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Involvement of autocrine CXCL12/CXCR4 system in the regulation of ovarian carcinoma cell invasion

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ABSTRACT

Ovarian carcinomas are often highly invasive, especially in the peritoneal cavity; however, the mechanism involved in invasion is not yet fully understood. In the present research, we studied the role of NF-κB in the invasiveness of ovarian carcinoma cells by using (−)-DHMEQ, a specific inhibitor of NF-κB. (−)-DHMEQ inhibited invasion *in vitro* and the expression of CXCL12 and CXCR4. We found that neutralizing antibody against CXCR4 or knockdown of CXCR4 suppressed the invasion. Proteomic analysis revealed that CXCR4-siRNA treatment lowered the secretion of several invasion-related proteins, such as MMP-9 and uPA. These data imply that (−)-DHMEQ suppressed ovarian cell invasion via inhibition of the NF-κB-regulated autocrine system of CXCL12–CXCR4.

1. Introduction

Ovarian cancer is the second most common gynecological cancer, and it is the leading cause of death from gynecological cancers in the United States [1]. Generally, the main cause of treatment failure and death is metastasis. Ovarian cancer can metastasize to neighboring tissues including the liver and lungs. Moreover, one of the characteristics of ovarian cancer is that it easily metastasizes into the peritoneal cavity [2]. Therefore, in the case of ovarian cancer, the intraperitoneal (IP) administration of anticancer drugs is considered to be more effective than intravenous (IV) administration; and, therefore, this administration route is often employed clinically [3].

Metastasis and invasion are activated by matrix metalloproteinases (MMPs) that facilitate the invasion by degrading tissues. MMPs have been implicated in both primary and metastatic cancer growth and angiogenesis. MMPs are a family of zinc-dependent proteolytic enzymes that are capable of degrading multiple components of the extracellular matrix [4]. Among these MMPs, the type IV collagenases, such as MMP-2 and MMP-9, are considered to be the main ones involved in cancer cell invasion and metastasis [5]. Elevated expression of MMP-2 and MMP-9 has been detected in ovarian cancer ascites, tissues, and cancer cells in culture [6,7].

One of the factors that enhance metastasis is the chemokine/chemokine receptor system. Chemokines are small protein molecules that attract cells, thus inducing cellular migration. They are secreted from normal tissues and sometimes from cancer cells. Chemokines attract cells by engaging receptor molecules located on the cell surface. Many types of cancer cells have been reported to express chemokine receptors [8]. Chemokines usually bind to multiple receptors, and the same receptor may bind to more than one chemokine. However, CXCL12, also known as stromal cell-derived factor 1 (SDF-1), binds exclusively to the CXCR4 receptor [9]. Muller and colleagues showed that CXCR4 is highly expressed in malignant breast cancer cells but not in normal breast tissue. CXCL12 has been reported to be highly expressed in bone marrow, lungs, and lymph nodes, where breast cancer cells metastasize preferentially [10]. Also, at least 23 different types of human cancer cells express CXCR4, and this receptor–ligand pair appears to be involved in the directed migration of cancer cells to sites of metastasis [11].

Nuclear factor-κB (NF-κB) is a transcription factor that promotes the transcription of inflammatory cytokines, cell-adhesion molecules, and inhibitors of apoptosis proteins. NF-κB might also regulate tumor angiogenesis and invasiveness. VEGF-C, MMP-9, and MT1-MMP possess an NF-κB binding site in their promoter regions and CXCR4 expression is also activated by NF-κB [12,13]. Several years ago we designed (−)-dehydroxymethylepoxyquinomicin ((−)-DHMEQ) as an NF-κB inhibitor, based on the structure of epoxyquinomicins, which are weak antibiotics isolated from *Amicolatopsis* [14]. Thereafter we showed that (−)-DHMEQ inhibits the NF-κB activity by inhibiting its binding to DNA [15]. Being a specific inhibitor, it does not affect activities of other transcription factors including AP-1, NFAT, and STAT1 [16]. Human carcinomas and leukemia often possess constitutively activated NF-κB. (−)-DHMEQ inhibits the constitutively activated NF-κB in various carcinoma and leukemia cells, and it also inhibits the

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secretion of inflammatory cytokines from cancer cells [17]. Moreover, (−)-DHMEQ inhibits the growth of human carcinoma and leukemia cells in which NF-κB is constitutively activated *in vivo*. It suppresses prostate carcinoma, thyroid carcinoma, breast carcinoma, multiple myeloma, and adult T-cell leukemia [17–19] in nude or SCID mice. Thus, (−)-DHMEQ shows potent anticancer activity without any toxicity in mice.

Using our chemical library, we screened for compounds that inhibited *in vitro* invasion by ovarian cancer cells. As a result, we found that (−)-DHMEQ strongly inhibited the cellular invasion. By performing a mechanistic study, we found that an NF-κB-regulated CXCL12/CXCL4 autocrine system was essentially involved in the invasiveness of ovarian cancer RMG1 cells.

2. Materials and methods

2.1. Materials

(−)-DHMEQ was synthesized in our laboratory as described before [20]. Mouse monoclonal anti-CXCR4 antibody was purchased from R&D Systems. GM6001 was purchased from Calbiochem.

2.2. Cell culture

Human ovarian carcinoma RMG1 and ES-2 cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 200 units/mL penicillin G, 200 mg/L kanamycin, 600 mg/L L-glutamine, and 2.25 g/L $NaHCO_3$ at 37 °C in a humidified incubator with 5% CO_2.

2.3. RNA isolation and semi-quantitative RT-PCR analysis

Total RNA was extracted from RMG1 cells by using Trizol reagent (Invitrogen). Reverse transcription was carried out at 37 °C for 120 min with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was used for PCR amplification with rTaq DNA polymerase (Takara). The number of PCR cycles for each product was determined after confirmation of the efficacy of amplification and after having defined the linear exponential portion of the amplification. The sequences of the primers used for semi-quantitative RT-PCR, the numbers of cycles, and the annealing temperatures were as follow: CXCR4, 5′-GGTGGTCTATGTTGGCGTCT-3′ (forward) and 5′-TGGAGTGTGACAGCTTGGAG-3′ (reverse), 25 cycles, 57 °C; CXCL12, 5′-TTCCATGGTGTGATCGTCTG-3′ (forward) and 5′-ACTGAGAGTCCAGCGAGGTT-3′ (reverse), 25 cycles, 55 °C; MMP-9, 5′-CTCGAACTTTGACAGCGACA-3′ (forward) and 5′-GCCATTCACGTCGTCCTTAT-3′ (reverse), 35 cycles, 55 °C; and β-actin, 5′-CTTCGAGCAAGAGATGGCCA-3′ (forward) and 5′-CCAGACAGCACTGTGTGTTGGC-3′ (reverse), 25 cycles, 57 °C. PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized with a UV illuminator.

2.4. In vitro invasion assay

An *in vitro* invasion assay was performed by using a 24-well Matrigel invasion chamber having a pore size of 8.0 μm (BD Biosciences). The Matrigel Matrix-coated filters were incubated in serum-free Dulbecco's modified Eagle's medium for 2 h before the assay. The lower compartment was filled with 750 μl of the same medium containing 10% FBS. RMG1 cells (2.5×10^5) were resuspended in 500 μl of the same medium containing 1% FBS and placed in the upper part of the Transwell plate. The cells were then incubated for 48 h. Cells were fixed and stained with Diff-Quik (Sysmex). Cells on the upper surface of the filter were removed mechanically by wiping with a cotton swab; and, under light microscopic observation, the invasive phenotypes were determined by counting the cells that had migrated to the lower side of the filter.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method of Andrews and Faller [21]. Cells were grown in 60-mm dishes and incubated with the desired chemicals. They were then harvested, washed with phosphate-buffered saline (PBS), suspended in 400 μl of buffer A (10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF), and incubated on ice for 15 min. Nuclei were precipitated, resuspended in 40 μl of buffer C (50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 25% glycerol (v/v)), incubated on ice for 20 min, and centrifuged. The supernatant was used as the nuclear extract.

The binding reaction mixture contained the nuclear extract (5 μg of protein), 2 μg poly(dI-dC), and 10,000-cpm ^{32}P-labeled probe (oligonucleotide containing NF-κB binding site) in a binding buffer (75 mM NaCl, 1.5 mM EDTA, 1.5 mM DTT, 7.5% glycerol, 1.5% NP-40, 15 mM Tris–HCl; pH 7.0). DNA/protein complexes were separated from protein-unbound DNA on a 4% native polyacrylamide gel. The DNA probes used for NF-κB binding were purchased from Promega. These oligonucleotides were labeled with [γ-^{32}P]-ATP (3000 Ci/mmol; GE Healthcare) by use of T4 polynucleotide kinase (Takara), and purified by passage through a Nick column (GE Healthcare). Mouse monoclonal anti-p65 and anti-RelB antibodies were purchased from Santa Cruz Biotechnology.

2.6. Knockdown of CXCR4 by siRNA

The siRNA against CXCR4 used was 5′-CUAUUCCCGACUUCAUCUUUG-3′. A scrambled oligonucleotide (designated as control-siRNA), 5′-CCAUUUCACUCUCUAUGUC-3′, was used as a negative control. Transfection of cells with siRNAs was performed by using Lipofectamine™ RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded in 6-well plates with opti-MEM supplemented with 10% FBS. The cells were incubated with siRNAs for 24–72 h.

2.7. Focused protein array

After transfection of cells with siRNA against CXCR4 or the scrambled negative control for 24 h, the medium was changed to fresh medium; and cells were cultured for another 24 h. The culture media were then collected, and the focused protein array analyses were performed according to the manufacturer's instructions. Each culture medium was analyzed with a human angiogenesis array kit (proteome profiler™, R&D Systems). Array data were developed on X-ray film following exposure to chemiluminescent reagents.

3. Results

3.1. Inhibition of cellular invasion by (−)-DHMEQ

Ovarian carcinoma RMG1 cells showed constitutively active NF-κB, whereas treatment with TNF-α was required for NF-κB activation in ES-2 cells, another ovarian carcinoma (Fig. 1A). (−)-DHMEQ inhibited the constitutively activated NF-κB in RMG1 cells in a dose-dependent manner (Fig. 1B). Using the Matrigel chamber assay, we found that the RMG1 cells showed high invasive activity and that (−)-DHMEQ inhibited the invasion at the concentration that was inhibitory toward NF-κB, as shown in Fig. 1C. Treatment

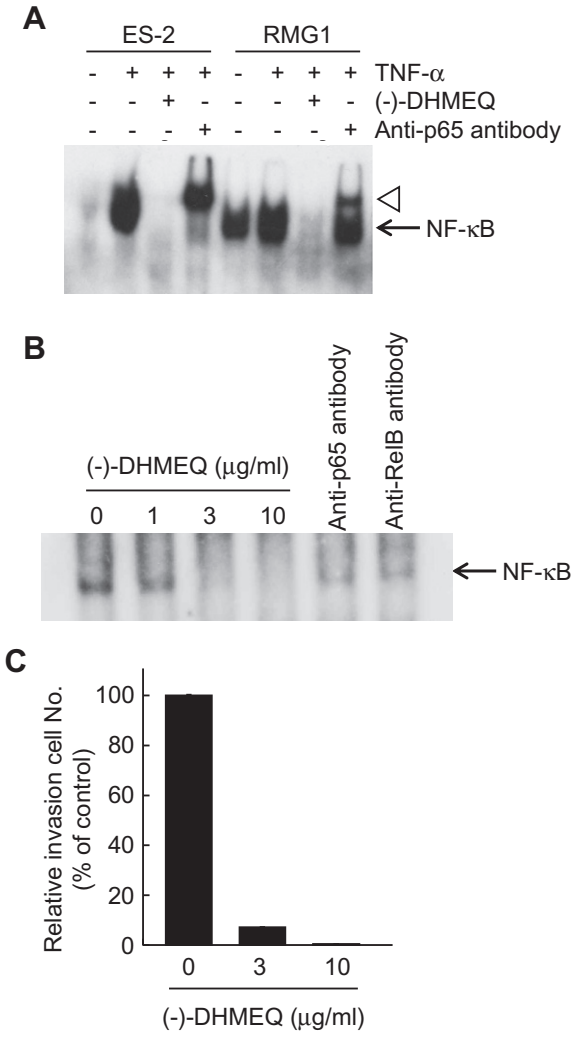

Fig. 1. (−)-DHMEQ-mediated inhibition of cellular invasion by RMG1 cells. (A) Continuous activation of NF-κB without stimulation in RMG1 cells. ES-2 and RMG1 cells were cultured with 10 μg/ml (−)-DHMEQ in the presence or absence of 10 ng/ml TNF-α for 60 min. Then, the nuclear extract was assayed by EMSA. Open triangle indicates the super-shifted complex. (B) Inhibition of NF-κB activation by (−)-DHMEQ in RMG1 cells. RMG1 cells were cultured with 1–10 μg/ml (−)-DHMEQ for 120 min. Then, the nuclear extract was assayed by EMSA. (C) (−)-DHMEQ-induced suppression of cellular invasion by RMG1 cells. RMG1 cells were incubated with (−)-DHMEQ in a chamber for 48 h. After removing the cells that remained on the upper side, cells that had migrated to the lower side of the membrane were counted.

with 10 μg/ml (−)-DHMEQ was not toxic at all to RMG1 cells. Thus, these results suggest that NF-κB is constitutively activated in RMG1 cells and (−)-DHMEQ suppressed the invasion of RMG cells through the inhibition of NF-κB activity.

3.2. Inhibition of CXCL12 and CXCR4 expressions by (−)-DHMEQ

CXCL12 is a chemokine that attracts cancer cells having CXCR4 on their cell surface, and this system is considered to be important for metastasis into various organs. Treatment of RMG1 cells with (−)-DHMEQ resulted in decreased CXCL12 expression over time (Fig. 2A), which occurred in a dose-dependent manner (Fig. 2B). Moreover, the expression of CXCR4 was also inhibited by the treatment with (−)-DHMEQ in both time- (Fig. 3A) and dose-dependent (Fig. 3B) manners. Thus, the above data indicate that the expression of CXCR4 and its ligand CXCL12 would be regulated by NF-κB.

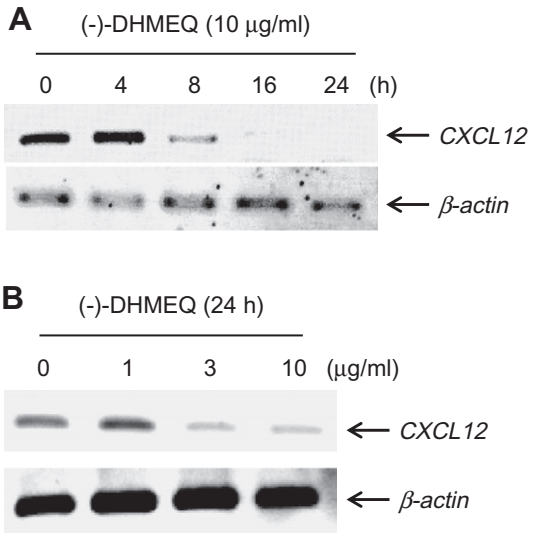

Fig. 2. Inhibition of *CXCL12* expression by (−)-DHMEQ. (A) Time-dependent inhibition of *CXCL12* expression by (−)-DHMEQ. RMG1 cells were treated with 10 μg/ml (−)-DHMEQ for the indicated times. Then, total RNA was extracted, and the expression of each gene was measured by semi-quantitative RT-PCR. (B) Dose-dependent inhibition of *CXCL12* expression by (−)-DHMEQ. RMG1 cells were treated with various concentrations of (−)-DHMEQ for 24 h. Then, total RNA was extracted, and the expression of each gene was measured by semi-quantitative RT-PCR.

To examine the role of this CXCL12-CXCR4 system in ovarian cancer cell migration, we used a neutralizing anti-CXCR4 antibody and siRNA targeted against CXCR4 (designated as CXCR4-siRNA). As shown in Fig. 3C, treatment of RMG1 cells with neutralizing antibody to CXCR4 or (−)-DHMEQ suppressed the invasiveness of RMG1 cells, whereas control IgG did not affect the migration through the filter. We next employed siRNA to knockdown CXCR4 expression. The CXCR4-siRNA-mediated decrease in the expression level by CXCR4 was confirmed by performing semi-quantitative RT-PCR (Fig. 3D). Treatment with CXCR4-siRNA or (−)-DHMEQ decreased the invasion activity of RMG1 cells (Fig. 3E), suggesting that this NF-κB-regulated CXCL12–CXCR4 autocrine system may be involved in the invasiveness of RMG1 cells.

3.3. Requirement of MMP-9 for RMG1 cell invasion

To investigate which factor(s) regulated by the CXCL12-CXCR4 autocrine system would be involved in the invasion, we carried out proteomic analysis of CXCR4 knockdown cells compared with control-siRNA-treated cells. As shown in Table 1, the expression of several proteins, such as MMP-9, uPA, amphiregulin, DPPIV, CXCL16, VEGF, and HB-EGF, was decreased by the CXCR4-siRNA treatment. Indeed, we confirmed the lower expression of MMP-9 and of uPA by semi-quantitative RT-PCR after treatment with CXCR4-siRNA (Fig. 4A). Moreover, treatment of RMG1 cells with (−)-DHMEQ also resulted in decreased levels of *MMP-9* and *uPA* mRNAs (Fig. 4B). Thus, these results suggest that *MMP-9* and *uPA* expressions were regulated by both NF-κB directly and the NF-κB-mediated CXCL12–CXCR4 autocrine system. Finally, to determine the role of MMP-9 in the cellular invasion, we investigated the effect of an MMP inhibitor on the invasiveness of RMG1 cells. As shown in Fig. 4C, treatment of the cells with GM6001, an inhibitor of MMPs, resulted in suppressed invasion. Therefore, these results indicate that MMP-9 may be at least one factor responsible for the ovarian cancer cell invasion regulated by the NF-κB-mediated CXCL12–CXCR4 autocrine system.

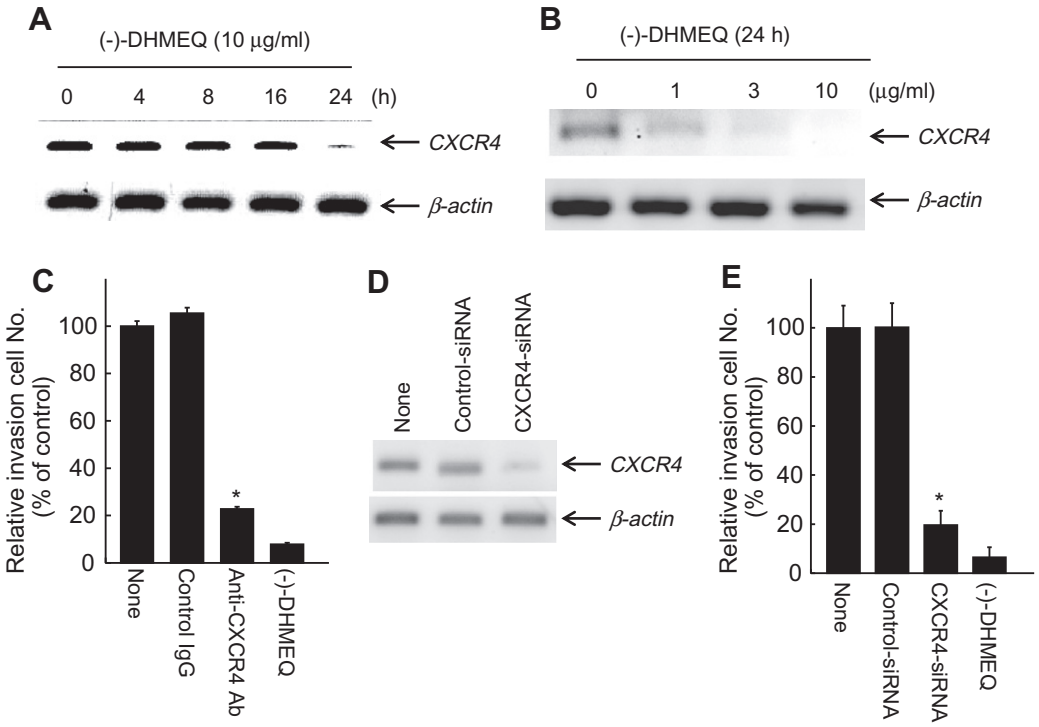

Fig. 3. Involvement of NF-κB-regulated CXCR4 expression for cellular invasion of RMG1 cells. (A) Time-dependent inhibition of *CXCR4* expression by (−)-DHMEQ. RMG1 cells were treated with 10 μg/ml (−)-DHMEQ, and the expression of each gene was measured by semi-quantitative RT-PCR. (B) Dose-dependent inhibition of *CXCR4* expression by (−)-DHMEQ. RMG1 cells were treated with various concentrations of (−)-DHMEQ for 24 h. (C) Suppression of cellular invasion by RMG1 cells by neutralizing anti-CXCR4 antibody. RMG1 cells were incubated with 10 μg/ml control IgG, 10 μg/ml anti-CXCR4 antibody or 10 μg/ml (−)-DHMEQ for 48 h. Asterisk denotes a statistically significant ($P < 0.05$, Student's *t*-test) difference between anti-CXCR4 antibody treatment and control IgG treatment. (D) Knockdown of *CXCR4* expression by CXCR4-siRNA. RMG1 cells were transfected with control-siRNA or CXCR4-siRNA, and the expression of each gene was measured by semi-quantitative RT-PCR. (E) Suppression of cellular invasion by RMG1 cells by treatment with CXCR4-siRNA. RMG1 cells were transfected with control-siRNA or CXCR4-siRNA for several days. RMG1 cells were then harvested, and incubated in a chamber for 48 h. Asterisk denotes a statistically significant ($P < 0.05$, Student's *t*-test) difference between CXCR4-siRNA treatment and control-siRNA treatment.

Table 1
List of proteins decreased in expression by treatment with CXCR4-siRNA.

Protein names	Expression level (% of control)
Amphiregulin	3.9
VEGF	8.6
HB-EGF	10.6
CXCL16	15.9
MMP-9	16.9
EG-VEGF	34.8
TIMP-4	42.3
DPPIV	44.9
PTX3	45.6
uPA	48.3

RMG1 cells were treated as described in Section 2 (under focused protein array). The expression levels were calculated by using the following formula: (expression level (%) = (protein level after treatment with CXCR4-siRNA − background value)/(protein level after treatment with control-siRNA − background value) × 100). The top 10 proteins whose levels were decreased by the treatment are listed.

4. Discussion

CXCR4 is the most common chemokine receptor that has been demonstrated to be over-expressed in many types of human cancers, including ovarian cancer and prostate cancer, and is of particular importance in the metastatic behavior and destination of solid cancers [22]. CXCL12 is a highly efficient chemoattractant for cells that express its receptor, CXCR4. Prominent CXCR4-positive tumors include breast cancer, ovarian cancer, prostate cancer, hepatocellular carcinoma, and hematologic malignancies [23,24]. In epithelial ovarian cancer, positive expression of CXCR4 has been associated with poor prognosis [25]. CXCR4 and CXCL12 are also important for peritoneal dissemination of ovarian cancer cells [26]. Therefore, these facts imply that the CXCL12–CXCR4 axis would be an attractive target for cancer metastasis therapy.

Bioactive compounds are usually evaluated by several methods, such as enzyme assays, cell-based assays, and chemical arrays [27]. To obtain compounds that inhibit invasion by ovarian cancer cells, we screened our chemical library for such compounds by using a cell-based assay system. In this present study, we found that RMG1, an ovarian cancer cell line, expressed constitutively activated NF-κB without stimulation (Fig. 1B) and that (−)-DHMEQ strongly inhibited the cellular invasion by RMG1 cells *in vitro* (Fig. 1C). Then, by conducting a mechanistic study, we found that treatment of the cells with (−)-DHMEQ resulted in decreased expressions of *CXCL12* and *CXCR4* (Figs. 2 and 3), suggesting that the κB sequence located in the promoter region of both ligand and receptor may be involved in their expression. It was earlier reported that CXCR4 expression is activated by NF-κB [13]. In the case of CXCL12 it is not clear whether its expression is κB dependent. It was reported that the promoter of the CXCL12 gene may be recognized by noncanonical NF-κB consisting of RelB and p52 and not by canonical NF-κB consisting of p65 and p50 [28]. These observations are consistent with our results shown in Fig. 1B, in which RMG1 cells were seen likely to possess RelB as one of their NF-κB components.

It has been known that the CXCL12–CXCR4 axis plays important roles in cancer metastasis by alteration of host immune response [22]. In the present research, treatment with a neutralizing anti-CXCR4 antibody or CXCR4-siRNA directly suppressed the invasion by RMG1 cells (Fig. 3C and E); however, the responsible factor(s)

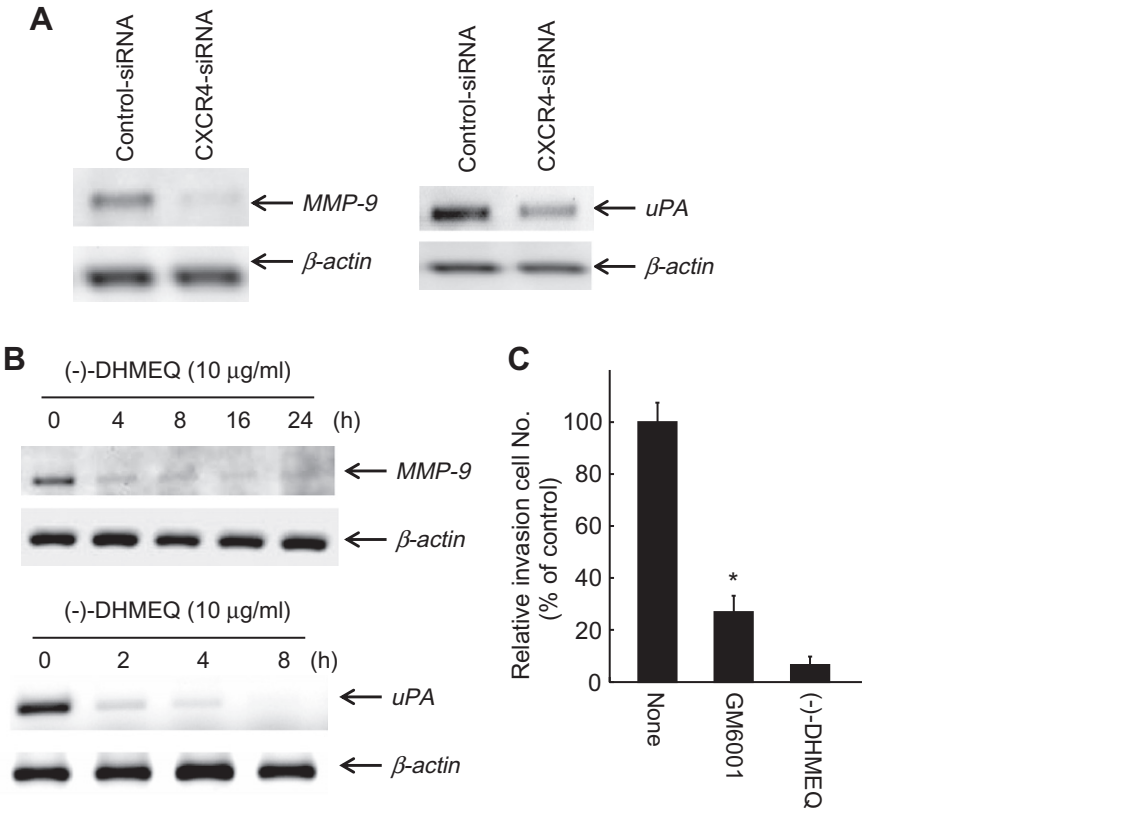

Fig. 4. Requirement of MMP-9 for cellular invasion mediated by the CXCR4–CXCL12 autocrine system. (A) Knockdown of CXCR4 resulted in decreased expression of MMP-9 and uPA. RMG1 cells were transfected with control-siRNA or CXCR4-siRNA, and the mRNA expression of each gene was measured by semi-quantitative RT-PCR. (B) Inhibition of *MMP-9* expression by (−)-DHMEQ. RMG1 cells were treated with 10 μg/ml (−)-DHMEQ, and the expression of MMP and uPA genes was measured by semi-quantitative RT-PCR. (C) GM6001-mediated suppression of cellular invasion by RMG1 cells. RMG1 cells were incubated in the presence or absence of 10 μM GM6001 or 10 μg/ml (−)-DHMEQ in a chamber for 48 h. The asterisk denotes a statistically significant ($P < 0.001$, Student's *t*-test) difference between GM6001-treated cells and control cells.

regulated by the CXCL12–CXCR4 axis for enhancement of cellular invasion by ovarian cancer cells had not yet been identified. Our proteomics approach demonstrated that knockdown of CXCR4 decreased the level of several metastasis-related proteins, such as MMP-9 and uPA (Table 1). To confirm whether their expression was regulated by NF-κB and the CXCL12–CXCR4 axis, we treated the RMG1 cells with CXCR4-siRNA or (−)-DHMEQ. As the result, the expression of *MMP-9* and of *uPA* (Fig. 4A and B) was decreased after either treatment. Furthermore, an inhibitor of MMPs resulted in suppressed invasion by RMG1 cells (Fig. 4C). Taken together, our present results indicate that NF-κB would positively regulate the expression of both *CXCL12* and *CXCR4*, which would then up-regulate the expression of several metastasis-related genes, thereby enhancing cellular invasion by ovarian cancer cells.

CXCL12 is often secreted from tissues, and it attracts CXCR4-expressing cancer cells to the secondary sites [10,23]. Our observations suggest that the CXCL12/CXCR4 axis would be important not only for the chemokine-induced local invasion by cancer cells but also for the autocrine activation towards metastasis.

Acknowledgments

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