Inhibition by a Selective IκB Kinase-2 Inhibitor of Interleukin-1–Induced Collagen Degradation by Corneal Fibroblasts in Three-Dimensional Culture

Yukiko Kondo, Ken Fukuda, Tadafumi Adachi, and Teruo Nishida

Purpose. Corneal ulcer results from excessive collagen degradation in the corneal stroma. Interleukin (IL)-1 promotes this process by activating signaling molecules that include nuclear factor (NF)-κB and stimulating the synthesis of matrix metalloproteinases (MMPs) in corneal fibroblasts. NF-κB activation is mediated by phosphorylation of the inhibitor IκB by IκB kinase (IKK)-2 and consequent IκB degradation. The authors investigated the effects of the IKK-2 inhibitor [5-(*p*-fluorophenyl)-2-ureido]thiophene-3-carboxamide (TPCA-1) on collagen degradation by corneal fibroblasts.

Methods. Rabbit corneal fibroblasts were cultured in three-dimensional collagen gels. Collagen degradation was evaluated by spectrophotometric quantitation of hydroxyproline in culture supernatants subjected to acid-heat hydrolysis. Expression of MMPs was evaluated by immunoblot analysis, gelatin zymography, and real-time reverse transcription polymerase chain reaction analysis. The phosphorylation and degradation of IκBα and the subcellular localization of NF-κB were examined by immunoblot and immunofluorescence analyses, respectively.

Results. IL-1β–induced collagen degradation by corneal fibroblasts was inhibited by TPCA-1 in a concentration- and time-dependent manner. TPCA-1 inhibited the IL-1β–induced expression of MMP-1, -3, and -9 in these cells at both the mRNA and protein levels and the IL-1β–induced activation of pro-MMP-2. In contrast to dexamethasone, TPCA-1 inhibited the phosphorylation and degradation of IκBα and the nuclear translocation of NF-κB induced by IL-1β.

Conclusions. An IKK-2 inhibitor blocked IL-1β–induced collagen degradation by corneal fibroblasts by inhibiting the activation of the NF-κB signaling pathway and the upregulation of MMPs. IKK-2 inhibitors are thus potential alternatives to dexamethasone for the treatment of corneal ulcer. (*Invest Ophthalmol Vis Sci.* 2008;49:4850–4857) DOI:10.1167/iovs.08-1897

Corneal ulcer is the result of excessive degradation of collagen in the corneal stroma by collagenolytic enzymes derived from infectious microorganisms or by matrix metalloproteinases (MMPs) released from activated keratocytes. Pathologic examination has also revealed the presence of infiltrated neutrophils and other leukocytes in or surrounding corneal ulcers. Enzymes released from neutrophils activate MMPs in various inflammatory conditions.[1] Free lysosomes derived from infiltrated neutrophils have also been detected amid degraded collagen in the corneal stroma, and neutrophils phagocytose such amorphous collagen material in corneal ulcers.[2] The functions of keratocytes, including cell proliferation and the synthesis of collagen and MMPs, are continuously modulated by interaction of the cells with extracellular collagen. We have previously shown that three-dimensional culture of corneal fibroblasts in a collagen gel mimics the in vivo situation more closely than does monolayer culture.[3] We have also established an assay system with which to measure the collagenolytic activity of corneal fibroblasts in such three-dimensional cultures.[4,5]

Interleukin (IL)-1 is a major proinflammatory cytokine produced by immune cells and corneal cells.[6] We have shown that IL-1 stimulates collagen degradation by corneal fibroblasts[7] and is responsible for the stimulatory effect of neutrophils on this process.[8] In addition, the inhibition of IL-1 activity by administration[9] or overexpression[10] of IL-1 receptor antagonist reduces the severity of keratitis in animal models, indicating the importance of this cytokine in corneal ulceration. Indeed, the concentration of IL-1 in tear fluid is increased in persons with corneal ulcer.[11] We have also shown that *Pseudomonas aeruginosa* elastase,[12] in addition to neutrophils[8] and IL-1,[7] stimulates collagen degradation by corneal fibroblasts, whereas dexamethasone[13] and triptolide[14] inhibit such IL-1–induced collagen degradation.

Transcription factors play an important role in cytokine signal transduction, with nuclear factor (NF)-κB a major mediator of signaling by IL-1.[7,15,16] In the inactive state, most NF-κB complexes consist of the subunits p50 and p65 and are restricted to the cytoplasm as a result of their interaction with the inhibitory protein IκB.[17] Cell stimulation with IL-1 triggers the activation of NF-κB by inducing the phosphorylation and ubiquitination of IκB and its consequent degradation by the proteasome; the released NF-κB is then able to translocate to the nucleus and to regulate the expression of its target genes.[18] IκB is phosphorylated by the IκB kinase (IKK) complex, which consists of the catalytic subunits IKK-1 (also known as IKK-α) and IKK-2 (also known as IKK-β) and the regulatory subunit IKK-γ.[19–21] The specific activity of IKK-2 with regard to phosphorylation of IκB is 20 times that of IKK-1,[22] suggesting that IKK-2 plays the dominant role in the activation of NF-κB in vivo.[23,24] A small molecule inhibitor of IKK-2 has recently been investigated for its therapeutic potential in the treatment of inflammatory diseases such as asthma[25] and arthritis.[26]

To provide insight into the mechanism of corneal ulceration and to explore new treatment options, we investigated the role of IKK-2 in IL-1–induced collagen degradation by corneal fibroblasts. In particular, we examined the effects of a selective IKK-2 inhibitor on such collagen degradation and on NF-κB activation and the expression of MMPs in these cells. The effects of the IKK-2 inhibitor were compared with those of dexamethasone, which is currently used to treat corneal ulcer.

From the Department of Ophthalmology, Yamaguchi University Graduate School of Medicine, Ube City, Yamaguchi, Japan.

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Corresponding author: Ken Fukuda, Department of Ophthalmology, Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube City, Yamaguchi 755-8505, Japan; k.fukuda@yamaguchi-u.ac.jp.

METHODS

Materials

Eagle minimum essential medium (MEM), phosphate-buffered saline (PBS), dispase, and trypsin-EDTA were obtained from Invitrogen-Gibco (Grand Island, NY); 24-well culture plates were from Corning Glass (Corning, NY); four-well chamber slides were from Becton Dickinson (Franklin Lakes, NJ); native porcine type 1 collagen (acid solubilized), 5× Dulbecco modified Eagle medium (DMEM), and reconstitution buffer were from Nitta Gelatin (Osaka, Japan); fetal bovine serum was from Cansera International (Toronto, ON, Canada); and bovine plasminogen, collagenase, and dexamethasone were from Sigma-Aldrich (St. Louis, MO). [5-(*p*-Fluorophenyl)-2-ureido]thiophene-3-carboxamide (TPCA-1) was obtained from Calbiochem (San Diego, CA); recombinant human IL-1β and biotinylated antibodies to human MMP-1 were from R&D Systems (Minneapolis, MN); mouse monoclonal antibodies to the phosphorylated form of (phospho-) c-Jun and rabbit polyclonal antibodies to human IκBα, to the p65 subunit of NF-κB, and to c-Jun were from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal antibodies to human phospho-IκBα and to p38 mitogen-activated protein kinase (MAPK) and rabbit polyclonal antibodies to human extracellular signal–regulated kinase (ERK), to phospho-ERK, to c-Jun NH_2-terminal kinase (JNK), to phospho-JNK, and to phospho–p38 MAPK were from Cell Signaling (Beverly, MA); mouse monoclonal antibodies to rabbit MMP-3 were from Daiichi Fine Chemicals (Toyama, Japan); Alexa Fluor 488–conjugated goat antibodies to rabbit immunoglobulin G were from Molecular Probes (Eugene, OR); mounting medium was from Vector Laboratories (Vectashield; Burlingame, CA); an enhanced chemiluminescence (ECL) kit was from GE Healthcare (Piscataway, NJ); and Coomassie brilliant blue and gelatin were from Bio-Rad (Hercules, CA). Kits for RNA purification (RNeasy Mini Kit) and polymerase chain reaction (PCR) analysis (QuantiTect SYBR Green PCR Kit) were obtained from Qiagen (Hilden, Germany), and a reverse transcription (RT) system and cytotoxicity assay (CytoTox 96 Non-Radioactive) were from Promega (Madison, WI). All media and reagents used for cell culture were endotoxin minimized.

Cell Isolation

Male Japanese albino rabbits (weight range, 2.0–2.5 kg) were obtained from Biotec (Saga, Japan). This study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Animal Experimental Committee of Yamaguchi University School of Medicine. Rabbit corneal fibroblasts were isolated and maintained as described previously.[12] In brief, the endothelial layer of the cornea was removed mechanically, and the remaining tissue was incubated with dispase (2 mg/mL, in MEM) for 1 hour at 37°C. After mechanical removal of the epithelial sheet, the remaining tissue was treated with collagenase (2 mg/mL, in MEM) at 37°C until a single-cell suspension of corneal fibroblasts was obtained. Isolated corneal fibroblasts were maintained under a humidified atmosphere containing 5% CO_2 at 37°C in MEM supplemented with 10% fetal bovine serum. Cells were used for experiments after four to eight passages and were harvested at subconfluence in the actively proliferating state.

Three-Dimensional Culture of Corneal Fibroblasts in a Type 1 Collagen Gel

Collagen gels were prepared as described.[12] In brief, corneal fibroblasts were harvested by exposure to trypsin-EDTA, collected by centrifugation, and resuspended in serum-free MEM. Acid-solubilized type 1 collagen (3 mg/mL), 5× DMEM, reconstitution buffer (0.05 M NaOH, 0.26 M Na_2CO_3, 0.2 M HEPES [pH 7.3]), and corneal fibroblast suspension (2.2×10^6 cells/mL in MEM) were mixed on ice in a ratio of 7:2:1:1. Portions (0.5 mL) of the resultant mixture were added to the wells of a 24-well culture plate and were allowed to solidify in an incubator under 5% CO_2 at 37°C, after which 0.5 mL serum-free MEM containing test agents and plasminogen (360 μg/mL) was overlaid and the cultures were returned to the incubator for the indicated times. The IKK-2 inhibitor was dissolved and diluted in dimethyl sulfoxide; the final concentration of solvent was 0.1% in all cultures containing TPCA-1, and the same amount of vehicle was added to control cultures. At the concentrations used in this study, the IKK-2 inhibitor did not have a cytotoxic effect on corneal fibroblasts (data not shown).

Assay of Collagenolytic Activity

Measurement of degraded collagen in culture supernatants was performed as described previously.[8,12] In brief, the supernatants from collagen gel incubations were collected, and native collagen fibrils with molecular sizes greater than 100 kDa were removed by ultrafiltration. The filtrate was subjected to hydrolysis with 6 M HCl for 24 hours at 110°C. The amount of hydroxyproline in the hydrolysate was then measured spectrophotometrically, and the amount of degraded collagen was expressed as micrograms of hydroxyproline per well.

Immunoblot Analysis

Immunoblot analysis of MMP-1 and -3 was performed as described previously.[12] In brief, culture supernatants were subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel under reducing conditions, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. Nonspecific sites of the membrane were blocked, and it was then incubated with antibodies to MMP-1 or to MMP-3. Immune complexes were detected with the use of ECL reagents. Immunoblot analysis of total and phosphorylated forms of IκBα, c-Jun, and MAPKs in cell lysates was also performed as described previously.[27]

Gelatin Zymography

Gelatin zymography of culture supernatants was performed as described previously.[12] In brief, culture supernatants (4 μL) were mixed with 2 μL nonreducing SDS sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 2% SDS, 0.002% bromophenol blue) and then fractionated by SDS-PAGE at 4°C on a 10% gel containing 0.1% gelatin. The gel was then washed in 2.5% Triton X-100 for 1 hour (to promote recovery of protease activity) before incubation for 18 hours at 37°C in a reaction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, and 1% Triton X-100. Finally, the gel was stained with Coomassie brilliant blue.

RT and Quantitative PCR Analysis

Total RNA was isolated from cells and subjected to RT with the use of kits. The abundance of MMP-1, -2, -3, -9, procollagen type 1, fibronectin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs was quantified by real-time PCR analysis with an automated PCR system (LightCycler; Roche Molecular Biochemicals, Indianapolis, IN), as described previously.[8] Sequences of the PCR primers for rabbit MMP-1, -2, -3, -9, and GAPDH cDNAs were also as described previously.[14] Those for rabbit procollagen α1 (I) cDNA were 5′-CCACACAGCCCAGCATTG-3′ (forward) and 5′-TCGCTGCCGTACTCGAACT-3′ (reverse), and those for rabbit fibronectin cDNA were 5′-AGCCCTTACAGTTCCGAGT-3′ (forward) and 5′-GTAGGGATCGAAGCATGAG-3′ (reverse). These primers yielded PCR products of the expected sizes of 649, 313, 306, 271, 293, 118, and 200 bp, respectively.

Immunofluorescence Staining of NF-κB

Immunostaining for NF-κB in corneal fibroblasts was performed as described previously.[7] In brief, cells cultured in four-well chamber slides were fixed and permeabilized, and nonspecific sites were blocked. The cells were then incubated overnight at 4°C with antibodies to the p65 subunit of NF-κB (1:100 dilution in PBS containing 1% bovine serum albumin), washed, and incubated for 1 hour at room temperature with Alexa Fluor 488–conjugated secondary antibodies (1:500 dilution in the same solution). They were finally washed, mounted in mounting medium, and examined under a fluorescence

microscope (Axiovert; Carl Zeiss Meditec, München-Hallbergmoos, Germany).

Statistical Analysis

Data are presented as mean ± SEM and were analyzed with Dunnett or Scheffé test. $P < 0.05$ was considered statistically significant.

Results

Inhibitory Effect of an IKK-2 Inhibitor on Collagen Degradation by Rabbit Corneal Fibroblasts

We first examined the effect of the IKK-2 inhibitor TPCA-1 on collagen degradation by corneal fibroblasts. The cells were cultured in three-dimensional collagen gels for 48 hours in the absence or presence of IL-1β (1.0 ng/mL) and in the presence of various concentrations of TPCA-1. In the absence of IL-1β, TPCA-1 had no significant effect on collagen degradation by corneal fibroblasts at any concentration examined (Fig. 1). In contrast, TPCA-1 inhibited IL-1β–induced collagen degradation in a concentration-dependent manner; this effect was significant at concentrations ≥0.1 μM and was maximal at 1.0 μM.

We also examined the time course of collagen degradation by corneal fibroblasts in the absence or presence of IL-1β (1.0 ng/mL) or TPCA-1 (1.0 μM). In the absence of IL-1β, the amount of degraded collagen increased to reach a plateau at 24 hours (Fig. 2) and was not affected by the presence of TPCA-1 for up to 48 hours (data not shown). IL-1β increased the amount of degraded collagen in a time-dependent manner for at least 48 hours, and this effect was blocked by TPCA-1 at all time points examined (Fig. 2). Together, these results thus demonstrated that the IKK-2 inhibitor inhibits IL-1β–induced collagen degradation by corneal fibroblasts.

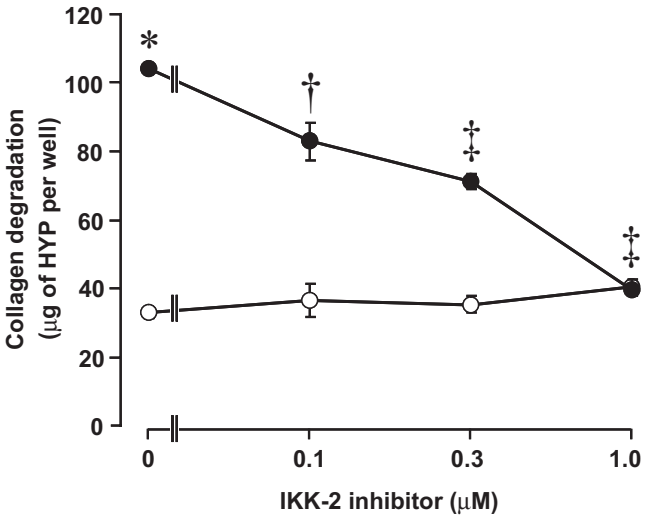

Figure 1. Concentration-dependent inhibition by an IKK-2 inhibitor of IL-1β–induced collagen degradation by corneal fibroblasts. Cells were cultured in collagen gels in the presence of plasminogen, in the absence (*open symbols*) or presence (*closed symbols*) of IL-1β (1.0 ng/mL) and in the presence of the indicated concentrations of TPCA-1 for 48 hours, after which the amount of degraded collagen was determined. Data are expressed as micrograms of hydroxyproline (HYP) per well and are mean ± SEM of values from three experiments. $^*P <$ 0.00001 (Dunnett test) versus the corresponding value for cells cultured without IL-1β; †$P < 0.01$ and ‡$P < 0.0001$ (Dunnett test) versus the corresponding value for cells cultured without TPCA-1.

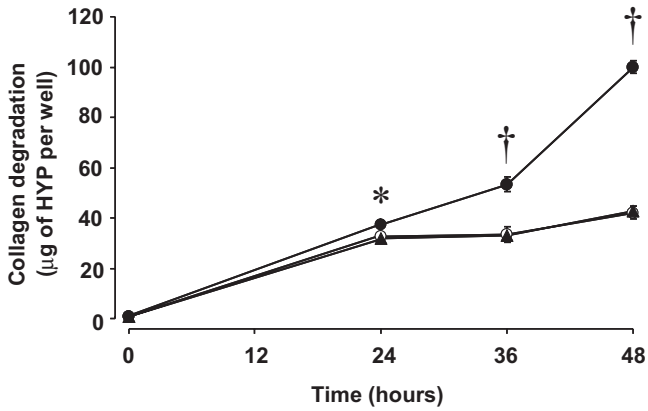

Figure 2. Time-dependent inhibition by an IKK-2 inhibitor of IL-1β–induced collagen degradation by corneal fibroblasts. Cells were cultured in collagen gels for the indicated times in the presence of plasminogen and in the absence (*open circles*) or presence of IL-1β (1.0 ng/mL) either alone (*closed circles*) or with 1.0 μM TPCA-1 (*closed triangles*), after which the amount of degraded collagen was determined. Data are mean ± SEM of values from three experiments. $^*P <$ 0.05 and †$P < 0.0001$ (Dunnett test) versus the corresponding value for cells cultured with both IL-1β and TPCA-1.

Inhibitory Effects of an IKK-2 Inhibitor on MMP Expression in Corneal Fibroblasts

To investigate the mechanism by which the IKK-2 inhibitor inhibited IL-1β–induced collagen degradation by corneal fibroblasts, we next examined its effects on the expression of MMPs by these cells. Corneal fibroblasts were cultured in collagen gels for 48 hours in the absence or presence of IL-1β (1.0 ng/mL) and in the presence of various concentrations of TPCA-1. Immunoblot analysis with antibodies to MMP-1 did not detect pro-MMP-1 or active MMP-1 in the culture supernatants of cells incubated in the absence of IL-1β (Fig. 3A). In contrast, a 57-kDa immunoreactive protein corresponding to pro-MMP-1 and 49- and 45-kDa immunoreactive proteins corresponding to active MMP-1 were detected in the culture supernatants of cells incubated with IL-1β. TPCA-1 inhibited the IL-1β–induced increases in pro-MMP-1 and active MMP-1 in a concentration-dependent manner.

Immunoblot analysis with antibodies to MMP-3 did not detect pro-MMP-3 or active MMP-3 in culture supernatants of cells incubated in the absence of IL-1β (Fig. 3A). In contrast, 57- and 45-kDa immunoreactive proteins corresponding to pro-MMP-3 and active MMP-3, respectively, were apparent in the culture supernatants of cells incubated with IL-1β. Again, TPCA-1 inhibited the IL-1β–induced increases in the pro-MMP-3 and active MMP-3 in a concentration-dependent manner.

We then examined the abundance of MMP-1 and MMP-3 mRNAs in corneal fibroblasts. Cells were cultured in monolayers for 12 hours in the absence or presence of IL-1β (1.0 ng/mL) or TPCA-1 (1.0 μM), after which the amounts of these mRNAs were determined by RT and real-time PCR analysis. IL-1β induced a 43-fold increase in the amount of MMP-1 mRNA, and this effect was inhibited by 87% in the presence of TPCA-1 (Fig. 3B). Similarly, IL-1β induced a 22-fold increase in the amount of MMP-3 mRNA in corneal fibroblasts, and TPCA-1 inhibited this effect of IL-1β by 75% (Fig. 3B).

We examined the effects of IL-1β and the IKK-2 inhibitor on the expression of MMP-2 and -9 by gelatin zymography. Analysis of culture supernatants of corneal fibroblasts incubated without IL-1β for 48 hours revealed two major bands of 65 and 57 kDa corresponding to pro-MMP-2 and active MMP-2, respec-

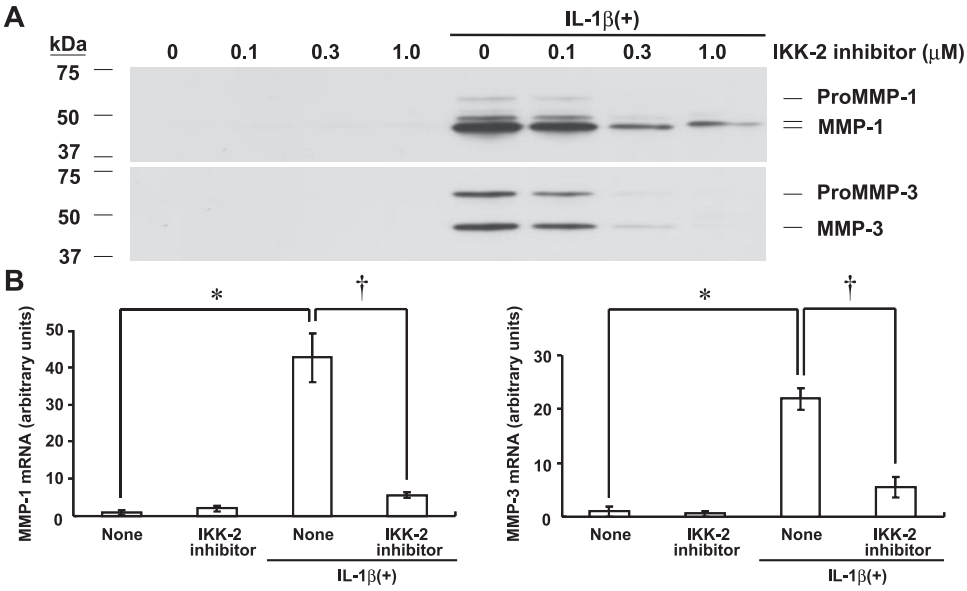

FIGURE 3. Effects of an IKK-2 inhibitor on the expression of MMP-1 and -3 by corneal fibroblasts. (**A**) Cells were cultured in collagen gels for 48 hours in the presence of plasminogen and in the absence or presence of IL-1β (1.0 ng/mL) and the indicated concentrations of TPCA-1. Culture supernatants were then subjected to immunoblot analysis with antibodies to MMP-1 (*top*) or to MMP-3 (*bottom*). The positions of bands corresponding to the pro-MMP-1 and -MMP-3 and active MMP-1 and MMP-3 are indicated on the *right*, and those of molecular size standards are shown on the *left*. Data are representative of three independent experiments. (**B**) Cells were cultured as monolayers for 12 hours in the presence of plasminogen and in the absence or presence of IL-1β (1.0 ng/mL) or TPCA-1 (1.0 μM), as indicated. The amounts of MMP-1 mRNA (*left*) and MMP-3 mRNA (*right*) in the cells were then determined by RT and real-time PCR analysis. Data were normalized on the basis of the abundance of GAPDH mRNA, are expressed in arbitrary units, and are means ± SEM of values from three experiments. $^*P < 0.0001$ and $\dagger P < 0.001$ (Scheffé test) for the indicated comparisons.

tively (Fig. 4A). These bands were not affected by incubation of the cells with TPCA-1 alone. Cell culture in the presence of IL-1β (1.0 ng/mL) resulted in an increase in the intensity of the band corresponding to active MMP-2 and the appearance of bands at 92 and 77 kDa corresponding to pro-MMP-9 and active MMP-9, respectively. TPCA-1 inhibited the IL-1β–induced increases in the amounts of the pro-MMP-9 and active MMP-9 and active MMP2 in a concentration-dependent manner.

RT and real-time PCR analysis revealed that culture of corneal fibroblasts with IL-1β (1.0 ng/mL) for 12 hours induced a 24-fold increase in the abundance of MMP-9 mRNA and that TPCA-1 inhibited this effect by 51% (Fig. 4B). Neither IL-1β nor TPCA-1 had a significant effect on the abundance of MMP-2 mRNA (Fig. 4B).

Consistent with our previous observations,[13] these results showed that IL-1β stimulates the synthesis of MMP-1, -3, and -9

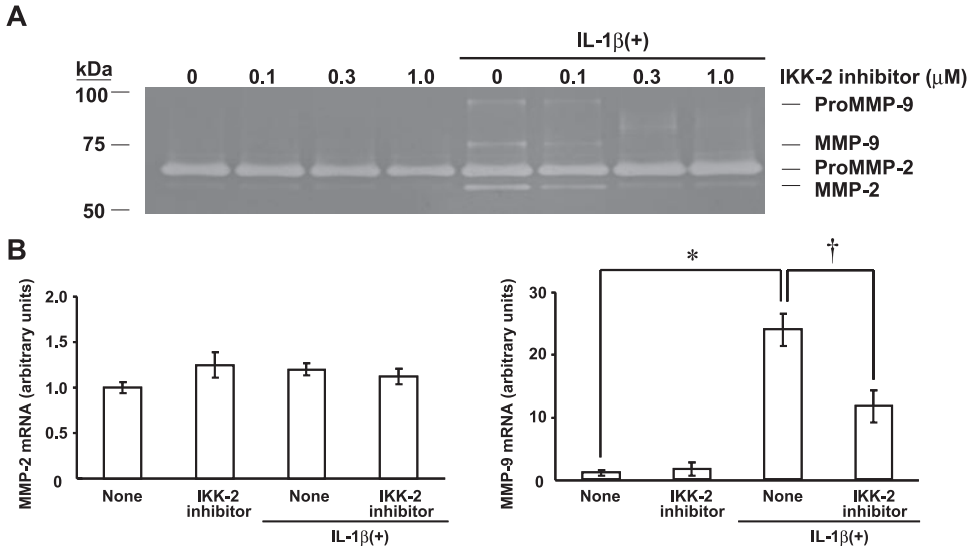

FIGURE 4. Effects of an IKK-2 inhibitor on the expression of MMP-2 and -9 by corneal fibroblasts. (**A**) Cells were cultured in collagen gels for 48 hours in the presence of plasminogen and in the absence or presence of IL-1β (1.0 ng/mL) and the indicated concentrations of TPCA-1. Culture supernatants were then subjected to gelatin zymography. The positions of bands corresponding to pro-MMP-2 and pro-MMP-9 and active MMP-2 and -9 are indicated on the *right*, and those of molecular size standards are shown on the *left*. Data are representative of three independent experiments. (**B**) Cells were cultured as monolayers for 12 hours in the presence of plasminogen and in the absence or presence of IL-1β (1.0 ng/mL) or TPCA-1 (1.0 μM), as indicated, after which the amounts of MMP-2 mRNA (*left*) and MMP-9 mRNA (*right*) in the cells were determined by RT and real-time PCR analysis. Data were normalized on the basis of the abundance of GAPDH mRNA, are expressed in arbitrary units, and are mean ± SEM of values from three experiments. $^*P < 0.0001$ and $\dagger P < 0.01$ (Scheffé test) for the indicated comparisons.

in corneal fibroblasts and stimulates the conversion of pro-MMP-2 to active MMP-2. They further show that these effects of IL-1β are inhibited by the IKK-2 inhibitor.

We also investigated the effects of the IKK-2 inhibitor on the expression of extracellular matrix genes in corneal fibroblasts. RT and real-time PCR analysis revealed that the abundance of procollagen type 1 mRNA was 1.0 ± 0.1, 0.8 ± 0.2, 1.4 ± 0.2, and 0.8 ± 0.1 arbitrary units (mean ± SEM of values from three experiments) after culture of cells for 12 hours either alone or in the presence of IL-1β (1.0 ng/mL), TPCA-1 (1.0 μM), or both IL-1β and TPCA-1. Corresponding values for fibronectin mRNA were 1.0 ± 0.1, 0.8 ± 0.4, 1.1 ± 0.2, and 0.9 ± 0.1 arbitrary units, respectively. These results thus showed that neither IL-1β nor TPCA-1 had a significant effect on the expression of these extracellular matrix genes in corneal fibroblasts.

Comparison of the Effects of an IKK-2 Inhibitor and Dexamethasone on IL-1β–Induced Collagen Degradation by Corneal Fibroblasts

We next compared the effect of the IKK-2 inhibitor on IL-1β–induced collagen degradation with that of dexamethasone, which we previously showed inhibited this effect of IL-1β.[13] Neither TPCA-1 (1.0 μM) nor dexamethasone (0.1 nM) affected collagen degradation in the absence of IL-1β (Fig. 5). In contrast, TPCA-1 and dexamethasone each completely inhibited the stimulatory effect of IL-1β on collagen degradation by corneal fibroblasts.

Inhibitory Effects of an IKK-2 Inhibitor on the Phosphorylation of IκBα and Nuclear Translocation of NF-κB

To investigate the mechanism by which the IKK-2 inhibitor blocks IL-1β–induced MMP expression in corneal fibroblasts, we examined its effects on the NF-κB signaling pathway in comparison with those of dexamethasone. We first examined the phosphorylation and degradation of IκBα in corneal fibroblasts by immunoblot analysis. Cells were cultured for 24 hours in serum-free medium, incubated in the absence or presence of TPCA-1 (1.0 μM) or dexamethasone (0.1 nM) for 6 hours, and incubated in the absence or presence of IL-1β (1.0 ng/mL) for 30 minutes. Consistent with our previous observations,[7] immunoblot analysis with antibodies to total or phosphorylated forms of IκBα revealed that IL-1β induced the phosphorylation and degradation of this NF-κB inhibitor (Fig. 6). These effects of IL-1β were blocked by TPCA-1 but not by dexamethasone. Neither TPCA-1 nor dexamethasone affected the abundance or phosphorylation of IκBα in the absence of IL-1β.

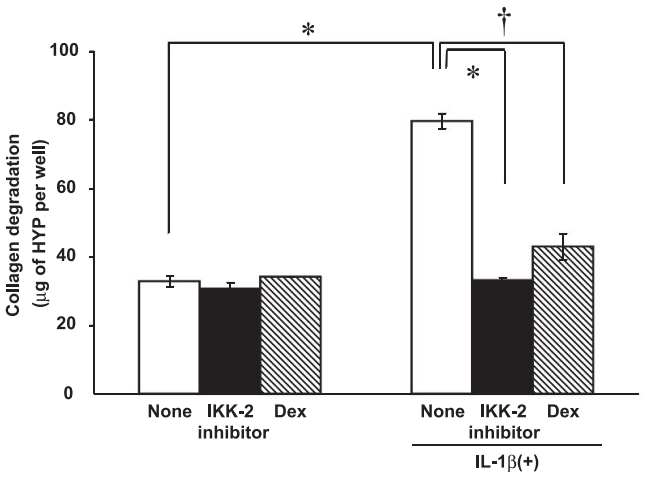

FIGURE 5. Comparison of the effects of an IKK-2 inhibitor and dexamethasone on collagen degradation by corneal fibroblasts. Cells were cultured in collagen gels for 48 hours in the presence of plasminogen and in the absence or presence of IL-1β (1.0 ng/mL), TPCA-1 (1.0 μM), or dexamethasone (Dex; 0.1 nM), as indicated, after which the amount of degraded collagen was determined. Data are mean ± SEM of values from three experiments. *$P < 0.00001$ and †$P < 0.001$ (Dunnett test) for the indicated comparisons.

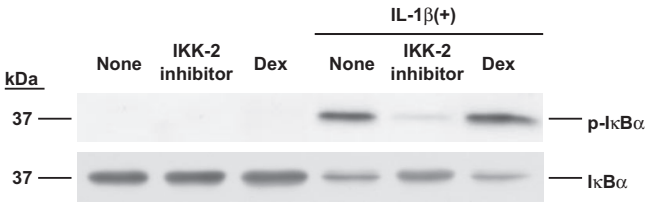

FIGURE 6. Effects of an IKK-2 inhibitor and dexamethasone on IκBα phosphorylation and degradation in corneal fibroblasts. Cell monolayers were deprived of serum for 24 hours, incubated in the absence or presence of TPCA-1 (1.0 μM) or dexamethasone (0.1 nM) for 6 hours, and incubated in the additional absence or presence of IL-1β (1.0 ng/mL) for 30 minutes. Cell lysates were then subjected to immunoblot analysis with antibodies to IκBα or to phosphorylated (p-) IκBα. Data are representative of three independent experiments.

Immunofluorescence analysis revealed that the p65 subunit of NF-κB was localized predominantly to the cytoplasm of corneal fibroblasts and that IL-1β induced the translocation of this protein to the nucleus (Fig. 7), as shown previously.[7] Neither TPCA-1 nor dexamethasone affected the subcellular distribution of p65 in the absence of IL-1β. However, the IKK-2 inhibitor, but not dexamethasone, inhibited the nuclear translocation of p65 induced by IL-1β.

To investigate further the differences in the mechanism of action of TPCA-1 and dexamethasone, we examined the effects of these agents on signaling by MAPKs and activator protein (AP)–1. Immunoblot analysis with antibodies to total or phosphorylated forms of the AP-1 component c-Jun revealed that IL-1β induced the phosphorylation of c-Jun and that this effect was blocked by dexamethasone but not by TPCA-1 (Fig. 8). Neither TPCA-1 nor dexamethasone affected the abundance or phosphorylation of c-Jun in the absence of IL-1β. Similar analysis revealed that IL-1β induced the phosphorylation of the MAPKs ERK, p38 MAPK, and JNK (Fig. 8). Dexamethasone inhibited the IL-1β–induced phosphorylation of JNK, but not that of ERK or p38 MAPK, whereas TPCA-1 had no effect on the IL-1β–induced phosphorylation of any of these MAPKs.

DISCUSSION

We have shown here that an IKK-2 inhibitor blocked IL-1–induced collagen degradation by corneal fibroblasts and have shown the IL-1–induced expression of MMPs at the mRNA and protein levels in these cells. We previously showed that dexamethasone inhibits these effects of IL-1 in corneal fibroblasts.[13] In addition, our present results show that the IKK-2 inhibitor blocked the IL-1β–induced activation of NF-κB in corneal fibroblasts by inhibiting the phosphorylation and degradation of IκBα, whereas dexamethasone had no such effects. The mechanisms by which the IKK-2 inhibitor and dexamethasone inhibit collagen degradation by corneal fibroblasts thus appear to differ. Our observations suggest that IKK-2 inhibitors are a potential alternative to dexamethasone for the treatment of corneal ulcer.

The proinflammatory cytokine IL-1 plays an important role in corneal ulceration, which is characterized by excessive degradation of collagen in the corneal stroma. IL-1 stimulates collagen degradation not only by corneal fibroblasts[7,13] but by

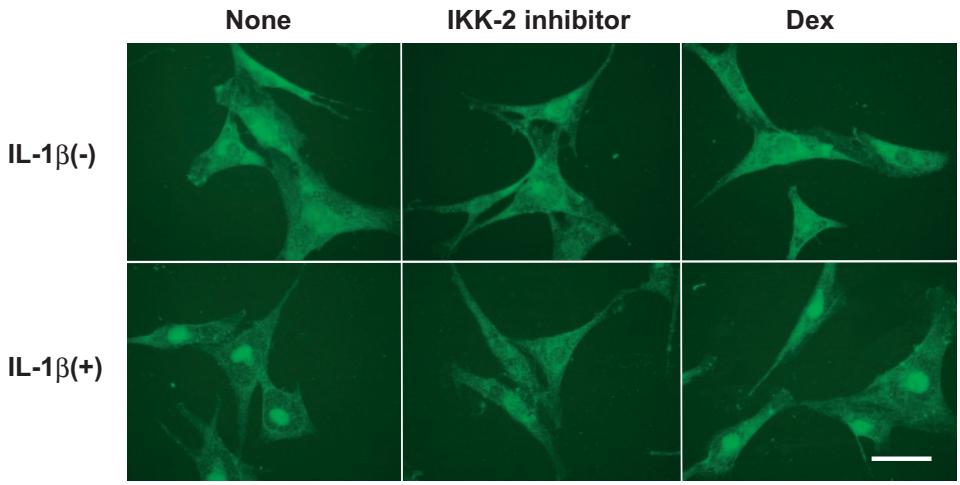

FIGURE 7. Effects of an IKK-2 inhibitor and dexamethasone on the subcellular localization of the p65 subunit of NF-κB in corneal fibroblasts. Cell monolayers were deprived of serum for 24 hours, incubated in the absence or presence of TPCA-1 (1.0 μM) or dexamethasone (0.1 nM) for 6 hours, and incubated in the additional absence or presence of IL-1β (1.0 ng/mL) for 30 minutes. They were then subjected to immunostaining with antibodies to the p65 subunit of NF-κB. Data are representative of three independent experiments. Scale bar, 50 μm.

fibroblasts from other tissues, including lung, bronchus,[28] and skin.[29] Corneal fibroblasts express receptors for IL-1.[30] The binding of IL-1 to its receptors induces the activation of NF-κB and of MAPKs including ERK, p38 MAPK, and JNK.[31] We previously showed that the activation of NF-κB by IL-1 is important for IL-1–induced collagen degradation by corneal fibroblasts.[7] We have also shown previously, and have confirmed in the present study, that IL-1 upregulates the expression of MMP-1, -3, and -9, but not that of MMP-2, in corneal fibroblasts.[13] Similar results have been obtained with lung fibroblasts[28] and vascular smooth muscle cells.[32] In addition, we have shown here that IL-1 induced the conversion of pro-MMP-2 to active MMP-2 in corneal fibroblasts, as it does in cardiac fibroblasts.[33]

Activation of NF-κB is mediated by the IKK-2–dependent phosphorylation and consequent degradation of IκB.[22] We

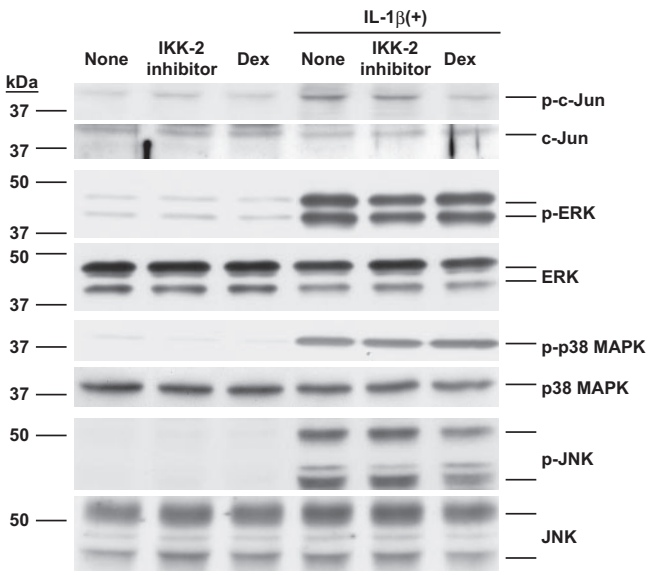

FIGURE 8. Effects of an IKK-2 inhibitor and dexamethasone on the phosphorylation of c-Jun and MAPKs in corneal fibroblasts. Cell monolayers were deprived of serum for 24 hours, incubated in the absence or presence of TPCA-1 (1.0 μM) or dexamethasone (0.1 nM) for 6 hours, and incubated in the additional absence or presence of IL-1β (1.0 ng/mL) for 30 minutes. Cell lysates were then subjected to immunoblot analysis with antibodies to total or phosphorylated (p-) forms of c-Jun, ERK, p38 MAPK, or JNK. Data are representative of three independent experiments.

have now shown that the IKK-2 inhibitor TPCA-1 inhibited the IL-1–induced upregulation of MMP-1, -3, and -9 expression in corneal fibroblasts at the mRNA and protein levels and the IL-1–induced activation of pro-MMP-2. It also blocked the IL-1–induced phosphorylation and degradation of IκBα and the translocation of NF-κB from the cytoplasm to the nucleus. These effects of TPCA-1 likely underlie its inhibition of collagen degradation by corneal fibroblasts. In contrast, neither IL-1 nor TPCA-1 affected the expression of genes for the extracellular matrix proteins procollagen type 1 and fibronectin in these cells.

We previously showed that dexamethasone inhibits IL-1–induced MMP synthesis in corneal fibroblasts and thereby inhibits IL-1–induced collagen degradation by these cells.[13] We now show that dexamethasone did not inhibit the phosphorylation and degradation of IκBα or the nuclear translocation of NF-κB induced by IL-1 in corneal fibroblasts. Dexamethasone appears to exert its biological effects by various mechanisms,[34] with diverse mechanisms thought to contribute to glucocorticoid-mediated inhibition of the NF-κB signaling pathway.[35] Dexamethasone thus inhibits NF-κB–dependent transcriptional activity without affecting IκB levels or the nuclear translocation of NF-κB in primary endothelial cells.[35] Our present results suggest that a similar mechanism may be operative in corneal fibroblasts.

The IKK-2 inhibitor was as effective as dexamethasone in inhibiting IL-1–induced collagen degradation by corneal fibroblasts, but these two agents appear to achieve their effects by distinct mechanisms. In addition to NF-κB, the transcription factor AP-1 also contributes to IL-1 signaling and MMP expression.[36–38] AP-1 is composed of c-Jun and c-Fos proteins and is a major target of mitogen-activated signaling pathways.[39] We found that dexamethasone, but not TPCA-1, inhibited the IL-1–induced phosphorylation of JNK—the principal mediator of AP-1 activation—and that of c-Jun in corneal fibroblasts. Dexamethasone was previously shown to antagonize AP-1 function by inhibiting the phosphorylation of JNK in various cell types.[40,41] Binding sites for AP-1 are present in the promoters of several MMP genes, including those for MMP-1, -3, and -9,[42] and dexamethasone has been shown to suppress MMP expression by inhibiting AP-1 signaling.[38] The inhibitory effect of dexamethasone on activation of the JNK–AP-1 signaling pathway may therefore also contribute to its inhibition of IL-1–induced collagen degradation by corneal fibroblasts. IKK-2 plays a central role in the activation of NF-κB in vivo.[23,24] Small molecule inhibitors of IKK-2 were recently found to show promise for the treatment of inflammatory diseases such as

asthma[25] and arthritis[26] and of ocular conditions such as corneal alkali burn[43,44] in animal models. We have now shown that the selective IKK-2 inhibitor TPCA-1 was as effective as dexamethasone in blocking IL-1–induced collagen degradation by corneal fibroblasts maintained in three-dimensional culture. Given that treatment with dexamethasone is associated with various side effects, such as increased intraocular pressure, posterior subcapsular cataract development, and increased susceptibility to infections,[45] there is a need for new and potent nonsteroidal anti-inflammatory drugs. Our data show that an IKK-2 inhibitor inhibits NF-κB–dependent MMP expression in corneal fibroblasts, likely as a result of its inhibition of IκBα phosphorylation and degradation and the consequent nuclear translocation of NF-κB. IKK-2 is thus a potential target in the development of drugs for the treatment of corneal ulcer.

References

1. Okada Y, Nakanishi I. Activation of matrix metalloproteinase 3 (stromelysin) and matrix metalloproteinase 2 ("gelatinase") by human neutrophil elastase and cathepsin G. *FEBS Lett*. 1989;249:353–356.
2. Steuhl KP, Döring G, Henni A, Thiel HJ, Botzenhart K. Relevance of host-derived and bacterial factors in *Pseudomonas aeruginosa* corneal infections. *Invest Ophthalmol Vis Sci*. 1987;28:1559–1568.
3. Nishida T, Ueda A, Fukuda M, et al. Interactions of extracellular collagen and corneal fibroblasts: morphologic and biochemical changes of rabbit corneal cells cultured in a collagen matrix. *In Vitro Cell Dev Biol*. 1988;24:1009–1014.
4. Hao JL, Nagano T, Nakamura M, et al. Galardin inhibits collagen degradation by rabbit keratocytes by inhibiting the activation of pro-matrix metalloproteinases. *Exp Eye Res*. 1999;68:565–572.
5. Hao JL, Nagano T, Nakamura M, et al. Effect of galardin on collagen degradation by *Pseudomonas aeruginosa*. *Exp Eye Res*. 1999;69:595–601.
6. Wilson SE, Liu JJ, Mohan RR. Stromal-epithelial interactions in the cornea. *Prog Retin Eye Res*. 1999;18:293–309.
7. Lu Y, Fukuda K, Li Q, Kumagai N, Nishida T. Role of nuclear factor-κB in interleukin-1-induced collagen degradation by corneal fibroblasts. *Exp Eye Res*. 2006;83:560–568.
8. Li Q, Fukuda K, Lu Y, et al. Enhancement by neutrophils of collagen degradation by corneal fibroblasts. *J Leukoc Biol*. 2003;74:412–419.
9. Thakur A, Xue M, Stapleton F, et al. Balance of pro- and anti-inflammatory cytokines correlates with outcome of acute experimental *Pseudomonas aeruginosa* keratitis. *Infect Immun*. 2002;70:2187–2197.
10. Biswas PS, Banerjee K, Kim B, Rouse BT. Mice transgenic for IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis: possible role for IL-1 in herpetic stromal keratitis pathogenesis. *J Immunol*. 2004;172:3736–3744.
11. Fukuda M, Mishima H, Otori T. Detection of interleukin-1β in the tear fluid of patients with corneal disease with or without conjunctival involvement. *Jpn J Ophthalmol*. 1997;41:63–66.
12. Nagano T, Hao JL, Nakamura M, et al. Stimulatory effect of pseudomonal elastase on collagen degradation by cultured keratocytes. *Invest Ophthalmol Vis Sci*. 2001;42:1247–1253.
13. Lu Y, Fukuda K, Liu Y, Kumagai N, Nishida T. Dexamethasone inhibition of IL-1-induced collagen degradation by corneal fibroblasts in three-dimensional culture. *Invest Ophthalmol Vis Sci*. 2004;45:2998–3004.
14. Lu Y, Fukuda K, Seki K, et al. Inhibition by triptolide of IL-1-induced collagen degradation by corneal fibroblasts. *Invest Ophthalmol Vis Sci*. 2003;44:5082–5088.
15. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev*. 2002;13:413–421.
16. Fukuda K, Fujitsu Y, Kumagai N, Nishida T. Inhibition of matrix metalloproteinase-3 synthesis in human conjunctival fibroblasts by interleukin-4 or interleukin-13. *Invest Ophthalmol Vis Sci*. 2006;47:2857–2864.
17. Adcock IM, Chung KF, Caramori G, Ito K. Kinase inhibitors and airway inflammation. *Eur J Pharmacol*. 2006;533:118–132.
18. Auron PE. The interleukin 1 receptor: ligand interactions and signal transduction. *Cytokine Growth Factor Rev*. 1998;9:221–237.
19. Courtois G, Smahi A, Israël A. NEMO/IKK gamma: linking NF-κB to human disease. *Trends Mol Med*. 2001;7:427–430.
20. Karin M. The beginning of the end: IκB kinase (IKK) and NF-κB activation. *J Biol Chem*. 1999;274:27339–27342.
21. Scheidereit C. Signal transduction: docking IκB kinases. *Nature*. 1998;395:225–226.
22. Mercurio F, Zhu H, Murray BW, et al. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. *Science*. 1997;278:860–866.
23. Li Q, Lu Q, Hwang JY, et al. IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes Dev*. 1999;13:1322–1328.
24. Takeda K, Takeuchi O, Tsujimura T, et al. Limb and skin abnormalities in mice lacking IKKα. *Science*. 1999;284:313–316.
25. Birrell MA, Hardaker E, Wong S, et al. Iκ-B kinase-2 inhibitor blocks inflammation in human airway smooth muscle and a rat model of asthma. *Am J Respir Crit Care Med*. 2005;172:962–971.
26. Schopf L, Savinainen A, Anderson K, et al. IKKβ inhibition protects against bone and cartilage destruction in a rat model of rheumatoid arthritis. *Arthritis Rheum*. 2006;54:3163–3173.
27. Lu Y, Liu Y, Fukuda K, et al. Inhibition by triptolide of chemokine, proinflammatory cytokine, and adhesion molecule expression induced by lipopolysaccharide in corneal fibroblasts. *Invest Ophthalmol Vis Sci*. 2006;47:3796–3800.
28. Fang Q, Liu X, Al-Mugotir M, et al. Thrombin and TNF-α/IL-1β synergistically induce fibroblast-mediated collagen gel degradation. *Am J Respir Cell Mol Biol*. 2006;35:714–721.
29. Fineschi S, Cozzi F, Burger D, et al. Anti-fibroblast antibodies detected by cell-based ELISA in systemic sclerosis enhance the collagenolytic activity and matrix metalloproteinase-1 production in dermal fibroblasts. *Rheumatology (Oxford)*. 2007;46:1779–1785.
30. Li DQ, Tseng SC. Differential regulation of cytokine and receptor transcript expression in human corneal and limbal fibroblasts by epidermal growth factor, transforming growth factor-α, platelet-derived growth factor B, and interleukin-1β. *Invest Ophthalmol Vis Sci*. 1996;37:2068–2080.
31. Liang KC, Lee CW, Lin WN, et al. Interleukin-1β induces MMP-9 expression via p42/p44 MAPK, p38 MAPK, JNK, and nuclear factor-κB signaling pathways in human tracheal smooth muscle cells. *J Cell Physiol*. 2007;211:759–770.
32. Bond M, Chase AJ, Baker AH, Newby AC. Inhibition of transcription factor NF-κB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. *Cardiovasc Res*. 2001;50:556–565.
33. Xie Z, Singh M, Singh K. Differential regulation of matrix metalloproteinase-2 and -9 expression and activity in adult rat cardiac fibroblasts in response to interleukin-1β. *J Biol Chem*. 2004;279:39513–39519.
34. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-κB or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev*. 2003;24:488–522.
35. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. *J Clin Invest*. 2001;107:135–142.
36. Li W, Zheng S, Tang C, Zhu Y, Wang X. JNK-AP-1 pathway involved in interleukin-1β-induced calcitonin gene-related peptide secretion in human type II alveolar epithelial cells. *Peptides*. 2007;28:1252–1259.
37. Liacini A, Sylvester J, Li WQ, Zafarullah M. Inhibition of interleukin-1-stimulated MAP kinases, activating protein-1 (AP-1) and nuclear factor kappa B (NF-κB) transcription factors down-regulates matrix metalloproteinase gene expression in articular chondrocytes. *Matrix Biol*. 2002;21:251–262.
38. Eberhardt W, Schulze M, Engels C, Klasmeier E, Pfeilschifter J. Glucocorticoid-mediated suppression of cytokine-induced matrix

metalloproteinase-9 expression in rat mesangial cells: involvement of nuclear factor-κB and Ets transcription factors. *Mol Endocrinol*. 2002;16:1752–1766.

39. Shaulian E, Karin M. AP-1 in cell proliferation and survival. *Oncogene*. 2001;20:2390–2400.
40. Hirasawa N, Sato Y, Fujita Y, Mue S, Ohuchi K. Inhibition by dexamethasone of antigen-induced c-Jun N-terminal kinase activation in rat basophilic leukemia cells. *J Immunol*. 1998;161:4939–4943.
41. González MV, Jiménez B, Berciano MT, et al. Glucocorticoids antagonize AP-1 by inhibiting the activation/phosphorylation of JNK without affecting its subcellular distribution. *J Cell Biol*. 2000; 150:1199–1208.
42. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem*. 2003;253:269–285.
43. Saika S, Miyamoto T, Yamanaka O, et al. Therapeutic effect of topical administration of SN50, an inhibitor of nuclear factor-κB, in treatment of corneal alkali burns in mice. *Am J Pathol*. 2005;166: 1393–1403.
44. Saika S, Ikeda K, Yamanaka O, et al. Expression of Smad7 in mouse eyes accelerates healing of corneal tissue after exposure to alkali. *Am J Pathol*. 2005;166:1405–1418.
45. David DS, Berkowitz JS. Ocular effects of topical and systemic corticosteroids. *Lancet*. 1969;2:149–151.