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# Dexamethasone Inhibits Interleukin-1β-Induced Corneal Neovascularization

## *Role of Nuclear Factor-κB-Activated Stromal Cells in Inflammatory Angiogenesis*

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**Dexamethasone, a synthetic corticosteroid, is widely used as a potent anti-inflammatory drug in various diseases including corneal angiogenesis. However, dexamethasone's impact on interleukin (IL)-1β-dependent inflammatory angiogenesis is unknown. Here, we show that dexamethasone inhibits IL-1β-induced neovascularization and the expression of the angiogenesis-related factors, vascular endothelial growth factor-A, KC, and prostaglandin $E_2$ in the mouse cornea 2 days after IL-1β implantation. IL-1β caused IκB-α phosphorylation in corneal stromal cells but not in infiltrated $CD11b^+$ cells 2 days after IL-1β implantation. In contrast, both cell types were positive for phosphorylated IκB-α 4 days after IL-1β implantation. Dexamethasone significantly inhibited IκB-α phosphorylation 2 and 4 days after IL-1β implantation. Furthermore, dexamethasone inhibited IL-1β-induced expression of vascular endothelial growth factor-A, KC, and prostaglandin $E_2$, and signaling of nuclear factor (NF)-κB in corneal fibroblasts *in vitro*. A selective NF-κB inhibitor attenuated IL-1β-induced corneal angiogenesis. These findings suggest that NF-κB activation in the corneal stromal cells is an important early event during IL-1β-induced corneal angiogenesis and that dexamethasone inhibits IL-1β-induced angiogenesis partially via blocking NF-κB signaling.** *(Am J Pathol 2007, 171:1058–1065; DOI: 10.2353/ajpath.2007.070172)*

Dexamethasone, a synthetic corticosteroid analogue, is a potent anti-inflammatory drug that is used in the treatment of various immune and inflammatory diseases, including those of the eye. Folkman and Ingber[1] reported the anti-angiogenic function of a class of steroids, including dexamethasone, which they named the angiostatic steroids. The anti-angiogenic effect of dexamethasone has been confirmed in various animal models.[2,3] For instance, Ishibashi and colleagues[2] showed that dexamethasone reduces laser-induced subretinal neovascularization in monkey. Furthermore, dexamethasone inhibits cauterization-induced corneal neovascularization.[4] Despite the widespread use of dexamethasone and other steroids in the clinical practice, little is known about the detailed mechanisms by which these molecules exert their anti-angiogenic effects *in vivo*.[5]

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The cornea, a transparent and avascular tissue, encompasses extracellular matrix, keratocytes,[6] and leukocytes.[7] Corneal neovascularization occurs in a number of corneal disorders and causes significant loss of visual acuity. In corneal diseases, a number of cytokines and growth factors are up-regulated and induce infiltration of neutrophils, macrophages, and lymphocytes.[8] Infiltration of inflammatory cells is often accompanied by an angiogenic response. However, the mechanistic role of inflammatory cells in corneal angiogenesis is only beginning to be understood.

Corneal stromal cells, known as keratocytes or corneal fibroblasts, are normally quiescent but can readily respond to injury and transit into activated phenotypes under pathological conditions.[6] After corneal injury, corneal stromal cells are activated and migrate to the site of injury.[9] In fibroblast growth factor-2-implanted corneas, stromal cells but not leukocytes express vascular endothelial growth factor (VEGF).[10] However, it is unknown whether corneal stromal cells contribute to inflammation-induced neovascularization.

Interleukin (IL)-1$\beta$, a multipotent cytokine, is critically involved in the acute inflammatory response, activation of inflammatory and antigen-presenting cells, chemotaxis, up-regulation of adhesion molecules and costimulatory factors on cells, and neovascularization.[11] In the eye, IL-1 activity has been correlated with corneal neovascularization.[12,13] The expression of IL-1$\beta$ in the cornea is increased in various corneal diseases including chemical burns[14] and herpetic stromal keratitis.[15–17] Previously, we reported that IL-1$\beta$ causes corneal angiogenesis by inducing VEGF-A, COX-2/prostanoids, and CXC chemokines (such as KC and MIP-2).[18,19] Furthermore, IL-1$\beta$ induces infiltration of various inflammatory cells, including neutrophils and macrophages into the cornea.[19] We demonstrated that infiltration of the $CD11b^+$ inflammatory cells, a major source of angiogenic factors, is important in IL-1$\beta$-induced corneal neovascularization.[19] Dexamethasone blocks the transcription of inflammatory proteins by prohibiting the activity of the transcription factor nuclear factor (NF)-$\kappa$B.[20] NF-$\kappa$B plays an important role in IL-1$\beta$-related inflammatory diseases, including various corneal diseases.[21,22] Blockade of NF-$\kappa$B reduces corneal epithelial defects during healing in a model of corneal injury.[21] However, it is unknown whether dexamethasone has an impact on IL-1$\beta$-dependent angiogenesis. In this work we investigate dexamethasone's potential in inhibiting IL-1$\beta$-induced angiogenesis and candidate mechanisms both *in vivo* and *in vitro*.

## Materials and Methods

### Animals

All animal experiments were approved by the Committee on the Ethics of Animal Experiments at the Kyushu University Graduate School of Medical Sciences and the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Male BALB/c mice, 6 to 10 weeks old, were purchased from Kyudo (Saga, Japan) and Taconic (Hudson, NY).

### Corneal Micropocket Assay in Mice

BALB/c mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). Hydron pellets (0.3 $\mu$l) containing 30 ng of human IL-1$\beta$ (201-LB; R&D Systems, Minneapolis, MN) were prepared and implanted into the corneas. Pellets were positioned at 1 mm to the corneal limbus. Implanted eyes were treated with Levofloxacin eye drops (Santen Pharmaceuticals, Osaka, Japan) to prevent infection. Dexamethasone (5 mg/kg) (D2915; Sigma Chemical Co., St. Louis, MO) was injected intraperitoneally daily, starting 1 day before ($-1$) and continued until the 5th day after implantation. A peptide inhibitor of NF-$\kappa$B, SN50 (P-600; Biomol International, Plymouth Meeting, PA), or the control peptide SN50M (P-601; Biomol International) was applied topically to IL-1$\beta$-implanted eyes twice a day from days $-1$ to 5. Two, 4, and 6 days after implantation, digital images of the corneal vessels were obtained and recorded using Viewfinder 3.0 (Pixera, San Jose, CA) or OpenLab software, version 2.2.5 (Improvision Inc., Lexington, MA) with standardized illumination and contrast and were saved to disks. The quantitative analysis of neovascularization in the mouse corneas was performed using Scion Image software (version 4.0.2; Scion Corp., Frederick, MD).

### Isolation of the Cornea-Infiltrating Cells

On days 2 and 4 after IL-1$\beta$ implantation, five corneas including limbal vessel were harvested, pooled, and dissected with microscissors. The tissues were then treated twice at 37°C for 30 minutes with 0.5 mg/ml collagenase type D (Boehringer-Mannheim, Indianapolis, IN) in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) containing 10% fetal calf serum (Life Technologies, Inc.), 10 mg/ml gentamicin, 50 $\mu$mol/L 2-mercaptoethanol, and 5 mg/ml HEPES buffer. The supernatants were collected, passed through a stainless-steel mesh sieve, and washed three times.

### Flow Cytometry

Infiltrated cells into the cornea were stained with phycoerythrin-conjugated anti-CD11b mAb (1:50, RM2804; Caltag Laboratory, Burlingame, CA) for 30 minutes on ice. After washing with phosphate-buffered saline (PBS) twice, flow cytometry was performed using the FACSCaliber system (Becton-Dickinson, Mountain View, CA).

### Enzyme-Linked Immunosorbent Assay (ELISA) of VEGF-A, KC, MIP-2, and Prostaglandin $E_2$ in Mouse Corneas

Four corneas with or without dexamethasone treatment were harvested at the indicated time points after pellet implantation. The corneas were pooled in 200 $\mu$l of media, dissected with scissors, extracted with 200 $\mu$l of Triton X-100 buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, and 10% glycerol containing 1 mmol/L

phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mmol/L sodium orthovanadate), and centrifuged. The supernatants were used in ELISA kits (R&D Systems) for mouse KC (MKC00B), mouse MIP-2 (MM200), mouse VEGF-A (MMV00), and prostaglandin $E_2$ ($PGE_2$; DE0100).

### *Immunohistochemistry*

Mice were sacrificed under deep anesthesia with pentobarbital sodium (60 mg/kg i.p.). The eyes were harvested, snap-frozen in optimal cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan), and 10-μm sections were prepared, air-dried, and fixed in ice-cold acetone for 10 minutes. The sections were blocked with 3% skim milk and stained with anti-phospho-IκB-α (1:100, no. 9246; Cell Signaling, Beverly, MA) and anti-CD11b mAb (1:100, 550282; BD Pharmingen, San Diego, CA). After an overnight incubation, sections were washed and stained for 20 minutes with secondary antibodies (Abs) (Chemicon International, Temecula, CA), fluorescein isothiocyanate-conjugated goat anti-rat (1:100, AP136F), Cy5-conjugated donkey anti-rabbit (1:100, AP182S), and Cy5-conjugated goat anti-mouse (1:100, AP181S).

### *Cell Culture*

Immortalized keratocytes from corneal stroma of C57BL/6 WT mice (MK/T-1 cells)[23] were grown in low-glucose Dulbecco's minimum essential medium (no. 11885-084; Life Technologies, Inc.), supplemented with 10% fetal bovine serum at 37°C in 5% $CO_2$.

### *Western Blot Analysis*

After culture for 12 hours in serum-free medium with or without the 100 nmol/L dexamethasone, MK/T-1 cells were stimulated with 1 ng/ml IL-1β for 15 minutes (whole cell lysates) or 30 minutes (nuclear extracts) at 37°C. After rinsing with ice-cold PBS, the cells were lysed in a mammalian cell lysis kit (MCL1; Sigma Chemical Co.). Nuclear extracts were prepared with a nuclear extract kit (no. 40010; Active Motif, Carlsbad, CA). Lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon membranes (Millipore, Bedford, MA). Blots were incubated with anti-IκB-α (1:1000, no. 9242; Cell Signaling), anti-phospho-IκB-α (1:1000, no. 9241; Cell Signaling), anti-phospho-NF-κB p65 (1:1000, no. 3033; Cell Signaling), anti-NF-κB p65 (1:1000, no. 3034; Cell Signaling), or anti-β-tubulin (1:1000, ab11308; Abcam, Cambridge, UK) and visualized with a secondary antibody coupled to horseradish peroxidase (Amersham, Arlington Heights, IL) and enhanced chemiluminescence system.

### *DNA-Binding Activity of NF-κB*

After culture for 12 hours in serum-free medium with or without the 100 nmol/L dexamethasone, MK/T-1 cells were stimulated with 1 ng/ml IL-1β for 30 minutes at 37°C. After preparation of whole cell lysates, the activity of NF-κB p65 transcription in MK/T-1 cells was measured using the Transfactor NF-κB p65 colorimetric kit (631930; Clontech, Palo Alto, CA), which allows identification of DNA-protein interactions.

### *Quantification of VEGF, KC, and $PGE_2$*

The concentrations of VEGF, KC, and $PGE_2$ in the conditioned media from MK/T-1 cells were measured using ELISA kits as described previously.[24] In brief, MK/T-1 cells were seeded in 24-well dishes at $2.5 \times 10^4$ cells in a 2-ml volume per well, and when subconfluent, the medium was replaced with serum-free medium for 24 hours, with or without 100 nmol/L dexamethasone, with or without 1 ng/ml IL-1β at 37°C.

### *Statistical Analysis*

Comparisons were evaluated by the two-tailed unpaired Student's *t*-test. N-numbers per group were as indicated. Data are presented as mean ± SD. The differences between the groups were considered statistically significant for values of $P < 0.05$.

## *Results*

### *Dexamethasone Inhibits IL-1β-Induced Angiogenesis in the Mouse Cornea*

To investigate whether dexamethasone affects IL-1β-induced angiogenesis, we implanted IL-1β-containing pel-

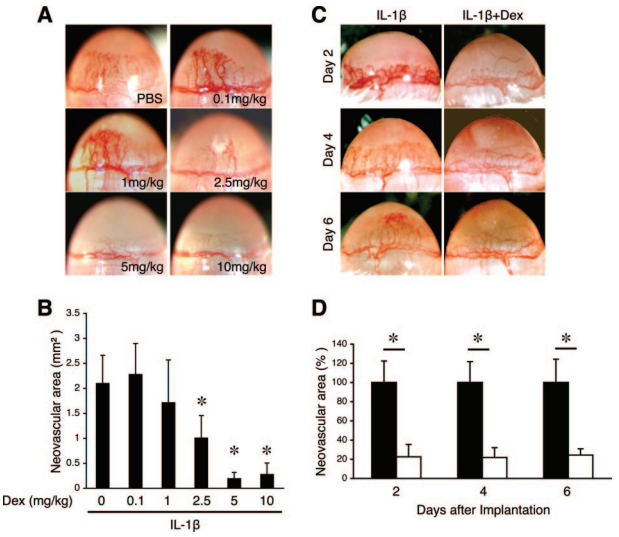


**Figure 1.** Dexamethsone's effect on IL-1β-induced corneal neovascularization. **A:** Hydron pellets containing 30 ng of IL-1β were implanted into the corneas of male BALB/c mice treated with vehicle or various concentrations of dexamethasone (Dex). **B:** Quantitative analysis of neovascularization was performed on day 6 after implantation (*$P < 0.01$ versus untreated using the two-tailed Student's *t*-test). **C:** Photomicrographs from vessels in the proximity of the sites of the pellets on days 2, 4, and 6 after implantation. **D:** Quantitative analysis of IL-1β-induced corneal neovascularization in dexamethasone-treated ($n = 6$) and control mice ($n = 8$) was performed on days 2, 4, and 6 (77.5, 78.2, and 75.7% reduction, respectively) (*$P < 0.01$).

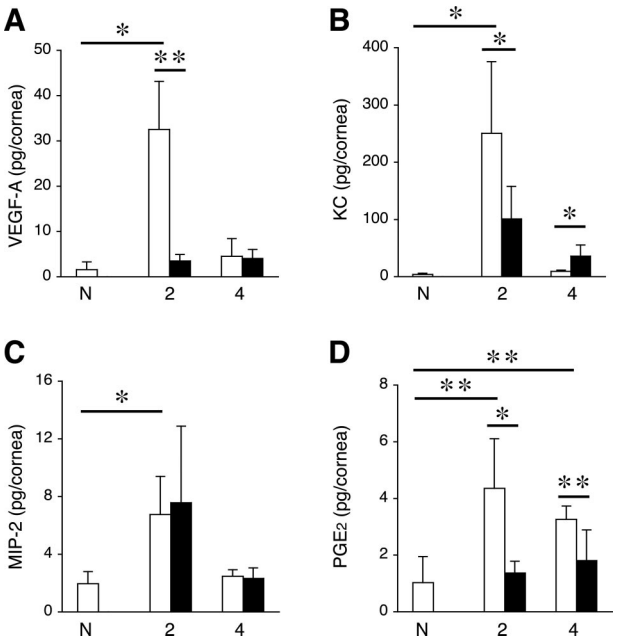


**Figure 2.** Kinetics of the levels of angiogenesis-related factors after IL-1$\beta$ pellet implantation. Lysates of four corneas with (black) or without (gray) Dex treatment were prepared and individually assayed using VEGF (**A**), KC (**B**), MIP-2 (**C**), or $PGE_2$ (**D**) ELISA at the indicated times ($n = 3$ to 8; $^*P < 0.05$, $^{**}P < 0.01$).

lets into the corneas of BALB/c mice, treated them with different doses of dexamethasone or vehicle, and quantified the amount of angiogenesis 6 days after implantation. Dexamethasone inhibited IL-1$\beta$-induced angiogenesis in a dose-dependent manner (Figure 1, A and B). Five mg/kg dexamethasone completely blocked IL-1$\beta$-induced angiogenesis on day 6 (Figure 1, A and B). Furthermore, dexamethasone inhibited IL-1$\beta$-induced corneal angiogenesis by 77.5, 78.2, and 75.7% on day 2 ($P < 8 \times 10^{-8}$, $n = 6$ and 8), day 4 ($P < 9 \times 10^{-6}$, $n = 6$ and 8), and day 6 ($P < 3 \times 10^{-8}$, $n = 6$ and 8), respectively (Figure 1, C and D), suggesting that dexamethasone has an impact on IL-1$\beta$-induced corneal angiogenesis from an early stage.

## *Dexamethasone Inhibits IL-1β-Induced Expression of Angiogenesis-Related Factors*

To investigate whether dexamethasone changes the expression of angiogenesis-related factors, such as VEGF-A[25] and CXC chemokines[26] (KC and MIP-2), we quantified these factors in corneas of mice during IL-1$\beta$-induced angiogenesis. VEGF-A, KC, and MIP-2 levels were significantly increased in IL-1$\beta$-implanted corneas compared with those of controls on day 2 ($P < 0.05$, $n = 3$) (Figure 2, A–C). VEGF-A and KC protein levels were significantly reduced by dexamethasone on day 2 ($P = 0.004$ and 0.03; $n = 3$ and 4, respectively) (Figure 2, A and B). However, dexamethasone did not significantly affect MIP-2 protein levels in IL-1$\beta$-implanted corneas ($P = 0.8$, $n = 3$ and 4) (Figure 2C). Interestingly, dexamethasone increased KC protein levels in IL-1$\beta$-implanted corneas on day 4 ($P = 0.03$, $n = 4$). These data show that dexamethasone inhibits IL-1$\beta$-induced VEGF-A and KC but not MIP-2 expression 2 days after implantation.

Furthermore, to examine whether dexamethasone impacts IL-1$\beta$-induced $PGE_2$ expression, we quantified its concentration in corneal extracts by ELISA. Dexamethasone treatment showed a significant decrease in $PGE_2$ levels 2 ($P = 0.04$, $n = 3$) and 4 days ($P = 0.004$, $n = 7$ and 8) after IL-1$\beta$ implantation (Figure 2D).

## *Dexamethasone Inhibits IL-1β-Induced Infiltration of CD11b$^+$ Cells*

To examine the effect of dexamethasone on IL-1$\beta$-induced infiltration of inflammatory cells, we quantified the number of $CD11b^+$ cells in corneas of implanted animals using flow cytometry and histology. Corneas from mice treated with dexamethasone or vehicle control were harvested, and the percentage of $CD11b^+$ cells were determined by FACScan. The percentage of the infiltrating $CD11b^+$ cells was increased to 55 ± 10.9% and 28.1 ± 9.9% on days 2 and 4 after IL-1$\beta$ implantation, respectively (Figure 3A). In comparison, the percentage of infiltrating $CD11b^+$ cells in dexamethasone-treated mice was 51.3 ± 6.9% and 33.3 ± 5.8% on days 2 and 4 after IL-1$\beta$ implantation, respectively (Figure 3A). There was no statistical difference between the results in the dexamethasone-treated and untreated IL-1$\beta$-implanted mice ($P = 0.6$, $n = 4$ on day 2; and $P = 0.2$, $n = 7$ and 8 on day 4). To analyze the effect of dexamethasone on the number of infiltrated $CD11b^+$ cells into corneas, we next performed immunostaining for CD11b. On day 2, in corneas of dexamethasone-treated mice significantly less $CD11b^+$ cells were found when compared with those of control mice ($P < 0.05$, $n = 5$), whereas there was no significant difference between the number of $CD11b^+$ cells in dexamethasone-treated and untreated control ($P = 0.8$, $n = 4$ and 5). These data indicate that 5 mg/kg dexamethasone inhibits IL-1$\beta$-induced infiltration of $CD11b^+$ into cornea on day 2 but not day 4.

## *Dexamethasone Inhibits NF-κB Activation of Stromal Fibroblasts in IL-1β-Implanted Corneas*

To elucidate the molecular events underlying the inhibition of IL-1$\beta$-induced angiogenesis by dexamethasone, we examined whether dexamethasone affects NF-$\kappa$B signaling in IL-1$\beta$-implanted corneas. We performed immunohistochemistry with Abs against CD11b and phosphorylated I$\kappa$B-$\alpha$, a key signaling molecule upstream of NF-$\kappa$B. Surprisingly, I$\kappa$B-$\alpha$ phosphorylation was observed mainly in stromal cells (94 ± 29.3 cells/field at ×200) but not in the $CD11b^+$ cells (13 ± 4.69 cells/field) in IL-1$\beta$-implanted corneas on day 2 (Figure 4, A and B). On day 4, most (91.9 ± 6.7%, $n = 8$) of $CD11b^+$ cells (38.8 ± 14.0 cells/field) as well as stromal cells (49.5 ± 10.9 cells/field) were positive for phosphorylated I$\kappa$B-$\alpha$ (Figure 4, A and B). In dexamethasone-treated mice, we observed infiltration of $CD11b^+$ cells in corneas both on day 2 and day 4. On day

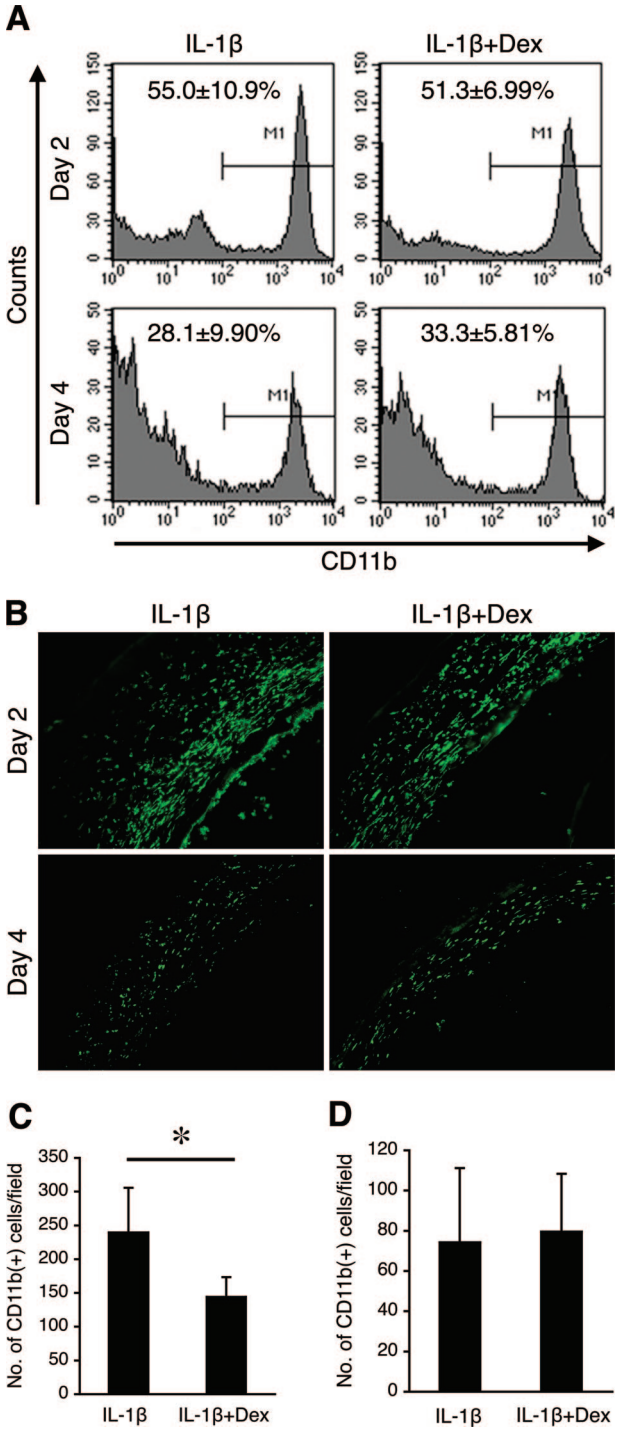


**Figure 3.** IL-1$\beta$-induced cell infiltration into the cornea. The analysis of infiltrating cells obtained after IL-1$\beta$ implantation and treatment with or without dexamethasone (Dex) on days 2 and 4. **A:** FACS analysis (CD11b) of infiltrating cells obtained from five IL-1$\beta$-implanted corneas treated with Dex or control on day 2 ($n = 4$, **A** and **B**) and day 4 ($n = 7$ to 8, **C** and **D**). **B:** Representative photomicrographs of CD11b-stained sections from IL-1$\beta$-implanted corneas with or without Dex on days 2 and 4. **C** and **D:** Quantification analysis of the number of CD11b$^+$ cells from IL-1$\beta$-implanted corneas treated with Dex or vehicle control on day 2 (**C**; 39.8% reduction, $^*P = 0.019$) and day 4 (**D**, $P = 0.8$). Original magnifications, ×200.

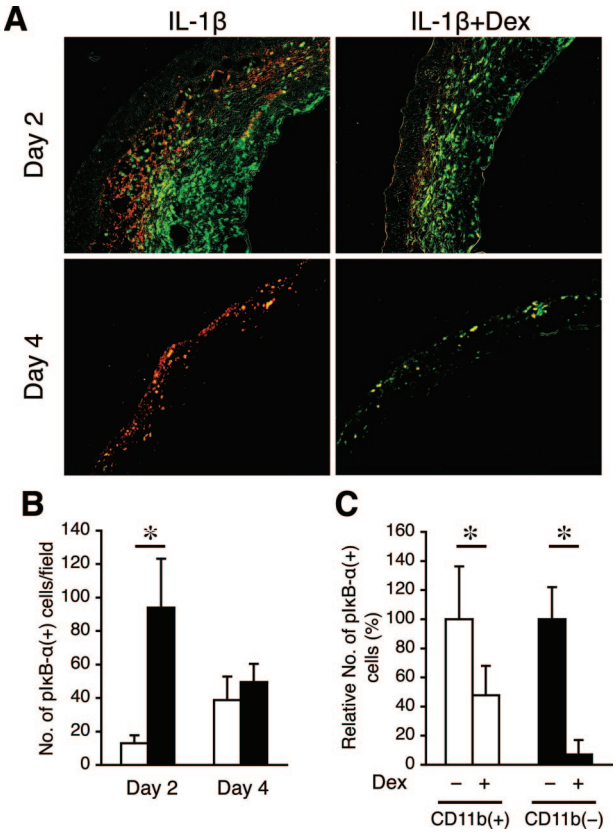


**Figure 4.** Histological detection of cell activation markers. **A:** Immunohistochemical detection of CD11b (green) and phosphorylated I$\kappa$B-$\alpha$ (red) in IL-1$\beta$-implanted corneas of Dex or vehicle control-treated mice on days 2 and 4. **B:** The number of phospho-I$\kappa$B-$\alpha^+$CD11b$^+$ cells (white) and phospho-I$\kappa$B-$\alpha^+$CD11b$^-$ cells (black) in the stroma of IL-1$\beta$-implanted corneas on day 2 ($n = 6$, $^*P < 0.0001$) and day 4 ($n = 8$, $P = 0.1$). Each value represents the mean number of cells from six to eight randomly selected microscopic fields ± SD. **C:** Comparison of the number of phospho-I$\kappa$B-$\alpha^+$CD11b$^+$ (white, 52.3% inhibition) or phospho-I$\kappa$B-$\alpha^+$CD11b$^-$ cells (black, 92.1% inhibition) in IL-1$\beta$-implanted corneas with or without Dex treatment (day 4, $n = 8$; $^*P < 0.01$). Original magnifications, ×200.

2, phosphorylated I$\kappa$B-$\alpha$ cells were not observed in dexamethasone-treated mice (Figure 4A). On day 4, some of CD11b$^+$ cells were stained with Ab against phosphorylated I$\kappa$B-$\alpha$, whereas CD11b$^-$ stromal cells were negative for phosphorylated I$\kappa$B-$\alpha$ (Figure 4, A and C). These results show that IL-1$\beta$ induces I$\kappa$B-$\alpha$ phosphorylation mainly in stromal cells but not infiltrated CD11b$^+$ cells on day 2 (Figure 4B, $P < 6 \times 10^{-6}$, $n = 6$) and in both CD11b$^+$ cells and stromal cells on day 4 (Figure 4C) and that dexamethasone inhibits NF-$\kappa$B signaling predominantly in the CD11b$^-$ stromal cells (Figure 4C).

## *Dexamethasone Inhibits IL-1β-Induced NF-κB Activation in Corneal Stromal Fibroblasts*

To investigate the impact of dexamethasone on IL-1$\beta$-induced expression of various angiogenesis-related factors and its relation to NF-$\kappa$B signaling, we cultured corneal stromal fibroblasts, MK/T1, and treated them with dexamethasone or control and measured in these cells the concentration of various angiogenic factors and NF-$\kappa$B activity. Dexamethasone significantly inhibited IL-

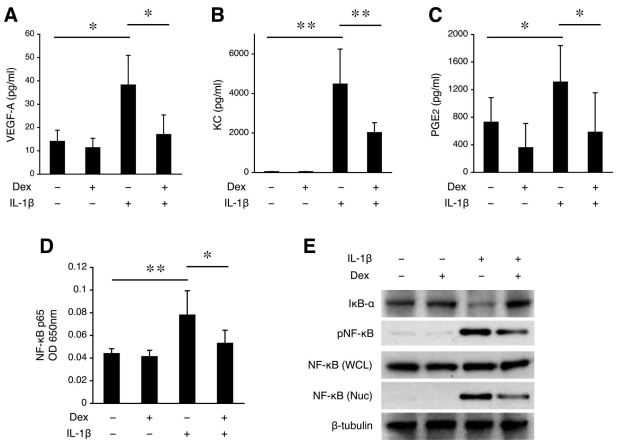


**Figure 5.** Dexamethasone's effect on IL-1$\beta$-induced NF-$\kappa$B activation and expression of angiogenesis-related factors in corneal fibroblasts. **A–C:** Detection of VEGF (**A**), KC (**B**), and $PGE_2$ (**C**) expression in MK/T1 cells after a 24-hour treatment with Dex (100 nmol/L), IL-1$\beta$ (1 ng/ml), or vehicle by ELISA ($n = 6$ to 7; $^*P < 0.05$, $^{**}P < 0.01$). **D:** MK/T1 cells were incubated with Dex (100 nmol/L) or vehicle for 12 hours and subsequently with IL-1$\beta$ (1 ng/ml) for 30 minutes. DNA-binding activity was measured by an ELISA-based assay at 650 nm. Data are representative of three separate experiments and show means ± SD from experiments performed in duplicate wells. $^*P < 0.05$, $^{**}P < 0.01$. **E:** Western blot analysis with anti-I$\kappa$B-$\alpha$, anti-pNF-$\kappa$B, anti-NF-$\kappa$B, or $\beta$-tubulin Abs using whole cell lysates (WCLs) or with anti-NF-$\kappa$B Ab using nuclear extracts (Nuc) of MK/T1 cells treated with Dex (100 nmol/L) or vehicle for 12 hours and subsequently with IL-1$\beta$ (1 ng/ml).

1$\beta$-induced production of VEGF-A, KC, and $PGE_2$ by MK/T1 cells ($P = 0.02$, 0.008, and 0.04, respectively; $n = 6$) (Figure 5, A–C). To understand how dexamethasone modulates IL-1$\beta$-induced angiogenesis, we next examined the effect of dexamethasone on IL-1$\beta$-dependent NF-$\kappa$B-p65 activity in MK/T1 cells. Dexamethasone significantly inhibited IL-1$\beta$-induced DNA-binding activity and nuclear localization of NF-$\kappa$B-p65 in MK/T1 cells ($P = 0.03$, $n = 6$) (Figure 5D). Furthermore, Western blots using nuclear extracts from dexamethasone- and vehicle control-treated MK/T1 cells revealed that dexamethasone inhibited IL-1$\beta$-induced translocation of NF-$\kappa$B-p65 into the nucleus (Figure 5E). To detect the signaling molecule that is targeted by dexamethasone, we performed immunoblot analysis of whole cell lysates with antibodies against NF-$\kappa$B signaling molecules. Dexamethasone inhibited IL-1$\beta$-induced I$\kappa$B-$\alpha$ degradation as well as NF-$\kappa$B phosphorylation (Figure 5E). These data indicate that dexamethasone inhibits IL-1$\beta$-induced NF-$\kappa$B signaling through blockade of I$\kappa$B-$\alpha$ degradation in corneal stromal cells.

### *Specific Blockade of NF-κB Inhibits IL-1β-Induced Angiogenesis in the Mouse Cornea*

To examine the role of NF-$\kappa$B in the IL-1$\beta$-induced angiogenesis in the mouse cornea, we treated the animals with the specific NF-$\kappa$B inhibitor peptide SN50 ($n = 6$) or the control peptide SN50M ($n = 5$) and quantified their corneal angiogenesis after IL-1$\beta$ implantation. SN50 significantly reduced IL-1$\beta$-induced angiogenesis on day 6, whereas the animals treated with the control SN50M peptide showed regular levels of corneal angiogenesis ($P = 0.002$) (Figure 6, A and B).

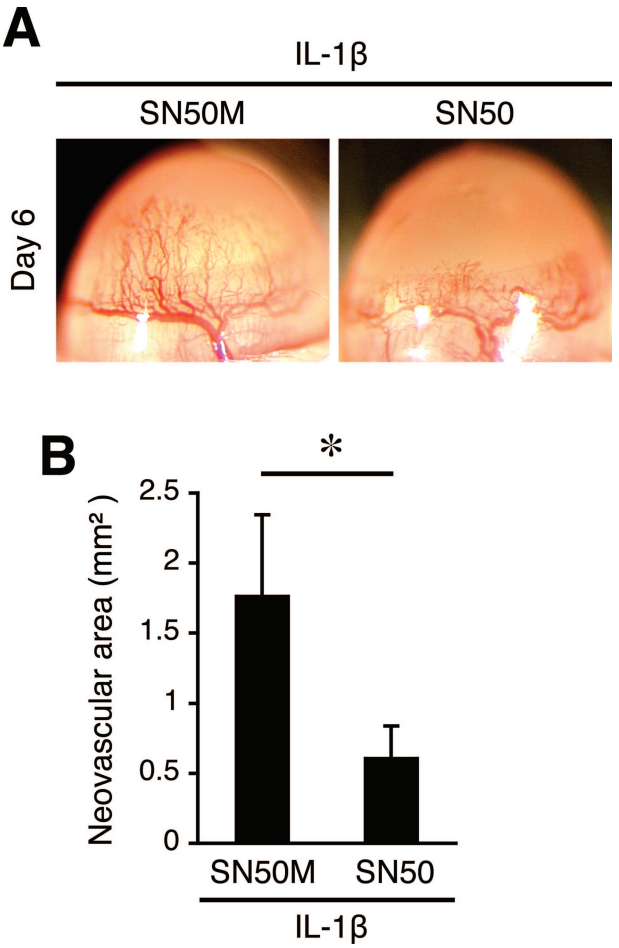


**Figure 6.** The effect of NF-$\kappa$B inhibition on IL-1$\beta$-induced corneal neovascularization. **A:** IL-1$\beta$-implanted corneas of BALB/c mice topically treated (3-$\mu$l eye drops) with the NF-$\kappa$B inhibitor peptide (SN50) or the control peptide (SN50M) (day 6). **B:** Quantitative analysis of IL-1$\beta$-induced corneal neovascularization in mice with SN50 ($n = 6$) and with SN50M ($n = 5$) treatment on day 6 (65.7% reduction, $^*P = 0.002$).

## *Discussion*

Dexamethasone potently suppresses the immunity and is commonly used in the treatment of a wide variety of immune and inflammatory diseases.[20] IL-1$\beta$, an inflammatory cytokine, is up-regulated in various corneal diseases.[14–17] Recently, we showed that IL-1$\beta$ induces corneal neovascularization via induction of various angiogenesis-related factors including VEGF, CXC chemokines, and COX-2/prostanoids.[18,19] In this study, we demonstrate that dexamethasone inhibits IL-1$\beta$-dependent corneal neovascularization partly through regulation of NF-$\kappa$B signaling and inhibition of VEGF, CXC chemokines, and $PGE_2$ production in corneal stromal fibroblast (Figure 7).

Corticosteroids inhibit inflammation through various different pathways.[20] For instance, corticosteroid-induced MAPK phosphatase 1 dephosphorylates and inactivates Jun N-terminal kinase, thereby inhibiting c-Jun-mediated transcription.[20] Corticosteroid-glucocorticoid receptor complex also interacts with NF-$\kappa$B to block its transcription activity.[20] Recent work suggests that glucocorticoids also have rapid nongenomic effects on inflammation. Hafezi-

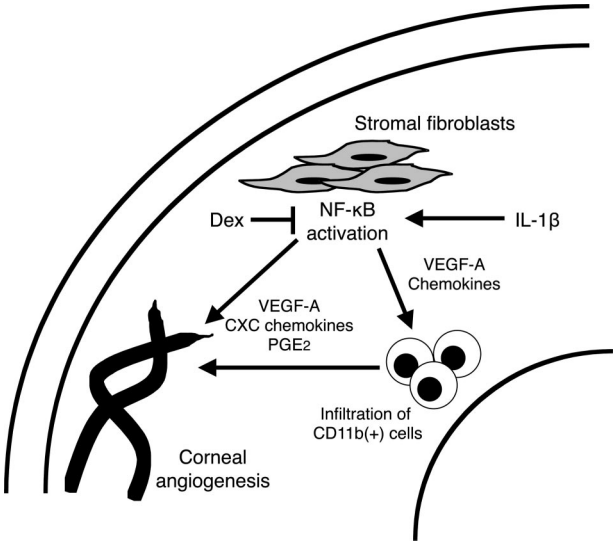


**Figure 7.** Schematic of how dexamethasone impacts IL-1$\beta$-induced corneal angiogenesis. Dexamethasone inhibits IL-1$\beta$-induced corneal angiogenesis by suppression of NF-$\kappa$B activation and the expression of angiogenesis-related factors in stromal cells during the early phase of the injury (day 2).

Moghadam and colleagues[27] reported that high-dose corticosteroids exert cardiovascular protection through nontranscriptional mechanisms involving rapid activation of endothelial nitric oxide synthase. In this study we examined the effect of dexamethasone on NF-$\kappa$B signaling and demonstrated that dexamethasone mainly inhibited NF-$\kappa$B activation. However, whether the angiostatic effects of dexamethasone involves nontranscriptional mechanisms remains to be investigated.

We first demonstrated that IL-1$\beta$ induces NF-$\kappa$B signaling in the mouse cornea during neovascularization. In the corneal alkali burns model, NF-$\kappa$B is activated in the corneal epithelial and stromal cells.[21] In our corneal angiogenesis model, I$\kappa$B-$\alpha$ was mainly phosphorylated in the corneal stromal cells, suggesting that NF-$\kappa$B-activated corneal stromal fibroblasts play an important role in corneal inflammatory conditions, such as angiogenesis and wound healing.

Dexamethasone is widely used in the treatment of corneal inflammation; however, steroid therapy in the management of some corneal diseases remains controversial because of its side effects.[28,29] Dexamethasone inhibits NF-$\kappa$B but not AP-1 activity in transfected human corneal fibroblasts.[30] We show that IL-1$\beta$-induced NF-$\kappa$B activation is inhibited by dexamethasone and that a selective NF-$\kappa$B inhibitor diminishes inflammatory corneal angiogenesis. These findings indicate that NF-$\kappa$B inhibition may be an effective therapeutic option for inflammatory corneal angiogenesis.[31–33] Further studies will be necessary to assess the safety and side effects for the treatment of human corneal diseases.

CXC chemokines containing the ELR motif mediate angiogenesis through G protein-coupled receptor CXCR2 on endothelial cells.[26,34] Previously, we showed that CXCR2 blockade partially inhibits IL-1$\beta$-induced corneal angiogenesis.[19] NF-$\kappa$B is known to play an important role as a master switch in the transactivation of angiogenic CXC chemokines.[26] Members of the CXC chemokines, such as KC and MIP-2, induce corneal angiogenesis[34] and have been implicated in the pathogenesis of inflammatory corneal diseases.[30,35] We observed that dexamethasone inhibits IL-1$\beta$-induced NF-$\kappa$B signaling and the level of KC protein but not that of MIP-2. These data suggest that the contribution of MIP-2 may be less than that of KC in IL-1$\beta$-induced corneal angiogenesis.

A recent report shows that fibroblasts produce SDF-1 and promote tumor progression via SDF-1-CXCR4-mediated angiogenesis.[36] Another report demonstrated that human corneal fibroblasts express SDF-1 mRNA.[37] We examined whether SDF-1 protein expression was increased in IL-1$\beta$-implanted corneas during neovascularization using ELISA. Unexpectedly, however, we did not detect SDF-1 overexpression in IL-1$\beta$-implanted corneas (data not shown), suggesting that SDF-1 may not be necessary in our IL-1$\beta$-induced corneal angiogenesis model.

The IL-1 receptor, which binds both IL-1$\alpha$ and IL-1$\beta$, is constitutively expressed in corneal fibroblasts.[38] After corneal injury, IL-1 protein is detectable in corneal fibroblasts. IL-1 up-regulates the expression of various cytokines or enzymes by corneal fibroblasts,[8,22,39] and it also contributes to wound healing. In our model, IL-1$\beta$ binds to the IL-1 receptor on corneal fibroblasts, up-regulates various angiogenesis-related factors, and induces corneal neovascularization.

Cancer cells are known to alter their adjacent stroma to form a permissive and supportive microenvironment by producing various growth factors and cytokines.[40] Recent studies reported that experimentally induced genetic alterations in stromal fibroblasts cause epithelial neoplasia and invasive carcinoma.[36,41] Various angiogenesis-related factors, including CXC chemokines and growth factors, are produced in IL-1$\beta$-treated corneal stromal fibroblasts. Activation of stromal fibroblasts by IL-1$\beta$ plays a central role in tumor angiogenesis as well as in corneal angiogenesis.[42]

To date, steroid therapy has been the standard anti-inflammatory and angiostatic treatment in the cornea.[28,29] Our results suggest that specific molecular or cellular targeting strategies, such as blockade of NF-$\kappa$B or regulation of the activation status of corneal stromal cells, may offer novel approaches in the treatment of inflammatory angiogenesis in the cornea.

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## *References*

1. Folkman J, Ingber DE: Angiostatic steroids. Method of discovery and mechanism of action. Ann Surg 1987, 206:374–383
2. Ishibashi T, Miki K, Sorgente N, Patterson R, Ryan SJ: Effects of intravitreal administration of steroids on experimental subretinal neo-

vascularization in the subhuman primate. Arch Ophthalmol 1985, 103:708–711
3. Gross J, Azizkhan RG, Biswas C, Bruns RR, Hsieh DS, Folkman J: Inhibition of tumor growth, vascularization, and collagenolysis in the rabbit cornea by medroxyprogesterone. Proc Natl Acad Sci USA 1981, 78:1176–1180
4. Proia AD, Hirakata A, McInnes JS, Scroggs MW, Parikh I: The effect of angiostatic steroids and β-cyclodextrin tetradecasulfate on corneal neovascularization in the rat. Exp Eye Res 1993, 57:693–698
5. Longenecker JP, Kilty LA, Johnson LK: Glucocorticoid influence on growth of vascular wall cells in culture. J Cell Physiol 1982, 113:197–202
6. West-Mays JA, Dwivedi DJ: The keratocyte: corneal stromal cell with variable repair phenotypes. Int J Biochem Cell Biol 2006, 38:1625–1631
7. Nakamura T, Ishikawa F, Sonoda KH, Hisatomi T, Qiao H, Yamada J, Fukata M, Ishibashi T, Harada M, Kinoshita S: Characterization and distribution of bone marrow-derived cells in mouse cornea. Invest Ophthalmol Vis Sci 2005, 46:497–503
8. Wilson SE, Mohan RR, Mohan RR, Ambrosio Jr R, Hong J, Lee J: The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. Prog Retin Eye Res 2001, 20:625–637
9. Fini ME, Stramer BM: How the cornea heals: cornea-specific repair mechanisms affecting surgical outcomes. Cornea 2005, 24:S2–S11
10. Chang LK, Garcia-Cardena G, Farnebo F, Fannon M, Chen EJ, Butterfield C, Moses MA, Mulligan RC, Folkman J, Kaipainen A: Dose-dependent response of FGF-2 for lymphangiogenesis. Proc Natl Acad Sci USA 2004, 101:11658–11663
11. Dinarello CA: Biologic basis for interleukin-1 in disease. Blood 1996, 87:2095–2147
12. Dana MR, Zhu SN, Yamada J: Topical modulation of interleukin-1 activity in corneal neovascularization. Cornea 1998, 17:403–409
13. BenEzra D, Hemo I, Maftzir G: In vivo angiogenic activity of interleukins. Arch Ophthalmol 1990, 108:573–576
14. 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
15. Staats HF, Lausch RN: Cytokine expression in vivo during murine herpetic stromal keratitis. Effect of protective antibody therapy. J Immunol 1993, 151:277–283
16. 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
17. Biswas PS, Banerjee K, Kim B, Kinchington PR, Rouse BT: Role of inflammatory cytokine-induced cyclooxygenase 2 in the ocular immunopathologic disease herpetic stromal keratitis. J Virol 2005, 79:10589–10600
18. Kuwano T, Nakao S, Yamamoto H, Tsuneyoshi M, Yamamoto T, Kuwano M, Ono M: Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. FASEB J 2004, 18:300–310
19. Nakao S, Kuwano T, Tsutsumi-Miyahara C, Ueda S, Kimura YN, Hamano S, Sonoda KH, Saijo Y, Nukiwa T, Strieter RM, Ishibashi T, Kuwano M, Ono M: Infiltration of COX-2-expressing macrophages is a prerequisite for IL-1β-induced neovascularization and tumor growth. J Clin Invest 2005, 115:2979–2991
20. Rhen T, Cidlowski JA: Antiinflammatory action of glucocorticoids—new mechanisms for old drugs. N Engl J Med 2005, 353:1711–1723
21. Saika S, Miyamoto T, Yamanaka O, Kato T, Ohnishi Y, Flanders KC, Ikeda K, Nakajima Y, Kao WW, Sato M, Muragaki Y, Ooshima A: 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
22. 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
23. Gendron RL, Liu CY, Paradis H, Adams LC, Kao WW: MK/T-1, an immortalized fibroblast cell line derived using cultures of mouse corneal stroma. Mol Vis 2001, 7:107–113
24. Ueda S, Basaki Y, Yoshie M, Ogawa K, Sakisaka S, Kuwano M, Ono M: PTEN/Akt signaling through epidermal growth factor receptor is prerequisite for angiogenesis by hepatocellular carcinoma cells that is susceptible to inhibition by gefitinib. Cancer Res 2006, 66:5346–5353
25. Loureiro RM, D'Amore PA: Transcriptional regulation of vascular endothelial growth factor in cancer. Cytokine Growth Factor Rev 2005, 16:77–89
26. Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA: Cancer CXC chemokine networks and tumour angiogenesis. Eur J Cancer 2006, 42:768–778
27. Hafezi-Moghadam A, Simoncini T, Yang Z, Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS, Thomas KL, Prorock AJ, Laubach VE, Moskowitz MA, French BA, Ley K, Liao JK: Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. Nat Med 2002, 8:473–479
28. Leibowitz HM, Kupferman A, Stewart RH, Kimbrough RL: Evaluation of dexamethasone acetate as a topical ophthalmic formulation. Am J Ophthalmol 1978, 86:418–423
29. Raizman M: Corticosteroid therapy of eye disease. Fifty years later. Arch Ophthalmol 1996, 114:1000–1001
30. Lu Y, Fukuda K, Nakamura Y, Kimura K, Kumagai N, Nishida T: Inhibitory effect of triptolide on chemokine expression induced by proinflammatory cytokines in human corneal fibroblasts. Invest Ophthalmol Vis Sci 2005, 46:2346–2352
31. Olivier S, Robe P, Bours V: Can NF-κB be a target for novel and efficient anti-cancer agents? Biochem Pharmacol 2006, 72:1054–1068
32. Miller JW, Stinson WG, Folkman J: Regression of experimental iris neovascularization with systemic α-interferon. Ophthalmology 1993, 100:9–14
33. Manna SK, Mukhopadhyay A, Aggarwal BB: IFN-α suppresses activation of nuclear transcription factors NF-κB and activator protein 1 and potentiates TNF-induced apoptosis. J Immunol 2000, 165:4927–4934
34. Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY, Buechi L, Walz A, Richmond A, Strieter RM: The CXC chemokine receptor 2, CXCR2, is the putative receptor for $ELR^+$ CXC chemokine-induced angiogenic activity. J Immunol 2000, 165:5269–5277
35. Yamagami S, Miyazaki D, Ono SJ, Dana MR: Differential chemokine gene expression in corneal transplant rejection. Invest Ophthalmol Vis Sci 1999, 40:2892–2897
36. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 2005, 121:335–348
37. Bourcier T, Berbar T, Paquet S, Rondeau N, Thomas F, Borderie V, Laroche L, Rostene W, Haour F, Lombet A: Characterization and functionality of CXCR4 chemokine receptor and SDF-1 in human corneal fibroblasts. Mol Vis 2003, 9:96–102
38. Wilson SE, Lloyd SA, He YG: Glucocorticoid receptor and interleukin-1 receptor messenger RNA expression in corneal cells. Cornea 1994, 13:4–8
39. Hong JW, Liu JJ, Lee JS, Mohan RR, Mohan RR, Woods DJ, He YG, Wilson SE: Proinflammatory chemokine induction in keratocytes and inflammatory cell infiltration into the cornea. Invest Ophthalmol Vis Sci 2001, 42:2795–2803
40. Mueller MM, Fusenig NE: Friends or foes—bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 2004, 4:839–849
41. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR: Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 1999, 59:5002–5011
42. Saijo Y, Tanaka M, Miki M, Usui K, Suzuki T, Maemondo M, Hong X, Tazawa R, Kikuchi T, Matsushima K, Nukiwa T: Proinflammatory cytokine IL-1β promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: in vivo analysis of tumor-stromal interaction. J Immunol 2002, 169:469–475