The Effect of Monocyte Chemoattractant Protein-1/CC Chemokine Ligand 2 on Aqueous Humor Outflow Facility

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PURPOSE. To investigate the effect of monocyte chemoattractant protein-1 (MCP-1)/CC chemokine ligand 2 on aqueous humor outflow facility.

METHODS. Aqueous humor outflow facility was measured in enucleated porcine eyes in a constant pressure perfusion system with or without MCP-1 (1600 ng/mL). Expression of CCR2, an MCP-1 receptor, in Schlemm’s canal endothelial (SCE) cells was examined by reverse transcription–polymerase chain reaction (RT-PCR) assay. The effect of MCP-1 (0–1600 ng/mL) on SCE cell viability was evaluated using a WST-8 assay. The effect of MCP-1 (0–800 ng/mL) on SCE-cell monolayer permeability was evaluated with or without a CCR2 antagonist (10 nM) by measuring transendothelial electrical resistance (TEER). The intracellular localization of the gap junction protein ZO-1 was analyzed by immunofluorescence staining of SCE cells.

RESULTS. The aqueous humor outflow facility increased significantly from basal levels at 80 minutes after perfusion with MCP-1 compared with control eyes (160.2% ± 6.6% [MCP-1] vs. 5.7 ± 2.5% [control]; P = 0.048). CCR2 was detected by RT-PCR. Cell viability was not affected by MCP-1 treatment. TEER of SCE-cell monolayer at 3 hours after treatment with 800 ng/mL MCP-1 decreased by 21.6 ± 1.7% compared with controls (P = 0.014), and the TEER-decreasing effects of MCP-1 were attenuated by a CCR2 antagonist. Immunocytochemical staining revealed a modest disruption of ZO-1 in MCP-1-treated SCE cells.

CONCLUSIONS. The present results revealed that MCP-1 increased aqueous humor outflow facility and decreased TEER via CCR2. These findings suggest that MCP-1 modulates aqueous humor outflow through the conventional pathway. (Invest Ophthalmol Vis Sci. 2012;53:6702–6707) DOI:10.1167/iovs.12-10376

Cataract surgery affects postoperative intraocular pressure (IOP). Although phacoemulsification has become the standard choice for surgical treatment of cataracts using minimally invasive procedures, the effects on IOP reduction after classical large incision cataract surgery are clinically similar.1 The reduction in IOP after cataract surgery is positively related to preoperative IOP, and inversely related to preoperative anterior chamber depth.2,3 Interestingly, IOP after phacoemulsification is decreased more in glaucomatous eyes compared with non-glaucomatous eyes, and remains reduced for a long period.4 The exact mechanisms related to the IOP-reducing effects of phacoemulsification, however, are not yet thoroughly understood.

Interleukin (IL)-1α increases outflow facility.5,6 In addition, in cultured trabecular meshwork (TM) cells, phacoemulsification ultrasound upregulates interleukin IL-1α and endothelial leukocyte adhesion molecule-1,7 suggesting potential effects of IL-1α on IOP reduction after phacoemulsification. On the other hand, our previous study showed that aqueous humor obtained from eyes with open-angle glaucoma contained increased monocyte chemoattractant protein-1 (MCP-1)/CC chemokine ligand 2 and IL-8 levels after phacoemulsification.8 In another study, we also observed elevated MCP-1 and IL-8 levels in the aqueous humor obtained from non-glaucomatous eyes after phacoemulsification compared with that before surgery (Kawai M, et al. IOVS 2012;53:ARVO E-Abstract 2503). Regarding the effects of IL-8 on the conventional outflow route, IL-8 decreases the resistance of the Schlemm’s canal epithelial (SCE) cell sheet.7 Thus, among the elevated cytokine levels induced by phacoemulsification, MCP-1 is the remaining factor to be addressed from the viewpoint of outflow facility. MCP-1 is a member of the cysteine–cysteine ligand chemokine family that regulates the recruitment and activation of monocytes (and macrophages), and is believed to play a role in inflammatory responses.10 In addition, MCP-1 regulates cytoskeletal changes in some cell lines via its receptor, cysteine–cysteine chemokine receptor 2 (CCR2).11 Taken together, we hypothesized that MCP-1 modulates outflow facility, especially after phacoemulsification.

In the present study, we focused on how MCP-1, via its receptor CCR2, alters the permeability of the SCE cell monolayer and outflow facility in perfused porcine eyes.

MATERIALS AND METHODS

Aqueous Humor Outflow Facility

Whole eye perfusion was conducted as previously described.1 Briefly, fresh porcine eyes (obtained from a local abattoir) were perfused with 1600 ng/mL MCP-1 in perfusion medium containing Dulbecco’s phosphate-buffered saline (DPBS) and 5.5 mM D-glucose at 25°C using a constant-pressure (15 mm Hg) perfusion system after the initial baseline outflow facility was established. As controls, the contralateral fellow eyes were perfused with DPBS alone. Outflow volume was measured every 30 seconds for 3 hours, and drug effects were calculated as a percentage change in outflow facility from baseline.
values. Following drug perfusion, the eyes were fixed by perfusion (Super Fix; Kurabo Industries, Osaka, Japan) at 15 mm Hg for 12 hours, and histologically examined by hematoxylin staining.

**Cell Culture**
Porcine TM cells were isolated as previously described, and cultured at 37°C under 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; WAKO Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), penicillin (100 U/mL), streptomycin (100 g/mL), and amphotericin B (0.5 μg/mL). Monkey SCE cells were isolated from the eyes of 6- to 12-month-old Cynomolgus monkeys obtained from Shin Nippon Biomedical Laboratories (Kagoshima, Japan) as previously described. Briefly, Schlemm’s canal was identified by cannulating its lumen with a 9-0 nylon suture. The corneoscleral and uveoscleral TM tissues were removed completely, and explants of the inner wall of Schlemm’s canal were cut out and placed on plates coated with collagen gel. Primary SCE cells were expanded in DMEM supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 g/mL), and amphotericin B (0.5 μg/mL) at 37°C in 5% CO2. Monkey SCE cells were used between passage 3 and passage 7, porcine TM cells were used between passage 4 and passage 7, and all experiments were conducted using confluent cell cultures.

**Reverse Transcription–Polymerase Chain Reaction Assay**
SCE and TM cells were plated in 35-mm culture dishes and grown to confluence. Total RNA was isolated (NucleoSpin RNA II; MACHEREY-NAGEL, Düren, Germany), and reverse-transcribed (RT) (Prime Script RT Master Mix; Takara Bio Inc., Shiga, Japan). The transcribed cDNA was amplified by polymerase chain reaction (PCR) (Platinum Taq DNA Polymerase High Fidelity; Invitrogen, Carlsbad, CA) according to the manufacturer’s protocols. The gene-specific primer pairs were as follows: monkey CCR2: forward (F), 5’-CAT GCT GTC GAC ATC TCG TFG-3’, reverse (R), 5’-TCA TTT GCA GAG TGA GC-3’; monkey GAPDH: (F), 5’-ACC ACA GTC CAT GCC ATC AC-3’, (R), 5’-TCC ACC ACC GTG TTG CTG TA-3’; porcine CCR2: (F), 5’-TTG TGT GAC CCA AGA GAG ACT TAG-3’, (R), 5’-GGT TCA GGC AAA CCA TCT TAA AGC-3’; porcine GAPDH: (F), 5’-ACC ACA GTC CAT GCC ATC AC-3’, (R), 5’- TCC ACC ACC CTG TTG CTG TA-3’. The thermal cycling conditions were 94°C for 15 minutes (followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 70°C for 50 seconds; then 70°C for 5 minutes). The PCR products were analyzed by electrophoresis using 1.5% agarose gels in 1× Tris base-boric acid-EDTA buffer containing ethidium bromide. To avoid the contamination of genomic DNA, the obtained total RNA samples were treated with DNase I and RT-PCR experiments without the use of reverse transcriptase enzyme were conducted.

**Cell Viability Assay**
The effects of MCP-1 on viability of SCE and TM cells were evaluated using the WST8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). SCE and TM cells were plated in 96-well plates (5 × 104 cells/well), and incubated at 37°C under 5% CO2 overnight. Cells were treated with MCP-1 at various concentrations (0–800 ng/mL) for 24 hours, then 10 μL of medium containing reagents added in each well and incubated for 2 hours at 37°C under 5% CO2. The absorbance at 450 nm was determined using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA).

**Measurement of Transendothelial Electron Resistance**
SCE cells were grown to confluence on a polyester membrane insert (0.4 μm pore size and 6.5 mm diameter; Transwell, Corning, NY) on 24-well culture plates. Transendothelial electrical resistance (TEER) was measured by MILLICELL-ERS (Millipore, Billerica, MA) according to the manufacturer’s instructions. MCP-1 (0–800 ng/mL) was added with or without CCR2 inhibitor (10 μM; Merck KGaA, Darmstadt, Germany) was placed in the lower and upper compartment of the dual chamber system (Transwell). The TEER was measured every hour for 5 hours at room temperature. TEER values were normalized by subtracting the background resistance from the filter alone. Time-dependent changes after treatments were followed and compared as a percentage change from baseline values. Each experiment was conducted in duplicate and repeated six times.

**Immunofluorescence Staining**
SCE cells were grown to confluence on gelatin-coated glass coverslips. Cells were treated with MCP-1 (800 ng/mL) for 3 hours, were washed with PBS, and fixed with 4% paraformaldehyde in PBS for 15 minutes. Cells were then washed with cytoskeletal buffer (10 mM 2-morpholinoethanesulfonic acid potassium salt, 150 mM NaCl, 5 mM EGTA, MgCl2, 5 mM glucose, pH 6.1), permeabilized with 0.5% Triton X-100 in PBS pH7.5, and blocked with 10% FBS and 0.2 mg/mL sodium azide in PBS. After blocking, cells were incubated with rabbit anti-ZO-1 antibody (1.25 μg/mL; Invitrogen) diluted in 10% bovine serum albumin solutions for 12 hours at 4°C, followed by incubation with goat anti-mouse IgG (Alexa Fluor 488; Invitrogen) for 30 minutes at room temperature. Samples were mounted with commercial mounting medium with 4’,6-diamidino-2-phenylindole (VECTASHIELD; Vector Laboratories, Burlingame, CA) and were observed using a fluorescence microscope (BX51; Olympus, Tokyo, Japan). Phalloidin-TRITC (Sigma-Aldrich, St. Louis, MO) was added to the secondary antibody solution to stain filamentous actin (F-actin).

**RhoA Activation Assay**
TM cells were cultured to confluence on 10-cm dishes and serum starved overnight. These cells were treated with various concentrations (0–800 ng/mL) of MCP-1 for 20 minutes. The effect of MCP-1 on RhoA activation was evaluated by pull-down assay (Rho Activation Assay Biochem Kit #BK056; Cytoskeleton, Denver, CO) according to the manufacturer’s instruction. Active RhoA (GTP binding form) immunoreactive bands were visualized with ECL advance Western blotting detection reagent (GE Healthcare, Little Chalfont, UK) and determined using a luminescent image analyzer (LAS-4000mini; Fujifilm, Tokyo, Japan). Densitometry of immunoreactive bands was performed using Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsweb.nih.gov/ij/index.html).

**Gelatin Zymography**
TM cells were cultured to confluence in 6-well plates and serum starved overnight. After cells were stimulated with MCP-1 (0–800 ng/mL) for 3 hours, the culture medium was collected and concentrated using ultracentrifugal filters (Millipore) with a cutoff of 10 kDa. Equal volumes of concentrated medium was mixed with Tris-glycine SDS sample buffer, incubated for 10 minutes at room temperature, and separated in gelatin zymography gels (Invitrogen) by electrophoresis. The gels were subsequently incubated in renaturing buffer (Invitrogen) for 30 minutes at room temperature and then overnight at 37°C in developing buffer (Invitrogen). The processed gels were stained with 0.25% Coomassie blue and destained in 5% MeOH and 7% acetic acid to reveal the lytic bands and scanned into digital images.

**Statistical Analysis**
Data were analyzed using commercial software (Prism 5.0; GraphPad Software Inc., San Diego, CA) and are presented as means and SE values. Statistical comparisons of multiple groups used ANOVA.
followed by Dunnett’s multiple comparison test. Student’s t-test was used for comparisons of two groups. Spearman’s correlation test was used to describe the relationship among variables. Differences were considered statistically significant at \( P < 0.05 \).

RESULTS

Effects of MCP-1 on Aqueous Outflow Facility

Enucleated porcine eyes were perfused with 1600 ng/mL MCP-1 at a constant pressure of 15 mm Hg, after establishing the baseline outflow facility with DPBS buffer containing glucose at 25°C. Baseline outflow facility range was 0.39 ± 0.04 μL/min/mm Hg in control, which was not significantly different from that at the beginning of MCP-1 perfusion 0.35 ± 0.05 μL/min/mm Hg (\( P = 0.798 \)). Following the MCP-1 perfusion, outflow facility increased progressively, and by 80 minutes it had significantly increased by 16% over control eyes (\( P = 0.048 \)). Outflow facility continued to increase and reached 32% over control eyes at 3 hours (Fig. 1). Fellow paired control eyes showed the expected washout response with an increase in outflow facility of 14% over the corresponding initial baseline outflow facility value. Histologic examinations of the experimental eyes with MCP-1 treatments revealed no changes compared with the fellow paired control eyes (data not shown).

CCR2 Gene Expression in SCE Cells and TM Cells

Our RT-PCR experiments using monkey SCE cells (315 bp) and porcine TM cells (529 bp) showed positive mRNA expression for CCR2, a MCP-1 receptor, as well as GAPDH (452 bp), a positive control, in both monkey SCE and porcine TM cells (Fig. 2).

Effects of MCP-1 on Viability of SCE and TM Cells

Monkey SCE and porcine TM cells were treated for 24 hours with or without various concentrations (0, 800, and 1600 ng/mL) of MCP-1, and were subjected to experiments evaluating cell viability using a WST-8 assay. Viabilities of SCE and TM cells was not changed by any of the examined concentrations of MCP-1 (Fig. 3).
Effects of MCP-1 on Barrier Function of SCE Cell Monolayer

To assess the effects of MCP-1 on the permeability of SCE cell monolayer, TEER was measured. In the experiments using confluent monolayer, the TEER value was decreased in a time-dependent manner after treatment with 800 ng/mL MCP-1 (Fig. 4A). The mean ± SE relative levels in TEER were 6.1 ± 6.5% (P = 0.370), 15.4 ± 6.2% (P = 0.452), 22.9 ± 7.4% (P = 0.014), 24.9 ± 8.0% (P = 0.035), and 27.9 ± 7.5% (P = 0.018) from the baseline levels 1, 2, 3, 4, and 5 hours after the treatment with 800 ng/mL MCP-1, respectively. Treatment with 100, 200, and 400 ng/mL MCP-1 also tended to decrease the TEER in a time-dependent manner, but the differences were not significant (data not shown). Data are shown as mean values ± SE, *P < 0.03, compared with control by Dunnett’s test (n = 12). (B) Effect of MCP-1 with a CCR2 antagonist on TEER in SCE cell monolayers. SCE cells monolayers were treated with MCP-1 (0–800 ng/mL) and CCR2 antagonist (10 nM). The TEER-decreasing effects of MCP-1 were attenuated by the CCR2 antagonist (n = 6).

Effect of MCP-1 on Junctional Protein

To investigate the mechanisms related to increased outflow facility, we examined immunoreactivities for the junctional protein, ZO-1, using immunocytochemical staining of cultured SCE cells. After 800 ng/mL MCP-1 treatment for 3 hours, the immunoreactive signal for ZO-1 exhibited a discontinuous pattern in the cell junctions, and was more prominent in the cytosol (Fig. 5).

Activation of Rho-GTP in TM Cells

To investigate the effects of MCP-1 stimulation on activation of RhoA in TM cells, we conducted a pull-down assay for the binding active form of RhoA (RhoA-GTP). In TM cells, the relative RhoA activities were not significantly changed, even after stimulation with 0 to 800 ng/mL MCP-1 (data not shown).

Discussion

Intraocular pressure is significantly reduced after cataract surgery, especially in glaucomatous eyes, but the mechanisms are unknown. Some studies have suggested the possibility that inflammatory cytokines are associated with alterations in conventional outflow. In our previous studies using aqueous humor samples obtained from the patients with glaucoma, elevated levels of MCP-1 were found in pseudophakic eyes after cataract surgery. In addition, a previous study evaluating aqueous MCP-1 levels in the same patients before and after phacoemulsification also demonstrated increased MCP-1 levels postoperatively in cataract eyes (non-glaucomatous eyes; Kawai M, et al. IOVS 2012;53:ARVO E Abstract 2503). Interestingly, the postsurgical percentage change from the baseline aqueous MCP-1 level was negatively correlated with the baseline of IOP in the study. Thus, we hypothesized that MCP-1 increases outflow facility in the eyes after cataract surgery.

In our perfusion studies using enucleated porcine eyes, the outflow facility after the perfusion with MCP-1 solution significantly increased for at least 3 hours in a time-dependent manner.
manner. This result suggested that MCP-1 is a potential factor that causes reduced IOP after surgical intervention. In cultured TM cells, phacoemulsification ultrasound upregulates the proinflammatory cytokine, IL-1α, suggesting potential effects on IOP reduction after phacoemulsification. In perfusion studies, IL-1α increases outflow facility in enucleated porcine eyes. Thus, similar to MCP-1 in the present study, the inflammatory cytokine IL-1α potentially causes IOP reduction after phacoemulsification. Additionally, another inflammatory cytokine, IL-8, decreases resistance of the SCE cell monolayer. Thus, proinflammatory cytokines including MCP-1 might synergistically contribute to IOP reduction during postoperative periods in the eye.

Our results showed that endothelial resistance was reduced by 30% in SCE cell monolayers after treatment with MCP-1. The fact that a CCR2 inhibitor blocked this effect indicated that MCP-1 changed the properties of SCE cells via CCR2. Our immunocytochemistry study demonstrated the localization and discontinuous changes in the expression of ZO-1, which is an important protein component of the tight junction. Because the junctional complexes between SCE cells contain tight junctions and very likely restrict paracellular flow, our findings suggest that changed paracellular flow may be the mechanism underlying the MCP-1–induced increase in outflow facility. The previous study indicated that MCP-1 increases permeability in the blood–brain barrier associated with changes in ZO-1 expression, consistent with the results of the present study. In the brain–blood vessels, however, Rho activation and actin stress fiber formation occur, in addition to the redistribution of tight junctions. Because we observed no changes in Rho activation nor an increase in F-actin stress fibers in TM cells, the mechanisms related to the increased outflow facility might differ among tissues. Additionally, other investigators reported that, in nephropathies, MCP-1 changes the behaviors of podocytes, resulting in increased permeability in the kidney.

In TM cells, inhibition of Rho-ROCK signal induces alterations in the cytoskeleton and focal adhesions, resulting in increased outflow facility and IOP reduction. Because, in the present study, we did not detect any alterations in Rho activity and F-actin in TM cells, we excluded the possibility of altered Rho-ROCK signaling to explain the increased outflow facility. In addition, our zymography experiments demonstrated no change in MMP-2 or MMP-9 (gelatinase A and B) activities. Thus, we cannot explain the increased outflow facility induced by MMP-2 or MMP-9 activation and resultant alterations in extracellular matrix metabolism. Taken together, we concluded that MCP-1 could increase outflow facility (and potentially reduce IOP levels) via altered cell–cell contact in SCE cells, but not by changing TM cell behaviors.

In conclusion, we demonstrated that MCP-1 increased the aqueous humor outflow facility and also that it decreased the TEER via CCR2. These data suggest that MCP-1 modulates aqueous humor outflow through the conventional pathway.
References


