Overexpression of Angiogenin in Pterygium Body Fibroblasts and Its Association With Proliferative Potency

Kyoung Woo Kim, Soo Hyun Park, Sung Wook Wee, and Jae Chan Kim

Department of Ophthalmology, Chung-Ang University Hospital, Seoul, Korea

Correspondence: Jae Chan Kim, Department of Ophthalmology, Chung-Ang University Hospital, 224-1, Heukseok-dong, Dongjak-gu, Seoul 156-755, Republic of Korea; jck50ey@kornet.net.

Submitted: April 1, 2013
Accepted: August 20, 2013

Citation: Kim KW, Park SH, Wee SW, Kim JC. Overexpression of angiogenin in pterygium body fibroblasts and its association with proliferative potency. *Invest Ophthalmol Vis Sci*. 2013;54:6355–6362. DOI:10.1167/iovs.13-12141

Purpose. Angiogenin (ANG) originally was identified as an angiogenic tumor factor, and recently its biologic activity is extended to stimulating cell proliferation. With viewing pterygium as a tumorigenic mimicry, we investigated ANG profiles within pterygia.

Methods. Expression levels of ANG were assessed using immunohistochemistry, RT-PCR, and Western blotting through examination of excised specimens and cultured fibroblasts from pterygium and conjunctiva tissues. The phenotypes of pterygia were classified by four grading indices, including recurrence, growth activity, pterygium body translucency (T), and vascularity (V). Then, ANG levels in pterygia were differentiated according to phenotypes of pterygia, and were compared to levels in normal conjunctiva. Furthermore, to investigate ANG-related acquisition of proliferative potency in fibroblasts, the correlation between ANG and α-smooth muscle actin (α-SMA) levels was evaluated.

Results. In immunohistochemistry, ANG was expressed strongly in pterygium stroma with all four severe phenotypes (with recurrence, active growth, thick body [T3], and marked vascularization [V3]), especially at the perivascular areas. There was a trend toward higher ANG expression in cultured fibroblasts of pterygia with severe phenotype, compared to those without and with normal conjunctiva. However, pterygium body V had a weak association with ANG expression. Additionally, Western blotting revealed a significant positive correlation between the expression levels of α-SMA and ANG.

Conclusions. Overexpression of ANG in pterygium body fibroblasts might be involved in active pterygium growth with thick pterygium body formation and increased risk of recurrence. A possible mechanism for this finding includes ANG-related transition of pterygium fibroblasts to the proliferative state.

Keywords: angiogenin, pterygium, fibroblast

Pterygium is a triangular-shaped ocular surface disease consisting of hypertrophic subconjunctival connective tissue and overlying conjunctival epithelium. Pterygia are divided into two parts, "head" at the apex and "body" over the sclera extended from the canthal region. Although benign, pterygium is difficult to treat because of the propensity for recurrence and lack of understanding of the disease.[1]

With improved understanding of molecular mechanisms, clearer insights into the pathogenesis of pterygium have been found. Current evidence implicates cellular proliferation,[2] antiapoptosis,[3] inflammation,[4,5] immune activity,[6] oxidative stress,[7] extracellular matrix (ECM) modulation,[8] viral involvement,[9,10] and inheritance.[11] Above all, one of the main factors leading to the development of pterygium is considered to be cell cycle dysregulation,[12,13] that is, the aberrance of cell cycle kinetics causing proliferation and escape of apoptosis likely has a significant role in pterygium formation and growth. Mutation of the p53 tumor suppression gene and the absence of wild-type p53 also are found in pterygium,[14,15] and most likely promote the mitogenic potency. In addition, excessive solar radiation–mediated genetic trauma may affect the expression of various growth factors and mitogens, such as VEGF,[16] basic fibroblast growth factor (bFGF),[17] insulin-like growth factor binding protein-2 (IGFBP-2),[18] matrix metalloproteinases (MMPs),[19] and heparin-binding epidermal growth factor (HB-EGF),[20] for construction of new fibrovascular tissue and ECM remodeling. Involvement of cell proliferation and antiapoptosis in pterygia led researchers to consider pterygium as a growth disorder analogous to a feature of tumor rather than degeneration.[2,21,22] Therefore, in-depth investigations of biochemical processes controlling cellular mitogenicity, which take place in the growth of pterygium, may lead to the development of a medical cure or more effective treatments for pterygia.

Angiogenin (ANG) originally was isolated as an angiogenic protein in colon adenocarcinoma cells.[23] Its expression is upregulated in various cancers,[24–29] and is reported in some studies to be associated with cancer progression or poor prognosis.[25,26,29] Recently, the biological activity of ANG has been extended to include the promotion of cell survival, which can be supported by Akt activation,[30] inhibition of p53 function, and blockage of proapoptotic Bax and p21 expression via ANG to mediate antiapoptosis and proliferation in tumor cells.[31] Furthermore, ANG-induced activation of the Erk1/2 pathway[32] possibly may be involved in Erk1/2-mediated MMP-1 induction and ECM remodeling, which is a known process in pterygia.[19] Moreover, ANG is necessary for angiogenesis induced by other angiogenic factors, such as bFGF and VEGF.[33] The multiple roles of ANG in tumors implicate that it may be an essential pathogenic factor of pterygium, which is a tumori-

Table 1. Clinical Grading System According to Body Characteristics of Pterygium

Grade	Characteristics
Grade T, translucency	
T1	Lesion with unobscured and clearly distinguished episcleral vessels underlying its body
T2	Lesion with indistinctly seen or partially obscured episcleral vessel details underlying its body
T3	Thick pterygium in which episcleral vessels underlying its body were obscured totally by fibrovascular tissue
Grade V, vascularity	
V1	Minimal vascularization with unidirectional pattern
V2	Moderate vascularization with unidirectional and enlarged vessels
V3	Marked vascularization with unidirectional, engorged vessels

genic mimicry. Uncovering the function of ANG in pterygia has never been studied before to our knowledge.

Several characteristics of pterygia may reflect their proliferative levels. Deviated body morphology of pterygium possibly is linked to acquisition of tumor-like nature.[34] The overgrowth of stromal fibroblasts and blood vessels accompanied by an ECM accumulation is a well-known prerequisite for pterygium genesis. Additionally, fibrovascular in-growth is more extensive in recurrent pterygia than in primary disease.[12] In the aspect of proliferative gain of function of fibroblasts, myofibroblasts with tumor-promoting phenotypes express α-smooth muscle actin (α-SMA)[35–37] and its expression in pterygia was identified in a previous study.[38] Besides, in vascular smooth muscle cells, it was reported that ANG immunoreactivity colocalized with α-SMA immunoreactivity.[39]

Therefore, in our study, we analyzed the expression of ANG in excised pterygium tissue and in cultured stromal fibroblasts according to growth and proliferative indices, including recurrence, clinical growth activity of pterygium, pterygium body morphology, and vascularity. These parameters then were compared to those of normal conjunctiva. Furthermore, to investigate the pathogenetic transdifferentiation of stromal fibroblasts in pterygium, we analyzed the correlation pattern between the expression of ANG and α-SMA of cultured pterygium body fibroblasts.

Methods

Patient Background and Classification of Pterygia

In this prospective study, pterygium samples were obtained intraoperatively from 42 eyes of 35 consecutive patients with mean age 51.9 ± 10.1 years (mean ± SD; range, 18–81 years) who underwent surgical excision between September 2011 and February 2013. In 7 patients among the whole 35 patients, pterygia were harvested from eyes due to the existence in bilateral eyes. Normal conjunctiva tissues additionally were obtained from 8 eyes of 8 healthy patients with mean age 52.6 ± 13.9 years (mean ± SD; range, 33–66 years) who were found to have no evidence of ocular surface disorders during cataract surgery. All surgeries were performed by one surgeon (JCK) to ensure consistent tissue harvest technique. Patients with a history of ischemic cardiovascular disease, hematologic disorder, neurodegenerative disease, or malignant disease were excluded.

Preoperatively, each patient underwent complete ocular examination by one ophthalmologist and all pterygia were

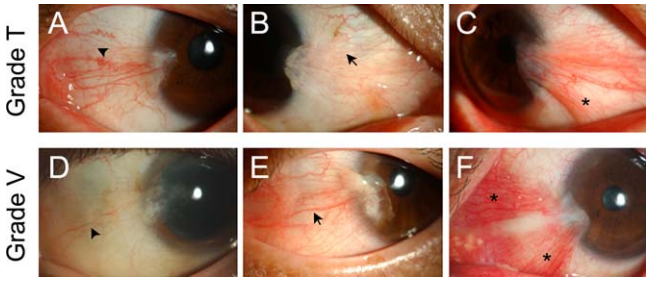

Figure 1. Standard photographs classified by translucency (grade T, [**A**–**C**]) and vascularity (grade V, [**D**–**F**]) of the pterygium body. According to grade T classification, each pterygium was classified into T1, atrophic pterygium (**A**); T2, intermediate pterygium (**B**); and T3, fleshy pterygium (**C**). In contrast to the lesion with unobscured episcleral vessels underlying its body in grade T1 pterygium ([**A**], *arrowhead*), partially obscured lesion in grade T2 pterygium ([**B**], *arrow*), and thick fibrovascular tissue totally obscuring the episcleral vessels in grade T3 pterygium ([**C**], *asterisk*) are noted. In addition, according to grade V classification, each pterygium was classified into V1, pterygium with minimal vascularization (**D**); V2, pterygium with moderate vascularization with enlarged vessels (**E**); and V3, marked vascularization with engorged vessels (**F**). In grade V1 pterygium, its vascularity is minimal and does not exceed remarkably the normal conjunctival vascularity ([**D**], *arrowhead*). However, the enlarged vessel is noted with higher density in grade V2 ([**E**], *arrow*); furthermore, dense and engorged multiple vessels are concentrated and densely distributed in grade V3 pterygium body ([**F**], *asterisks*).

classified by each of a 4-point grading system: (1) recurrence of disease, (2) growth activity, (3) translucency of the pterygium body (grade T), and (4) vascularity of the pterygium body (grade V) for data analysis. The grading, according to pterygium body translucency by the Tan classification[34] and vascularity, was evaluated based on the grading criteria (Table 1) and by aid of standard photographs (Fig. 1). Rapidly occurring pterygia or those with recurrence within the last 6 months were considered as having proliferative growth patterns and regarded as active forms, while growth-quiescent forms were considered to be inactive. Among the 4 characteristics according to the grading indices, each characteristic of (1) with recurrence, (2) active form, (3) grade T3, and (4) grade V3 was designated as a severe phenotype.

The study protocol and consent form were approved by the institutional review board of the Chung-Ang University Hospital. All procedures were performed according to the tenets of the Declaration of Helsinki, and informed consent was obtained from all patients.

Evaluation of ANG Expression in Excised Tissues of Pterygium and Normal Conjunctiva

To investigate and differentiate the expression pattern of ANG in pterygia and normal conjunctiva, 6 pterygial tissue samples, including 3 specimens with all of four severe phenotypes (with recurrence, active form, grade T3, and grade V3), and 3 specimens with none of the severe phenotypes (primary, inactive form, grade T1, and grade V1), and an additional 2 normal conjunctiva samples were obtained among the enrolled patients for immunohistochemical (IHC) staining of ANG. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Briefly, all paraffin sections (4 μm) were deparaffinized in xylene, rehydrated, and quenched with endogenous peroxidase. Cryostat sections were placed on gelatinized slides and fixed in cold acetone. Tissue sections were equilibrated in tris-buffered saline (TBS), blocked in nonimmune serum (Zymed Laboratories, South San Francisco, CA), and incubated with mouse monoclonal antibody against human ANG (1:500;

Table 2. Demographics of Patients With Pterygia

Grading Index of Pterygium	*N* of Eyes	Sex, Male/Female	Mean Age ± SD, y
Recurrence			
Primary	20 (47.6%) from 16 patients	9/7	55.4 ± 10.0
Recurrent	22 (52.4%) from 19 patients	10/9	48.9 ± 11.9
Growth activity			
Inactive	28 (66.7%) from 24 patients	12/12	52.0 ± 12.7
Active	14 (33.3%) from 12 patients	8/4	52.1 ± 8.0
Grade T			
T1, non-T3	7 (16.7%) from 7 patients	3/4	56.4 ± 12.6
T2, non-T3	10 (23.8%) from 8 patients	5/3	53.6 ± 8.0
T3	25 (59.5%) from 22 patients	13/9	49.6 ± 11.5
Grade V			
V1, non-V3	9 (21.4%) from 8 patients	4/4	52.5 ± 5.9
V2, non-V3	15 (35.7%) from 13 patients	8/5	56.2 ± 11.0
V3	18 (42.9%) from 16 patients	8/8	47.9 ± 12.3

Abcam, Inc., Cambridge, MA) overnight at 4°C. Sections were washed in TBS before adding the biotinylated secondary antibody, rewashed, and incubated for 1 hour with peroxidase-conjugated streptavidin, and the presence of peroxidase was revealed by adding substrate-chromogen (3-amino-9-ethycarbazole) solution. The sections then were counterstained with hematoxylin, examined under an optical microscope (Axioskop 40; Carl Zeiss, Göttingen, Germany) and photo-documented.

Specimen Collection and Culture of Stromal Fibroblasts From Pterygium and Normal Conjunctiva

Intraoperatively excised tissues for culture were collected from all cases enrolled in this study. Pterygium specimens were obtained at the central portion of the pterygium body at the time of excision, and samples of normal 2 × 2 mm conjunctiva were harvested from the superonasal bulbar conjunctiva during cataract surgery. As a rule, within 1 hour of excision, all samples were placed in sterile tubes containing Dulbecco's modified Eagle's medium (DMEM; WelGENE, Daegu, South Korea) and transferred to the laboratory for culture. These specimens were used for explant cultures to generate normal human conjunctival fibroblasts (HCJFs) and human pterygium body fibroblasts (HPBFs).

For fibroblast isolation, each specimen was cut into explants (1 mm^2) and placed in 6-well plates. After 10 minutes of adhesion, each explant was covered with alpha-MEM (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; WelGENE) and 100 units/mL penicillin/streptomycin (WelGENE), and then placed in a humidified incubator (37°C, 5% CO_2). The medium was changed every 4 to 5 days thereafter. Fibroblasts were subcultured with 0.05% trypsin and 0.85 mM EDTA in a calcium-free MEM at 80% to 90% confluence with a 1:3 to 1:4 split for 3 passages.

RT-PCR Analysis of ANG mRNA

Expression level of ANG mRNA was evaluated with HCJFs and HPBFs by semiquantitative RT-PCR with β-actin as an internal control. RNA extractions were performed using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (500 ng) was subjected to reverse transcription into cDNA (cDNA synthesis kit; Takara Bio, Inc., Otsu, Japan). Equal amounts of samples were used for PCR amplification of cDNA, with specific primers for human ANG. Human ANG primers were 5′-CCTGGGCGTTTTGTTGTTGG-3′ (sense) and 5′-TGTGGCTCGGTACTGGCATG-3′ (antisense) with expected ANG PCR products of 352 base pairs (bp). The PCR amplification was performed (EmeraldAmp GT PCR Master Mix; Takara Bio, Inc.) with 1 μL cDNA product in a total volume of 20 μL. The PCR cycling conditions were as follows: 30 cycles of denaturation at 95°C for 1 minute, annealing at

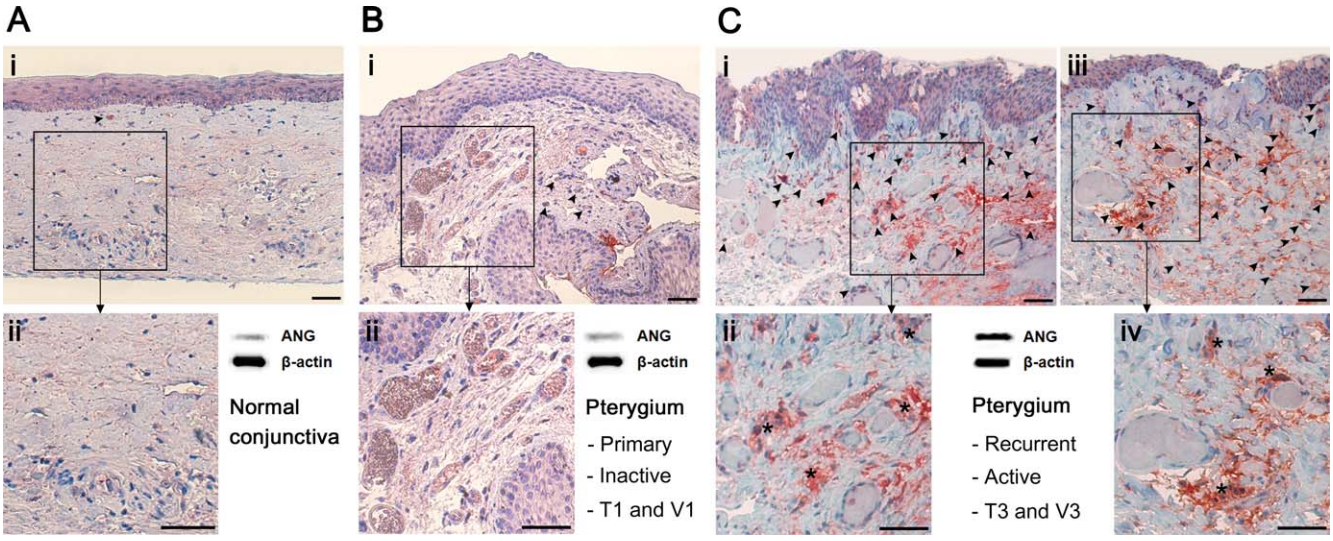

Figure 2. Immunostain of ANG from one normal conjunctival tissue (**A**) and two specimens of pterygia (**B**, **C**). While there is very sparse staining of ANG in the normal conjunctiva (*i* in [**A**], *arrowhead*) and inactive primary pterygium with grade T1 and V1 (*i* in [**B**], *arrowheads*), ANG expression is prominent in the whole layer of the stroma of the pterygium with all of four severe phenotypes (*i* and *iii* in [**C**], *arrowheads*). Moreover, high power photomicrographs show that multiple ANG-immunopositive cells are concentrated surrounding the stromal vessels in the highly ANG-expressing pterygium (*ii* and *iv* in [**C**], *asterisks*), compared to no existence of those cells in the others (*ii* in [**A**, **B**]). These findings correspond with Western immunobands of ANG protein from cultured fibroblasts from each specimen. *Scale bars*: 100 μm.

Investigative Ophthalmology & Visual Science

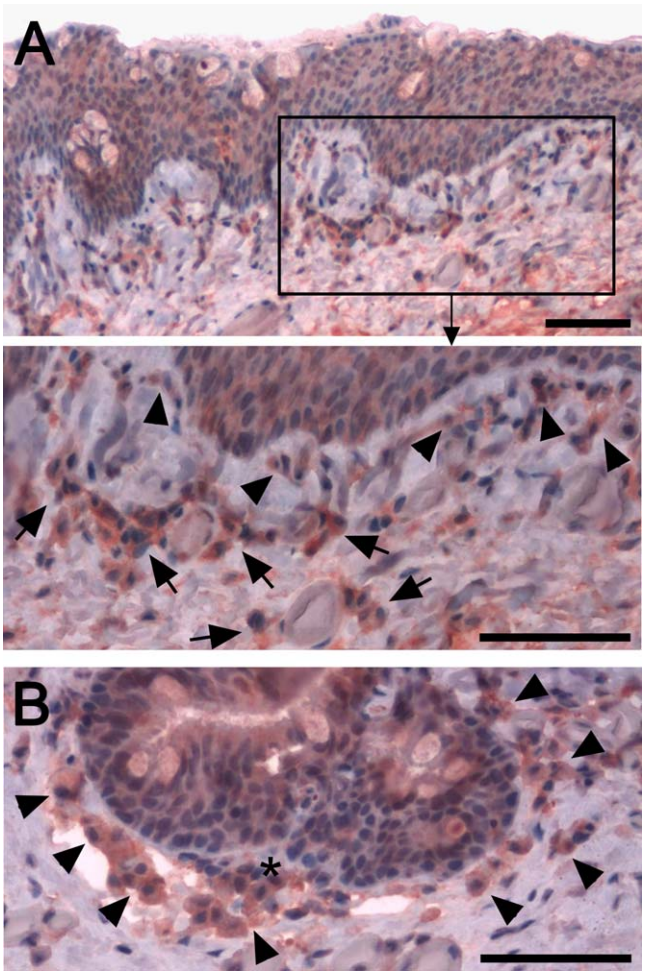

Figure 3. Expression of ANG in two representative pterygium tissue samples. (**A**, **B**) ANG-stained cells are noted at the subepithelial fibrovascular layer adjacent to the epithelium (*arrowheads*) and perivascular area in the stroma (*arrows*). ANG expression in the stroma and basal layer of the epithelium are quite contiguous (*asterisk* in [**B**]) and is noted in one section. *Scale bars*: 100 μm.

57°C for 1 minute, and extension at 72°C for 1 minute. Amplified products were verified by electrophoresis in 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet (UV) transillumination.

Western Blot Analysis of ANG Expression

To identify the expression of the ANG protein in HCJFs and HPBFs, Western blot analysis was performed using specific antibodies. Total protein concentration was measured with a NanoDrop spectrophotometer (NanoDrop 1000; Thermo Scientific, Wilmington, DE). The individual protein samples were run through SDS-PAGE and transferred to a polyvinylidene fluoride membrane (PVDF; Merck Millipore, Billerica, MA). The membrane then was blocked with 5% BSA in TBS (50 mM Tris-HCl, pH 7.5; 150 mM NaCl). Primary mouse monoclonal antibodies against human ANG (1:500; Abcam, Inc.) were diluted in TBS, applied to the membrane, and incubated overnight at 4°C. Secondary antibodies were diluted in TBS (1:2000), applied to the membrane, and incubated for 1 hour at room temperature. After each step, the PVDF membrane was washed 4 times with 0.1% Tween 20 in TBS buffer (TBS-T) for 10 minutes. The protein signal after the application of secondary antibody was visualized using an enhanced chemiluminescence Western blotting detection kit (Pierce Biotechnology, Inc., Rockford, IL). β-actin was used as an internal control.

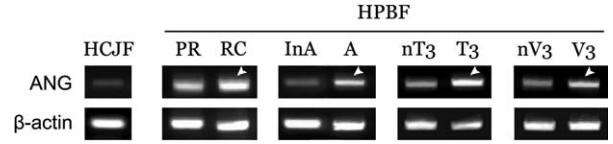

Figure 4. Representative data of ANG mRNA expression by RT-PCR from cultured normal HCJFs and HPBFs according to recurrence, growth activity, grade T, and grade V. ANG mRNA showed tendencies of higher expression in pterygia with recurrence, active growth, and grade T3 or grade V3 (*arrowheads*) compared to normal conjunctiva, and pterygia with no history of recurrence, inactive growth patterns, grade non-T3, or grade non-V3. PR, primary pterygium; RC, recurrent pterygium; InA, pterygium with inactive growth; A, pterygium with active growth; nT3, pterygium with grade non-T3; nV3, pterygium with grade non-V3.

Analysis of Correlation Between ANG and α-SMA Protein Expression in Cultured Fibroblasts

In addition, primary mouse monoclonal antibodies against human α-SMA (1:500; Dako, Glostrup, Denmark) were used in the same manner as anti-ANG antibodies in 5 normal conjunctiva and 26 pterygium tissue samples selected randomly among all enrolled samples. Image analysis of the immunobands of Western blot was performed using ImageJ version 1.46 (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using 1-way ANOVA followed by a post hoc pairwise comparison adjusted with a Bonferroni correction and univariate regression analysis. Statistical analyses were performed using SPSS software version 19.0 (SPSS, Inc., Chicago, IL). *P* values less than 0.05 were considered to be statistically significant.

Results

Totals of 42 eyes of 35 patients (19 males and 16 females) with pterygia, and an additional 8 eyes of 8 patients (3 males and 5 females) with normal conjunctiva were enrolled in this study. Detailed demographic data of patients and pterygia according to the grading indices are shown in Table 2.

ANG Expression in Pterygium and Normal Conjunctival Tissues

In IHC, ANG-immunopositive cells were detected distinctly in the stromal layer in all pterygium cases with all of four severe phenotypes. In contrast, ANG was stained sparsely in all samples from normal conjunctiva and pterygia with none of the severe phenotypes (Fig. 2). In pterygia with strong expression of ANG, expression was confined preferentially to the subepithelial fibrovascular layer adjacent to the basement membrane of the epithelium and near vascular structures (Fig. 3). The staining of ANG showed a diffuse pattern in the epithelium and was not as substantial as in the stromal layer (Fig. 2C). There was no ANG staining in vascular endothelial cells of conjunctiva or pterygium tissues.

Investigative Ophthalmology & Visual Science

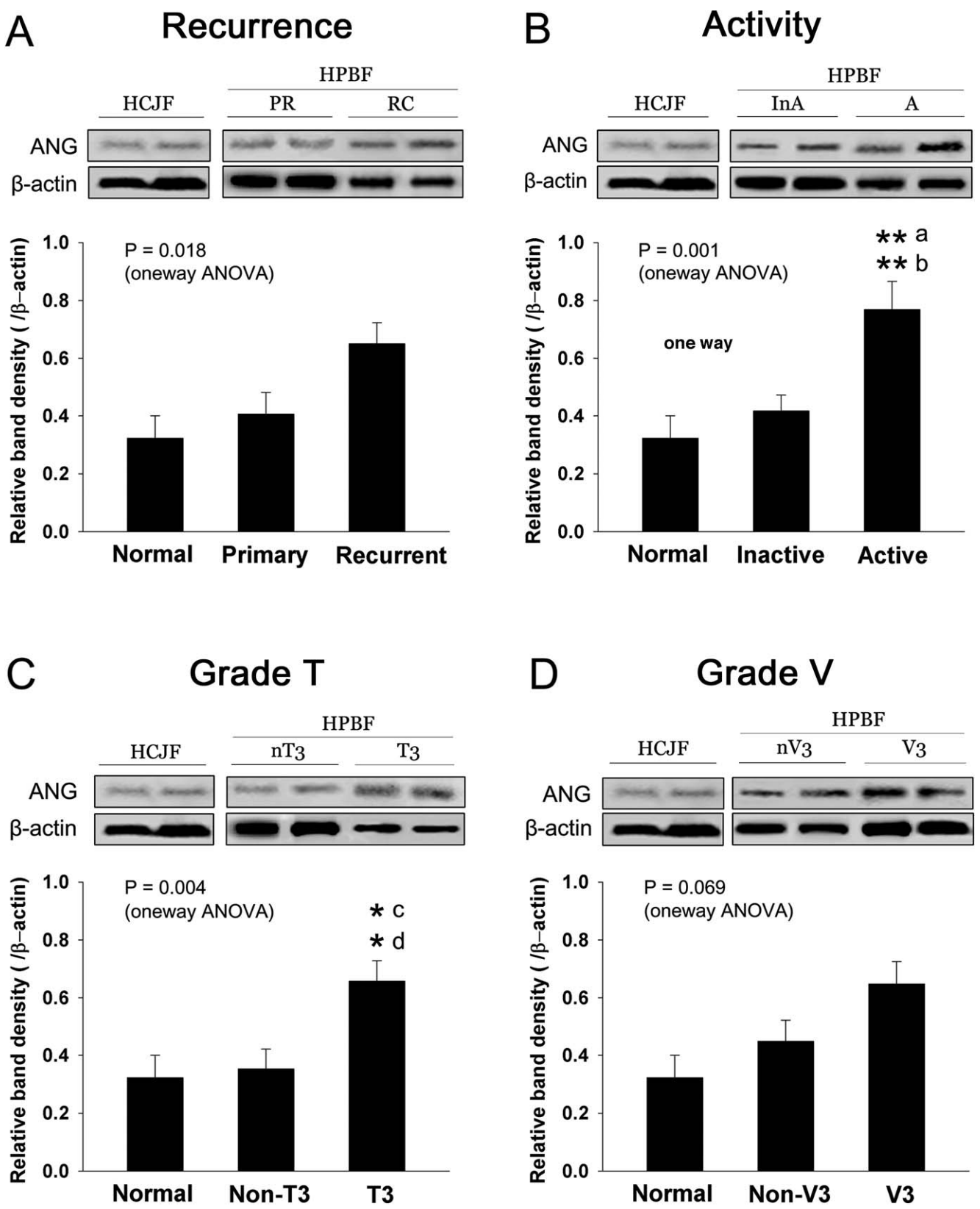

FIGURE 5. Western blot analysis of ANG protein expression in cultured normal HCJFs and HPBFs according to recurrence, growth activity, grade T, and grade V. (**A–C**) A significant association of ANG protein expression was noted with recurrence ($P = 0.018$), growth activity ($P = 0.001$), and grade T ($P = 0.004$). (**B**) In particular, HPBFs from active forms of pterygia showed higher ANG expression compared to HCJFs (*a*) and HPBFs from inactive pterygia (*b*). (**C**) HPBFs from pterygia with grade T3 showed higher ANG expression compared to HCJFs (*c*) and HPBFs from pterygia with grade non-T3 (*d*). (**D**) There was a trend toward higher ANG expression in pterygia with more vessels, but without statistical significance ($P = 0.069$). $^{**}P < 0.01$; $^{*}P < 0.05$; *a*, active versus normal; *b*, active versus inactive; *c*, T3 versus normal; *d*, T3 versus non-T3.

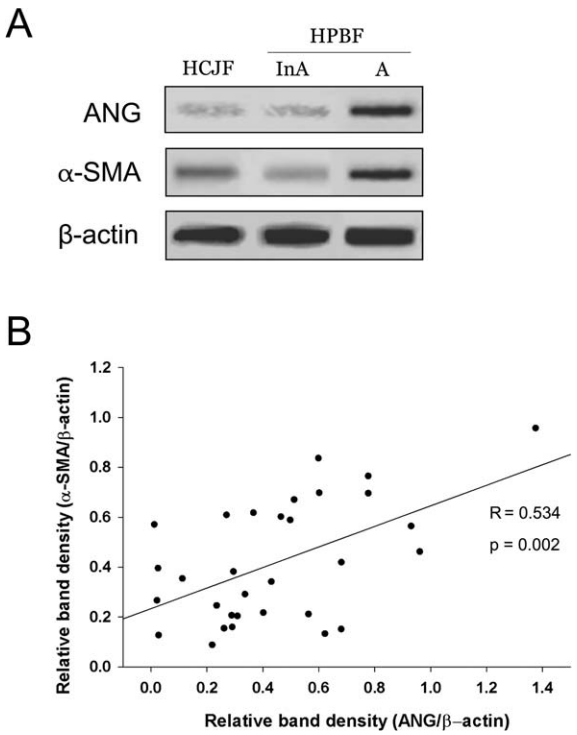

FIGURE 6. Association of ANG protein with proliferative transition of HPBFs. (**A**) Western blot analysis showed upregulation of α-SMA and ANG protein in HPBFs from pterygium with active growth patterns relative to the HPBFs from inactive pterygium and normal HCJFs. (**B**) Higher protein levels of α-SMA revealed a significant positive trend toward a higher level of ANG (Pearson's coefficient = 0.534, $P = 0.002$).

ANG Expression in Cultured Fibroblasts From Pterygium Bodies and Normal Conjunctiva

To analyze expression of ANG in fibroblasts from human pterygia and normal conjunctiva, cellular mRNA levels of ANG were examined by RT-PCR, and Western blot analysis was performed using an anti-ANG antibody. We hypothesized that pterygia with severe phenotypes are tumorigenic mimicries, and are different from normal conjunctiva and pterygia without severe phenotypes from the aspect of ANG expression. Thus, ANG expression in cultured fibroblasts of pterygia with each severe phenotype was analyzed and compared to those of pterygia without severe phenotype and also to normal conjunctival fibroblasts.

A trend toward higher ANG expression was found in pterygia from samples with severe phenotype at mRNA (Fig. 4) and protein levels (Fig. 5). Specifically, statistically significant associations of ANG expression were noted with growth activity ($P = 0.029$) and grade T ($P = 0.041$) by RT-PCR, and with recurrence ($P = 0.018$), growth activity ($P = 0.001$), and grade T ($P = 0.004$) by Western blotting. Moreover, active forms showed significantly higher expression of ANG compared to normal conjunctiva ($P = 0.003$) or inactive forms of pterygia ($P = 0.006$, Fig. 5B). Pterygia with grade T3 also expressed higher levels of ANG than normal conjunctiva ($P = 0.036$) or pterygia with grade non-T3 ($P = 0.011$, Fig. 5C) at protein levels. Differences of ANG protein expression between recurrent pterygia and either primary forms or normal conjunctiva were not statistically significant, but were noted to have marginal significance ($P = 0.058$ and $P = 0.056$, respectively). Interestingly and contrary to expectations, there was no significant association between ANG expression and grade V at mRNA ($P = 0.078$) and protein levels ($P = 0.069$). This finding was in contrast with the well-known fact that ANG is a potent factor of angiogenesis. However, there was a trend toward greater ANG expression in pterygia with more evidence of vascularization. Fibroblasts of pterygium with no previous recurrence, inactive growth patterns, or grade non-T3 or non-V3 according to grading indices had no considerable differences of ANG expression compared to normal conjunctiva.

Effect of ANG on Proliferative Transition of Pterygium Body Fibroblasts

To investigate the ANG-related proliferative transition of HPBFs, the presentation of α-SMA, a well-known marker for mesenchymal differentiation and highly invasive characteristics, was identified and paired with ANG expression. Western blot analysis noted upregulated expression of α-SMA protein in HPBFs of pterygium with active growth relative to the HPBFs of pterygium with inactive patterns or control HCJFs (Fig. 6A). Additionally, higher protein levels of α-SMA revealed a trend toward higher ANG levels, which was found to be statistically significant (Fig. 6B, Pearson's coefficient = 0.534, $P = 0.002$).

DISCUSSION

Although still under investigation, several studies have revealed a considerable degree of pathogenesis of pterygium as a proliferative disorder.[2,3,21,22] However, in some patients, pterygium remains quiescent for decades, while others occasionally experience rapid growth with multiple episodes of recurrence and repeated complications, such as symblepharon and motility restriction in practice. These intrinsic aspects of unpredictability and interindividual inconsistency of pterygium complicate the establishment of therapeutic strategies. Therefore, understanding the process determining active and aggravated growth patterns of pterygium may aid in the development of more promising methods to prevent or disrupt pterygium in earlier stages. In our study, we first demonstrated a potential link between ANG and the pathogenesis of pterygium. ANG expression in fibroblasts had a close correlation with disease recurrence, active growth, pterygium thickness, and vascularization. Additionally, we identified a significant association between high expression of ANG and possible pheno-transdifferentiation of body fibroblasts of pterygium by analyzing α-SMA expression.

Previously, it has been reported that the stomal layer was quite important in the pathogenesis of pterygium.[40,41] Moreover, pterygium fibroblasts were known to have acquired many of the properties of the transformed phenotype of neoplastic cells compared to conjunctival fibroblasts.[42] To specify the distinct characteristics of invasive and proliferative types of pterygium, we investigated the role of ANG more than any other in this study. In general, cellular proliferation is regulated primarily by cell cycle regulation, which is controlled by positive and negative regulators. The main mechanism of ANG, which is upregulated in several types of cancers, includes cell survival and apoptosis via inhibition of p53 phosphorylation, and blockage of proapoptotic Bax and p21 expression.[31] Our comprehensive results, especially the finding of strong ANG expression with postoperative recurrence and active growth of pterygium, corresponded with the emerging role of ANG as a cell survival and antiapoptotic factor.[43]

The MMP expression has a role in the migration and growth of pterygium, and the variable expression of MMP may explain differences in the growth of pterygium seen clinically.[12,44] Specifically, MMP-1 is reported to be induced via Erk1/2 in the aspect of ECM remodeling.[19] Strong expression of ANG in

grade T3 pterygium, which has a fleshy and sparsely translucent appearance, made investigators presume the possible involvement of ANG in ECM remodeling. Coincidentally, ANG was reported to activate Erk1/2 signaling, although this was found in human umbilical endothelial cells.[32] Further studies are needed to investigate ANG-mediated activation of the Erk pathway in pterygium.

Although there was a positive correlation tendency, there was no significant correlation between the expression levels of ANG mRNA or protein in pterygium fibroblasts and the vascularization levels of pterygium. We speculated that this is because previously known angiogenic factors of pterygium, such as VEGF, thrombospondin-1 and substance P,[45–47] were not coanalyzed and not adjusted in the molecular analysis of this study. However, copious staining of ANG in the perivascular cells, which we presumed to be immature fibroblasts, may suggest a possible role of ANG in the angiogenesis of pterygium. Supporting this speculation includes the finding of a requirement for fibroblasts in active angiogenesis accentuated in the tumor environment.[48]

Interestingly, ANG expression also was prominent at the subepithelial fibrovascular layer adjacent to the basement membrane of the epithelium. Coexpression of ANG in the fibrovascular and basal layers of the epithelium, which are contiguous (Fig. 3B, asterisk), suggested that the potential interaction of the epithelium and stroma may promote proliferative capability in pterygium.

Stromal myofibroblasts have an ability to promote invasive tumor growth substantially.[49] Moreover, the existence of myofibroblasts in pterygium has been identified in a previous study.[38] One important finding of this study is the upregulation of α-SMA protein expression, a hallmark of activated fibroblasts, in HPBFs with strong ANG expression. Besides, there was a significant positive correlation between ANG and α-SMA levels. These results indicated that overexpression of ANG in HPBFs may participate in the proliferative gain of function of pterygium fibroblasts.

There are several limitations to our study. The association of ANG with the proliferation potency in fibroblasts was analyzed indirectly using cross-sectional investigations, such as RT-PCR, Western blotting, and IHC analysis. Several growth factors and cytokines are known to be related to proliferation of HPBFs, such as VEGF, bFGF, TGF-β, and so forth. Because this analysis was limited, we cannot rule out the possibility of concurrent effects from other factors in the analysis of ANG relevance. To overcome this problem, cell proliferation assays or verification of proliferative transition of inactive HPBFs using α-SMA after the treatment of ANG protein may be helpful through further studies.

To our knowledge, there has never been a study of the role of ANG in ocular surface disorders, including pterygium. The role of ANG is being expanded recently to cell survival and proliferation more than just angiogenesis. In the same vein, the findings presented here showed a strong association of overexpressed ANG in fibroblasts of pterygium with characteristics of active growth. This newly uncovered mechanism of pterygium pathogenesis will motivate further investigation targeting ANG to establish new strategies for the management of pterygium.

Acknowledgments

Supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012-0005222), and by a grant from the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A121487). The authors alone are responsible for the content and writing of the paper.

Disclosure: **K.W. Kim**, None; **S.H. Park**, None; **S.W. Wee**, None; **J.C. Kim**, None

References

1. Krachmer JH, Mannis MJ, Holland EJ. *Cornea*. St. Louis, MO: Mosby Elsevier; 2011;1625.
2. Liang K, Jiang Z, Ding BQ, Cheng P, Huang DK, Tao LM. Expression of cell proliferation and apoptosis biomarkers in pterygia and normal conjunctiva. *Mol Vis*. 2011;17:1687–1693.
3. Tan DT, Tang WY, Liu YP, Goh HS, Smith DR. Apoptosis and apoptosis related gene expression in normal conjunctiva and pterygium. *Br J Ophthalmol*. 2000;84:212–216.
4. Chiang CC, Cheng YW, Lin CL, et al. Cyclooxygenase 2 expression in pterygium. *Mol Vis*. 2007;13:635–638.
5. Di Girolamo N, Kumar RK, Coroneo MT, Wakefield D. UVB-mediated induction of interleukin-6 and –8 in pterygia and cultured human pterygium epithelial cells. *Invest Ophthalmol Vis Sci*. 2002;43:3430–3437.
6. Beden U, Irkec M, Orhan D, Orhan M. The roles of T-lymphocyte subpopulations (CD4 and CD8), intercellular adhesion molecule-1 (ICAM-1), HLA-DR receptor, and mast cells in etiopathogenesis of pterygium. *Ocul Immunol Inflamm*. 2003;11:115–122.
7. Kau HC, Tsai CC, Lee CF, et al. Increased oxidative DNA damage, 8-hydroxydeoxy- guanosine, in human pterygium. *Eye (Lond)*. 2006;20:826–831.
8. Naib-Majani W, Eltohami I, Wernert N, et al. Distribution of extracellular matrix proteins in pterygia: an immunohistochemical study. *Graefes Arch Clin Exp Ophthalmol*. 2004;242:332–338.
9. Gallagher MJ, Giannoudis A, Herrington CS, Hiscott P. Human papillomavirus in pterygium. *Br J Ophthalmol*. 2001;85:782–784.
10. Detorakis ET, Sourvinos G, Spandidos DA. Detection of herpes simplex virus and human papilloma virus in ophthalmic pterygium. *Cornea*. 2001;20:164–167.
11. 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.
12. Bradley JC, Yang W, Bradley RH, Reid TW, Schwab IR. The science of pterygia. *Br J Ophthalmol*. 2010;94:815–820.
13. 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.
14. Reisman D, McFadden JW, Lu G. Loss of heterozygosity and p53 expression in pterygium. *Cancer Lett*. 2004;206:77–83.
15. Tsai YY, Cheng YW, Lee H, Tsai FJ, Tseng SH, Chang KC. P53 gene mutation spectrum and the relationship between gene mutation and protein levels in pterygium. *Mol Vis*. 2005;11:50–55.
16. Lee DH, Cho HJ, Kim JT, Choi JS, Joo CK. Expression of vascular endothelial growth factor and inducible nitric oxide synthase in pterygia. *Cornea*. 2001;20:738–742.
17. Kria L, Ohira A, Amemiya T. Growth factors in cultured pterygium fibroblasts: immunohistochemical and ELISA analysis. *Graefes Arch Clin Exp Ophthalmol*. 1998;236:702–708.
18. Solomon A, Grueterich M, Li DQ, Meller D, Lee SB, Tseng SC. Overexpression of insulin-like growth factor-binding protein-2 in pterygium body fibroblasts. *Invest Ophthalmol Vis Sci*. 2003;44:573–580.
19. Di Girolamo N, Coroneo MT, Wakefield D. UVB-elicited induction of MMP-1 expression in human ocular surface epithelial cells is mediated through the ERK1/2 MAPK-

dependent pathway. *Invest Ophthalmol Vis Sci*. 2003;44: 4705–4714.

20. Nolan TM, Di Girolamo N, Coroneo MT, Wakefield D. Proliferative effects of heparin-binding epidermal growth factor-like growth factor on pterygium epithelial cells and fibroblasts. *Invest Ophthalmol Vis Sci*. 2004;45:110–113.
21. Tan DT, Lim AS, Goh HS, Smith DR. Abnormal expression of the p53 tumor suppressor gene in the conjunctiva of patients with pterygium. *Am J Ophthalmol*. 1997;123:404–405.
22. Ueda Y, Kanazawa S, Kitaoka T, et al. Immunohistochemical study of p53, p21 and PCNA in pterygium. *Acta Histochem*. 2001;103:159–165.
23. Fett JW, Strydom DJ, Lobb RR, et al. Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry*. 1985;24:5480–5486.
24. Barton DP, Cai A, Wendt K, Young M, Gamero A, De Cesare S. Angiogenic protein expression in advanced epithelial ovarian cancer. *Clin Cancer Res*. 1997;3:1579–1586.
25. Chopra V, Dinh TV, Hannigan EV. Serum levels of interleukins, growth factors and angiogenin in patients with endometrial cancer. *J Cancer Res Clin Oncol*. 1997;123:167–172.
26. Chopra V, Dinh TV, Hannigan EV. Circulating serum levels of cytokines and angiogenic factors in patients with cervical cancer. *Cancer Invest*. 1998;16:152–159.
27. Li D, Bell J, Brown A, Berry CL. The observation of angiogenin and basic fibroblast growth factor gene expression in human colonic adenocarcinomas, gastric adenocarcinomas, and hepatocellular carcinomas. *J Pathol*. 1994;172:171–175.
28. Montero S, Guzman C, Cortes-Funes H, Colomer R. Angiogenin expression and prognosis in primary breast carcinoma. *Clin Cancer Res*. 1998;4:2161–2168.
29. Shimoyama S, Gansauge F, Gansauge S, Negri G, Oohara T, Beger HG. Increased angiogenin expression in pancreatic cancer is related to cancer aggressiveness. *Cancer Res*. 1996; 56:2703–2706.
30. Kim HM, Kang DK, Kim HY, Kang SS, Chang SI. Angiogenin-induced protein kinase B/Akt activation is necessary for angiogenesis but is independent of nuclear translocation of angiogenin in HUVE cells. *Biochem Biophys Res Commun*. 2007;352:509–513.
31. Sadagopan S, Veettil MV, Chakraborty S, et al. Angiogenin functionally interacts with p53 and regulates p53-mediated apoptosis and cell survival. *Oncogene*. 2012;31:4835–4847.
32. Liu S, Yu D, Xu ZP, Riordan JF, Hu GF. Angiogenin activates Erk1/2 in human umbilical vein endothelial cells. *Biochem Biophys Res Commun*. 2001;287:305–310.
33. Kishimoto K, Liu S, Tsuji T, Olson KA, Hu GF. Endogenous angiogenin in endothelial cells is a general requirement for cell proliferation and angiogenesis. *Oncogene*. 2005;24:445–456.
34. Tan DT, Chee SP, Dear KB, Lim AS. Effect of pterygium morphology on pterygium recurrence in a controlled trial comparing conjunctival autografting with bare sclera excision. *Arch Ophthalmol*. 1997;115:1235–1240.
35. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature*. 2004;432:332–337.
36. Serini G, Gabbiani G. Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res*. 1999;250:273–283.
37. Shimoda M, Mellody KT, Orimo A. Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression. *Semin Cell Dev Biol*. 2010;21:19–25.
38. Touhami A, Di Pascuale MA, Kawatika T, et al. Characterisation of myofibroblasts in fibrovascular tissues of primary and recurrent pterygia. *Br J Ophthalmol*. 2005;89:269–274.
39. Pavlov N, Hatzi E, Bassaglia Y, Frendo JL, Evain Brion D, Badet J. Angiogenin distribution in human term placenta, and expression by cultured trophoblastic cells. *Angiogenesis*. 2003;6:317–330.
40. Seet LF, Tong L, Su R, Wong TT. Involvement of SPARC and MMP-3 in the pathogenesis of human pterygium. *Invest Ophthalmol Vis Sci*. 2012;53:587–595.
41. Solomon A, Li DQ, Lee SB, Tseng SC. 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.
42. Chen JK, Tsai RJ, Lin SS. Fibroblasts isolated from human pterygia exhibit transformed cell characteristics. *In Vitro Cell Dev Biol Anim*. 1994;30A:243–248.
43. Li S, Hu GF. Emerging role of angiogenin in stress response and cell survival under adverse conditions. *J Cell Physiol*. 2012; 227:2822–2826.
44. Yang SF, Lin CY, Yang PY, Chao SC, Ye YZ, Hu DN. Increased expression of gelatinase (MMP-2 and MMP-9) in pterygia and pterygium fibroblasts with disease progression and activation of protein kinase C. *Invest Ophthalmol Vis Sci*. 2009;50:4588–4596.
45. Aspiotis M, Tsanou E, Gorezis S, et al. Angiogenesis in pterygium: study of microvessel density, vascular endothelial growth factor, and thrombospondin-1. *Eye (Lond)*. 2007;21: 1095–1101.
46. Chui J, Di Girolamo N, Coroneo MT, Wakefield D. The role of substance P in the pathogenesis of pterygia. *Invest Ophthalmol Vis Sci*. 2007;48:4482–4489.
47. Marcovich AL, Morad Y, Sandbank J, et al. Angiogenesis in pterygium: morphometric and immunohistochemical study. *Curr Eye Res*. 2002;25:17–22.
48. Newman AC, Nakatsu MN, Chou W, Gershon PD, Hughes CC. The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Mol Biol Cell*. 2011;22:3791–3800.
49. De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer*. 2008;123:2229–2238.

Investigative Ophthalmology & Visual Science