MCP-1 Promotes Invasion and Adhesion of Human Ovarian Cancer Cells

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Abstract. *Background: Monocyte chemoattractant protein-1 (MCP-1) can accelerate tumor progression by attracting tumor-associated macrophages. We studied the effects of MCP-1 on SKOV-3 cells in order to investigate MCP-1 biological activity ovarian cancer. Materials and Methods: A SKOV-3 cell invasion assay (Transwell assay) and cell adhesion assay (96-well assay) were performed. Immunohistochemical staining for C-C motif chemokine receptor-2 (CCR2), a receptor for MCP-1, was also performed on cultured SKOV-3 cells. Results: Migration and adhesion of MCP-1-treated SKOV-3 cells were significantly increased compared to untreated cells ($p<0.01$). A CCR2 antagonist attenuated the invasion and adhesion of MCP-1-treated cells. CCR2 was expressed in the cytoplasm of SKOV-3 cells. Conclusion: MCP-1 promoted invasion and adhesion of ovarian cancer cells, and a CCR2 antagonist attenuated the effects of MCP-1 in vitro. These data suggest that MCP-1 is a potential therapeutic target for ovarian cancer therapy.*

Ovarian cancer is the fifth most common malignancy in females in the United States, accounting for 15,500 deaths in 2012 (1). In Japan, 8,000 women are diagnosed with ovarian cancer each year, and 4,000 died of ovarian cancer in 2009 (2). One-half of ovarian cancer cases in Japan are classified as stage III or IV, and almost all of them are complicated by peritoneal carcinomatosis (3). Although the standard therapy for epithelial ovarian cancer consists of debulking surgery followed by taxane and platinum chemotherapy, the 5-year survival for patients with ovarian cancer remains less than 30% (4). Therefore, development of more effective chemotherapeutic agents is required.

In several malignant tumor types, tumor-associated monocytes (TAMs) are detected in tumor stroma. Monocyte chemoattractant protein-1 (MCP-1; CCL2) is a member of the chemokine family and plays an important role in attracting TAMS and other inflammatory cells to the cancer stroma (5). Studies of prostatic cancer, hepatocellular cancer, and breast cancer have demonstrated that MCP-1 promotes invasion, migration and proliferation of cancer cells, thereby contributing to the progression of tumors (6-8). In ovarian cancer, reports have shown that the levels of MCP-1 in the ascites or serum of patients with ovarian cancer are higher than those of healthy people or patients with benign tumors (9, 10). Additionally, expression of MCP-1 is higher in cancerous ovarian tissues than in healthy ovarian tissues (11). These data suggest that MCP-1 may play a role in ovarian cancer growth. The goal of this study was to investigate whether MCP-1 promotes tumor invasion or adhesion of ovarian cancer cells *in vitro*.

Materials and Methods

Cell culture and reagents. Recombinant MCP-1 was purchased from R&D Systems, Inc. (Tokyo, Japan). C-C motif chemokine receptor-2 (CCR2) antagonist was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and a monoclonal antibody to CCR2 was purchased from Abcam (Tokyo, Japan). The SKOV-3 cell line, established from ovarian serous carcinoma cells, were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI-1640 medium (GIBCO Life Technologies Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; GIBCO Life Technologies Japan) at 37˚C in 5% CO_2. Cells were cultured every 10 days in 100-mm tissue culture dishes. The medium was changed twice per week.

Invasion assays. A SKOV-3 cell invasion assay was performed using 8.0-μm pore 24-well Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA). The porous membranes in the wells were coated with Matrigel that was reconstituted with the addition of 0.5

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Key Words: Ovarian cancer, SKOV-3 cell line, tumor-associated macrophages (TAMs), monocyte chemoattractant protein-1 (MCP-1), C-C motif chemokine receptor-2 (CCR2) antagonist.

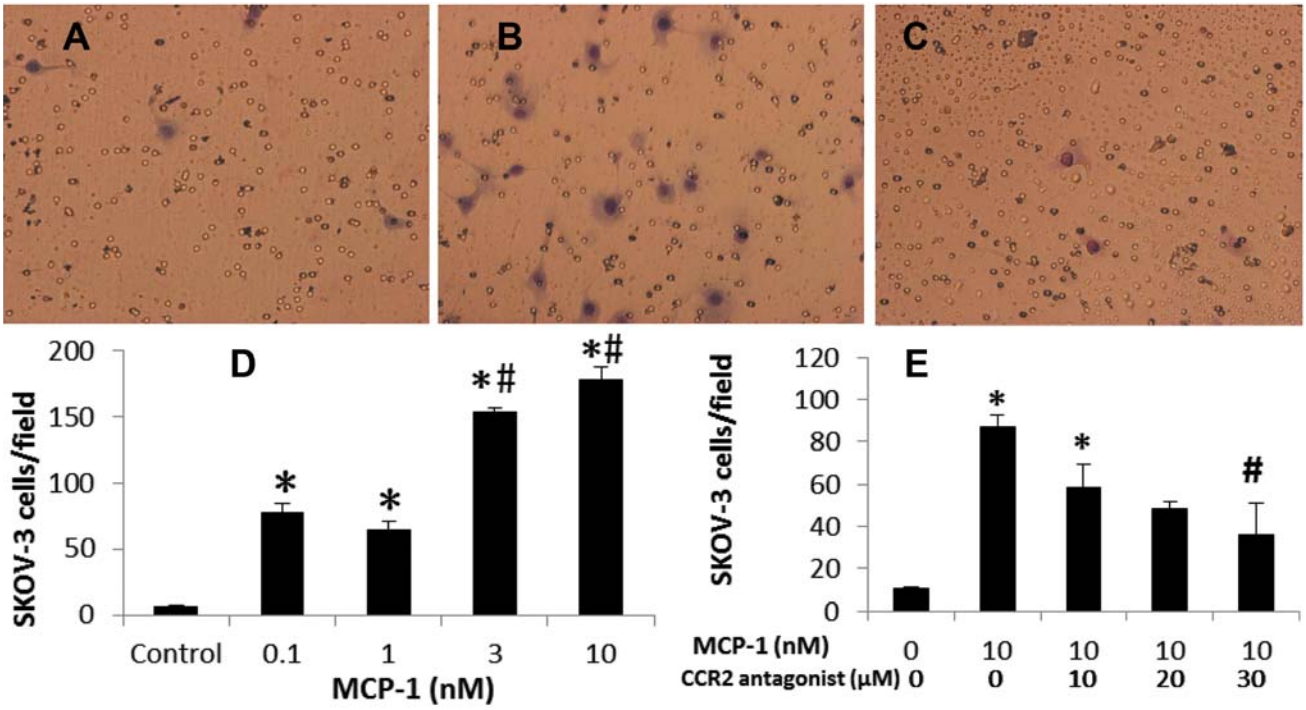

Figure 1. *Transwell invasion assay. A: Control, untreated SKOV-3 cells; B: Cells treated with 10 nM monocyte chemoattractant protein-1 (MCP-1). C: Cells treated with 10 nM MCP-1 with 30 μM C-C motif chemokine receptor-2 (CCR2) antagonist (×200). D: MCP-1 induced an increase in invasion by SKOV-3 cells in a dose-dependent manner. E: CCR2 antagonist inhibited the invasion of SKOV-3 cells treated with 10 nM of recombinant MCP-1 (rMCP-1). SKOV-3 cells were cultured with recombinant MCP-1 (rMCP-1) at the indicated concentrations. *p≤0.001 compared with control (0 nM MCP-1). #p≤0.001 compared to 0.1 nM MCP-1-treated cells. Results are expressed as the mean±SD of SKOV-3 cells counted by field for three independent experiments.*

ml of serum-free RPMI-1640 to the wells for 2 h. To assess the cells' ability to cross the membrane, 1.0×10^4 cells with and without CCR2 antagonist (10 μmol/l, sc-202525, Santa Cruz Biotechnology) in 0.5 ml RPMI-1640 containing 0.1% FBS were added to the upper compartment. In the lower compartment, 0.75 ml of RPMI-1640 containing 5% FBS with recombinant MCP-1 (0.1-10 nmol/l) was added. The chambers were incubated for 24 h at 37˚C in 95% air and 5% CO_2. After incubation, cells from the top chamber were removed using a cotton-tipped swab. The cells that penetrated through the membrane were detected by staining the cells on the porous membrane with a Diff-Quick stain kit (Sysmex International Reagents Co., Kobe, Japan) and were quantified by counting the numbers of cells in three microscopic fields (×200 magnification) per filter. Data are presented as the mean±standard deviation (SD) from triplicates.

Adhesion assays. Adhesion assays of SKOV-3 cells were performed in 96-well plates. Cells with and without the CCR2-antagonist (1-25 μmol/l) were plated in 100 μl of RPMI-1640 in a 96-well plate at a density of 50,000/well. Recombinant MCP-1 (1-100 μmol/l) was then added to the medium. The cells were incubated at 37˚C in 5% CO2 for 20 minutes. After incubation, the plate was washed. Adhered cells were then fixed with 95% ethanol and stained. The cells were counted, and data were presented in the same manner as that used for the invasion assay.

Immunostaining. Immunohistochemical staining for CCR2 was performed on SKOV-3 cells. SKOV-3 cells were cultured in two-well chamber slides (Nunc Lab-Tek II Chamber Slide; Thermo Scientific, West Palm Beach, MA, USA). After incubation for 48 h, the supernatant was removed, and adhered cells were stained using the labeled streptavidin-biotin method. The slides were incubated with monoclonal antibodies for 120 min at room temperature. The primary antibody was rabbit monoclonal antibody against CCR2 at 1:100 dilution (Abcam, Tokyo, Japan). A negative control experiment was performed by omitting the primary antibody. After incubation with the primary antibody, the slides were treated with biotinylated anti-rabbit immunoglobulin G (IgG) for 20 min, followed by incubation with avidin-biotinylated antibody. The cells on the slides were soaked in 3, 3'-diaminobenzidine (DAB) and H_2O_2 for 5 min. Finally, staining of the cells was achieved by soaking them in hematoxylin. The CCR2 positivity was estimated by calculating the percentage of stained area.

Statistical analysis. Data from the invasion assay and adhesion assay were statistically analyzed by one-way analysis of variance (ANOVA) with Bonferroni's post hoc test, using SPSS software for Windows (SPSS Japan, Inc., Tokyo, Japan). $p<0.05$ was considered to indicate statistical significance.

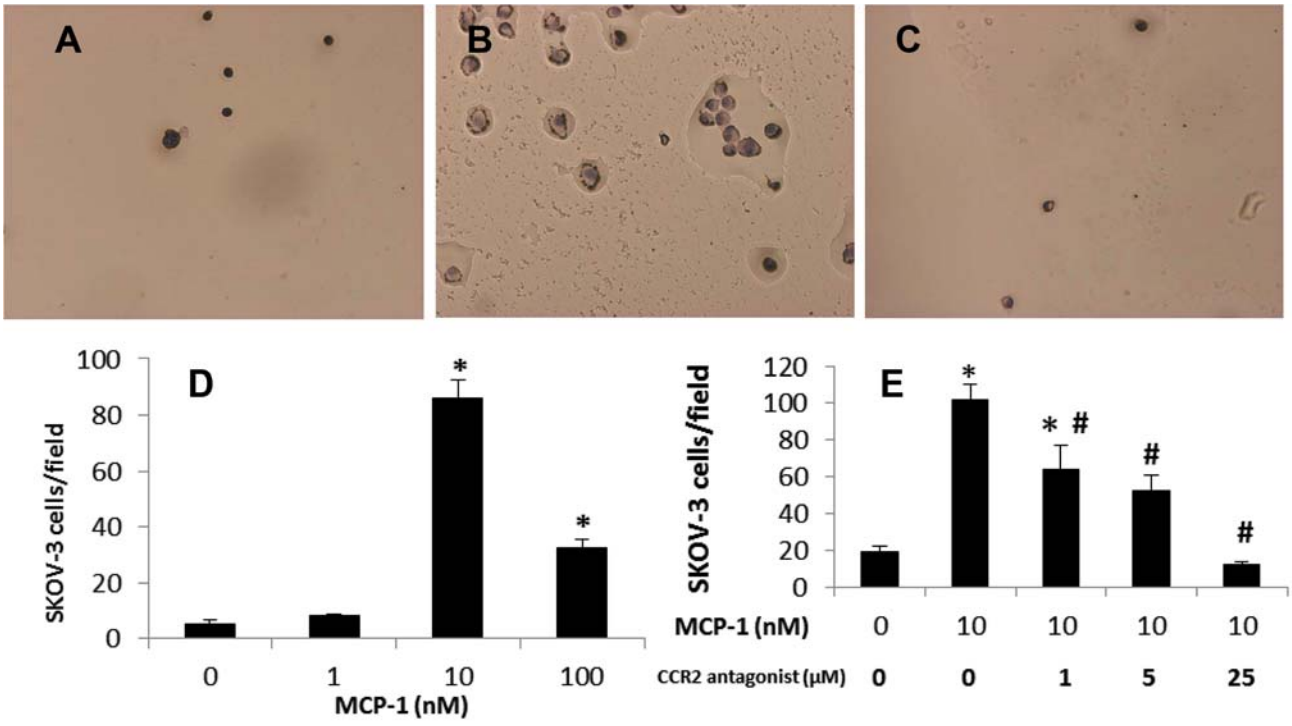

Figure 2. *96-Well adhesion assay. A: Control cells, 0 nM monocyte chemoattractant protein-1 (MCP-1). B: Cells treated with 10 nM MCP-1. C: 10 nM MCP-1 with 25 μM C-C motif chemokine receptor-2 (CCR2) antagonist (×200). D: The number of SKOV cells adhering to the well. MCP-1 induced the adhesion of SKOV-3 cells stimulated by MCP-1. E: CCR2 antagonist inhibited the migration of SKOV-3 cells treated with 10 nM of recombinant MCP-1 (rMCP-1) in a dose-dependent manner. *p≤0.001 compared with control (MCP-1 0 nM). #p≤0.001 compared to 0.1 nM MCP-1-treated cells. SKOV-3 cells were incubated with rMCP-1 at the indicated concentrations. Results are expressed as the mean±SD of SKOV-3 cells counted by field for three independent experiments.*

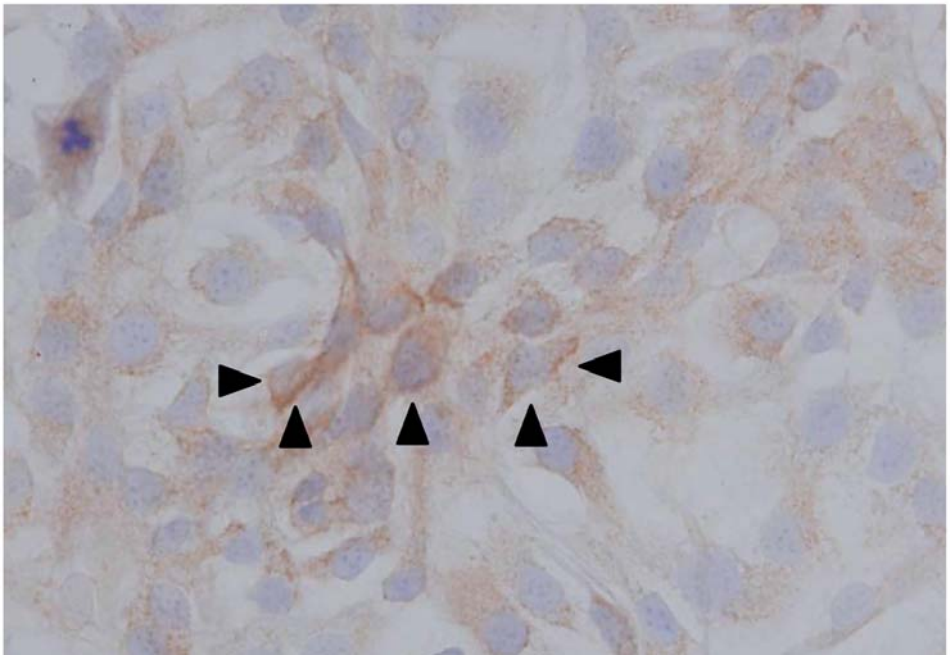

Figure 3. *Avidin-biotin-peroxidase complex staining of the SKOV-3 cells (×400). Arrows show CCR2-positive cells.*

Results

MCP-1 induced SKOV-3 cell invasion and adhesion. MCP-1 induced the invasion of SKOV-3 cells in a dose-dependent manner (Figure 1). Significant effects were observed at 0.1 nmol/l MCP-1, and the maximal effect was obtained at 3 nmol/l MCP-1. In addition, high concentrations of MCP-1 induced the adhesion of SKOV-3 cells (Figure 2). Significant effects were observed at 10 and 100 nmol/l of MCP-1.

CCR2 antagonist attenuated the MCP-1-induced increase in SKOV-3 cell invasion and adhesion. The CCR2 antagonist attenuated the invasiveness of the SKOV-3 cells incubated in a medium containing 10 nmol/l of MCP-1. Significant effects were observed at 10 μmol/l and 30 μmol/l of the CCR2 antagonist (Figure 1). Furthermore, the CCR2 antagonist attenuated the adhesion of SKOV-3 cells incubated in medium with MCP-1 (10 nmol/l). Significant effects of the CCR2 antagonist were observed in a dose-dependent manner (Figure 2).

Expression of CCR2 in SKOV-3 cells. Expression of CCR2 in SKOV-3 cells was assessed by immunohistochemical staining. CCR2 was expressed in the cytoplasm of SKOV-3 cells. CCR2-positive cells are shown in Figure 3.

Discussion

MCP-1 is a chemokine that is secreted by TAMs (12). TAMs promote tumor growth and progression *via* enhancement of angiogenesis and inflammation in the tumor microenvironment (13, 14). The CCL2–CCR2 axis also acts as a direct mediator of angiogenesis and tumor progression (5, 15, 16), and some reports have shown that MCP-1 expression is regulated by the mitogen-activated protein (MAP) kinase and the Janus kinase–signal transducer and activator of transcription (JAK/STAT) pathways (17-19).

For several types of cancer, some reports showed that the interaction between TAMs and MCP-1 contributes to tumor progression (20-23). In gynecological tumors, studies have reported that the presence of TAMs correlates with poor prognosis (24, 25). However, relatively few studies have characterized the role of MCP-1 in tumor progression. In ovarian cancer, the roles of MCP-1 and TAMs for tumor growth remain controversial. Wu *et al*. reported that macrophage migration-inhibitory factor (MIF) was expressed in ovarian cancer tissues and promoted migration and invasion of ovarian cancer cell lines (26). On the other hand, another study reported that human ovarian carcinoma cells do not express CCR2 in macrophages (27). Fader *et al*. demonstrated that MCP-1 affected the tumor microenvironment and its expression was associated with good clinical outcomes (28).

Thus, whether the CCL2–CCR2 axis or the TAMs affect the development of ovarian cancer remains controversial. The current study showed CCR2 expression in the SKOV-3 ovarian cancer cell line. Levina *et al*. used flow cytometry to show CCR2 expression in about 20% of SKOV-3 cells (29). Furthermore, data from the present study showed that MCP-1 promotes invasion and adhesion of SKOV-3 ovarian cancer cells in a manner unrelated to TAMs. There have been no prior reports to suggest that MCP-1 directly promotes ovarian tumor progression *in vitro*. Several studies have shown that CCR2 activates the JAK/STAT pathway to promote tumor progression (30, 31). Although in the current study did not investigate signaling pathways, we suspect that MCP-1/CCR2 acts *via* the JAK/STAT pathway in the ovarian cell line.

Use of an antibody against CCL2 or CCR2 antagonist to block MCP-1 can suppress tumor growth or inflammation. Jerath *et al*. showed that CCR2 antagonist attenuated vascular inflammation in murine models (32). In prostate cancer, some reports have shown that the CNTO888 antibody against CCL2 induced tumor regression *in vivo* and suppressed migration of cancer cells *in vitro* (33, 34). Furthermore, clinical studies have confirmed the efficacy of CNTO888 (35, 36), including in ovarian cancer (36). The present study demonstrated that the CCR2 antagonist inhibited invasion and adhesion of SKOV-3 ovarian cancer cells co-cultured with MCP-1, suggesting that the CCR2 antagonist can act as a tumor suppressor. CCR2 is a receptor for other ligands such as CCL7 and CCL8 (15). Because CCR2 antagonist also inhibits these ligands, it may have stronger antitumor effects compared to the antibody against CCL2.

In summary, we predict that CCR2 antagonist can inhibit growth of ovarian cancer cells by blocking the action of MCP-1. A deeper understanding of the specific functions of CCL2 in tumor growth might provide a new therapeutic strategy for the treatment of ovarian cancer.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

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