned culture medium was collected and studied by zymography. (**A**) Zymography (MMP-2 activity: 72 kDa, activated MMP-2: 62 kDa and MMP-9: 92 kDa). (**B**) Levels of MMP-2 (latent and activated) and MMP-9 activities in conditioned media of pterygium fibroblasts cultured with various concentrations of TPA. Different letters on TPA levels indicate significant difference ($P < 0.05$).

gium tissue/fibroblasts increase with the progression of pterygium. It is possible that the conflicting results in previous reports could be explained at least in part by the different stages of specimens tested. It could be expected that significant differences of expression of MMP-2 exist between normal tissues and advanced pterygium specimens and that no or nonsignificant differences can be detected between normal tissues and early-stage pterygium.

MMP-9 is not constitutively expressed in ocular surface cells. MMP-9 mRNA and protein could not be detected in normal conjunctival or corneal fibroblasts.[7–9,18,19] However, MMP-9 mRNA expression and activity in scleral and conjunctival fibroblasts could be stimulated by inflammatory cytokines (e.g., IL-1).[37] MMP-9 protein and activity were absent or present in pterygium tissues in different reports.[7,8,9,18,19] In immunocytochemical studies, three sets of investigators reported that MMP-9 was detected in pterygium fibroblasts but not in normal fibroblasts.[7,18,19] In the present study, MMP-9 mRNA and protein could not be detected in normal tissue/fibroblasts and early-stage pterygium/fibroblasts and were present only in advanced stages of pterygium/fibroblasts. These findings may also provide an explanation for the conflicting results in previous studies that MMP-9 may be expressed only in tissues and fibroblasts of advanced-stage pterygium but not from early-stage pterygium.

The occurrence of pterygium shows similarity with tumorigenesis. The concept that tumorigenesis is a multistep process has been well documented and is widely accepted.[38] During tumor development, MMPs are among the important factors involved in the growth, invasion, and metastasis of tumors. The expression of MMPs in tumor cells and stromal cells begins at the earlier stage and progressively increases during the development of tumors.[38] It has been hypothesized that pterygium cells are tumorlike altered limbal epithelial cells and fibroblasts. Pterygium fibroblasts grow well in culture medium with only a low concentration of serum and can grow in semisolid sugar, indicative of anchorage-independent growth (i.e., a phenotype of transformed or tumor cells).[10] It has been reported that pterygium head fibroblasts express increased levels of mRNA, protein, and activity in MMP-1 and MMP-3.[8] The present study demonstrated that fibroblasts of advanced-stage pterygium produce and release MMP-9 and activated MMP-2. This can enhance the degradation of Bowman's membrane and ECM by MMP-1 and MMP-3. MMP-1 makes only a single cut in intact collagens (e.g., types 1 and 3 collagens). Then the gela-

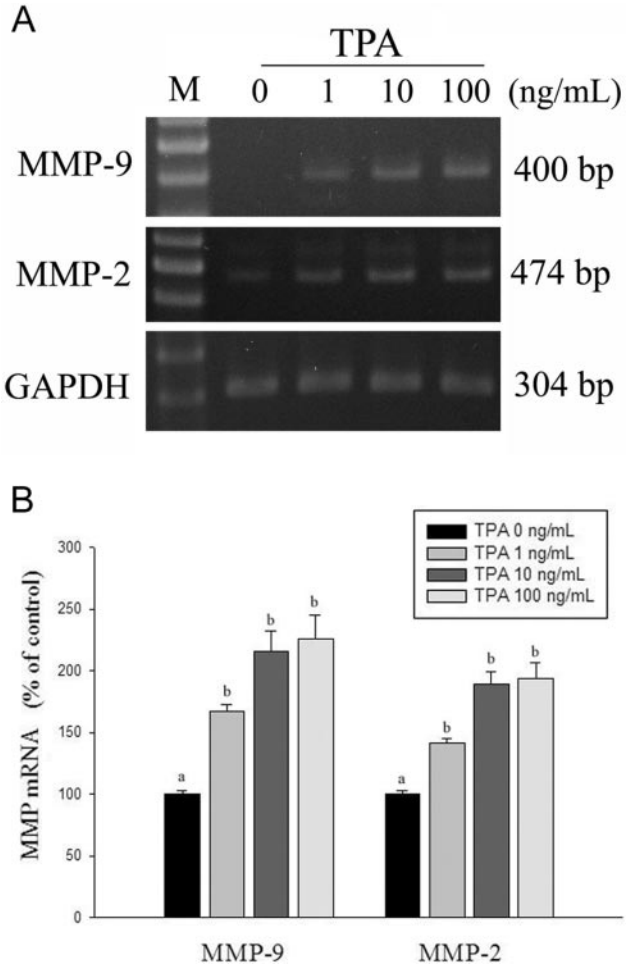

FIGURE 7. Effects of TPA on the expression of MMP-2 and MMP-9 mRNA by pterygium fibroblasts from early stages of pterygium. TPA at various concentrations (0–100 ng/mL) was added to the culture medium of pterygium fibroblasts. After culturing for 24 hours, the cells were collected and studied by RT-PCR. (**A**) RT-PCR (MMP-2 mRNA at 474 bp; MMP-9 mRNA at 400 bp). (**B**) MMP-2 and MMP-9 mRNA levels in pterygium fibroblasts cultured with various concentrations of TPA. Different letters on TPA levels indicate significant difference ($P < 0.05$).

tinases (MMP-2 and MMP-9) make successive cleavage in the degraded type 1 collagen and completely destroy it. Gelatinases can also release various growth factors (e.g., VEGF and bFGF) from the ECM to stimulate angiogenesis and the proliferation of pterygium cells.

Understanding of the role of pterygium fibroblasts in the development of pterygium has relevance to devising a new strategy of pterygium treatment. The pterygium and its underlying stroma containing the transformed fibroblasts must be excised completely during pterygium surgery; if they are not, residual fibroblasts may stimulate the recurrence of pterygium. Our finding that the expression of MMP-2 and MMP-9 plays a role in the progression of pterygium encourages the development of various novel treatments by targeting these MMPs. Pharmacologic intervention (e.g., using anti-inflammatory medications or selective inhibitors for distinct signaling pathways involving the promotion of expression of MMPs) or molecular biological intervention (e.g., siRNA) that seek to modulate the expression and activation of MMPs may have effects on the prevention and treatment of pterygium.

The occurrence of pterygium is relevant to the exposure of UV radiation.[1–6] UV radiation induces the overexpression of MMPs in cultured pterygium tissues.[17] Signal pathways involved in the occurrence and progression of pterygium are complicated. It has been reported that TGF-β signal pathways, TNF signal pathways, and EGF receptor signal pathways are involved.[7,9,22] In the MAPK pathway, ERK, but not p38 or JNK, have been demonstrated to play an important role in UV radiation-induced MMP-1 expression in pterygium epithelial cells.[17,22] It has been reported that UV-induced changes in several cell types are dependent on PKC activation.[28–30] Activation of PKC increased the expression of MMP in pterygium epithelial cells.[17] However, the effects of activation of the PKC signaling pathway on the expression of MMPs in pterygium fibroblasts have not been reported. In the present study, the effects of activation of the PKC signaling pathway on pterygium fibroblasts were tested in fibroblasts of early-stage pterygium with lower expression of MMPs. After the stimulation of TPA, a standard PKC activator, these cells expressed MMP-9 and released activated MMP-2 and a greater amount of latent MMP-2, which made these cells similar to the fibroblasts of advanced-stage pterygium. These findings are consistent with that of previous studies, which found TPA induces the expression of MMP-2 and MMP-9 and the activation of the MMP-2 proenzyme in various normal cell types (e.g., vascular endothelial cells, trabecular cells, astrocytes, corneal epithelial cells) and tumor cells (e.g., breast cancer cells, glioma cells, glioblastoma cells).[36,39–45] Our findings indicate that PKC may play a role in the transformation of pterygium fibroblasts because of exposure to UV radiation. Therefore, further studies in this field could be helpful for the elucidation of the pathogenesis of pterygium.

References

1. Coroneo MT. Pterygium as an early indicator of ultraviolet insolation: a hypothesis. *Br J Ophthalmol.* 1993;77:734–739.
2. Coroneo MT, Di Girolamo N, Wakefield D. The pathogenesis of pterygia. *Curr Opin Ophthalmol.* 1999;10:282–288.
3. Hilgers JHC. Pterygium: its incidence, heredity and etiology. *Am J Ophthalmol.* 1960;50:635–644.
4. Karai I, Horiguchi S. Pterygium in welders. *Br J Ophthalmol.* 1984;68:347–349.
5. Threlfall TJ, English DR. Sun exposure and pterygium of the eye: a dose-response curve. *Am J Ophthalmol.* 1999;128:280–287.
6. Chui J, Di Girolamo N, Wakefield D, Coroneo MT. The pathogenesis of pterygium: current concepts and their therapeutic implications. *Ocul Surf.* 2008 6:24–43.
7. Dushku N, John MK, Schultz GS, Reid TW. Pterygia pathogenesis: corneal invasion by matrix metalloproteinase expressing altered limbal epithelial basal cells. *Arch Ophthalmol.* 2001;119:695–706.
8. Li D, Lee S, Gunja-Smith Z, et al. Overexpression of collagenase (MMP-1) and stromelysin (MMP-3) by pterygium head fibroblasts. *Arch Ophthalmol.* 2001;119:71–80.
9. Solomon A, Li D, Lee S, Tseng SCG. Regulation of collagenase, stromelysin, and urokinase-type plasminogen activator in primary pterygium body fibroblasts by inflammatory cytokines. *Invest Ophthalmol Vis Sci.* 2000;41:2154–2163.
10. Chen JK, Tsai RJ, Lin SS. Fibroblasts isolated from human pterygia exhibit transformed cell characteristics. *In Vitro Cell Dev Biol Anim.* 1994;30:243–248.
11. Kria K, Ohira A, Amemiya T. Growth factors in cultured pterygium fibroblasts: immunohistochemical and ELISA analysis. *Graefes Arch Clin Exp Ophthalmol.* 1998;236:702–708.
12. Yong VW, Krekoski CA, Forsyth PA, Bell, Edwards D. Matrix metalloproteinases and diseases of the CNS. *Trends Neurosci.* 1998;21:75–80.
13. Kahari V, Saarialho-Kere U. Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Ann Med.* 1999;31:34–45.
14. Westermarck J, Kahari V. Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J.* 1999;13:781–792.
15. Romantic AM, Madri JA. Extracellular matrix-degrading proteinases in the nervous system. *Brain Pathol.* 1994;4:146–156.
16. Murphy G, Stanton H, Cowell S, et al. Mechanisms for pro matrix metalloproteinase activation. *APMIS.* 1999;107:38–44.
17. Di Girolamo N, Coroneo MT, Wakefield D. UVB-elicited induction of MMP-1 expression in human ocular surface epithelial cells is mediated through the ERK ½ MAPK-dependent pathway. *Invest Ophthalmol Vis Sci.* 2003;44:4705–4714.
18. Zeng J, Jiang D, Liu X, Tang L. Expression of matrix metalloproteinase in human pterygia. *Yan Ke Xue Bao.* 2004;20:242–245.
19. Di Girolamo N, Wakefield D, Coroneo MT. Differential expression of matrix metalloproteinases and their tissue inhibitors at the advancing pterygium head. *Invest Ophthalmol Vis Sci.* 2000;41:4142–4149.
20. Di Girolamo N, Coroneo MT, Wakefield D. Active matrilysin (MMP-7) in human pterygia: potential role in angiogenesis. *Invest Ophthalmol Vis Sci.* 2001;42:1963–1968.
21. Di Girolamo N, Chui J, Coroneo MT, Wakefield D. Pathogenesis of pterygia: role of cytokines, growth factors and matrix metalloproteinases. *Prog Retin Eye Res.* 2004;23:195–228.
22. Di Girolamo N, Coroneo MT, Wakefield D. Epidermal growth factor receptor signaling is partially responsible for the increased matrix metalloproteinase-1 expression in ocular epithelial cells after UVB radiation. *Am J Pathol.* 2005;167:489–503.
23. Di Girolamo N, McCluskey P, Lloyd A, Coroneo MT, Wakefield D. Expression of MMPs and TIMPs in human pterygia and cultured pterygium epithelial cells. *Invest Ophthalmol Vis Sci.* 2000;41:671–679.
24. Aimes RT, Quigley JP. Matrix metalloproteinase is an interstitial collagenase. *J Biol Chem.* 1995;270:5872–5876.
25. Okada Y, Naka K, Kawamura K, et al. Localization of matrix metalloproteinase-9 (92 kDa gelatinase/type IV collagenase-gelatinase B) in osteoclasts: implications for bone resorption. *Lab Invest.* 1995;72:311–322.
26. Basset P, Okada A, Chenard MP, et al. Matrix metalloproteinases as stromal effectors of human carcinoma progression: therapeutical implications. *Matrix Biol.* 1997;15:535–541.
27. Murphy G, Knauper V. Relating matrix metalloproteinase structure to function: why the 'hemopexin' domain? *Matrix Biol.* 1997;15:511–518.
28. Ryter SW, Kim HP, Hoetzel A, Park J, Nakahira K, Wang X, Choi A. Mechanisms of cell death in oxidative states. *Antioxid Redox Signal.* 2007;9:49–89.
29. He YY, Huang JL, Chingnell CF. Delayed and sustained activation of extracellular signal-regulated kinase in human keratinocytes by UVA: implications in carcinogenesis. *J Biol Chem.* 2004;279:53867–53874.

30. Sodhi A, Sethi G. Role of protein kinase C delta in UV-B-induced apoptosis of macrophages in vitro. *Cell Signal.* 2005;17:377–383.
31. Li D-Q, Tseng SCG. Three patterns of cytokine expression potentially involved in epithelial-fibroblast interactions of human ocular surface. *J Cell Physiol.* 1995;163:61–79.
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248–254.
33. Chu SC, Hu DN, Yang SF, et al. Uveal melanocytes produce matrix metalloproteinases-2 and -9 in vitro. *Pigment Cell Res.* 2004;17:636–642.
34. Wang PH, Ko JL, Tsai HT, et al. Clinical significance of matrix metalloproteinase-2 in cancer of uterine cervix: a semiquantitative study of immunoreactivities using tissue array. *Gynecol Oncol.* 2008;108:533–542.
35. Weng CJ, Chau CF, Hsieh YS, Yang SF, Yen GC. Lucidenic acid inhibits PMA-induced invasion of human hepatoma cells through inactivating MAPK/ERK signal transduction pathway and reducing binding activities of NF-κB and AP-1. *Carcinogenesis.* 2008;29:147–156.
36. Fini ME, Girard MT. The pattern of metalloproteinase expression by corneal fibroblasts is altered by passage in cell culture. *J Cell Sci.* 1990;97:373–383.
37. Di Girolamo N, Lloyd A, McCluskey P, Coroneo MT, Wakefield D. Increased expression of matrix metalloproteinases in vivo in scleritis tissue and in vitro in cultured human scleral fibroblasts. *Am J Pathol.* 1997;150:653–666.
38. Coussens LM, Werb Z. Matrix metalloproteinases and the development of cancer. *Chem Biol.* 1996;3:895–904.
39. Foda HD, George S, Conner C, Drews M, Tompkins DC, Zucker S. Activation of human umbilical vein endothelial cell progelatinase A by phorbol myristate acetate. *Lab Invest.* 1996;74:538–545.
40. Lee WJ, Shin CY, Yoo BK, et al. Induction of matrix metalloproteinases-9 (MMP-9) in lipopolysaccharide-stimulated primary astrocytes is mediated by extracellular signal-regulated protein kinase ½ (Erk1/2). *Glia.* 2003;41:15–24.
41. Park MJ, Park IC, Hur JH, et al. Modulation of phorbol ester-induced regulation of matrix metalloproteinases and tissue inhibitors of metalloproteinases by SB203580, a specific inhibitor of p38 mitogen-activated protein kinase. *J Neurosurg.* 2002;97:112–118.
42. Liu JF, Crepin M, Liu JM, Barritaulth D, Ledoux D. FGF-2 and TPA induce matrix metalloproteinases-9 secretion in MCF-7 cells through PKC activation of the Ras/ERK pathway. *Biochem Biophys Res Commun.* 2002;17:1174–1182.
43. da Rocha AB, Mans DR, Lenz G, et al. Protein kinase C-mediated in vitro invasion of human glioma cells through extracellular-signal-regulated kinase and ornithine decarboxylase. *Pathobiology.* 2000;68:113–123.
44. Shearer T, Crosson CE. Activation of extracellular signal-regulated kinase in trabecular meshwork cells. *Exp Eye Res.* 2001;73:25–35.
45. Fini ME, Girard MT, Matsubara M, Bartlett JD. Unique regulation of the matrix metalloproteinase, gelatinase B. *Invest Ophthalmol Vis Sci.* 1995;36:622–633.