The Nuclear-Factor κB Pathway Is Activated in Pterygium

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PURPOSE. Pterygium is a prevalent ocular surface disease with unknown pathogenesis. The authors investigated the role of nuclear factor kappa B (NF-κB) transcription factors in pterygium.

METHODS. Surgically excised primary pterygia were studied compared with uninvolved conjunctiva tissues. NF-κB activation was evaluated using Western blot analysis, ELISA, and DNA-binding assays. Primary pterygium fibroblasts were treated with TNF-α (20 ng/mL), and NF-κB activation was evaluated using immunocytochemistry. Western blot analysis, phospho-IκB ELISA, and DNA-binding assays. TNF-α stimulation of NF-κB target genes RelB, NFκB2, RANTES, MCP-1, ENA-78, MMP-1, MMP-2, and MMP-3 in pterygium fibroblasts was compared with that in primary tenon fibroblasts by real-time PCR.

RESULTS. Phosphorylation of IκBα (Ser32) was increased in pterygium tissues compared with uninvolved conjunctiva tissues, as determined by Western blot analysis and ELISA. IκBα expression was decreased, whereas nuclear ReLA and p50 DNA-binding capacities were increased. Within 30 minutes of treatment with TNF-α, pterygium fibroblasts showed increased IκBα phosphorylation and nuclear translocation of ReLA and p50. Treatment with TNF-α beyond 12 hours resulted in increased nuclear expression of ReLB, p100, and p52. Furthermore, the upregulation of RANTES, MCP-1, ENA-78, MMP-1, MMP-2, and MMP-3 expression was more pronounced in TNF-α-treated pterygium fibroblasts than in tenon fibroblasts.

CONCLUSIONS. The NF-κB pathway is shown for the first time to be activated in pterygia tissues compared with normal conjunctiva tissues. Stimulation by the inflammatory cytokine TNF-α can activate both canonical and noncanonical NF-κB pathways in pterygium fibroblasts with concomitant upregulation of NF-κB target genes. (Invest Ophthalmol Vis Sci. 2011;52: 230–236) DOI:10.1167/iovs.10-5735

Pterygium is a prevalent human ocular surface disease that manifests as a fibrovascular conjunctival outgrowth that encroaches the clear cornea in a centripetal fashion. It can lead to reduced vision when it involves the visual axis or to induced astigmatism,1 ocular irritation, and dryness.2 The exact pathogenesis of this condition is not understood, but processes leading to extracellular matrix dysfunction3 are thought to be involved. Epidemiologic studies2,4–6 have linked pterygium formation to various chronic inflammatory insults such as ultraviolet irradiation,4 sawdust exposure,7 and dry eye disease.2,6 However, the molecular mechanism, which may be common to all these inflammatory conditions, involved in the pathogenesis or progression of pterygium has yet to be elucidated.

Inflammatory stimuli induce many genes containing an important cis-acting regulatory sequence in their enhancers that binds members of the nuclear factor kappa B (NF-κB) family of transcription factors. Indeed, the NF-κB transcription factors are central mediators of cell stress.7 Five distinct NF-κB subunits are known,8 namely NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and c-Rel. Homodimers or heterodimers are formed in the cytoplasm, and differential activation of these dimers is involved in mediating specific target gene expression in response to various extracellular stimuli.8 For example, RelA/p50 dimers are involved in the canonical NF-κB pathway, which is activated as part of the acute inflammatory response to stimuli such as tumor necrosis factor (TNF)-α and interleukin (IL)-1. RelB/p52 dimers are involved in the noncanonical NF-κB pathway, which typically produces a delayed inflammatory response.10 Dimers are sequestered in the cytoplasm in resting cells through binding to inhibitors of κB (IκB) proteins.11 In the canonical pathway, stimulation by cytokines such as TNF-α results in IκB kinase (IKK) complex activation, which, in turn, leads to the phosphorylation and proteasomal degradation of IκB proteins (classically IκBα), thereby releasing RelA/p50 dimers into the nucleus.12 In the noncanonical pathway, stimulation by ligands such as lymphotoxin (LT)β results in NF-κB-inducing kinase being activated, which then mediates the processing of p100 into p52 in RelB/p100 dimers, leading to RelB/p52 nuclear translocation.12 Although differences in activating stimuli and participating proteins exist between the canonical and noncanonical pathways, considerable cross-talk has been reported.13 Cells control various biological processes such as proliferation, apoptosis, differentiation, and migration through the NF-κB pathway through the expression of more than 200 genes.7 Important target genes that may play a part in pterygium pathogenesis include chemokines such as epithelial neutrophil activating peptide 78 (ENA-78 or CXCL5), monocyte chemotactic protein 1 (MCP-1 or CCL2), and regulated on activation, normal T cell expressed and presumably secreted...
(RANTES or CCL5), which recruit neutrophils, monocytes, and lymphocytes during inflammation. Other target genes include matrix metalloproteinases (MMPs) because excessive fibroblast proliferation and invasion occur at the head of pterygia with Bowman’s membrane and cornea stroma destruction. To date, there has been no report on the regulation of target genes by NF-κB in pterygium.

We hypothesized that inflammation is involved in the pathogenesis of pterygium through the excessive activation of the NF-κB pathways. In the present study, we investigated the activation level of NF-κB in pterygia tissue compared with normal conjunctiva. We also examined the in vitro NF-κB response of primary pterygium fibroblasts to recombinant human TNF-α stimulation. The expressions of NF-κB target genes RANTES, MCP-1, ENA-78, MMP-1, MMP-2, and MMP-3 in pterygium fibroblasts compared with control tenon fibroblasts were also analyzed.

**PATIENTS AND METHODS**

**Patients and Specimens**
Primary pterygium and uninvolved superior temporal bulbar conjunctiva were obtained from patients who underwent pterygium excision and conjunctiva autograft surgery at the Singapore National Eye Centre (Singapore). Paired tissues were obtained from 20 patients. Superotemporal subconjunctival tenon tissues were also obtained during extra-capsular cataract extraction performed on three healthy patients without signs and symptoms of any ocular surface disorder for establishment of tenon fibroblasts cultured as normal controls. All protocols adhered to the tenets of the Declaration of Helsinki and were approved by the institutional review board of the Singapore Eye Research Institute. Written informed consent was acquired from all participating patients.

**Cell Cultures**
All tissue-culture reagents were from Gibco (Invitrogen Corporation, Carlsbad, CA) unless otherwise indicated. Primary pterygium fibroblast cultures were established using explants of pterygium head. Primary tenon fibroblasts cultures were established from explants of normal human tenon tissue. Specimens were first dissected into approximately 2 × 2-mm blocks and were kept in sterile phosphate-buffered saline (PBS) containing penicillin (100 U/mL), streptomycin (0.1 mg/mL), and amphotericin B (0.25 μg/mL; Sigma-Aldrich, St. Louis, MO) for 15 minutes before transfer to cell culture dishes with stromal aspect face down. Ten minutes later, each explant was covered with 1 drop of Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and placed overnight in an incubator at 37°C under 95% humidity with 5% CO₂. No additional supplements were added to the culture medium. One milliliter medium was added the next day, and the medium was changed every 2 days thereafter. By approximately 10 to 14 days, fibroblast culture in 60-mm cell culture plates were approximately 80% confluent, and they were subcultured into T-75 flasks and subsequently expanded in T-175 flasks at approximately 80% confluence. Fibroblasts of no more than six passages were used in this study.

For experiments requiring stimulation with TNF-α, cells were pre-incubated in serum-free DMEM overnight before treatment with 20 ng/mL recombinant human TNF-α (R&D Systems, Minneapolis, MN) in fresh serum-free DMEM.

**Protein Extraction**
Total protein extracts from conjunctiva and pterygium tissues were obtained by snap-freezing tissues in liquid nitrogen, followed by grinding using a pestle. Extraction buffer (T-PER; Pierce, Thermo Fisher Scientific Inc, Rockford, IL) was then added, and the tissue suspension was passed through a 22-gauge needle five times. The protein lysate was then centrifuged at 10,000g for 15 minutes at 4°C. Both total lysates and nuclear protein lysates from cultured cells were obtained (Nuclear Extract Kit; Active Motif, Carlsbad, CA) according to manufacturer’s instructions, and the protein concentration of all samples was determined (Coomassie Plus Assay; Pierce).

**Western Blot Analysis**
Each protein sample (10 μg) was electrophoresed under reducing conditions in 12% SDS-polyacrylamide gel. The resolved proteins were then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes at 4°C that were incubated overnight with primary antibodies to ReLA (4764; Cell Signaling Technology, Danvers, MA), p105/p50 (3035; Cell Signaling Technology), IkBα (9242; Cell Signaling Technology), phospho-IkBα (Ser32) (9246; Cell Signaling Technology), p100/p52 (3017; Cell Signaling Technology), RelB (AB35917; Abcam Inc., Cambridge, MA), β-actin (A1978; Sigma), TATA-binding protein (AB818; Abcam). Membranes were then incubated with secondary antibodies conjugated to horseradish peroxidase (HRP; Pierce) at room temperature. Immunoreactivity was visualized with chemiluminescence substrate (SuperSignal West Pico or West Dura; Pierce). Membranes were stripped by incubation in a buffer containing 15 g/L glycine, 1 g/L sodium dodecyl sulfate, and 10 mL/L Tween 20, pH 2.2, for 5 to 10 minutes at room temperature before reprobing with another antibody.

**Phosphorylated IkBα ELISA**
Total protein extracts were obtained as described with the exception of the buffer (Complete Lysis Buffer; Active Motif, Carlsbad, CA) supplied with the assay kit (Function ELISA IkBα; Active Motif) instead of T-PER. The assay was performed according to the manufacturer’s protocol.

**NF-κB Consensus DNA-Binding Assay**
NF-κB DNA binding was assessed with an assay kit (TransAM Flexi NF-κB Family Assay Kit; Active Motif) according to the manufacturer’s protocol. Briefly, 5 μg nuclear protein for each sample was loaded into each well, which was precoated with consensus binding site oligonucleotide. Bound NF-κB probes were probed with primary antibodies against ReLA, p50, RelB, or p52 and then probed with HRP-conjugated secondary antibodies. On addition of the substrate, the intensity of the colorimetric reaction was read using a microplate reader (TECAN Genios Pro; MTX Laboratory Systems, Inc., Vienna, VA) at an absorbance of 450 nm.

**Immunocytochemistry**
Cells grown on eight-well chamber slides (Laboratory-Tek II; Nunc, Thermo Fisher Scientific, Rochester, NY) were fixed with 4% paraformaldehyde for 15 minutes at room temperature after cytokine treatment, washed with 1× PBS, blocked with 10% normal goat serum in 1× PBS containing 0.3% Triton X-100 (PBS-T) for 1 hour at room temperature, and incubated with ReLA antibody (4764; Cell Signaling Technology) or RelB antibody (AB35917; Abcam Inc.) for 2 hours at room temperature. After washing with PBS-T, the cells were incubated with AlexaFluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA) at room temperature for 1 hour. After mounting (UltraCruz Mounting Medium, SC-24941; Santa Cruz Biotechnology, Inc. Santa Cruz, CA), cells were visualized and imaged with a fluorescence microscope (Axioplan 2; Zeiss, Oberkochen, Germany).

**Real-Time Polymerase Chain Reaction**
Total RNA was prepared with a purification kit (RNasy Mini Kit; Qiagen Inc., Valencia, CA) according to manufacturer’s protocol. RNA concentration was determined with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Wilmington, DE). Reverse transcription of 500 ng RNA for each sample was performed using primer (Oligo(dT) 15; Promega, Madison, WI) and polymerase (Superscript II; Invitrogen) according to the manufacturer’s protocol. Real-time poly-
were unable to demonstrate a significant difference in nuclear RelB and p52 levels in our tissue samples using consensus DNA-binding assay. In vivo steady state nuclear levels of these transcription factors may be too low for this assay to detect a statistically significant difference.

**Phosphorylation of IκBα Is Increased in Pterygium Compared with Normal Conjunctiva**

Expression of the NF-κB inhibitor IκBα and its phosphorylated counterpart, p-IκBα (Ser32), in pterygia compared with normal conjunctival tissue was examined by immunoblot analysis. As shown in Figure 2A, total IκBα was lower in pterygium tissue than in normal conjunctival tissue. Conversely, there was greater expression of p-IκBα (Ser32) in pterygia tissue than in normal conjunctival tissue. We confirmed the Western blot analysis by quantifying the level of p-IκBα by ELISA. As shown in Figure 2B, there was a 2.6-fold higher level of phospho-IκBα in pterygium compared with normal conjunctiva. These data suggest that there is greater IκBα degradation and therefore higher NF-κB activation in pterygia.

**TNF-α Activates NF-κB in Pterygium Fibroblasts In Vitro**

To elucidate whether the NF-κB pathway is activated in response to the inflammatory stimulus by TNF-α in pterygium, we performed experiments on fibroblasts derived from disease samples. The nuclear localization of RelA and RelB in pterygium fibroblasts on treatment with TNF-α was visualized by immunofluorescence. At 30 minutes after stimulation with TNF-α, RelA could be observed to translocate to the nucleus (Fig. 3A). This contrasted with untreated cells, which showed a predominantly cytosolic localization for RelA. Untreated fibroblasts also had a very low RelB level. After 24 hours of TNF-α stimulation, increased nuclear RelB level could be observed (Fig. 3B). These observations suggested that RelA was activated in pterygium fibroblasts in response to acute stimulation by TNF-α, whereas nuclear RelB expression was increased after prolonged TNF-α stimulation.

To further corroborate this observation, we performed Western blot analysis of the nuclear expression of RelA and p50 in response to TNF-α. As shown in Figure 3C, nuclear RelA expression was increased 30 minutes after TNF-α stimulation, and this increase was sustained 1 hour after stimulation. Nuclear p50 expression showed the same profile. In contrast, nuclear p105 protein was reduced at these two time points. Given that functional p50 is produced by adenosine triphosphate-dependent polyprotein of p105, the reduced levels of p105 may indicate increased p105 processing to p50. We also examined the expression patterns of RelB, p100, and p52. No increase in RelB and p52 was seen after 1 hour of TNF-α stimulation.
treatment (data not shown). Increased nuclear RelB, p100, and p52 were instead observed as a delayed response after 12 hours of TNF-α/H9251 treatment (Fig. 3D). The amount of nuclear p100 showed a progressive decrease from 12 hours to 24 hours and finally 48 hours of stimulation with TNF-α/H9251. The corresponding increase in the expression of nuclear p52 reflected the processing of p100, a precursor of p52, to its functional p52 form (Fig. 3D). In addition, because RelB and p100/p52 mRNA expressions were significantly increased in pterygium fibroblasts 12 hours after TNF-α/H9251 treatment, as measured by quantitative real time-PCR (Fig. 3E), the increases in nuclear RelB and p100 were likely the result of de novo synthesis of these proteins as a response to stimulation by TNF-α. This explains the delayed increase in RelB and p100/p52 in stimulated pterygium fibroblasts because the synthesis of new proteins requires more time than does immediate recruitment of

**FIGURE 2.** (A) Western blot analysis of IκBα and phospho-IκBα levels in human pterygium and uninvolved conjunctiva tissues. Ten micrograms of total protein extracted from pterygium and conjunctiva tissue, each pooled from three patients, were loaded in each lane of SDS-PAGE, transferred to PVDF membrane, and probed with antibodies against IκBα, phospho-IκBα (Ser32), and β-actin (loading control). (B) ELISA for phospho-IκBα (Ser32) levels in total protein extracted from human pterygium (n = 5) and uninvolved conjunctiva tissues (n = 5). Bar heights represent mean values, and error bars represent SD. *P values represent significance using Student’s t-test.

**FIGURE 3.** Activation of NF-κB in pterygium fibroblasts on treatment with TNF-α. (A) Translocation of RelA from the cytoplasm into the nucleus after TNF-α (20 ng/mL) treatment was observed by immunofluorescence analysis with antibody against RelA followed by AlexaFluor 488-conjugated secondary antibody (green). The nuclei were counterstained with DAPI (blue). (B) Increased nuclear RelB level was observed after 24 hours of TNF-α (20 ng/mL) treatment by immunofluorescence analysis with antibody against RelB followed by AlexaFluor 488-conjugated secondary antibody (green). The nuclei were counterstained with DAPI (blue). (C) Protein levels of nuclear RelA, p105, and p50 in primary pterygium fibroblasts were determined by Western blot analysis. Cells were lysed at 0, 30, and 60 minutes after TNF-α treatment, and 10 μg nuclear protein was resolved by SDS-PAGE and immunoblotted with antibodies against RelA, p105, p50, and TATA-binding protein (TBP; loading control). (D) Protein levels of nuclear RelB, p100, and p52 in primary pterygium fibroblasts were similarly determined by Western blot analysis. Cells were lysed at 0, 12, 24, and 48 hours after TNF-α, and the nuclear proteins were analyzed as in B with antibodies against RelB, p100, and p52 and TBP (loading control). (E) mRNA expressions of RelB and p100 were analyzed by real-time PCR. Total RNA from primary pterygium fibroblasts was extracted at the indicated times after TNF-α treatment, and 0.5 μg total RNA was subjected to real-time PCR with the appropriate primers. The GAPDH transcript was used for normalization. Data are shown as fold induction compared with unstimulated primary pterygium fibroblasts at time 0 hours. Bar heights represent mean value, and error bars represent SD. (F) Phospho-IκBα (Ser32) levels in primary pterygium fibroblasts were determined by ELISA. Total protein extracted from cells treated with TNF-α for 30 minutes was subjected to ELISA and compared against that from untreated cells (control). Values are the means of triplicates. Bar heights represent mean value, and error bars represent SD. *P values represent significance using Student’s t-test.
existing preformed proteins from the cytoplasm, as is likely with RelA and p105/p50.

Furthermore, we measured the levels of phosphorylated IκBα (Ser32) in unstimulated and TNF-α-stimulated pterygium cells by ELISA. A significant 1.5-fold increase in phospho-IκBα (Ser32) level in total protein extract from pterygium fibroblasts after 30 minutes of TNF-α treatment can be observed compared with unstimulated control (Fig. 3F). This result reinforced the emerging picture that the NF-κB pathway is activated in pterygium fibroblasts in response to stimulation by TNF-α.

**TNF-α Induces the Expression of Chemokines and Matrix Metalloproteinases That Are NF-κB Target Genes in Pterygium Fibroblasts**

Real-time PCR showed that TNF-α treatment of pterygium fibroblasts upregulated the transcriptional expression of the NF-κB target genes—RANTES by 25,444-fold \( (P < 0.001) \) (Fig. 4A), MCP-1 by 46-fold \( (P = 0.001) \) (Fig. 4B), ENA-78 by 241-fold \( (P < 0.001) \) (Fig. 4C), MMP-1 by 3-fold \( (P < 0.001) \) (Fig. 4D), MMP-2 by 1.6-fold \( (P = 0.001) \) (Fig. 4E), and MMP-3 by 2-fold \( (P = 0.004) \) (Fig. 4F)—compared with untreated cells at 12 hours after treatment with TNF-α. Interestingly, there was a continued increase in RANTES mRNA expression at 24 hours after stimulation (Fig. 4A), whereas the other mRNAs analyzed showed a decrease at 24 hours. Furthermore, the upregulation of the respective transcripts was significantly greater in pterygium fibroblasts than in tenon fibroblasts. These data suggest that pterygium fibroblasts respond to TNF-α induction with higher NF-κB activation than normal tenon fibroblasts.

**DISCUSSION**

To our knowledge, this is the first study describing the involvement of the NF-κB signaling pathway in the pathogenesis of pterygium. Our data showed that the NF-κB pathway is activated in pterygium tissue, with increased phosphorylation of inhibitor IκBα compared with normal conjunctiva. We further demonstrated that both canonical and noncanonical NF-κB pathways can be activated in primary pterygium fibroblasts in response to TNF-α stimulation. Increased phosphorylation of IκBα and nuclear translocation of RelA and p50, proteins belonging to the canonical pathway, occurred within 30 minutes of TNF-α stimulation, whereas delayed increase in nuclear RelB and p100/p52, which are involved in the non-canonical pathway, occurred 12 hours after stimulation.

Current theories of pterygium pathogenesis as a result of inflammatory stimuli are likely to involve activation of the NF-κB pathway. Ultraviolet irradiation can activate mitogen-activated protein kinase pathways, leading to NF-κB induction and release of IL-1, IL-6, and TNF-α from skin cells and cultured human corneal epithelial cells. Tears of patients with dry eyes have increased cytokines such as TNF-α. Moreover, TNF-α is observed by immunohistochemistry to be increased in pterygium tissue. Proinflammatory cytokines can stimulate the production of chemokines such as RANTES, MCP-1, and ENA-78, as shown in our study. The progressive increase in RANTES over time on TNF-α treatment, which was not observed for MCP-1 or ENA-78, is particularly intriguing.

We speculate that NF-κB pathway activation resulted in the upregulation of other RANTES-specific regulatory genes that further enhanced the expression of RANTES over time. These chemokines function to recruit lymphocytes, plasma cells, and mast cells to pterygium tissue, which, in turn, induce more cytokine and growth factor production and can result in a hyperproliferative state. NF-κB activation can also stabilize Snail protein by reducing its degradation, inducing its expression, and repressing E-cadherin, thereby increasing the motility and invasiveness of cells as part of the epithelial-mesenchymal transition (EMT). EMT has been suggested to play an important role in the pathogenesis of pterygium. NF-κB can further influence the expression of proapoptotic and antiapoptotic genes and cell-cycle proteins such as cyclin D1 and D3. It is likely that the NF-κB pathway also influences the expression of proapoptotic and antiapoptotic genes and cell-cycle proteins such as cyclin D1 and D3.
pathway is involved in more than one of these biological effects that may contribute to the pathogenesis of pterygium. Additional work is required to address the involvement of the NF-κB pathway in each of these possibilities in pterygium.

Inflammatory signals typically activate the canonical NF-κB pathway, whereas developmental signals use the noncanonical pathway. Developmental signals elicit a weaker and more delayed response than inflammatory signals, and the differential threshold of NF-κB activation may determine selective expression of cytokines, cell remodeling molecules, or survival genes. However, new findings indicate that there is cross-talk between the two pathways. Basak observed in mouse embryonic fibroblasts (MEFs) that TNF-primed cells would display hyperresponsive RelA/p50 dimer activation when subsequently stimulated by noncanonical pathway activator LT-β. LT-β receptor stimulation in MEF cells with altered IκB balance resulted in the expression of inflammatory cytokines not normally induced in wild-type cells. It would be interesting to investigate whether patients with ocular surface disease and chronically inflamed conjunctiva have altered levels of IκB and NF-κB proteins, which may lead to abnormal expression of cell remodeling or EMT genes and, hence, a predisposition to pterygium development.

Our findings are consistent with previous observations of increased mRNA and protein expressions of MMP-1 and MMP-3 in pterygium fibroblasts compared with normal conjunctiva fibroblasts. In particular, MMP-1 expression was observed in pterygium fibroblasts that migrated between the corneal epithelium and Bowman’s layer at the advancing pterygium edge. The NF-κB pathway is known to affect MMP expression based on a number of studies. With respect to MMP-1, NF-κB was found to dynamically interact with the promoter region of MMP-1 in combined chromatin immunoprecipitation and microarray analysis, whereas inhibition of the NF-κB pathway by either pharmacologic blockade or retroviral overexpression of IκBα repressed MMP-1 expression. MMP-2 expression was found to be increased in p100/p52 overexpressing mammary epithelium and in phosphatidic acid-induced glioblastoma by increased binding of NF-κB to the MMP-2 promoter. Conversely, an NF-κB decoy oligodeoxynucleotide suppressed high glucose-induced MMP-2 production in adventitial fibroblasts. MMP-3 expression may also be regulated by the NF-κB pathway because NF-κB binding activity was associated with MMP-3 gene expression in synovial tissues of mice with collagen-induced arthritis, although IκBα overexpression in vascular smooth muscle cells inhibited MMP-3 expression.

In conclusion, we found that the NF-κB signaling pathway is activated in pterygium. This has important clinical implications because we now have a new avenue by which novel therapeutics for the treatment or management of pterygium may be developed. With various NF-κB inhibitors currently in development, there is hope that the progression and recurrence of pterygium may be delayed or halted.

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References


