Cytokine, Chemokine, and Adhesion Molecule Expression Mediated by MAPKs in Human Corneal Fibroblasts Exposed to Poly(I:C)

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Purpose. Polyinosinic-polycytidylic acid [poly(I:C)], an analog of viral double-stranded RNA, interacts with Toll-like receptor (TLR)-3 and thereby elicits immunoinflammatory responses characteristic of viral infection. The effects of poly(I:C) on the expression of proinflammatory cytokines, chemokines, and adhesion molecules, as well as the signaling pathways that underlie such effects, were investigated in cultured human corneal fibroblasts.

Methods. Expression of the adhesion molecules intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 was evaluated by immunoblot and immunofluorescence analyses. Release of the proinflammatory cytokine IL-6 and of the chemokines interleukin (IL)-8, granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1β, eotaxin, and RANTES was measured with assay kits. Subcellular localization of the p65 subunit of the transcription factor nuclear factor (NF-κB) was examined by immunofluorescence analysis. Expression of TLR3, phosphorylation (activation) of mitogen-activated protein kinases (MAPKs), and phosphorylation and degradation of the NF-κB–inhibitory protein IκB-α was assessed by immunoblot analysis.

Results. Poly(I:C) induced the up-regulation of TLR3, the release of IL-6, IL-8, G-CSF, MIP-1β, eotaxin, and RANTES, and the expression of ICAM-1 and VCAM-1 in corneal fibroblasts. It also activated the MAPKs ERK, p38, and JNK and induced the phosphorylation and degradation of IκB-α and the nuclear translocation of p65 in these cells. Poly(I:C)-induced expression of IL-6, IL-8, G-CSF, MIP-1β, exotaxin, RANTES, and ICAM-1 was inhibited differentially by the MAPK inhibitors PD98059 and SB203580 and by JNK inhibitor II.

Conclusions. Poly(I:C) induces the up-regulation of TLR3, the MAPK-dependent expression of proinflammatory cytokines, chemokines, and adhesion molecules and the activation of NF-κB in human corneal fibroblasts. Corneal fibroblasts may thus play an important role in the modulation of local immune and inflammatory responses to viral infection in the corneal stroma. (*Invest Ophthalmol Vis Sci.* 2008;49:3336–3344) DOI: 10.1167/iovs.07-0972

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Supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science (TN).

Submitted for publication July 30, 2007; revised January 15 and March 5, 2008; accepted June 11, 2008.

Disclosure: **Y. Liu**, None; **K. Kimura**, None; **R. Yanai**, None; **T. Chikama**, None; **T. Nishida**, None

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Immunoinflammatory lesions of the corneal stroma that result from ocular infection with viruses such as herpes simplex virus are among the most common causes of blindness in humans in developed countries.[1] Viral stromal keratitis is a chronic immunopathologic disease characterized by complex interactions among infiltrating immune cells, including polymorphonuclear leukocytes (PMNs), macrophages, T cells, and corneal resident cells.[2,3] These cellular interactions are thought to result in the induction and perpetuation of a chronic inflammatory process that leads to corneal neovascularization, edema, injury, and opacity and, eventually, to blindness.[4] However, the factors responsible for these inflammatory events in the corneal stroma after virus infection remain incompletely understood.

Keratocytes, the major resident cells of the corneal stroma, maintain the precise organization of the extracellular matrix of the stroma that underlies corneal transparency. Corneal fibroblasts (activated keratocytes) are also thought to play an important role in the modulation of local inflammatory responses to stromal infection through the expression of adhesion molecules and the secretion of regulatory factors such as cytokines and chemokines.[5,6] We have previously shown that corneal fibroblasts produce proinflammatory cytokines, such as interleukin (IL)-6, and chemokines, including IL-8, granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1β, and eotaxin, in response to inflammatory stimulation.[7,8] The chemokine RANTES (regulated on activation, normal T expressed and secreted) is also synthesized by corneal fibroblasts after exposure to cytokines.[9] These factors together induce the infiltration of various types of leukocyte into the corneal stroma and their activation. Expression by corneal fibroblasts of intercellular adhesion molecule (ICAM)-1, a cell surface glycoprotein that binds leukocyte function-associated antigen-1 present on the surfaces of all leukocytes, and of vascular cell adhesion molecule (VCAM)-1, an immunoglobulin-like transmembrane adhesion molecule, also contributes to inflammatory corneal disease.[10]

Polyinosinic-polycytidylic acid [poly(I:C)], a synthetic analog of viral double-stranded RNA (dsRNA), is recognized by Toll-like receptor (TLR)-3 and has been used to study the effects of viral infection on several cell types.[11,12] Stimulation of human corneal epithelial cells with poly(I:C) induced the activation of nuclear factor (NF-κB) and the production of IL-6 and IL-8.[11] To clarify the role of corneal fibroblasts in viral stromal keratitis, we have examined the effects of poly(I:C) on the expression of TLR3, the production of proinflammatory cytokines (IL-6) and chemokines (IL-8, G-CSF, MIP-1β, eotaxin, RANTES), and the expression of ICAM-1 and VCAM-1 in human corneal fibroblasts. The possible signaling pathways that underlie such effects were also investigated.

Methods

Materials

Eagle minimum essential medium (MEM), fetal bovine serum (FBS), and trypsin-EDTA were obtained from Invitrogen-Gibco (Carlsbad, CA);

Investigative Ophthalmology & Visual Science, August 2008, Vol. 49, No. 8
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24-well culture plates, 60-mm culture dishes, and four-well chamber slides were from Corning-Costar (Corning, NY); and poly(I:C) was from Invivogen (San Diego, CA). Antibodies to TLR3 were obtained from Imgenex (Sorrento Valley, CA); those to ICAM-1, VCAM-1, the p65 subunit of NF-κB, and normal rabbit immunoglobulin G were from Santa Cruz Biotechnology (Santa Cruz, CA); and antibodies to ERK, phospho-ERK, to p38, phospho-p38, JNK, phospho-JNK, IκB-α, and phospho-IκB-α were from Cell Signaling (Beverly, MA). Antibodies to keratocan were kindly provided by Winston W. Y. Kao (Department of Ophthalmology, University of Cincinnati), and blocking antibodies to TLR3 (TLR3.7) were from Abcam (Cambridge, UK). A protein multiarray system and human cytokine assay were from Bio-Rad (Bio-Plex; Bio-Rad, Hercules, CA). PD98059, SB203580, and JNK inhibitor II were obtained from Calbiochem (La Jolla, CA), and a protease inhibitor cocktail and mouse monoclonal antibodies to β-actin were from Sigma-Aldrich (St. Louis, MO). Nitrocellulose membranes and an enhanced chemiluminescence (ECL) kit were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden), and Alexa Fluor 488–labeled goat antibodies to mouse or rabbit IgG and 4′,6-diamidino-2-phenylindole (DAPI) were from Molecular Probes (Eugene, OR). All media and reagents for cell culture were endotoxin minimized.

Isolation and Culture of Human Corneal Fibroblasts

Human corneas were obtained for corneal transplantation surgery from NorthWest Lions Eye Bank (Seattle, WA). Human tissue was used in strict accordance with the tenets of the Declaration of Helsinki. Corneal stroma and fibroblasts were prepared and cultured as described previously.[8] In brief, the endothelial layer of the rim of the cornea remaining after transplantation surgery was removed mechanically, and the tissue was then incubated with dispase (2 mg/mL in MEM) for 1 hour at 37°C. After mechanical removal of the epithelial sheet, the 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 of 5% CO_2 at 37°C in MEM supplemented with 10% FBS. The cells were used for experiments after four to seven passages. All the cells were positive for vimentin and negative for cytokeratin, suggesting the absence of contamination of the cultures by epithelial cells (data not shown).

Assay for IL-6, IL-8, G-CSF, MIP-1β, Eotaxin, and RANTES

Assays were performed as described previously.[7] Corneal fibroblasts were cultured in 24-well plates until they achieved confluence, after which the culture medium was replaced with serum-free MEM for 1 day. The cells were then incubated first for 1 hour in the absence or presence of mitogen-activated protein kinase (MAPK) inhibitors and then for an additional 24 hours in the same medium supplemented with poly(I:C). The medium was then collected and centrifuged at 120*g* for 5 minutes, and the resultant supernatant was frozen at −80°C for subsequent assay of proinflammatory cytokines and chemokines. The cells were detached from the culture plate by exposure to trypsin-EDTA, and their number was determined with a particle counter. The morphology and number of cells were not affected by incubation with poly(I:C) or MAPK inhibitors for 24 hours (data not shown). Simultaneous quantification of IL-6, IL-8, G-CSF, MIP-1β, eotaxin, and RANTES in culture supernatants was performed with a human cytokine assay system (Bio-Plex; Bio-Rad). The sensitivity of the assay was 1.95 pg/mL for each cytokine or chemokine. For determination of the effect of blocking antibodies to TLR3, the cells were incubated in the presence of the monoclonal antibodies (10 μg/mL) for 1 hour at 37°C before stimulation with poly(I:C) (10 μg/mL) for 24 hours; the concentration of IL-6 in culture supernatants was then measured with an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

Immunoblot Analysis

For immunoblot analysis of TLR3, ICAM-1, and VCAM-1, corneal fibroblasts were cultured in 60-mm dishes until they achieved confluence, after which the culture medium was replaced with serum-free MEM for 24 hours. For immunoblot analysis of MAPKs or IκB-α, the cells were cultured in 60-mm dishes first for 24 hours in MEM supplemented with 0.5% FBS and then for an additional 24 hours in serum-free medium. All serum-deprived cells were incubated with various concentrations of poly(I:C) for the indicated times at 37°C and were lysed in 100 μL of a solution containing 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail (lysis buffer). For immunoblot analysis of keratocan, corneal stromal tissue was lysed with lysis buffer. Cell lysates (10 μg protein) were then subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. After blocking of nonspecific sites, the membrane was incubated with antibodies to TLR3, ICAM-1, VCAM-1, ERK, p38, JNK, IκB-α, phosphorylated forms of MAPKs or IκB-α, or keratocan (or with normal rabbit IgG as a control), and immune complexes were then detected with the use of secondary antibodies and ECL reagents.

Immunofluorescence Staining

For immunostaining of ICAM-1 or VCAM-1, corneal fibroblasts were cultured on four-well chamber slides until they achieved confluence. The medium was changed to serum-free MEM, and the cells were cultured for an additional 24 hours, after which they were incubated for 24 hours with MEM in the absence or presence of poly(I:C) (10 μg/mL). The cells were washed twice with phosphate-buffered saline (PBS) and then fixed for 15 minutes at room temperature with PBS containing 1% paraformaldehyde. After three washes with PBS containing 1% bovine serum albumin (BSA), the cells were incubated for 1 hour at room temperature with PBS-BSA containing mouse monoclonal antibodies to ICAM-1 or to VCAM-1 or normal mouse IgG (control), washed three times with PBS-BSA, and incubated for 1 hour with Alexa Fluor 488–conjugated goat antibodies to mouse IgG.

Immunostaining for NF-κB was performed as described previously.[13] In brief, corneal fibroblasts grown on four-well chamber slides were incubated at 37°C first for 24 hours in serum-free MEM and then for 90 minutes with MEM containing poly(I:C). The cells were then washed twice with PBS, fixed with 4% paraformaldehyde in PBS, and washed three additional times with PBS before permeabilization with 100% methanol for 6 minutes at −20°C. Nonspecific adsorption of antibodies was blocked by incubation for 30 minutes with PBS containing 3% BSA, and the cells were then incubated for 1 hour at room temperature with rabbit polyclonal antibodies to the p65 subunit of NF-κB or normal rabbit IgG (control), washed, and incubated for 30 minutes at room temperature with Alexa Fluor 488–conjugated goat antibodies to rabbit IgG.

Finally, all cells were washed with PBS, mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA), and observed with a fluorescence microscope (Axioskop 50; Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Data are presented as mean ± SD. Statistical analysis was performed with the Dunnett multiple comparison test, Tukey-Kramer test, or Student unpaired *t*-test. $P < 0.05$ was considered statistically significant.

Results

Expression of Keratocan in Corneal Stroma and Cultured Corneal Fibroblasts

To investigate the difference between keratocytes and corneal fibroblasts, we first examined the expression of keratocan in human corneal stroma and cultured corneal fibroblasts by immunoblot analysis. Keratocan was expressed in the corneal

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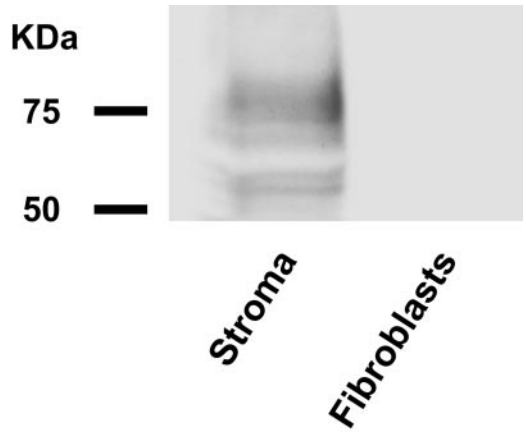

FIGURE 1. Expression of keratocan in human corneal stroma and cultured human corneal fibroblasts. Lysates of corneal stroma and cultured corneal fibroblasts were subjected to immunoblot analysis with antibodies to keratocan.

stroma, but its expression was not detected in corneal fibroblasts (Fig. 1).

Effects of Poly(I:C) on IL-6, IL-8, G-CSF, MIP-1β, Eotaxin, RANTES, ICAM-1, and VCAM-1 Expression in Corneal Fibroblasts

We next investigated the effects of poly(I:C) on the release of proinflammatory cytokines and chemokines and on the expression of adhesion molecules by corneal fibroblasts. Incubation of the cells with poly(I:C) (0.1–10 μg/mL) for 24 hours stimulated the release of IL-6, IL-8, G-CSF, MIP-1β, eotaxin, and RANTES in a concentration-dependent manner (Fig. 2). Immunoblot and immunocytofluorescence analyses also revealed that poly(I:C) induced a concentration-dependent increase in the expression of ICAM-1 (Fig. 3A) and VCAM-1 (Fig. 3B) in corneal fibroblasts. Immunofluorescence for ICAM-1 and VCAM-1 was most prominent around the edges of the cells, indicative of surface expression of these adhesion molecules. Slides exposed to control mouse IgG in place of the antibodies to ICAM-1 or to VCAM-1 did not exhibit such fluorescence.

Effect of Poly(I:C) on TLR3 Expression in Corneal Fibroblasts

We also examined the effect of poly(I:C) on the expression of TLR3 in corneal fibroblasts. Immunoblot analysis revealed that cells cultured in the presence of poly(I:C) (10 μg/mL) for 24 hours manifested an increase in the amount of TLR3 compared with those cultured in the absence of poly(I:C) (Fig. 4A). Densitometric analysis revealed that this effect of poly(I:C) was statistically significant. To investigate the role of TLR3 in the observed effects of poly(I:C) on corneal fibroblasts, we examined whether blocking antibodies to this receptor might inhibit poly(I:C)-induced IL-6 release by these cells. The blocking antibodies to TLR3 indeed inhibited poly(I:C)-induced IL-6 release, whereas control mouse IgG had no such effect (Fig. 4B).

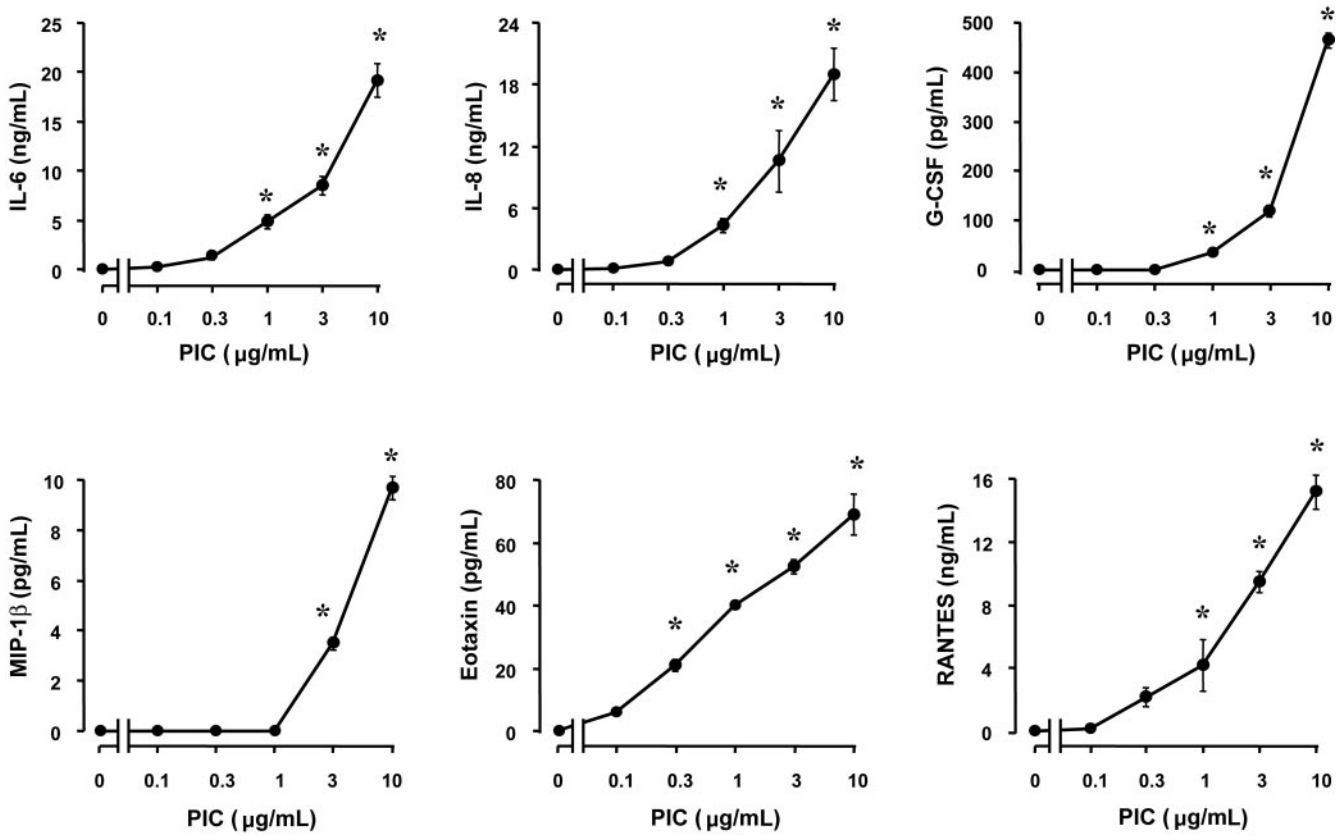

FIGURE 2. Effects of poly(I:C) on proinflammatory cytokine and chemokine release from corneal fibroblasts. Cells were incubated for 24 hours in the presence of the indicated concentrations of poly(I:C) (PIC), after which the amounts of the indicated proteins in culture supernatants were determined. Data are mean ± SD of triplicates from an experiment repeated three times with similar results. $^*P < 0.05$ (Dunnett test) versus the corresponding value for cells incubated without poly(I:C).

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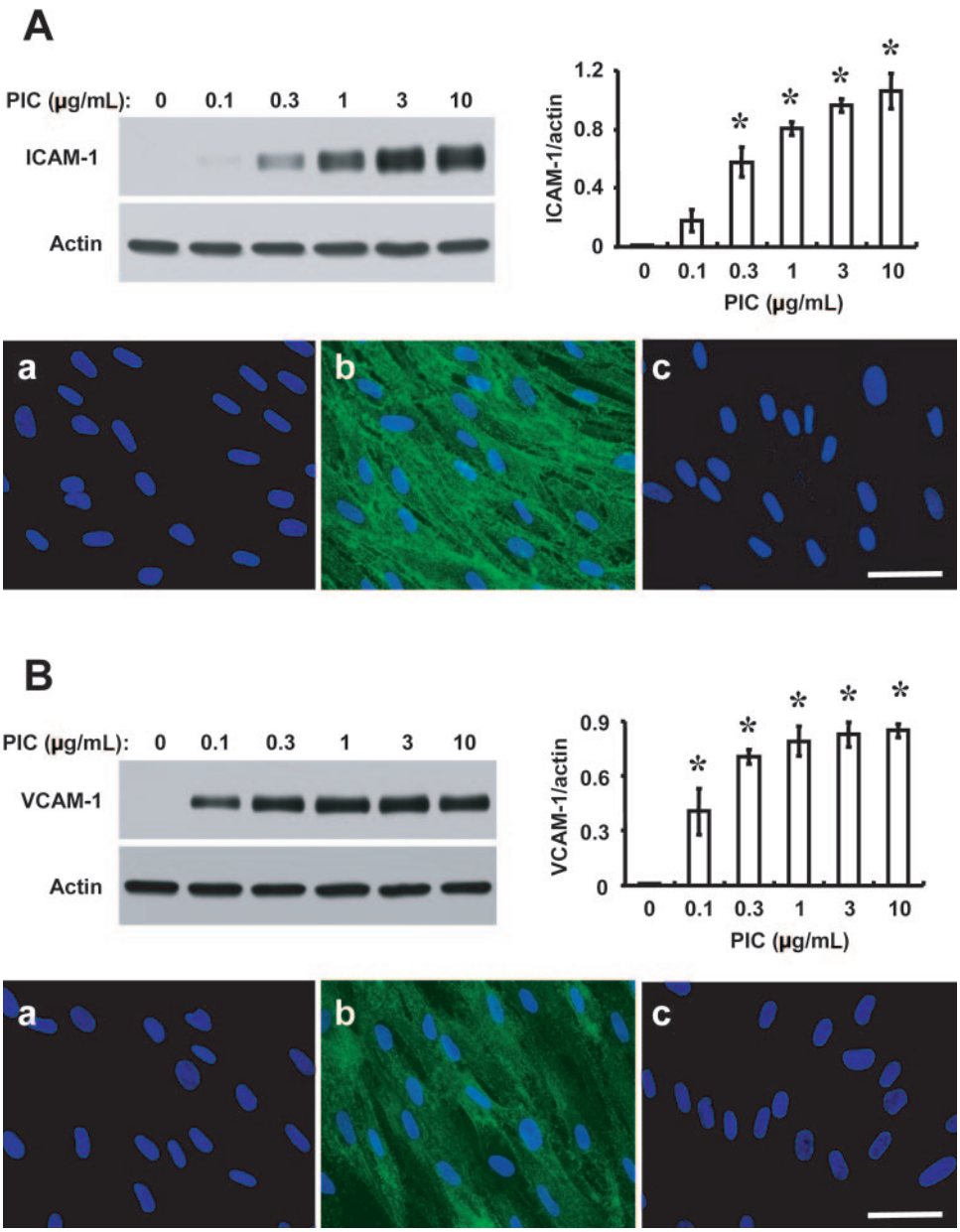

FIGURE 3. Effects of poly(I:C) on ICAM-1 and VCAM-1 expression in corneal fibroblasts. (**A**) Cells were incubated for 24 hours in the presence of the indicated concentrations of poly(I:C), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to ICAM-1 or to actin as a loading control (*upper left*). Immunoblots similar to those shown were subjected to densitometric analysis to determine the intensity of the bands corresponding to ICAM-1 (*upper right*). Data are expressed relative to the intensity of the corresponding band for actin and are mean ± SD of results from three independent experiments. $^*P < 0.01$ (Student's *t*-test) versus the value for cells incubated without poly(I:C). Alternatively, the cells were incubated for 24 hours in the absence (**a**) or presence (**b**, **c**) of poly(I:C) (10 μg/mL) and were then stained with antibodies to ICAM-1 (**a**, **b**) or normal mouse IgG (**c**) and with Alexa Fluor 488–conjugated secondary antibodies (*green*). Nuclei were stained with DAPI (*blue*). Scale bar, 50 μm. Data are representative of three independent experiments. (**B**) Cells were treated and analyzed as in (**A**) with the exception that the analyses were performed with antibodies to VCAM-1 instead of those to ICAM-1.

Effects of Poly(I:C) on Phosphorylation of MAPKs in Corneal Fibroblasts

Intracellular signaling by MAPK pathways contributes to regulation of the synthesis of proinflammatory cytokines, chemokines, and adhesion molecules by poly(I:C).[12,14] We therefore investigated the possible role of the MAPKs extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK) in the poly(I:C)-induced expression of these proteins in corneal fibroblasts. Immunoblot analysis showed that though the abundance of ERK, p38, and JNK was not affected by poly(I:C), this agent (10 μg/mL) induced the phosphorylation (activation) of each of these MAPKs in a time-dependent manner (Fig. 5). The phosphorylation level of each MAPK was thus increased after 60 minutes of stimulation. The activation of ERK, p38, and JNK induced by poly(I:C) was also concentration dependent (Fig. 5).

Effects of MAPK Inhibitors on IL-6, IL-8, G-CSF, MIP-1β, Eotaxin, RANTES, ICAM-1, or VCAM-1 Expression Induced by Poly(I:C) in Corneal Fibroblasts

We next examined the effects of MAPK inhibitors on poly(I:C)-induced expression of proinflammatory cytokines, chemokines, and adhesion molecules in corneal fibroblasts. Cells were incubated with the ERK inhibitor PD98059, the p38 inhibitor SB203580, or the JNK inhibitor II (each at 10 μM) for 1 hour before incubation for 24 hours in the presence of poly(I:C) (10 μg/mL). Poly(I:C)-induced release of IL-6, IL-8, or G-CSF was inhibited by 51%, 45%, or 68%, respectively, in the presence of PD98059; poly(I:C)-induced release of IL-6, IL-8, G-CSF, MIP-1β, or eotaxin was inhibited by 59%, 72%, 35%, 69%, or 38%, respectively, in the presence of SB203580; and poly(I:C)-induced release of IL-6, IL-8, G-CSF, MIP-1β, or RANTES was inhibited by 67%, 81%, 87%, 73%, or 68%, respec-

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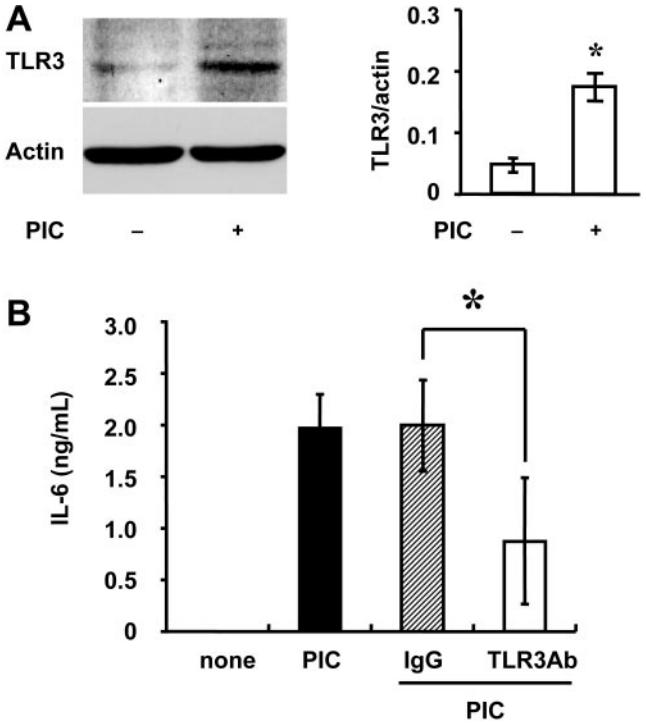

FIGURE 4. Effect of poly(I:C) on the expression of TLR3 and the effect of blocking antibodies to TLR3 on poly(I:C)-induced IL-6 expression in corneal fibroblasts. (**A**) Cells were incubated for 24 hours in the absence or presence of poly(I:C) at 10 μg/mL, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TLR3 or to actin (*left*). Immunoblots similar to those shown were subjected to densitometric analysis to determine the intensity of the bands corresponding to TLR3 (*right*). Data are expressed relative to the intensity of the corresponding band for actin and are mean ± SD of results from three independent experiments. $^*P < 0.05$ (Student's *t*-test) versus the value for cells incubated without poly(I:C). (**B**) Cells were incubated for 1 hour in the absence or presence of blocking antibodies to TLR3 (TLR3Ab) or control mouse IgG at 10 μg/mL and then for 24 hours in the additional absence or presence of poly(I:C) (10 μg/mL). Culture supernatants were then collected and assayed for IL-6. Data are mean ± SD of triplicates from an experiment repeated three times with similar results. $^*P < 0.05$ (X test).

tively, in the presence of JNK inhibitor II (Fig. 6). JNK inhibitor II also inhibited the poly(I:C)-induced expression of ICAM-1 by 44% (Fig. 7A). None of the MAPK inhibitors exhibited a significant effect on poly(I:C)-induced expression of VCAM-1 at the concentrations examined (Fig. 7B). The expression of these various proteins in the absence of poly(I:C) was not affected by the MAPK inhibitors (data not shown).

Activation of NF-κB by Poly(I:C) in Corneal Fibroblasts

Given that NF-κB signaling is implicated in the induction of genes for chemokines and adhesion molecules in several cell types,[11,15] we examined whether poly(I:C) affected the phosphorylation or abundance of the endogenous NF-κB inhibitor IκB-α in corneal fibroblasts. Immunoblot analysis revealed that poly(I:C) (10 μg/mL) induced a time-dependent increase in the phosphorylation of IκB-α that was first apparent at 30 minutes and was maximal at 90 minutes after the onset of stimulation; it also induced the degradation of this protein, with the amount of IκB-α greatly reduced at 30 minutes but somewhat recovered by 120 minutes (Fig. 8A). Furthermore, the effects of poly(I:C) on the phosphorylation and degradation of IκB-α were concentration dependent (Fig. 8B). Finally, immunofluorescence analysis showed that the incubation of cells with poly(I:C) (10 μg/mL) for 90 minutes induced translocation of the p65 subunit of NF-κB from the cytosol to the nucleus (Fig. 8C). No immunofluorescence was apparent in cells stained with normal rabbit IgG as a negative control.

DISCUSSION

We have shown that poly(I:C) induced the up-regulation of TLR3, the production of IL-6, IL-8, G-CSF, MIP-1β, eotaxin, and RANTES, and the surface expression of ICAM-1 and VCAM-1 in human corneal fibroblasts. Furthermore, poly(I:C) induced the activation of the MAPKs ERK, p38, and JNK, the phosphorylation and degradation of IκB-α, and the nuclear translocation of the p65 subunit of NF-κB in these cells. The poly(I:C)-induced expression of IL-6, IL-8, and G-CSF was inhibited by the MAPK inhibitors PD98059, SB203580, and JNK inhibitor II; that of MIP-1β by SB203580 and JNK inhibitor II; that of eotaxin by SB203580; and that of RANTES and ICAM-1 by JNK inhibitor II.

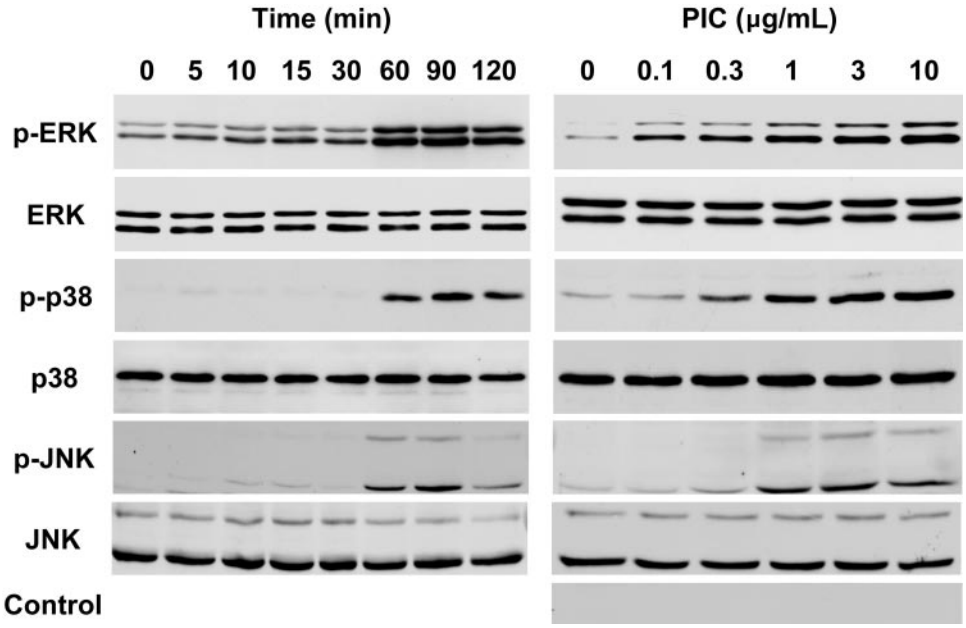

FIGURE 5. Time- and concentration-dependent effects of poly(I:C) on MAPK phosphorylation in corneal fibroblasts. Cells were incubated for the indicated times with poly(I:C) at 10 μg/mL (*left*) or for 90 minutes with the indicated concentrations of poly(I:C) (*right*), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to ERK, p38, JNK, or phosphorylated forms (p-) of these MAPKs. As a control, the blot on the *right* was probed with control rabbit IgG. Data are representative of three independent experiments.

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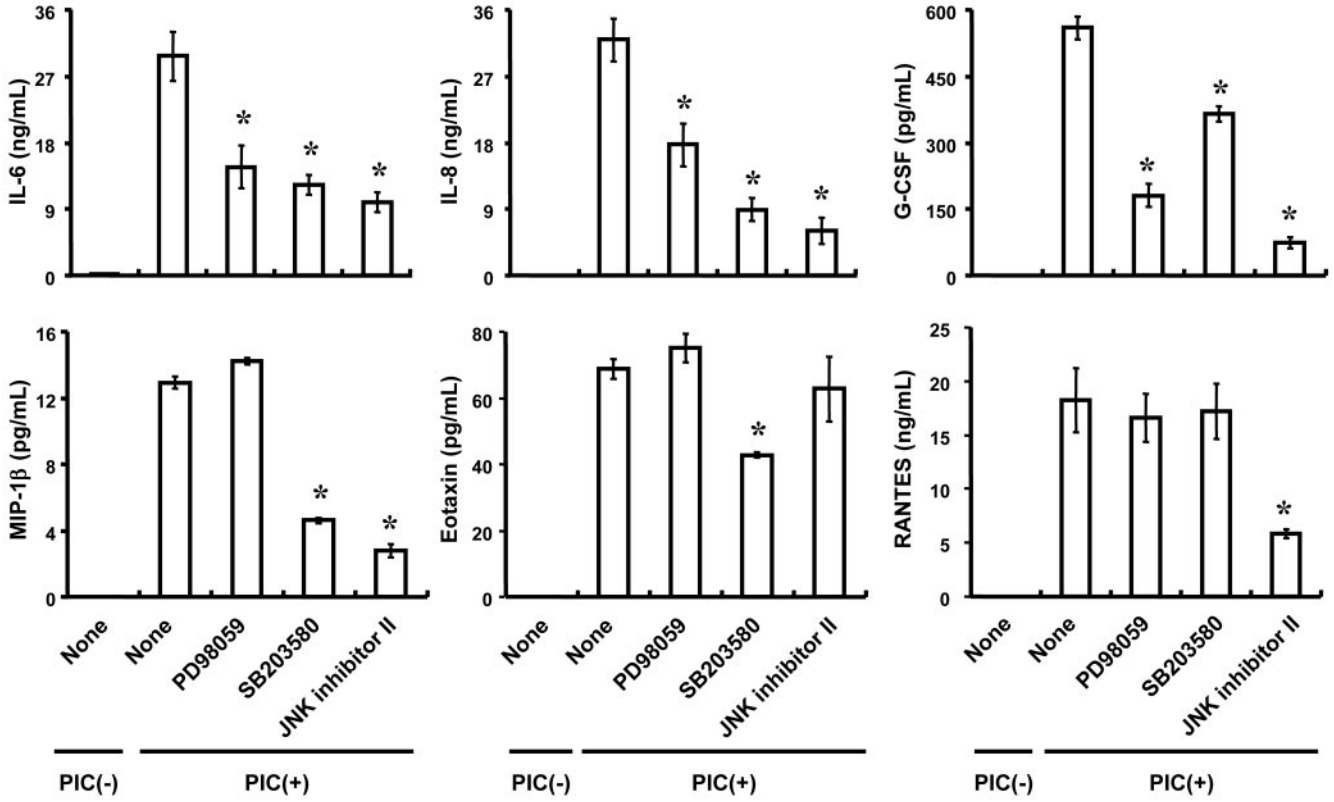

FIGURE 6. Effects of MAPK inhibitors on poly(I:C)-induced proinflammatory cytokine and chemokine release from corneal fibroblasts. Cells were incubated first for 1 hour in the absence or presence of PD98059, SB203580, or JNK inhibitor II (each at 10 μM) and then for 24 hours in the additional absence or presence of poly(I:C) (10 μg/mL), after which the amounts of the indicated proteins in culture supernatants were determined. Data are mean ± SD of results from four independent experiments. $^*P < 0.01$ (Tukey-Kramer test) versus the corresponding value for cells incubated with poly(I:C) alone.

Leukocytes are prominent participants in the immunoinflammatory pathogenesis of viral infection in the corneal stroma. Viral infection of the cornea is followed by early, marked infiltration of PMNs into the stroma,[16] whereas other cell types, including macrophages,[17] natural killer cells,[18] and T cells,[19] infiltrate the stroma at later stages. Migration to and

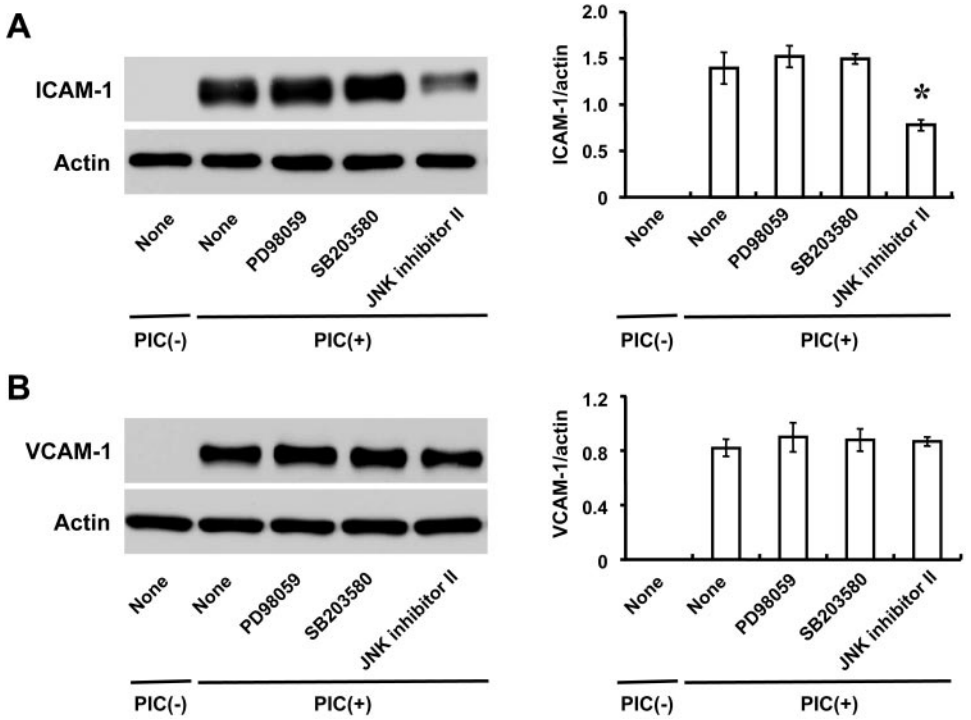

FIGURE 7. Effects of MAPK inhibitors on poly(I:C)-induced ICAM-1 or VCAM-1 expression in corneal fibroblasts. Cells were incubated first for 1 hour in the absence or presence of PD98059, SB203580, or JNK inhibitor II (each at 10 μM) and then for 24 hours in the additional absence or presence of poly(I:C) (10 μg/mL), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to ICAM-1 (**A**), VCAM-1 (**B**), or actin. Representative immunoblots are shown on the *left*, and quantitative data (expressed relative to the intensity of the corresponding band for actin) from three independent experiments are shown on the *right* as mean ± SD. $^*P < 0.01$ (Tukey-Kramer test) versus the corresponding value for cells incubated with poly(I:C) alone.

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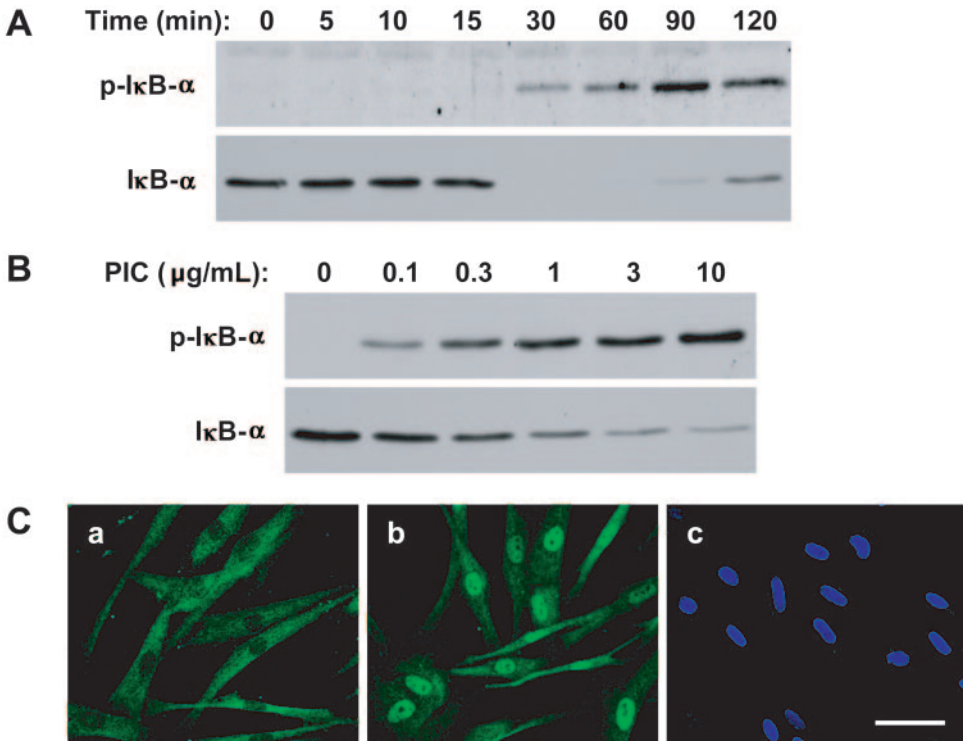

FIGURE 8. Effect of poly(I:C) on the activation status of NF-κB in corneal fibroblasts. (**A**, **B**) Cells were incubated in the presence of poly(I:C) (10 μg/mL) for the indicated times (**A**) or with various concentrations of poly(I:C) for 90 minutes (**B**), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to IκB-α or to the phosphorylated form (p-) of this protein. Data are representative of three independent experiments. (**C**) Cells were incubated for 90 minutes in the absence (**A**) or presence (**B**, **C**) of poly(I:C) (10 μg/mL), after which the cells were fixed, permeabilized, and subjected to immunofluorescence analysis with antibodies to the p65 subunit of NF-κB (**A**, **B**) or normal rabbit IgG (**C**) and with Alexa Fluor 488–conjugated secondary antibodies (*green*). Nuclei were stained with DAPI (**c**, *blue*) to show the existence of the cells. Data are representative of three independent experiments. Scale bar, 50 μm.

infiltration into tissues of various leukocytes and subsequent activation of these cells are regulated by cytokines and chemokines. IL-6 is a key molecule for PMN recruitment to the corneal stroma[20] and promotes the differentiation of B cells.[21] IL-8 is also a chemoattractant for PMNs,[22] whereas G-CSF induces the activation of neutrophilic granulocytes[23] and MIP-1β regulates monocytes and T cells.[24] Eotaxin and RANTES regulate the local accumulation of eosinophils during corneal allergic inflammation,[8] with the latter also acting as a chemotactic factor for monocytes and lymphocytes.[25] ICAM-1 contributes to the local infiltration of leukocytes during immune responses by mediating the adhesion and activation of these cells.[26] The role of VCAM-1 in the infiltration of inflammatory cells appears to be selective for eosinophils,[27] which adhere to fibroblasts and contribute to the pathogenesis of severe persistent allergic corneal ulcers.[10] In the present study, poly(I:C) induced the expression of IL-6, IL-8, G-CSF, MIP-1β, eotaxin, RANTES, ICAM-1, and VCAM-1 in corneal fibroblasts, suggesting that corneal fibroblasts may regulate the local infiltration and activation of various types of leukocyte during stromal viral infection through the production of these cytokines, chemokines, and adhesion molecules. An association of eosinophils with viral stromal keratitis has not been described, but our finding that poly(I:C) induced the expression of eotaxin in corneal fibroblasts suggests that eosinophil infiltration into the stroma may occur in response to viral infection. However, the amount of eotaxin and the amounts of G-CSF and MIP-1β released by corneal fibroblasts in response to poly(I:C) were relatively low. Additional studies with more physiologically relevant models may thus be necessary to demonstrate a role for these factors in the response of the corneal stroma to viral infection.

Toll-like receptors, a family of type 1 transmembrane proteins, play an important role in early host defense against invading pathogens.[28] We recently showed that human corneal fibroblasts express TLR4 in response to stimulation with lipopolysaccharide, a component of the cell membrane of Gram-negative bacteria.[26] TLR3 is a specific receptor for dsRNA, a molecular marker of viral infection produced by most viruses during the replication cycle.[29] In the present study, poly(I:C), a synthetic analog of viral dsRNA, induced the expression of TLR3 in corneal fibroblasts, suggesting that signaling by this receptor contributes to the immunoinflammatory response to viral infection in the corneal stroma. Our results are consistent with previous observations showing the expression of TLR3 in corneal fibroblasts.[30] We have also shown that blocking antibodies to TLR3 inhibited the poly(I:C)-induced expression of IL-6 in corneal fibroblasts, suggesting that these cells are directly activated by poly(I:C) acting at TLR3. Moreover, another type of ssRNA and dsRNA are recognized not only by TLR3 but also by TLR-7/8.[31,32] Given that IL-6 production is not completely inhibited by blocking antibodies to TLR3, whether other Toll-like receptors also mediate the actions of poly(I:C) in corneal fibroblasts remains to be determined.

TLR3 shares with the other members of the TLR family the ability to induce the activation of MAPKs and NF-κB, key signaling molecules that mediate cell activation and regulate cell proliferation in response to a variety of extracellular stimuli.[33,34] Stimulation of TLR3 by dsRNA also results in activation of the transcription factor interferon regulatory factor (IRF).[35] These signaling molecules mediate up-regulation of the expression of cytokines, chemokines, and adhesion molecules induced by stimulation of TLR3. For instance, IL-8 expression is increased by transcriptional activation of the IL-8 gene mediated by the JNK pathway and NF-κB and by stabilization of IL-8 mRNA mediated by the p38 pathway.[36] In contrast, the expression of RANTES is regulated by the combination of NF-κB and IRF.[37] In the present study, poly(I:C) induced the activation of the MAPKs ERK, p38, and JNK and that of NF-κB in corneal fibroblasts in a time- and concentration-dependent manner. Our experiments with specific inhibitors of MAPKs suggested that the poly(I:C)-induced expression of cytokines, chemokines, and adhesion molecules in these cells is mediated by ERK, p38, and JNK but that the relative contributions of these three MAPKs to the up-regulation of these various molecules may differ.

Keratocytes, the major resident cells of the corneal stroma, differentiate into fibroblasts when the integrity of the corneal stroma is disrupted, and the resultant fibroblasts are thought to

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play an important role in corneal wound healing.[38] Keratocan is expressed by keratocytes but not by corneal fibroblasts.[39,40] Our finding that keratocan was expressed by the human corneal stroma but not by cultured fibroblasts is consistent with previous observations showing that exposure of the stroma to serum induces fibroblast differentiation accompanied by the downregulation of keratocan expression.[38,41,42] However, the responses of keratocytes or cultured corneal fibroblasts of different passages to poly(I:C) remains to be determined because of the limitation of in vitro multiple passages. The observation that corneal fibroblasts, but not keratocytes, express TLR3 and TLR9 suggests that corneal fibroblasts may play an important role in the response of the cornea to viral or bacterial infection.[30] We recently showed that lipopolysaccharide interacts with human corneal fibroblasts to induce the activation of NF-κB and the consequent expression of chemokines and adhesion molecules,[15] with these effects inhibited by triptolide.[7] Together with these previous findings, our present results suggest that corneal fibroblasts contribute to induction of the inflammatory response to viral or bacterial keratitis.

In corneal infection, leukocytes clear viruses and bacteria from the corneal stroma, but they also amplify the inflammatory response. PMNs express vascular endothelial growth factor, which contributes to the neovascularization of the cornea,[43] and matrix metalloproteinase-9, which degrades the stromal matrix.[44] We recently showed that neutrophils stimulate collagen degradation mediated by corneal fibroblasts.[45] It is, therefore, possible that interactions of inflammatory cells with resident corneal fibroblasts play an important role in the inflammatory response of the stroma to viral infection.

In summary, we have shown that poly(I:C) induced the up-regulation of TLR3, the activation of NF-κB, and the MAPK-dependent expression of proinflammatory cytokines, chemokines, and adhesion molecules in human corneal fibroblasts. These results suggest that corneal fibroblasts play an important modulatory role in local immune and inflammatory responses associated with viral infection in the corneal stroma.

Acknowledgments

The authors thank Makoto Asashima and Satoshi Ebina (ICORP Organ Regeneration Project, Japan Science and Technology Agency) for helpful discussion on the manuscript, and Yasumiko Akamatsu and the staff of Yamaguchi University Center for Gene Research for technical assistance.

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