Conjunctival Expression of Toll-Like Receptor 3 Plays a Pathogenic Role in the Formation of Ultraviolet Light–Induced Pterygium

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Purpose. Toll-like receptor 3 (TLR3), as a damage-associated molecular pattern sensor, can detect self-RNA released from necrotic cells induced by ultraviolet B (UVB) radiation exposure. Pterygium formation is believed to be a tumorigenesis-like process induced by UVB exposure. In this study, we aimed to investigate the expression pattern of TLR3 in pterygium specimens and cultured pterygial epithelial cells (PECs).

Methods. Human pterygium and ipsilateral pterygium-free conjunctiva from the same patients were used in this study. The expression of TLR3 and nuclear factor-kappa B (NF-κB) was investigated in these specimens. PECs were exposed to UVB radiation to determine the effect of UVB on the expression of TLR3 and the activation of NF-κB.

Results. The immunofluorescence study showed stronger TLR3 expression in superficial epithelial cells in the pterygial epithelium in comparison with the normal conjunctival epithelium. The expression of TLR3 decreased in intensity from the superficial epithelium toward the basal cell layer, implying a correlation between UVB exposure and TLR3 expression. Differential TLR3 expression patterns in pterygial and conjunctival tissues were also found in quantitative PCR analyses. PECs after UVB irradiation had higher protein levels of TLR3 and phospho-NF-κB than those of the PECs without irradiation. Immunofluorescence studies showed that UVB irradiation induced the nuclear translocation of NF-κB in the PECs. In PECs with the targeted TLR3 gene silencing, the expression of phospho-NF-κB was not induced by UVB irradiation.

Conclusions. Our results indicate that UVB exposure, TLR3 expression, and NF-κB activation may be a critical sequence that leads to the formation of pterygium.

Keywords: pterygium, pterygial epithelial cells, Toll-like receptor 3, ultraviolet B, NF-κB

Pterygium is one of the most common corneal disorders in subtropical and tropical regions, with a higher prevalence in patients with prolonged exposure to ultraviolet (UV) radiation.[1–4] It resembles actinic disorders of other epithelial tissues that have proliferative changes resulting from radiation-activated fibroblasts.[4–7] Moreover, pterygium is believed to originate from the conjunctival tissue, especially the sunlight-exposed area.[8–10] Clinically, advanced pterygium encroaches the cornea and invades into the Bowman's layer, resembling the invasiveness of a neoplastic tumor.[11] P63, an important epithelial cell proliferation marker, was found to have higher expression at the advanced stage of pterygium than at the quiescent stage.[12]

Excessive exposure to solar radiation is a key risk factor for skin cancer, and the Toll-like receptor 3 (TLR3)-mediated cytokine response has been shown to have major implications for tumorigenesis.[13–15] Previous studies have shown that TLR3 mRNA is also expressed in corneal epithelial cells.[16] Because pterygium is a UV-related cell proliferation,[3] we surmise that the ultraviolet B (UVB)-related TLR3 pathway may be associated with the pathogenesis of pterygium, which may be similar to that of UVB-induced skin damage.[17] UVB irradiation can damage normal human skin epidermal keratinocytes and causes the cells to release intracellular noncoding RNAs, which become damage-associated molecular patterns (DAMPs) in the UVB-exposed tissues.[18] These RNAs, including U1 RNAs, may have a secondary structure comprised of double-stranded stem-loop regions and, similar to dsRNAs, can activate TLR3 and further stimulate the production of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6).[18,19] This process has been proposed to be the underlying mechanism of acute skin inflammation (i.e., sunburn).[18]

Like the skin, the ocular surfaces, including cornea and bulbar conjunctiva, also have a high opportunity for sunlight exposure.[20] In the present study, we investigated the

expression of TLR3, nuclear factor-kappa B (NF-κB), and p63 in pterygium in comparison with ipsilateral pterygium-free conjunctiva and correlated the expression results with the epithelial proliferation and UVB exposure to provide a molecular basis for the pathogenesis of pterygium formation.

Materials and Methods

Patients and Tissues

The study was approved by the Institutional Review Board and Ethics Committee of National Cheng Kung University Hospital. All surgeries were performed by two surgeons from October 20, 2013, to November 29, 2019, with pterygium excision and conjunctival autografting performed using fibrin glue. Specimens of pterygium and ipsilateral pterygium-free conjunctiva were taken from 45 patients during the surgical procedures. The conjunctival specimens were all harvested from the superior bulbar area. None of the patients had any other ocular surface diseases other than pterygium. None of them had a history of long-term topical eye drop use or an immunocompromised condition. Twenty-seven men and 18 women participated in this study, which was conducted according to the tenets of the Declaration of Helsinki. Written informed consent for publication was obtained from the patients.

Immunohistochemical Analysis

Formalin-fixed and paraffin-embedded tissue specimens were cut into 3-µm-thick sections. The samples were heated at 65°C for 30 minutes and then deparaffinized in xylene and rehydrated using concentrations of ethanol ranging from 100% to 75%. The slides were then washed three times for 3 minutes in PBS. For antigen retrieval, the samples were microwaved twice in 10-mM citrate (pH 6.0) for 10 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes. Nonspecific binding was blocked using a immunoblocking reagent with preformulated commercial buffers (BioTnA, Kaohsiung, Taiwan) for 30 minutes at room temperature. The samples were then reacted with rabbit polyclonal antibody anti-TLR3 (1:100, ab62566; Abcam, Cambridge, UK), anti-NF-κB (1:100, ab32536; Abcam), anti-p63 (1:100, ab735; Abcam), and anti-Ki-67 (1:100, AB_1661314; Spring Bioscience, Pleasanton, CA, USA) at 4°C overnight and were subsequently washed three times for 3 minutes with PBS. Then, the samples were detected with a Super Sensitive Polymer-HRP IHC Detection System (BioGenex, Fremont, CA, USA) according to the manufacturer's protocol. The slides were counterstained with hematoxylin before being mounted. The Mouse/Rabbit Double Stain Kit (with AEC/HRP Green, TADS03A; BioTnA) was utilized for the detection system. After the double-staining immunohistochemistry reaction, the p63, Ki-67, and NF-κB signals in the tissue samples were represented as brown, and the TLR3 signal was represented as green. Images were acquired with a C1si confocal microscope (Nikon, Tokyo, Japan) and analyzed with HistoQuest software (TissueGnostics, Vienna, Austria) to quantify the nuclear expression of NF-κB in both the pterygial and conjunctival specimens.

Pterygial Epithelial Cell Culture

Pterygium specimens were cut into small pieces (around 1 mm^3 in size) under a stereomicroscope, rinsed with PBS, and placed in a six-well plate with the epithelium up. Pterygial epithelial cells (PECs) with similar shapes grew out from the edges of the specimens after 3 days. Fibroblast contamination was minimized by removing the tissue when sufficient epithelial cells surrounded each explant. The epithelial cells were grown for up to three to seven passages before use. A purity of 98% was established using flow cytometry with cytokeratin antibodies. Conjunctival epithelial cells from ipsilateral pterygium-free conjunctiva were cultured in the same manner as the PECs.

UVB Irradiation of Cultured Cells

The epithelial cells were seeded at approximately 5×10^5 cells in a 60-mm culture dish (GeneDireX, Las Vegas, NE, USA) and grown in the presence of 10% fetal bovine serum–Dulbecco's modified Eagle medium. When the cells reached semiconfluence, the medium was aspirated, and the cells were washed three times with sterile PBS and left in a serum-free medium for 15 hours. This medium was replaced with PBS (2 mL), and the monolayers were irradiated with 20-mJ/cm^2 UVB light (G15T8E; SANKYO, Tokyo, Japan). The UVB light intensity was monitored and calibrated before each experiment with the aid of a radiometer/photometer (Solarmeter 6.0; Solar Light Company, Glenside, PA, USA). Some cells were incubated in PBS for an equivalent time without irradiation. After each exposure, the PECs were rinsed once with PBS and placed in 5 mL of PBS.

Western Blotting

After treatment, cells were lysed with a protein extraction solution containing protease inhibitors, and the protein concentrations were measured with a bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as the standard. Then, 30 µg of crude proteins were separated in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted overnight at 4°C with each primary antibody at the indicated dilution. The primary antibodies included TLR3 antibody (ab62566; Abcam), NF-κB p65 antibody (ab32536; Abcam), NF-κB (phosphor S536) antibody (ab76302; Abcam), and beta-actin antibody (ab8227; Abcam). The membranes were washed three times and exposed to horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Immunoreactive bands were detected using enhanced chemiluminescence with the Clarity Western ECL Substrate (170-5060; Bio-Rad, Hercules, CA, USA). The intensity of the bands was analyzed using the UVP Biospectrum imaging system (UVP LLC, Jena, Germany).

Quantitative Real-Time PCR

Total RNA was extracted from the pterygium and conjunctival tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). A reverse transcription kit (FYT501; Yeastern Biotech, Taipei City, Taiwan) was used for reverse transcription of total RNA (1 µg). SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) was used for the PCR according to the instructions supplied with the StepOne Real-Time PCR System (Applied Biosystems). Each mRNA expression was calculated as the relative expression with respect to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. All data are presented as fold changes

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against each control. Following are the gene primers used:

TLR3 forward: AGAGTTGTCATCGAATCAAATTAAAG
TKR3 reverse: AATCTTCCAATTGCGTGAAAA
p63 forward: TTGAGATTAGCATGGACTGTATCC
p63 reverse: GTTCTGAATCTGCTGGTCCAT
Ki-67 forward: CCAACCAAAAGAAAGTCTCTGG
Ki-67 reverse: TGATGGTTGAGGCTGTTCCT
GAPDH forward: AGCCACATCGCTCAGACAC
GAPDH reverse: GCCCAATACGACCAAATCC

Immunofluorescence

The epithelial cells were fixed and permeabilized in 4% paraformaldehyde and permeabilization buffer with 0.1% Triton X-100 and 0.05% SDS for 15 minutes, stained with antibodies to NF-κB p65 for 1 hour, and then incubated with Alexa Fluor 488 Conjugated Goat-anti-Rabbit Antibody (Thermo Fisher Scientific). The pterygium and conjunctival specimens were treated with the primary antibodies TLR3 (1:100, ab62566; Abcam) and p63 (1:50, ab735; Abcam) and then incubated at 4°C for 24 hours. The sections were incubated with Alexa Fluor 488 conjugated (1:200, A11008) and Alexa Fluor 594 (1:200, ab150116; Abcam) secondary antibodies at room temperature for 1 hour in a dark incubation chamber. The nuclei were visualized with 4′,6-diamidino-2-phenlindole (DAPI) staining. Images were acquired with a Nikon C1si confocal microscope and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) to quantify the mean fluorescence intensity in the cytoplasmic and nuclear regions of the cells.

Cell Proliferation Assay

The epithelial cells were seeded at a density of 2×10^4 cells/100 µL in 24-well plates and cultured overnight. The medium was changed, and the cells were then cultured in medium alone (control) or in medium containing various concentrations of polyriboinosinic–polyribocytidylic acid (poly[I:C]). After treatment, WST-1 reagent (630118; Clontech Laboratories, Mountain View, CA, USA) was added to each well, and the cells were incubated for another 2 hours at 37°C. The absorbance was determined using a microplate reader at a test wavelength of 450 nm.

Lentiviral-Based RNA Interference Transfection

The silencing of TLR3 expression in the primary PECs was performed via the lentiviral transduction of short hairpin RNAs (shRNAs). The shRNA clones in the pLKO.1 vector were provided by the National RNAi Core Facility (Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taipei, Taiwan). For cell infection, viral supernatants were supplemented with 8 µg/mL polybrene and incubated with the cells for 24 hours. The PECs were transduced by the lentiviral particles followed by puromycin selection (4 µg/mL) for 2 days. Cells that stably expressed shRNAs were maintained in puromycin (4 µg/mL). The target sequences for human TLR3 was 5′-CCTCTTCGTAACTTGACCATT-3′.

RNA-Seq Analysis

The purified RNA was used for preparation of the sequencing library by the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's recommendations. Briefly, mRNA was purified from total RNA (1 µg) by oligo(dT)-coupled magnetic beads and fragmented into small pieces under elevated temperature. The first-strand cDNA was synthesized using reverse transcriptase and random primers. After the generation of double-stranded cDNA and adenylation on the 3′ ends of DNA fragments, the adaptors were ligated and purified with the AMPure XP system (Beckman Coulter, Beverly, MA, USA). The quality of the libraries was assessed on a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) and a real-time PCR system. The qualified libraries were then sequenced on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA) with 150-bp paired-end reads generated by Genomics BioSci & Tech Co. (New Taipei City, Taiwan).

Statistical Analysis

All results are expressed as means ± standard errors of the mean (SEM). A Student's *t*-test was used for the analysis with SigmaPlot (Systat Software, San Jose, CA, USA). $P < 0.05$ was considered statistically significant.

Results

TLR3 Was Highly Expressed in Pterygium

The intensity of UVB radiation under direct exposure to the UVB light tube was 0.14 mW/cm^2. The UVB intensity decreased to only 0.05 mW/cm^2 under the same conditions when the detector was covered with part of a pterygial specimen (Supplementary Fig. S1). This indicates that the pterygium had a partial UVB-blocking effect.

An immunofluorescence study in the pterygial specimens of four patients revealed that TLR3 was highly expressed and was located mainly in the cytoplasm of the epithelial cells at stronger concentrations than in the tissue sections of ipsilateral pterygium-free conjunctiva harvested from the same eye ($P < 0.01$). It should be noted that the expression of TLR3 was strongest in the superficial epithelial layer and appeared to have a gradient indicating decreasing intensity from the superficial squamous epithelium toward the basal cell layer. In contrast, p63-positive cells were mainly distributed in the nuclei of the basal and suprabasal layers of the epithelium. A merged image of immunofluorescence pictures shows a comparison of the distribution tendency for the expression of TLR3 and p63 (Fig. 1A). Of note, some epithelial cells in the middle and superficial layers had a co-expression of TLR3 and p63. We hence analyzed the fluorescence intensity of nuclear p63 with HistoQuest analysis on the basal/suprabasal cell layer (blue) and the superficial epithelial layer (orange). We found that the pterygium, in general, had a higher expression of p63 in comparison with conjunctiva. The basal/suprabasal layer of pterygium had a markedly higher p63 expression when compared with the corresponding layer in ipsilateral pterygium-free conjunctiva and the superficial epithelial layer of pterygium (Fig. 1A, right lower panel).

Immunohistochemical staining was performed in tissues from eight patients. Similarly, we found a stronger expression of TLR3 in the cytoplasm of superficial epithelial cells as compared with the ipsilateral pterygium-free conjunctival epithelium (Fig. 1B). Consistent with the findings in immunofluorescent staining, we found that pterygium had a wider distribution of p63 immunostaining on all layers

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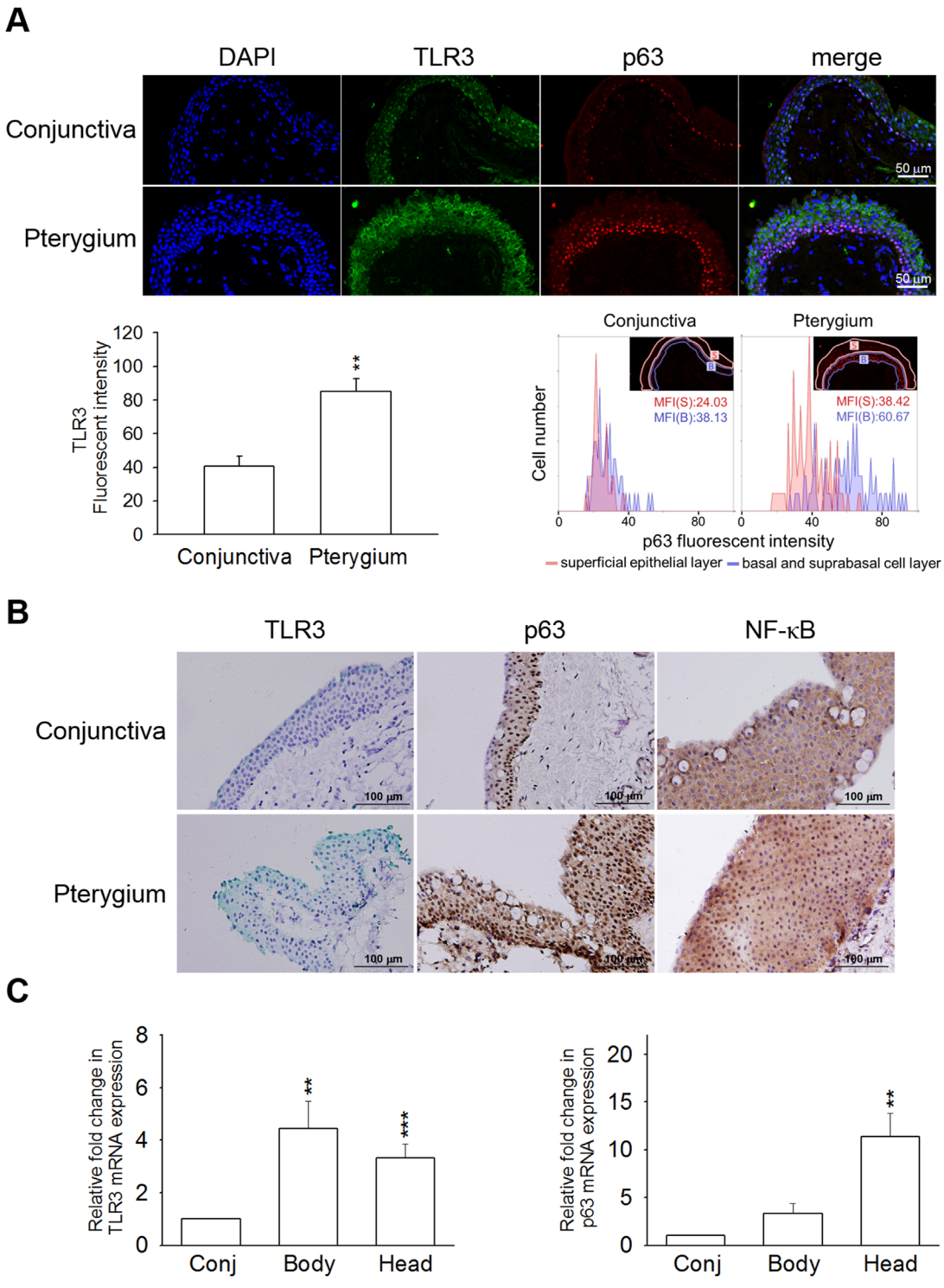

Figure 1. (A) Representative images of immunofluorescence staining of TLR3 (*green*) and p63 (*red*) in human pterygium and ipsilateral pterygium-free conjunctiva from the superior bulbar area. *Scale bar*: 50 µm. The nuclei of the cells were stained with DAPI (*blue*). Four images from each experiment were taken with a Nikon C1si confocal microscope, and the fluorescence intensities were quantified. The cytoplasmic expression of TLR3 was analyzed with ImageJ software to quantify the mean fluorescence intensity (MFI) in the cytoplasmic regions of the cells ($n = 4$; $^{**}P < 0.01$ vs. the conjunctiva). The distributive pattern of nuclear p63 was analyzed based on the fluorescence intensity of the nuclear regions of the cells in two different layers, the basal/suprabasal cell layer (*blue*) and the superficial epithelial layer (*orange*). Compared to the conjunctival tissue, the cells in the basal/suprabasal cell layer (*blue area*) exhibited stronger p63 staining, and more cells in the superficial epithelial layer (*orange area*) exhibited p63 staining in the pterygial tissue. (B) Representative images of immunohistochemical staining of TLR3 (*green*), p63 (*brown*), and NF-κB (*brown*) in human pterygium and ipsilateral pterygium-free conjunctiva. *Scale bar*: 100 µm. Four images from each experiment were taken with a Nikon C1si confocal microscope, and the nuclear expression of NF-κB was analyzed and calculated using HistoQuest software ($n = 8$). (C) Quantitative PCR analysis of TLR3 and p63 in human pterygium and ipsilateral pterygium-free conjunctiva from the superior bulbar area ($n = 5$; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the conjunctiva). Comparisons were made using Student's *t*-test. Data are presented as means ± SEM. All experiments were repeated with similar results. Conj, conjunctiva; Body, body portion of pterygium; Head, head portion of pterygium.

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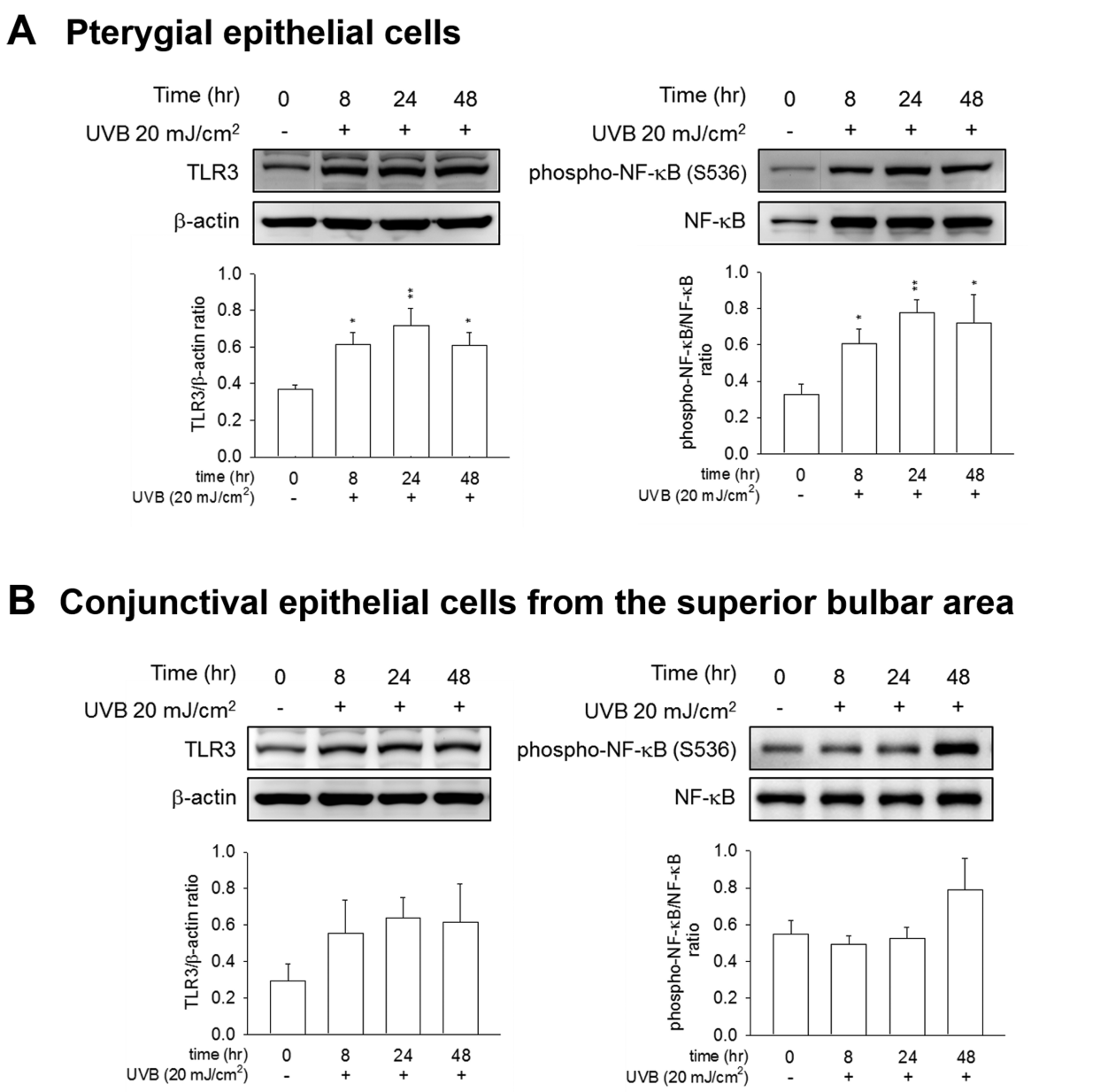

Figure 2. Western blotting analysis of TLR3 and phospho-NF-κB in cultured PECs (A) and in conjunctival epithelial cells (B) without UVB exposure (control groups) and those in PECs and CECs 8, 24, and 48 hours after each UVB irradiation (20 mJ/cm^2) ($n = 6$ in PECs; $n = 3$ in CECs; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the control groups). Comparisons were made using Student's t-test. Data are presented as means ± SEM. All experiments were repeated with similar results. Time, hours after UVB irradiation.

of the epithelium, with stronger staining on the basal and suprabasal layers, whereas ipsilateral pterygium-free conjunctiva had weaker staining of p63 on all layers.

We also investigated the expression of the proliferation marker Ki-67 along with TLR3. We found that, similar to p63, Ki-67 showed a higher expression in the pterygium compared with the ipsilateral pterygium-free conjunctiva. The double staining for TLR3 and Ki-67 showed distinctive distribution patterns in their expression, with stronger TLR3 staining in the superficial pterygial epithelium and stronger Ki-67 staining in the basal and suprabasal epithelial cells. Different from the wide distribution of p63 in the pterygium, the expression of Ki-67 was confined to the basal and suprabasal cell layers of the epithelium (Supplementary Fig. S2A).

A quantitative PCR (qPCR) study of five patients showed that the pterygium had higher TLR3 expression than did the ipsilateral pterygium-free conjunctiva from the superior bulbar area in both the head portion (the part of the pterygium invading the cornea) and the body portion (the part of the pterygium covering the sclera) of the same pterygium. However, the head portion, which is thought to have greater proliferative ability, showed stronger expression of p63 than was the case for the body portion, which is thought to be more silent; the ipsilateral pterygium-free conjunctiva had the weakest expression of p63 (Fig. 1C). Similarly, qPCR analysis of Ki-67 also showed the highest expression level in the head portion of the pterygium, followed by the body portion and normal conjunctiva (Supplementary Fig. S2B), even though it did not reach statistical significance.

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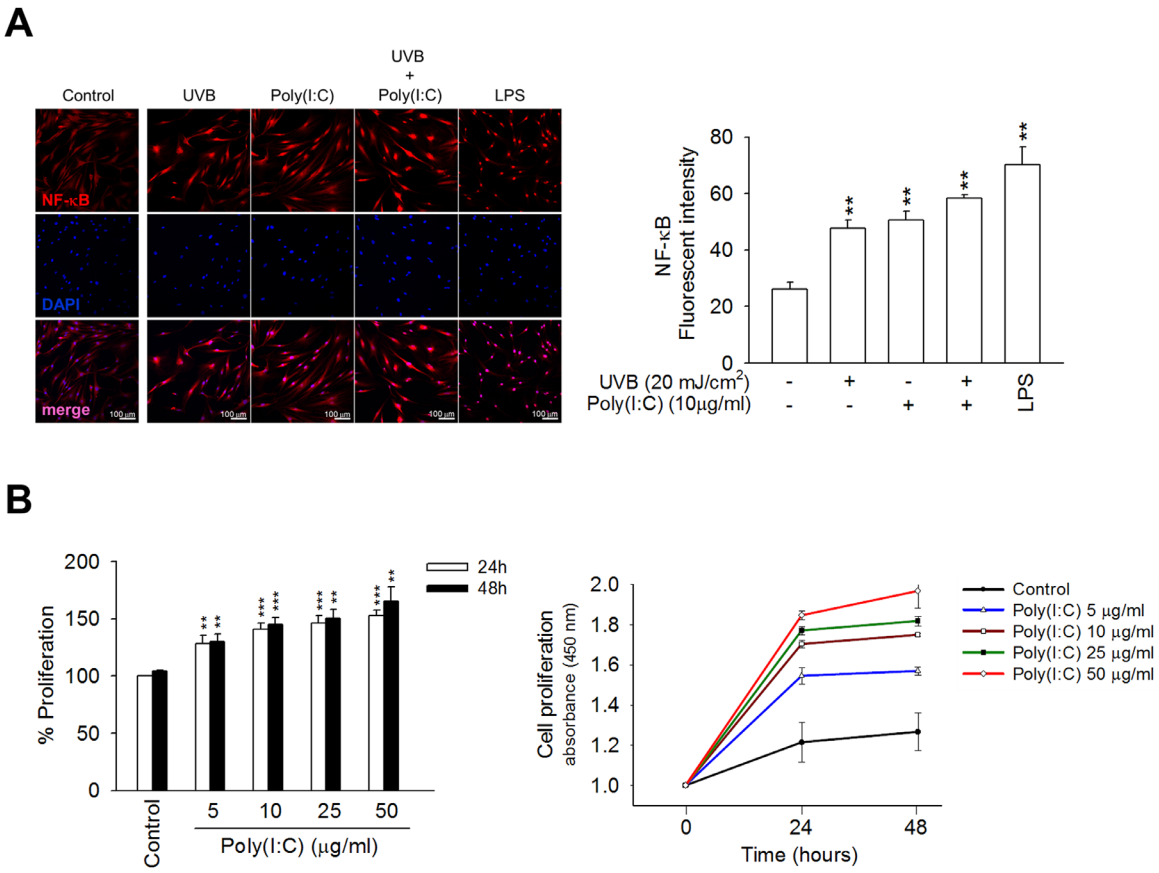

Figure 3. (A) Immunofluorescence study of NF-κB (*red*) in cultured PECs without UVB exposure (control group) and in PECs 24 hours after UVB irradiation (20 mJ/cm^2) or after pretreating with poly(I:C). *Scale bar*: 100 µm. The nuclei of the cells were stained with DAPI (*blue*). Four images from each experiment were taken with a Nikon C1si confocal microscope, and the fluorescence intensities were quantified. The nuclear expression of NF-κB was analyzed with ImageJ software to quantify the mean fluorescence intensity in the nuclear regions of the cells. The nuclear translocation of NF-κB induced by lipopolysaccharides (LPS) is shown here as a positive control ($n = 3$; $^{**}P < 0.01$ vs. the control group). (B) WST-1 cell proliferation assay for the cultured PECs after the addition of various concentrations of poly(I:C) (5, 10, 25, and 50 µg/mL) after incubation for 24 and 48 hours. Poly(I:C), a TLR3 ligand, enhanced cell proliferation in a dose-dependent manner ($n = 4$; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the control). Comparisons were made using a Student's *t*-test. Data are presented as means ± SEM. All experiments were repeated with similar results.

Nuclear Localization of NF-κB in Epithelial Cells of Pterygium

An immunohistochemistry study was performed on eight patients to analyze the percentage of nuclear expression of NF-κB in each tissue section (Fig. 1B). Four images of each experiment were taken with a Nikon C1si confocal microscope, and the nuclear expression of NF-κB was analyzed and calculated with HistoQuest software in these patients. The expression of NF-κB was mostly distributed in the nuclei of the pterygial epithelium, whereas NF-κB was expressed to a much greater degree in the cytoplasm of ipsilateral pterygium-free conjunctiva. The averaged proportion of nuclear localization of NF-κB was 80.69% in the pterygium and 42.2% in the conjunctiva ($n = 8$; $P < 0.01$).

UVB Irradiation Induced Upregulation of TLR3 and Nuclear Translocation of NF-κB in Pterygial Epithelial Cells

The cultured PECs and conjunctival epithelial cells (CECs) were exposed to UVB radiation (20 mJ/cm^2). A western blotting analysis showed a higher expression of TLR3 in the PECs after UVB irradiation than in the PECs without UVB irradiation. The expression of phospho-NF-κB in the PECs also increased after UVB irradiation. The peak levels of TLR3 and phospho-NF-κB were observed 24 hours after irradiation in the PECs (Fig. 2A). TLR3 expression also increased after UVB irradiation in the CECs but did not reach statistical significance compared with the CECs without irradiation. However, the expression of phospho-NF-κB in the CECs remained unchanged 24 hours after UVB irradiation but increased 48 hours after UVB irradiation, although this did not reach statistical significance (Fig. 2B).

An immunofluorescence study showed that the nuclear translocation of NF-κB significantly increased in the PECs 24 hours after UVB irradiation (20 mJ/cm^2) as compared with the PECs without UVB irradiation ($P < 0.01$). Pretreatment with the synthetic analog poly(I:C) (10 µg/mL), a TLR3 ligand, also significantly promoted the nuclear translocation of NF-κB in the PECs (Fig. 3A). When the PECs were pretreated with poly(I:C) and then irradiated with UVB, the combination appeared to have a tendency to enhance nuclear translocation of NF-κB in comparison with poly(I:C) only ($P = 0.138$) or UVB irradiation alone ($P = 0.0678$) (Fig. 3A).

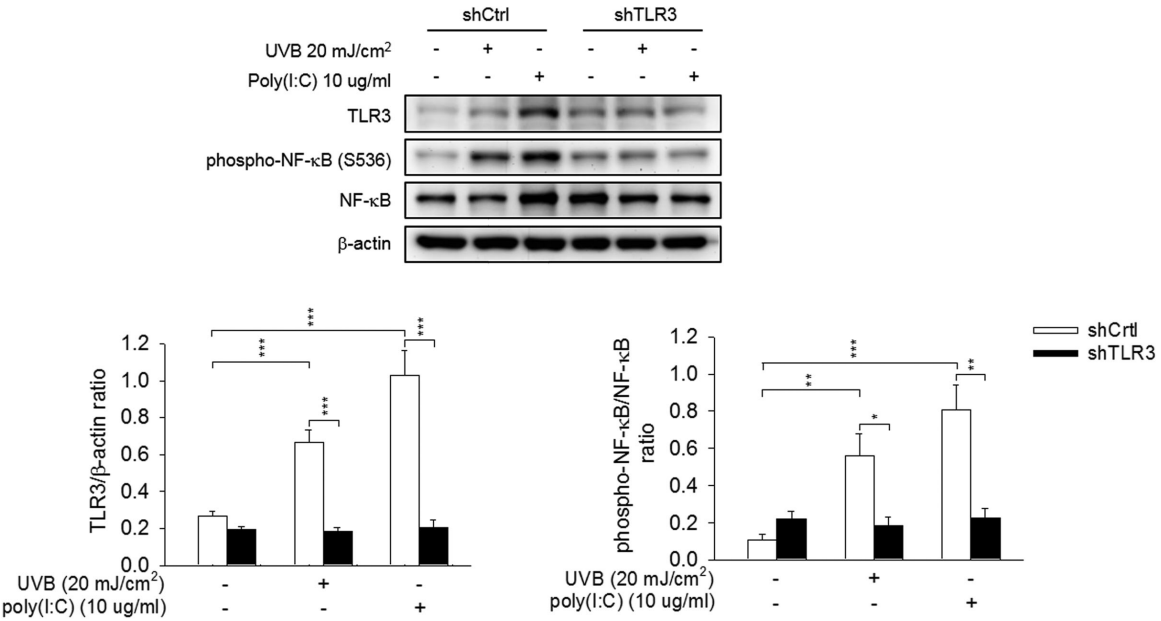

Figure 4. Western blotting analysis of TLR3 and phospho-NF-κB in cultured PECs with TLR3 knockdown using shTLR3 transfection and in PECs without TLR3 knockdown using non-targeting shRNA (shCtrl, control group) transfection. The expression of TLR3 and phospho-NF-κB was analyzed in these cells (shTLR3 and shCtrl) 24 hours after UVB irradiation (20 mJ/cm^2) or 24 hours after treatment with poly(I:C) (10 µg/mL) ($n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Comparisons were made using Student's t-test. Data are presented as means ± SEM. All experiments were repeated with similar results.

TLR3 Ligand Promoted Proliferation of PECs

Various concentrations of poly(I:C) (5, 10, 25, and 50 µg/mL) were added to cultured PECs followed by incubation for 24 and 48 hours. The WST-1 cell proliferation assay showed that poly(I:C) enhanced the cell proliferation in the PECs in a dose-dependent manner after 24 or 48 hours ($P < 0.01$) (Fig. 3B).

UVB-Induced Phosphorylation of NF-κB in PECs Was Dependent on TLR3 Pathway

The TLR3 expression in the PECs was suppressed using TLR3 shRNA (shTLR3) transfection, and the PECs without TLR3 suppression were transfected with non-targeting shRNA followed by puromycin selection and cloning. Both PECs with the targeted suppression of TLR3 and those without suppression were then exposed to UVB radiation (20 mJ/cm^2). A western blotting analysis showed that the expression of TLR3 increased 24 hours after UVB irradiation in the PECs without TLR3 suppression ($P < 0.001$), whereas PECs with TLR3 suppression were not induced to increase the expression of TLR3 after UVB irradiation ($P < 0.001$). In the PECs without TLR3 suppression, the expression of phospho-NF-κB was induced by either UVB radiation (20 mJ/cm^2) or poly(I:C) (10 µg/mL) 24 hours after treatment ($P < 0.01$). In contrast, the expression of phospho-NF-κB was not induced by either UVB radiation or poly(I:C) in PECs with TLR3 suppression as compared with the PECs without TLR3 suppression ($P < 0.05$) (Fig. 4).

Discussion

UVB exposure to skin can cause sunburn, carcinogenesis, and melanogenesis.[13,18,21] Some ocular surface diseases, including actinic conjunctivitis and pterygium, are also well known for being related to sunlight exposure.[20] Pterygium usually arises from bulbar conjunctiva, which is the most sunlight-exposed area on the eye ball.[2] Unlike the skin, conjunctiva is a non-keratinized, stratified squamous epithelium; however, both skin and conjunctiva are first-line barriers to environmental stimuli, including solar radiation.[11,22,23] In the present study, a stronger expression of TLR3 was found in the epithelium of the pterygial tissue than in the pterygium-free conjunctiva, suggesting that TLR3 may play a role in the pathogenesis of pterygium that may be similar to the role of TLR3 in UVB-induced skin damage.[18,24] The expression of TLR3 was mostly distributed in the superficial epithelial cells and decreased in intensity from the superficial squamous epithelium toward the basal cell layer, implying a positive relationship between UVB radiation and TLR3 because UVB from sunlight mostly damages surface cells.

UVB exposure was postulated to result in tumorigenesis in the skin and induce the formation of pterygium on ocular surface.[25–27] Interestingly, a previous study showed that the patients with pterygium were 1.64-fold more likely to develop non-melanoma skin cancer than those without pterygium,[20] implicating the role of UV exposure in both diseases. UVB light can cause superficial epithelial cell necrosis.[23] These necrotic cells may release some DAMPs, which in turn induces the upregulation and activation of TLR3 in neighboring live epithelial cells, which are also located on the superficial layer of the epithelium.[24] The activation of TLR3 can induce the production of inflammatory mediators, such as IL-6 and TNF-α.[16] Under the effects of chronic inflammation, the proliferative ability of basal cells may be enhanced via the upregulation of p63, which in turn induces uncontrolled cellular proliferation, leading to the growth and progress of pterygium.[12]

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Pterygium is postulated to be a stem cell proliferative disorder. A stem-cell marker of squamous epithelium, p63 is known to be a key regulator for keratinocyte differentiation and proliferation. Moreover, p63 was found to be specifically expressed by stem cells of human keratinocyte.[28,29] In many studies, p63 has been found to be expressed in both pterygium and normal conjunctiva.[30,31] However, pterygium has more widely distributed p63 not only in basal and suprabasal cell layers but also in the lower two-thirds of epithelium or even throughout all epithelial layers.[32] Consistent with this result, our immunofluorescence and immunohistochemistry studies showed stronger expression and wider distribution of p63 in the pterygium than in the ipsilateral pterygium-free conjunctiva. Our finding of many more cells expressing p63 staining in the superficial epithelial layer of pterygial tissue indicated that the proliferative activity in the pterygium occurred not only in the basal/supra-basal cell layer but also in the superficial epithelial layer. The co-expression of TLR3 and p63 in some epithelial cells in the middle and superficial layers suggests a biological crosstalk between these two molecules. Therefore, we surmise that the activation of TLR3 in the superficial epithelial layer may pass signals to induce the production of p63 in both the basal/suprabasal cell layer and the superficial epithelial layer in a paracrine or autocrine manner.

Surgical excision is still the mainstay of treatment for pterygium; however, with simple excision leaving a bare sclera, the recurrence risk has been reported to be as high as 80%. Pterygium excision combined with a tissue graft covering the bare sclera, such as conjunctival autograft, has a lower risk of recurrence.[11] We believed that, with a coverage of tissue graft on the bare sclera, direct sunlight exposure to the original pterygium area would be greatly reduced, which in turn would reduce the risk of pterygium recurrence. In the present study, both the head and body portions of the pterygial specimens had stronger expression of TLR3 than was the case in the ipsilateral pterygium-free conjunctiva in the qPCR study. The results indicated that both the head and body portions of the pterygium on the ocular surface had similar exposure to UVB radiation from sunlight. However, the ipsilateral pterygium-free conjunctiva harvested from the superior bulbar conjunctiva, which was covered by the upper eyelids, had a lower chance of being exposed to sunlight during daily activities. We believe this is the reason why the ipsilateral pterygium-free conjunctiva had lower expression of TLR3. We also found that the head portion had stronger expression of p63 than was the case in either the body portion or the ipsilateral pterygium-free conjunctiva. As the head portion of the pterygium is the area that encroaches on the cornea and is generally thought to have greater invasive and proliferative ability, whereas the body portion is located on the sclera and is believed to be more dormant,[11,12] our results indicated that p63 may play a role in the invasiveness and aggressiveness of a pterygium.

UV irradiation can activate NF-κB and induce subsequent release of IL-1, IL-6, and TNF-α from cultured human corneal epithelial cells.[33] TNF-α and IL-6 are both the downstream elements of TLR3.[18,19] Phospho-NF-κB is a protein involved in NF-κB heterodimer formation, nuclear translocation, and activation, and Siak et al.[10] first found involvement of the NF-κB signaling pathway in the pathogenesis of pterygium. For this reason, we investigated the expression of TLR3 and phospho-NF-κB in cultured PECs and found that their peak levels both occurred 24 hours after UVB irradiation, indicating a correlation between the upregulation of TLR3 and the activation of NF-κB. When we irradiated the PECs pretreated with poly(I:C), the NF-κB translocation was enhanced more than was the case for the PECs either treated with poly(I:C) only or irradiated with UVB alone. Taken together, our results indicate that UVB radiation induces TLR3 upregulation and that poly(I:C) induces the nuclear translocation of NF-κB by binding and activating its receptor, TLR3. Promotion of the nuclear translocation of NF-κB by either UVB radiation or TLR3 activation has been proven in the literature[18,24]; however, to our knowledge, our study is the first to link the correlation between UVB radiation and TLR3 to the nuclear translocation of NF-κB in PECs. In addition, using a WST-1 assay, we found that poly(I:C) promoted proliferation of PECs, and the findings indicate that cellular proliferation of pterygium may be enhanced by TLR3 activation. To directly address the response of PECs and CECs to UVB irradiation, we investigated the expression of p63 in cultured cells after UVB irradiation (20 mJ/cm^2), but it did not show a significant increase of p63 (Supplementary Fig. S3). This could be because the production of p63 after UVB irradiation may take a longer time, just like the tumorigenesis process.

The ocular surface epithelium serves as the defensive front line of the innate immune system; therefore, different types of TLRs are expressed on normal and diseased ocular surfaces for the defense against microbial invasion.[34,35] Data from our RNA-seq analysis showed that the TLR3 gene has the highest expression among those TLRs significantly expressed in the pterygial tissue (Supplementary Fig. S4), consistent with the suggestion that UVB-induced phosphorylation and nuclear translocation of NF-κB may depend on the TLR3 pathway in PECs. In the present study, we further provided direct evidence supporting the observation that suppression of TLR3 stimulation may be an effective means of non-surgical intervention for inhibiting pterygium formation and recurrence.

Conclusions

In the present study, pterygium had higher expression of TLR3, p63, and nuclear NF-κB than did ipsilateral pterygium-free conjunctiva. Furthermore, UVB radiation induced the upregulation of TLR3 and the nuclear translocation of NF-κB in PECs. The knockdown of TLR3 suppressed UVB-induced phosphorylation of NF-κB in PECs. Based on these observations, we suggest that TLR3 is an important contributor to the pathogenesis and progression of UV-related pterygium, in which the TLR3 in the superficial epithelial cells of the ocular surface may recognize some DAMPs, such as self-noncoding RNA, released from necrotic cells damaged by UV light. Then, the activation of TLR3 induces the nuclear translocation and activation of NF-κB, in turn promoting the uncontrolled proliferation of pterygial cells via the production of proliferative proteins such as p63. TLR3 and p63 may have a causal relationship during the differentiation of conjunctival cells and pterygial epithelial cells; however, the distributive correlation between TLR3 and p63 could not be fully elucidated in this study. More studies are thus needed to verify the relationship between the proliferative activity of the pterygium and the expression of TLR3.

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