Mitomycin C Upregulates IL-8 and MCP-1 Chemokine Expression via Mitogen-Activated Protein Kinases in Corneal Fibroblasts

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Purpose. To investigate the expression of chemokines and their signaling pathways after application of mitomycin C (MMC) to corneal fibroblasts.

Methods. Primary porcine and human corneal fibroblasts from passages 3 to 6 were treated with MMC at concentrations of 0.05, 0.1, or 0.2 mg/mL for 1, 2, 5, or 10 minutes. The relative expression of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) were investigated with reverse transcription, and quantitative real-time polymerase chain reaction (qRT-PCR), and enzyme-linked immunosorbent assay (ELISA). The effects of MMC on the activation of kinases were analyzed by Western blot analysis with specific antiphosphokinase antibodies. The signaling pathways by which MMC regulates the expression of IL-8 and MCP-1 were evaluated by pharmacological kinase-specific inhibitors.

Results. The expression of IL-8 and MCP-1 were upregulated after MMC treatment in a time- and concentration-dependent manner. Furthermore, the upregulated expression of IL-8 and MCP-1 increased with longer incubation time. MMC treatment enhanced the phosphorylation of p38, JNK, and ERK at different time points. The MMC-related IL-8 and MCP-1 expression was inhibited by both a p38 inhibitor (SB203580) and an ERK inhibitor (PD98059). A JNK inhibitor (SP600125) reduced the expression of MMC-induced MCP-1 but not of IL-8.

Conclusions. MMC treatment upregulated the expression of IL-8 and MCP-1 mRNA and protein secretion by the activation of mitogen-activated protein kinases (MAPKs) in corneal fibroblasts. (*Invest Ophthalmol Vis Sci.* 2007;48:2009–2016) DOI:10.1167/iovs.06-0835

Mitomycin C (MMC) is an antibiotic produced by *Streptomyces caespitosus* and has both antiproliferative and antimetabolic properties. It functions as an alkylating agent that causes cross-linking of DNA and inhibits RNA as well as protein synthesis. In addition to its well-known role as a chemotherapeutic agent, MMC has been widely used in ophthalmology, including in surgical treatment of glaucoma and pterygium and treatment of conjunctival and corneal intraepithelial neoplasia and ocular cicatricial pemphigoid. In addition, it has been used in such high-risk refractive surgery as photorefractive keratectomy (PRK) over previous laser in situ keratomileusis (LASIK),[1] over buttonhole LASIK flaps,[2] radial keratotomy, and penetrating keratoplasty,[3] to prevent subepithelial haze formation. Based on its success in the treatment of symptomatic haze, the application of MMC has been extended to the prophylaxis of corneal haze after refractive surgery in virgin eyes.[4–9] Although a single intraoperative application of MMC seems to be safe and effective in preventing corneal scar formation and recurrence of subepithelial fibrosis after refractive corneal surgery,[5,6,8] and thus of great potential usefulness in the next generation of refractive surgery,[10] such inflammation-related side effects as anterior and posterior scleritis and acute topical irritation and toxicity with late extensive fibrosis in the orbit have been reported.[11–14]

Clinical outcomes of refractive surgery depend in large part on the corneal wound-healing response, in which corneal fibroblasts play a crucial role. Under normal conditions, corneal fibroblasts are the major cellular component of the corneal stroma and contribute to the maintenance of the transparent structure of the cornea. After damage to the epithelium caused by surface ablation or other injury, corneal fibroblasts undergo apoptosis. The remaining fibroblasts begin to proliferate and migrate into the wound site, where they differentiate into myofibroblasts, which are associated with haze formation. Corneal fibroblasts also secrete chemokines to attract inflammatory cells into the cornea to participate in the wound-healing process.[15–17] Although inflammation is a host response to various insults, it may cause tissue destruction and fibrotic consequences.[18,19] Although MMC has been demonstrated to prevent corneal haze formation by both inhibiting fibroblast proliferation and inducing cellular apoptosis, thus reducing the number of fibroblasts and myofibroblasts,[7,20–23] little is known about its effects on the characteristics of the remaining cells. Investigation of MMC-related chemokine expression in corneal fibroblasts is thus warranted.

IL-8, a member of the C-X-C chemokine family, is a potent activator and chemoattractant of neutrophils.[24] MCP-1 belongs to the C-C chemokine family and functions as a chemoattractant and activator of lymphocytes and monocytes, causing monocyte/macrophage infiltration into tissues.[25] In this study, we examined the effect of MMC treatment on the expression of the chemokines IL-8 and MCP-1 in corneal fibroblasts. Mitogen-activated protein kinases (MAPKs) consist of three main subfamilies: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs. They are cell-signaling mediators essential in transmitting external insults into the cell nucleus. We have investigated the role of MAPKs in the expression of MMC-related chemokines in corneal fibroblasts. Understanding the effects of MMC on corneal fibroblasts and modifying its pharmacological application may further help in avoiding complications after refractive surgery.

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Materials and Methods

Culture of Corneal Stromal Fibroblasts and Treatment with MMC

Fresh porcine eyes were purchased from a local abattoir. The central portion of the porcine corneas was excised and sterilized with povidone iodine solution for 30 seconds. The rim of a human cornea was obtained from a domestic donor, in accordance with the Declaration of Helsinki, after corneal transplantation surgery. After removal of the corneal epithelium and endothelium by scraping with a surgical blade, the corneal stroma was incubated in collagenase (1.5 mg/mL in Dulbecco's modified Eagle's medium [DMEM] with 10% fetal calf serum [FCS]) at 37°C for 1 hour with gentle shaking to remove residual endothelium and epithelium contamination. The residual stroma was treated with 3 mg/mL collagenase for approximately 2 to 3 hours until the tissue was completely digested. The softened material was collected and centrifuged at 1000*g* for 5 minutes to obtain stromal cells. These cells were cultivated with DMEM supplemented with 10% fetal bovine serum, penicillin G (100 U/mL), and streptomycin sulfate (100 μg/mL), to expand the cell population. The fibroblasts were passaged by trypsinization (Trypsin-EDTA; Invitrogen-Gibco, Carlsbad, CA) and used in passages 3 through 6. The cells were treated with MMC at 0.05, 0.1, or 0.2 mg/mL for 1, 2, 5, or 10 minutes followed by three washings in DMEM. Samples were collected as described later at the indicated time points, for various purposes.

Measurement of IL-8 and MCP-1 mRNA Expression by Reverse Transcription and Quantitative Polymerase Chain Reaction

After treatment with MMC and incubation in DMEM containing 10% FCS for indicated periods, the total RNA was isolated (TRIzol Reagent; Invitrogen) according to the manufacturer's recommendations. The first-strand complementary DNA (cDNA) synthesis was performed from 2 μg of total RNA and reverse transcribed using oligo-$(dT)_{15}$ and Moloney murine leukemia virus (MoMuLV) reverse transcriptase (Promega, Madison, WI) in RT buffer at 25°C for 5 minutes, 42°C for 1 hour, and 70°C for 15 minutes. A fluorescein quantitative real-time PCR (qRT-PCR) detection system (LightCycler DNA Master SYBR Green I; Roche Molecular Biochemicals, Indianapolis, IN) was used to determine the change in messenger RNA (mRNA) levels of IL-8 and MCP-1 expression. The reactions were performed in a 10-μL volume containing 5 pM each of oligonucleotide primer, SYBR green I dye, *Taq* DNA polymerase (FastStart; Roche), reaction buffer, dNTP mixture, and 4 mM $MgCl_2$. The PCR conditions were set at 95°C for 10 minutes to activate the *Taq* DNA polymerase, followed by 45 cycles at 95°C for 10 seconds, primer-specific annealing temperature for 5 seconds, and 72°C for 10 to 26 seconds, depending on product length. The fluorescence was monitored at the end of each cycle to obtain a measure of the amount of PCR product formed. The thermocycler PCR data were analyzed with the dedicated software, which determines the cycle number at which each sample reaches this threshold. The cycle number corresponding to the fluorescence threshold is inversely related to the logarithm of the initial template concentration. Given that amplification occurs in an exponential manner, a difference of 1 in the cycle threshold corresponds approximately to a twofold difference in relative transcript abundance. The amount of IL-8 or MCP-1 mRNA was normalized by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and is presented in arbitrary units, with 1 unit corresponding to the value in cells treated with a sham procedure. The primer sequences used were as follows: GAPDH (GTCACCAGGGCTGCTTTT AA and ACGGAAGGCCATGCCAGTGA); IL-8 (TTTCTGCAGCTCTCTGTGAGG and CTGCTGTTGTTGTTGCTTCTC); and MCP-1 (ATTCTCCAGTCACCTGCTGC and CTTCAAGGCTTCGGAGTTTGG).

Enzyme-Linked Immunosorbent Assay

After treatment with MMC and/or kinase inhibitors, confluent human corneal fibroblasts were incubated in 0.1% FCS-DMEM for the times indicated earlier. Culture supernatants were collected and centrifuged at 14,000*g* for 10 minutes. The resultant supernatants were properly diluted with a reagent diluent (1% TopBlock in phosphate-buffered saline [PBS]; Fluka, Taufkirchen, Germany), and the levels of IL-8 and MCP-1 secreted into the supernatants were determined (DuoSet ELISA Development System for human IL-8 and MCP-1; R&D Systems, Minneapolis, MN) according to the instructions of the manufacturer. Briefly, the 96-well microplate was coated with 2 μg/mL of capture antibody (mouse anti-human IL-8 or MCP-1 antibody) overnight at room temperature. Nonspecific binding sites were blocked with reagent diluent. Diluted supernatants were then added and incubated for 2 hours at room temperature. The plates were incubated with detection antibody (biotinylated goat anti-human IL-8 or MCP-1 antibody) for 2 hours at room temperature followed by streptavidin conjugated to horseradish-peroxidase (streptavidin-HRP) for 20 minutes. Peroxidase substrate (SureBlue Reserve ELISA substrate; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added, and the plates were incubated for 20 minutes at room temperature. The reaction was terminated with 1 N HCl. Absorbance for each well at 450 nm was read in an ELISA reader.

Assessment of Kinase Phosphorylation by Western Blot Analysis

To measure the short-term response, porcine corneal fibroblasts that had been serum-deprived for 24 hours were treated with 0.2 mg/mL MMC. The cells were lysed (PhosphoSafe reagent; Novagen, Madison, WI), either immediately or at 15 minutes or 30 minutes or 1, 2, or 3 hours after treatment. Ten micrograms of cell lysate were resolved on 10% polyacrylamide gels and blotted onto a nitrocellulose membrane. The nitrocellulose membrane was incubated with a blocking buffer (5% skim milk in Tris-buffered saline Tween-20 [TBS-T] containing 0.1% Tween-20, 140 mM NaCl, and 25 mM Tris-HCl [pH 7.4]) for 1 hour. It was then incubated with the primary antibodies overnight at 4°C. The following primary antibodies were used to measure the phosphorylation status of p38, ERK1/2, and JNK1/2 MAPKs: anti-phospho-p38 (Thr-180/Tyr-182; p-p38 MAPK), anti-phospho-p44/p42 (Thr-202/Tyr-204; p-ERK1/2; Cell Signaling Technology, Beverly, MA), and anti-phospho-JNK (Thr-183/Tyr-185; p-JNK1/2; Sigma-Aldrich, St. Louis, MO) antibodies. Horseradish peroxidase–labeled secondary antibody was added to the membrane, which was further incubated for 1 hour at room temperature. Immunoreactive bands were visualized with enhanced chemiluminescence reagents (ECL; GE Healthcare, Amersham, UK).

To measure the long-term effects on kinase activation, we incubated the cells for an additional period of 24, 48, or 72 hours after MMC treatment. The cell lysates were then collected for detecting the phosphorylated form of these kinases by Western blot analysis as just described. In addition, the total amount of kinase protein was detected by using antibodies specific to p38 MAPK (anti-p38 MAPK antibody; Cell Signaling Technology), ERK1/2 (anti-p44/p42; anti-ERK1/2 antibody; Cell Signaling Technology), or JNK1/2 (anti-stress-activated protein kinase/JNK antibody; Santa Cruz Biotechnology, Santa Cruz, CA) to detect any changes in their expression after MMC treatment.

Treatment with Kinase Inhibitors

To evaluate which kinase(s) is (are) involved in the effect of MMC on IL-8 and MCP-1 expression, the cells were pretreated with the kinase inhibitors SB203580, a p38 inhibitor[26] (1, 10, and 20 μM; Calbiochem-Novabiochem International, Inc., San Diego, CA); PD98059, an ERK MAPK inhibitor[27] (1, 10, and 20 μM; Calbiochem-Novabiochem International, Inc.); and SP600125, a JNK/SAPK inhibitor[28] (1, 10, and 20

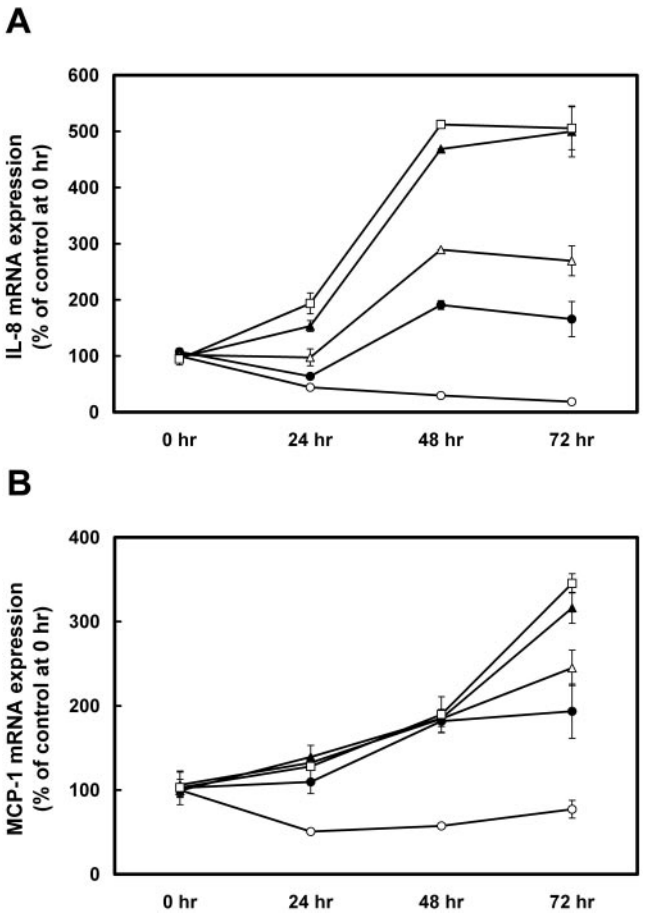

FIGURE 1. MMC-stimulated IL-8 and MCP-1 expressions increase with length of incubation time after exposure to MMC. Porcine corneal fibroblasts were treated with 0.2 mg/mL MMC for 1 (●), 2 (△), 5 (▲), and 10 (■) minutes or with a control sham procedure (○), followed by three washings in DMEM. After additional incubation for 24, 48, and 72 hours, the total RNA was isolated, and cDNA was synthesized. The mRNA expressions of (**A**) IL-8 and (**B**) MCP-1 were determined by qRT-PCR with porcine IL-8- and MCP-1-specific primers. The expressions of target genes were normalized to that of GAPDH. The mRNA level analyzed immediately after MMC treatment or sham procedure and the DMEM washing is represented as 0 hour. The abundance of MMC-stimulated IL-8 and MCP-1 mRNA was expressed as a percentage of the control group at 0 hour. Data are the mean ± SD of duplicates from an experiment that was repeated with similar results.

μM; Sigma-Aldrich, St. Louis, MO) for 1 hour. SB203580 is a selective inhibitor of p38 MAPK, which acts by competitively inhibiting adenosine triphosphate (ATP) binding.[26] PD98059 is a selective and potent inhibitor of MAPK (also known as MAPK/ERK or MEK kinase). It mediates its inhibitory properties by binding to the ERK-specific MAPK MEK, thereby preventing phosphorylation and activation of ERK1/2 (p44/p42 MAPK) by MEK1/2.[27] SP600125 is a selective and potent inhibitor of JNK1, -2, -3 and a reversible ATP competitive inhibitor with >300-fold selectivity compared with related MAPKs.[28] After pretreatment with kinase inhibitors, the cells were stimulated with MMC, as described earlier, and were then incubated with the inhibitors for a further 48 (for qRT-PCR analysis) and 72 (for ELISA) hours.

Statistical Analysis

The difference in the mRNA expression levels of IL-8 and MCP-1 among various conditions at a specific time point was examined with one-way analysis of variance (ANOVA). The change in expression over time after a specific MMC treatment regimen was also evaluated by one-way ANOVA. $P < 0.05$ was considered statistically significant. Pair-wise comparisons were made with the Tukey multiple comparisons test, when an overall significance was obtained.

RESULTS

MMC's Effect on IL-8 and MCP-1 Expressions

We first examined the effects of MMC treatment on the abundance of the C-X-C chemokine IL-8 and the C-C chemokine MCP-1 mRNAs in porcine corneal fibroblasts by qRT-PCR analysis. After MMC treatment, the abundance of IL-8 mRNA in corneal fibroblasts was upregulated and increased along with incubation time in comparison with a control group (Fig. 1A). The extent of upregulation was dependent on the length of treatment time. MMC treatment also upregulated the abundance of MCP-1 mRNA (Fig. 1B). Compared with the group without MMC treatment, the induction also increased along with incubation time. Although longer durations of MMC treatment did not result in higher levels of MCP-1 expression at 24 and 48 hours after treatment, MMC treatment for 5 and 10

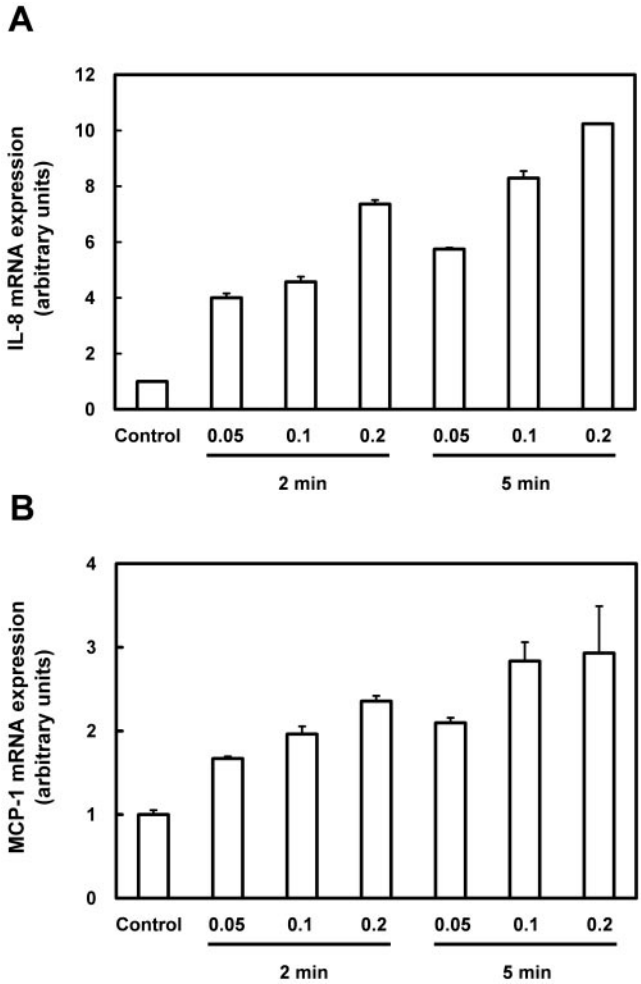

FIGURE 2. Dose-dependent effect of MMC on the abundance of IL-8 and MCP-1 expression in porcine corneal fibroblasts. Cells were treated with 0.05, 0.1, and 0.2 mg/mL MMC for 2 or 5 minutes, followed by three washings in DMEM. After 72 hours, the total RNA was isolated, and the mRNA expressions were analyzed by qRT-PCR. The expression of target genes was normalized to that of GAPDH and presented as arbitrary units compared with the control group. Data are the mean ± SD of duplicates from an experiment that was repeated with similar results.

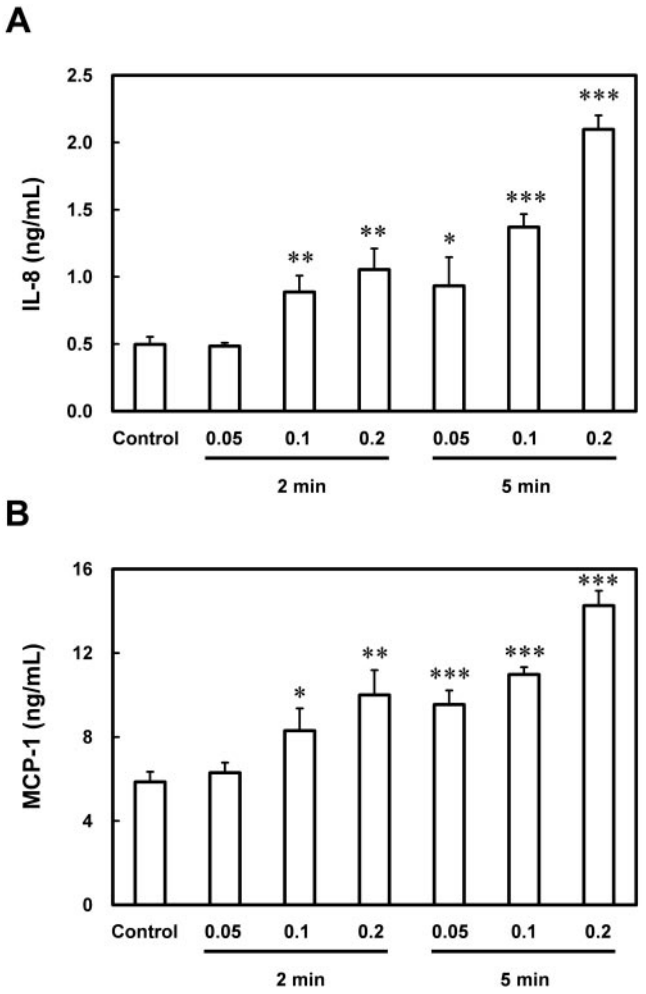

FIGURE 3. Effects of MMC on chemokine secretion in human corneal fibroblasts. Confluent cells were treated with 0.05, 0.1, and 0.2 mg/mL MMC for 2 or 5 minutes and then cultivated for an additional 72 hours in serum-free medium. The amount of (**A**) IL-8 and (**B**) MCP-1 in culture supernatants was determined by ELISA. Data are the mean ± SD of triplicates from an experiment that was repeated three times with similar results. The mean differences are significant at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the control group.

minutes induced a significantly higher level of MCP-1 expression at 72 hours. To evaluate the effect of a specific MMC dose on the expression of IL-8 and MCP-1 mRNA, porcine corneal fibroblasts were treated with 0.05, 0.1, and 0.2 mg/mL MMC for 2 or 5 minutes. The upregulation of IL-8 and MCP-1 mRNA was also dependent on the concentration of MMC (Fig. 2).

The effects of MMC on IL-8 and MCP-1 protein secretion in corneal fibroblasts were determined by ELISA. MMC also upregulated IL-8 and MCP-1 secretion in a concentration- and time-dependent manner in human corneal fibroblasts (Fig. 3).

MMC-Induced Activation of MAPKs

We used Western blot analysis with phosphospecific antibodies to test the effects of MMC treatment on the activation of MAPKs in porcine corneal fibroblasts. This analysis revealed that the phosphorylated forms of JNK-1 (p-JNK-1) and JNK-2 (p-JNK-2) increased in corneal fibroblasts 30 minutes after MMC treatment and peaked at 1 hour (Fig. 4A). The level of phosphorylated p38 also increased as early as 15 minutes after exposure to MMC and reached peak levels at 1 hour (Fig. 4A). In contrast, there was no significant change in the intensity of the total JNK and p38 bands at these time points. The phosphorylation of p38 in MMC-treated cells was attenuated in the presence of the p38 inhibitor SB203580, whereas that of JNK was not reduced (Fig. 4A). This represented that SB203580 inhibited the activation of p38 but not that of JNK. Similarly, the phosphorylation of JNK in MMC-treated cells was diminished in the presence of the JNK inhibitor SP600125, whereas that of p38 was not suppressed. This result provided evidence that SP600125 decreases the activation of JNK but not of p38. ERK was massively activated immediately by experimental manipulation in all groups, so that there was no significant difference in the phosphorylated form intensities between control and MMC treatment groups at early time points of 0 minutes to 3 hours (data not shown). However, MMC did exert a sustained effect on ERK phosphorylation, so that a difference in the phosphorylated form intensity was observed up to 72 hours as shown in Figure 4B. In contrast to the increase in the levels of the phosphorylated form, MMC slightly decreased the total expression of ERK protein. The phosphorylated form of ERK in MMC-treated cells was attenuated in the presence of ERK inhibitor PD98059.[27] At 24 to 72 hours, the signals from phosphorylated p38 and JNK were faint (data not shown). According to these results, we suggest that MMC activates different MAPKs at different time points.

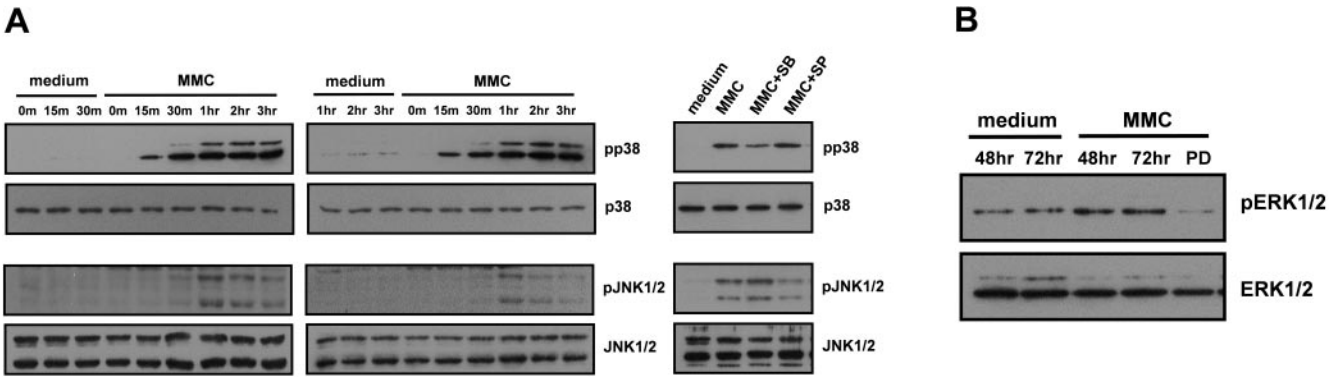

FIGURE 4. Effects of MMC on phosphorylation of p38, JNK, and ERK1/2. After treatment with 0.2 mg/mL MMC or sham treatment, cell lysate from porcine corneal fibroblasts was collected at the indicated time points and subjected to Western blot analysis to detect the levels of total and activated forms of (**A**) p38 and JNK and (**B**) ERK proteins. Data are representative of results from one of three independent experiments. The cells were harvested at 1 hour after MMC treatment in the presence of 20 μM SB203580 (SB) and 10 μM SP600125 (SP) and at 48 hours in the presence of 20 μM PD98059 (PD).

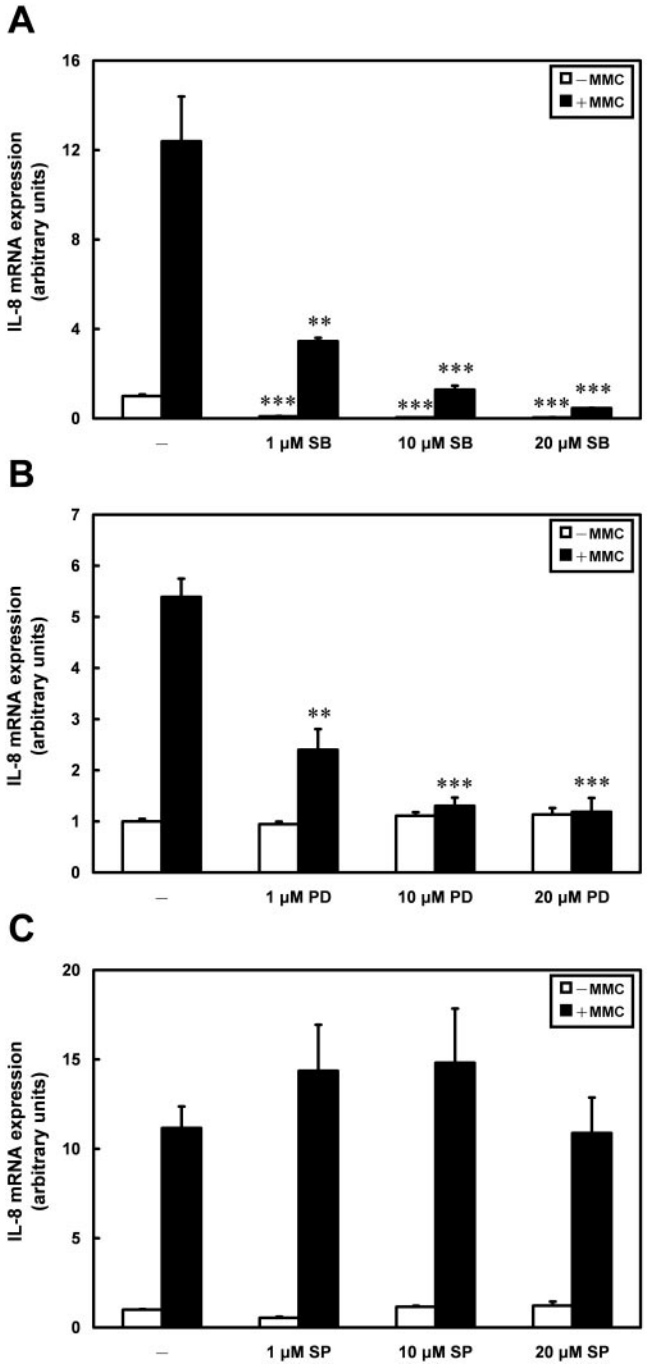

FIGURE 5. Effect of kinase-specific inhibitors on MMC-induced IL-8 mRNA expression in porcine corneal fibroblasts. Cells were pretreated with the inhibitors (**A**) SB203580, (**B**) PD98059, and (**C**) SP600125 for 1 hour, followed by treatment with 0.2 mg/mL MMC for 5 minutes and then were incubated for an additional 48 hours in inhibitor-containing media. The mRNA expression of IL-8 was determined by qRT-PCR, normalized to that of GAPDH, and presented as an arbitrary unit compared with a control group that was treated neither with MMC nor an inhibitor. Data are the mean ± SEM of triplicates from an experiment that was repeated with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the corresponding value for cells incubated without an inhibitor.

Effects of Kinase-Specific Inhibitors on MMC-Induced IL-8 and MCP-1 Expression

The signaling pathways mediating IL-8 and MCP-1 expression vary according to cell type and the nature of the stimulus.[29,30] To examine the MMC-activated kinases involved in IL-8 and MCP-1 induction in corneal fibroblasts, qRT-PCR was performed to compare the levels of IL-8 and MCP-1 expression in a control group and in MMC-treated

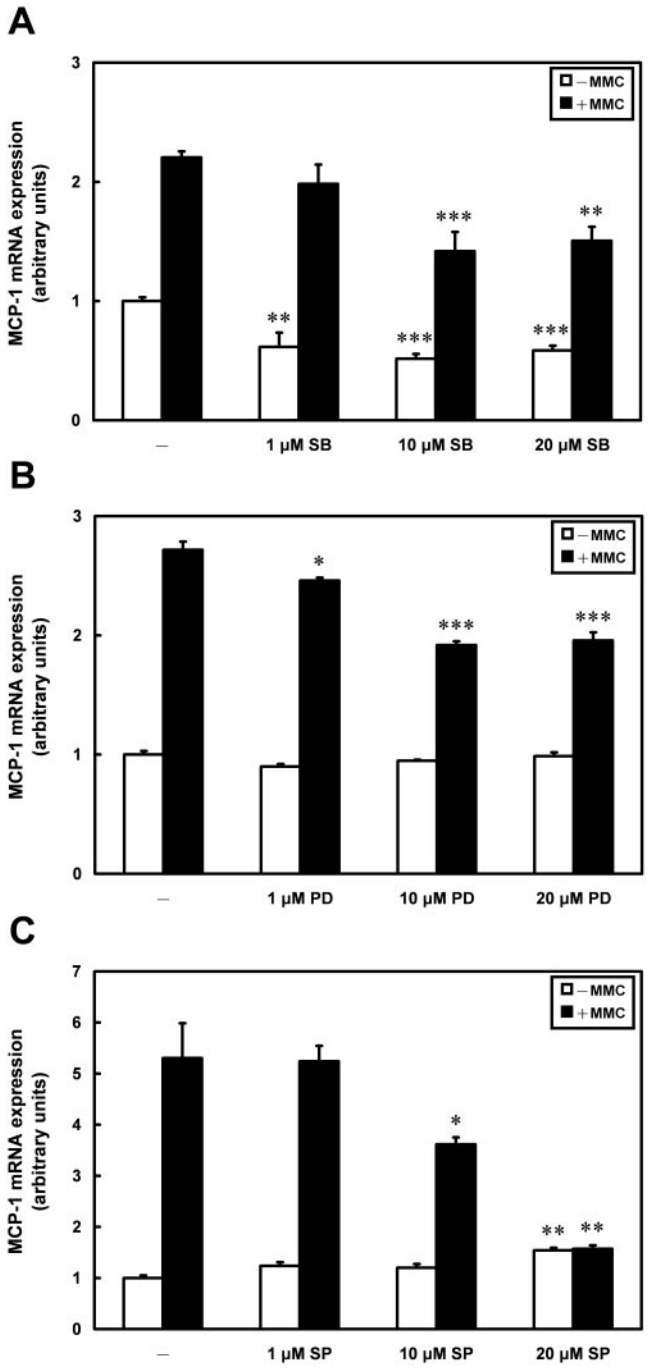

FIGURE 6. Effect of kinase-specific inhibitors on MMC-induced MCP-1 mRNA expression in porcine corneal fibroblasts. Cells were pretreated with inhibitors (**A**) SB203580, (**B**) PD98059, and (**C**) SP600125 for 1 hour, followed by treatment with 0.2 mg/mL MMC for 5 minutes and then were incubated for an additional 48 hours in an inhibitor-containing medium. The mRNA expression of MCP-1 was determined by qRT-PCR, normalized to that of GAPDH, and presented as an arbitrary unit compared with a control group that was treated neither with MMC nor an inhibitor. Data are the mean ± SEM of triplicates from an experiment that was repeated with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with the corresponding value for cells incubated in the absence of an inhibitor.

porcine corneal fibroblasts in the presence or absence of kinase-specific inhibitors.

SB203580, a p38-specific inhibitor,[26] almost abolished basal IL-8 mRNA expression in a dose-dependent manner (Fig. 5A). It also reduced the expression of MMC-stimulated IL-8 mRNA in a dose-dependent manner. In addition, SB203580 reduced the expression of both basal and MMC-induced MCP-1 mRNA; however, the extent of inhibition was less than that of IL-8 mRNA expression (Fig. 6A). These results suggest that p38 MAPK activation is involved in the expression of both IL-8 and MCP-1 mRNA in porcine corneal fibroblasts.

PD98059, an ERK-specific inhibitor,[27] reduced the abundance of IL-8 mRNA in MMC-treated cells and completely abolished the MMC-related expression at a concentration of 10 μM (Fig. 5B). The expression of MCP-1 mRNA was attenuated in the presence of PD98059 in MMC-treated cells (Fig. 6B). The extent of inhibition of MMC-induced IL-8 mRNA was higher than the inhibition of MCP-1 mRNA. In MMC-untreated cells, PD98059 had no significant effect on either IL-8 or MCP-1 mRNA. These results show that ERK activation is involved in the expression of both MMC-induced IL-8 and MCP-1 mRNA in porcine corneal fibroblasts.

SP600125, a JNK-specific inhibitor,[28] had no significant effect on constitutive MCP-1 mRNA expression but reduced MCP-1 mRNA expression in MMC-treated cells (Fig. 6C). The *x*-fold induction of MMC-induced MCP-1 mRNA expression was significantly attenuated in the presence of SP600125 and was abolished at a concentration of 20 μM. These results suggest that JNK is involved in the expression of MMC-induced MCP-1 mRNA. SP600125 did not, however, reduce the expression of MMC-induced IL-8 mRNA (Fig. 5C). These results suggest that MMC-stimulated IL-8 upregulation is not associated with JNK activation in porcine corneal fibroblasts.

The effects of these kinases on IL-8 and MCP-1 protein secretion in human corneal fibroblasts were further verified by ELISA. We confirmed that SB203580 and PD98059 also reduced the secretion of IL-8 and MCP-1 (Fig. 7), whereas SP600125 decreased the secretion of MCP-1 secretion but not of IL-8.

Although the effects of SB203580 and PD98059 on MCP-1 mRNA were modest, they abolished the secretion of MMC-upregulated MCP-1 protein. These results demonstrate that p38 MAPK and ERK are involved in MCP-1 expression at both the mRNA and protein levels. Given these results, we suggest that MMC activates MAPKs that are differentially involved in IL-8 and MCP-1 induction in corneal fibroblasts.

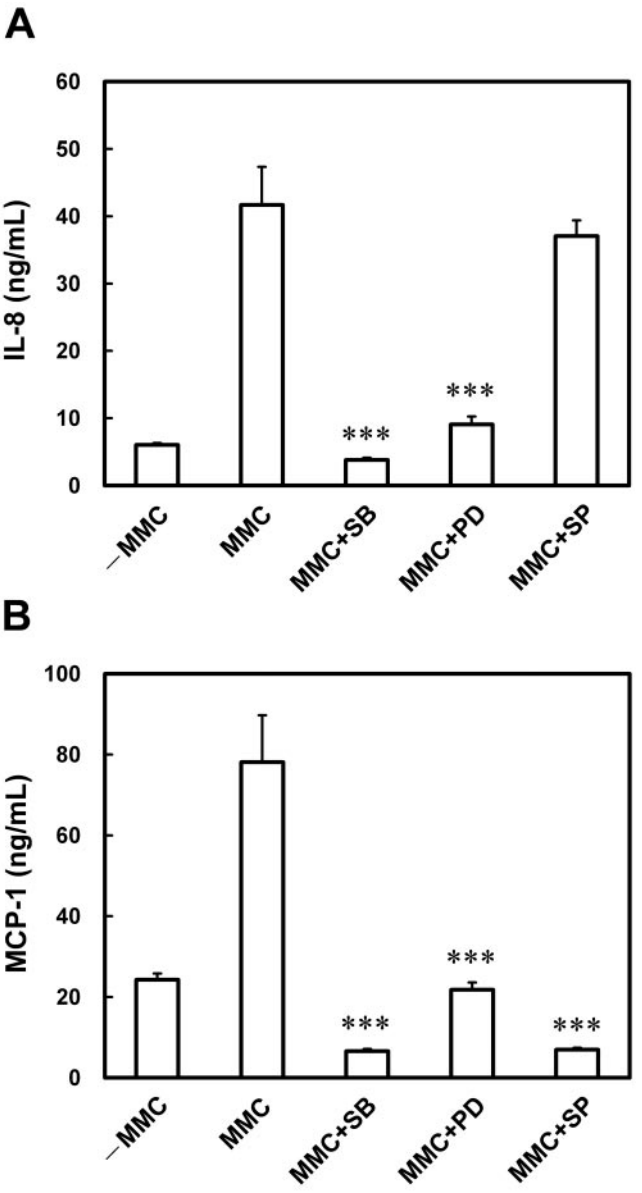

FIGURE 7. Effect of kinase-specific inhibitors on MMC-induced IL-8 and MCP-1 chemokine secretion in human corneal fibroblasts. After pretreatment with an inhibitor, cells were exposed to treatment with 0.2 mg/mL MMC for 5 minutes and then incubated for an additional 72 hours in a serum-free medium containing a specific inhibitor, 20 μM SB203580, 20 μM PD98059, or 10 μM SP600125. The amount of (**A**) IL-8 and (**B**) MCP-1 in culture supernatants was determined by ELISA. Data are the mean ± SEM of triplicates from an experiment that was repeated with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$ compared with the MMC group.

DISCUSSION

MMC acts as a modulator of the wound-healing process and has been shown to prevent haze formation after PRK.[4,8] Although MMC has been demonstrated to induce keratocyte apoptosis[20,21] and decrease the number of keratocytes and myofibroblasts,[7,22,23] little is known about its effects on the characteristics of live corneal fibroblasts. Corneal fibroblasts secrete the chemokines IL-8 and MCP-1 on stimulation by such environmental stimuli as glycated human serum albumin,[31] infection by bacteria[32] or viruses,[33,34] proinflammatory cytokines,[35,36] and injury.[17] High concentrations of and prolonged exposure to MMC cause marked inflammation and toxicity.[13,14,20] The dosage of MMC used in this study did not cause a significant level of cell apoptosis or death, as analyzed by an annexin V/PI assay but was sufficient to inhibit cell proliferation (data not shown), and resulted in increased IL-8 and MCP-1 secretion. The changes in the level of IL-8 and MCP-1 expression in corneal fibroblasts may modify the process of corneal wound healing or may influence their normal functions. Careful recommendation of an MMC concentration and an application time sufficient to inhibit keratocyte proliferation, but not to induce extensive cell death and chemokine expression, as a prophylactic against haze formation are desirable.

Chemotherapeutic agents have been shown to activate MAPKs in various cells.[37–39] In the present study, we demonstrated that MMC activates JNK, ERK, and p38 MAPKs in corneal fibroblasts and that the activation occurs at different time points. JNK and p38 MAPK were transiently activated at early time points, whereas the activation of ERK was a sequential event after MMC treatment. In addition to the finding that MMC induces the expression of IL-8 and MCP-1, further inves-

tigation is needed to elucidate whether the activation of ERK is subsequent to JNK and p38 activation after MMC treatment and whether it represents a sustained activation that might consequently affect cell characteristics.[40] Although MAPKs are involved in IL-8 and MCP-1 expression,[29,30,34,41–43] the signaling pathways mediating their expression may vary according to cell type and the nature of the stimulus. In the present study, we demonstrated that p38 and ERK activation were involved in both IL-8 and MCP-1 mRNA expression and secretion in corneal fibroblasts, whereas JNK activation was related only to that of MCP-1. The MCP-1 promoter contains binding sites for NF-αB, AP-1, and Sp1, whereas the IL-8 promoter contains binding sites for NF-αB, AP-1, and NF-IL6. We found that both ERK and p38 activation mediated the expression of MMC-related IL-8 and MCP-1 in corneal fibroblasts, suggesting that these signaling pathways may converge on AP-1 and/or NF-αB binding sites.[44] Although IL-8 and MCP-1 are coincidentally stimulated by a variety of stimuli through multiple signaling pathways, selective stimulation of either IL-8 or MCP-1 by a particular signaling pathway has been reported.[29,30,34,45] In human RPE cells, both IL-1β and TNF-α activated p38, ERK, and PI3K/AKT pathways, among which the PI3K/AKT signaling pathway was specifically involved in the upregulation of MCP-1 but not of IL-8.[29,45] In human corneal fibroblasts, adenovirus infection activated ERK, JNK, and p38 signaling pathways, among which ERK[34] was involved in the upregulation of IL-8, whereas JNK was involved in the upregulation of MCP-1.[30] The evidence suggests that the difference in IL-8 and MCP-1 promoters results in divergent regulation of gene expression. In human corneal epithelial cells, TNF-α induced the binding of the p65/p65 complex to the IL-8 promoter but not to the MCP-1 promoter. This resulted in the expression of IL-8 in the absence of MCP-1 production.[46] The MCP-1 promoter also contains an Sp1 binding site. The enhancement of the interaction of Sp1 and the Sp1 binding site, in cooperation with NF-αB, resulted in the upregulation of MCP-1.[47,48] Recently, the JNK signaling pathway has been shown to phosphorylate and activate Sp1 transcriptional factor, leading to the upregulation of Sp1-regulated gene expression.[49,50] In our study, the molecular mechanism underlying the selective induction of MCP-1 but not IL-8 expression by the JNK inhibitor SP600125 remains elusive. It is reasonable to suggest, however, that the JNK inhibitor selectively modulates MMC-related MCP-1 expression by inhibiting certain transcriptional factors, such as Sp1, that are required for MCP-1 expression.

In the present study, our results illustrated for the first time that exposure to MMC upregulated the expression and secretion of IL-8 and MCP-1 in corneal fibroblasts and that these effects continued after MMC removal. We also showed that IL-8 and MCP-1 expression were differentially mediated by MAPKs. Because cultured cells are different from native cells, confirmation of our results in situ and/or in vivo may be necessary. Understanding the molecular mechanisms of the effects of MMC on corneal fibroblasts would benefit adjustments in clinical application of this wound-healing modulator.

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