Inhibition of chemokine receptor CXCR4 as a novel therapeutic strategy in a prostate cancer xenograft model

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Directed by Professor Sung Joon Hong

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ABSTRACT

Inhibition of chemokine receptor CXCR4 as a novel therapeutic strategy in a prostate cancer xenograft model

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Purpose: The stromal derived factor-1 (SDF-1)/CXCR4 axis is associated with tumor aggressiveness and metastasis in prostate cancer. In this study, we explored whether a CXCR4-specific antagonist can abrogate the function of the SDF-1/CXCR4 axis and its downstream signaling. In addition, we tested the potential therapeutic effect of the CXCR4 antagonist in a prostate cancer xenograft model.

Materials and methods: A representative metastatic prostate cancer cell line, PC-3 was used for *in vitro* and *in vivo* experiments. First, we tested the effect of SDF-1 and the CXCR4-specific antagonist, AMD3100, on PC-3 cell proliferation using a proliferation assay. Second, we evaluated whether AMD3100 could influence SDF-1 induced cell migration and SDF-1/CXCR4-mediated Akt signaling in PC-3 cells by migration assay and western blot. Finally, we investigated the effect of AMD3100 on tumor growth in a PC-3 tumor xenograft model by performing H&E staining and immunohistochemical staining of bcl-2, Ki-67 and CD34 to identify the histological differences between AMD3100-treated and untreated groups.

Results: We found expression of CXCR4 protein in PC-3 cells. Cell proliferation was not significantly affected by SDF-1 (50 - 200 ng/ml) or AMD3100 (0.01 - 10 μg/ml) treatment. Further, the SDF-1/CXCR4 axis plays an important role in chemotactic migration in PC-3 cells, and AMD3100-treatment resulted in a 39.1 % reduction in SDF-1-induced migration. Western blot revealed that SDF-1 stimulation could enhance the expression of phosphorylated Akt in PC-3 cells, but the SDF-1-induced expression of phosphorylated Akt was abrogated in AMD3100-treated PC-3 cells. In the PC-3 tumor xenograft model, AMD3100 significantly inhibited tumor growth in nude mice inoculated with PC-3 cells, and AMD3100-treated PC-3 tumors had less microvessel formation and less immunoreactivity for the proliferation marker Ki-67 and the anti-apoptotic marker bcl-2 when compared to control tumors *in vivo*. Conclusions: The SDF-1/CXCR4 axis plays an important role in chemotactic migration and Akt signal pathway in PC-3 cells. The CXCR4-specific antagonist, AMD3100, effectively inhibits SDF-1-induced PC-3 cell migration and CXCR4/Akt signal transduction. Moreover, AMD3100 suppresses tumor growth in nude mice inoculated with PC-3 cells. We suggest that CXCR4 targeting might represent a novel strategy with potential utility for the treatment of human prostate cancer.

Key words: prostate cancer, SDF-1/CXCR4 axis, CXCR4 antagonist, xenograft model

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I. INTRODUCTION

Prostate cancer is the third most common malignancy in men worldwide.[1] It is the most commonly diagnosed cancer, and the second leading cause of cancer death, in American males.[2] In Korea, prostate cancer is the most rapidly increasing male malignancy by incidence, probably due to the Westernization of the Korean diet and increasingly elderly population. Despite numerous advances in treatment, the high mortality rate for prostate cancer is due to the fact that malignant cells typically spread into many other tissues. In advanced prostate cancer, the sites most frequently affected by metastasis are the bones and regional lymph nodes. Patients with these metastases not only have a poor prognosis, but also suffer pain and deterioration in quality of life.

Radical surgery or radiotherapy can be curative therapies for patients with localized prostate cancer, but there are no effective treatment modalities for the management of advanced or metastatic prostate cancer. Androgen deprivation treatment is the most effective systemic approach for patients with metastatic disease. Although 80-90% of patients initially respond favorably to this treatment, eventually they become unresponsive to androgen deprivation, developing hormone-

refractory prostate cancer (HRPC).[3, 4] Currently, the combination of docetaxel and prednisone is considered standard first-line therapy for HRPC, given its demonstrated ability to prolong survival. Unfortunately, the survival gain through docetaxel chemotherapy is also limited and unsatisfactory.[5] Mortality from prostate cancer is not due to the prostate cancer itself, but to progression of metastatic diseases. As a result, there is growing interest in early detection and screening for prostate cancer, and a greater understanding of the mechanisms that lead to metastasis. There are particular interests in the prevention of metastasis to bones, lymph nodes and lungs, and in the identification of effective therapeutic approaches for metastatic disease.

Metastasis is a complicated, multi-step process.[6] Malignant cells must initially escape from the primary tumor, invade the surrounding tissues and enter the vascular circulation. If they are able to survive in the blood stream, they must arrest at a secondary target site, cross the vascular barrier and migrate into extravascular connective tissues. Subsequently, the tumor cells must proliferate, thereby establishing a secondary (metastatic) colony. These events involve numerous cell-cell and cell-matrix adhesive interactions that are mediated by cell surface adhesion molecules.

The chemokines are a family of low molecular weight cytokines that mediate their chemical effects on target cells through seven transmembrane G-protein-coupled receptors. Chemokines are organized into four classes (CXC, CC, C and CX3C) based on their N-terminal cysteine motifs.[7-10] Stromal cell-derived factor-1 (SDF-1), which was initially cloned by Tashiro et al.[11], is a member of the CXC subfamily of chemokines and interacts with the seven transmembrane G-protein-coupled receptor CXCR4, a SDF-1-specific receptor.[12, 13] Later, SDF-1 was identified as a growth factor for B-cell progenitors and as a

chemotactic factor for T cells and monocytes relevant to B-cell lymphopoiesis and bone marrow myelopoiesis.[12, 14, 15] Furthermore, SDF-1 was found to play a critical role in directed cell migration,[12, 16] and embryonic development.[14, 17, 18] The functions of a subset of chemokine receptors that serve as co-receptors for the entry of HIV-1 has also been clarified.[19, 20] Meanwhile, CXCR4 is the most widely expressed chemokine receptor in many different cancers.[21] CXCR4 expression has been reported in at least 23 epithelial, mesenchymal and hematopoietic cancers, suggesting the importance of this ligand/receptor axis in tumor metastasis.[22] CXCR4 expression is known to be associated with tumor aggressiveness and poor prognosis in melanoma, esophageal cancer and ovarian cancer.[23-25]

There has been a lot of interest in the role of the SDF-1/CXCR4 axis in various malignancies. Activation of CXCR4 stimulates the directed migration of cancer cells, invasion through matrigel, endothelial cell, bone marrow stromal or fibroblast monolayers, towards a SDF-1 gradient.[22] The SDF-1/CXCR4 axis may influence the biology of tumors and direct the metastasis of CXCR4+ tumor cells by chemoattracting them to organs that highly express SDF-1 (e.g., lymph nodes, lungs, liver, or bones). Supporting this notion, it has been recently reported that several CXCR4+ cancers (e.g., breast cancer, ovary cancer, prostate cancer, rhabdomyosarcoma and neuroblastoma) metastasize to the bones from the bloodstream in an SDF-1-dependent manner.[26-33] In some types of cancer, SDF-1 can also stimulate cancer cell proliferation or survival under suboptimal conditions.[22]

The role of the SDF-1/CXCR4 axis in prostate cancer has been experimentally demonstrated. It is known that CXCR4 mRNA and protein are expressed in prostate cancer cell lines such as LNCaP, PC3, and DU145, and human prostate samples.[30] Recently, Akashi et al.

demonstrated that patients with high expression of CXCR4 in tumors had worse cancer-specific survival rates than those with low expression of CXCR4.[34] Several experimental models have provided critical evidence for the role of the SDF-1/CXCR4 axis in skeletal metastasis;[35-37] Darash-Yahana et al. reported that subcutaneous xenografts of prostate tumors that overexpressed CXCR4 in nude mice were two- to three-fold larger in volume and weight compared to controls. Moreover, blood vessel density, functionality, invasiveness of tumors and metastasis to the lymph node and lung were significantly increased in these tumors.[38]

In this study, we explored whether a CXCR4-specific antagonist can abrogate the function of the SDF-1/CXCR4 axis and its downstream signaling. In addition, we tested the potential therapeutic effects of the CXCR4 antagonist in a prostate cancer xenograft model.

II. MATERIALS AND METHODS

1. Cell culture and reagents

PC-3 and LNCaP cells were obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% l-glutamine. All cells were grown in a humidified incubator at 37°C and 5% CO_2. We used SDF-1 (R&D Systems, Minneapolis, MN, USA) as a specific ligand for CXCR4 and the bicyclam derivative, AMD3100 (Sigma-Aldrich, St. Louis, MO, USA), as a CXCR4-specific antagonist. In some experiments, cells were pretreated with filipin (Sigma-Aldrich) to deplete membrane cholesterol.[39]

2. *In vitro* proliferation assay and cell viability assay

Prostate cancer cells (PC-3, LNCaP) were seeded at a density of $2x10^3$ cells per well into 96-well plates in culture medium containing 10% FBS. After 24 hr, the cultures were washed and refed with serum-free medium alone (control) or with medium containing SDF-1 or AMD3100 at various concentrations. After 72 hr, the number of viable cells was counted using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. This assay is based upon the ability of viable cells to bioreduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium to formazon in the presence of phenazine methosulfate, an electron-coupling reagent. Formazon production is quantified by measuring absorbance at 490 nm, which is directly proportional to the number of living cells. Filipin is a polyene macrolide antibiotic that binds to cholesterol and disrupts the organization of the surrounding membrane. While treatment with low concentrations of filipin has minimal side effects, high concentrations

dramatically reduce cell viability.[39] For this reason, cell viability was tested after filipin treatment using trypan blue solution (Gibco, Grand Island, NY, USA). PC-3 cells were treated with filipin at concentrations ranging from 0 to 100 μg/ml at 37°C for 15 min. Cell viability decreased significantly at concentrations ≥ 2 μg/ml of filipin in a dose- dependent manner. Therefore, the following experiments were performed using filipin at a concentration of 1 μg/ml.

3. Western blot analysis

Prostate cancer cells were cultured to subconfluence (80-90%), and cells were washed and then incubated in serum-free media for 12hr. SDF-1 stimulation was performed with 0 - 200 ng/ml SDF-1 for various amounts of time. In some experiments, 1 μg/mL of AMD3100 or 1 μg/ml of filipin was pre-incubated with the cells. Cells were lysed with RIPA lysis buffer consisting of 50 mM HEPES (USB, Cleveland, Ohio, USA) (pH 7.6), 150 mM NaCl (Sigma-Aldrich), 1% NP-40 (Amresco, Solon, OH, USA), 10 ml/ml phenylmethylsulfonyl fluoride (Amresco) and 10 ml/ml aprotinin (Sigma-Aldrich), and cleared by centrifugation. Protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of cell lysates were separated by 10-12% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. Membranes were blocked for 1 hr at room temperature in 5% nonfat dried milk and then incubated for 2 hr at room temperature with anti-Akt1 (1:1000; Santa Cruz Biotechnology), anti-p-Akt1/2/3 (1:1,000; Santa Cruz Biotechnology), anti-CXCR4 (1:1000; Santa Cruz Biotechnology) or β-actin (1:2,000; Santa Cruz Biotechnology) antibodies. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or goat anti-mouse

IgG for 1 hr at room temperature. Protein signals were detected by chemiluminescence with ECL detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

4. *In vitro* migration assay

Tumor cell migration was assayed in 24-well Transwell chambers (Costar, New York, NY, USA). Transwell chambers with 6.5-mm polycarbonate filters of 8-μm pore size were used. Prostate cancer cells were suspended in serum-free media, and then 10^5 cells in 300 μl SFM were added to the upper chamber. Then, 500 μl serum-free media with various concentrations of SDF-1 was added to the lower chamber. In some experiments, 1 μg/mL of AMD3100 was preincubated with the cells, or the cells were pretreated with filipin. The chambers were incubated at 37℃ with 5% CO_2. After 16 hr of incubation, the non-migrating cells were removed from the upper surface of the membrane with a cotton swab, and the number of viable cells was counted using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay.

5. Xenografts in nude mice

Six-week-old male nude mice (BALB/c) were obtained from Japan SLC (Hamamatsu, Japan). PC-3 cells (5 x 10^6 cells per mouse) were injected subcutaneously into the right dorsal region. When tumors measured 40 mm^3, mice were randomized to receive AMD3100 (n = 6; 3 mg/kg) or vehicle alone (n = 6; PBS) by intraperitoneal injection for five consecutive days per week for 4 weeks. Tumor diameters were measured at regular intervals with calipers. Tumor volumes (V) were calculated with the following formula: $V = A \times B^2 / 2$ (A = axial diameter; B = rotational diameter). Tumors were excised and fixed overnight in neutral buffered formalin and processed by routine methods. All protocols for

animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at Yonsei University.

6. Immunohistochemistry

Immunohistochemical staining was performed using the mouse anti-human Ki-67 monoclonal antibody (1:50; DAKO, Carpinteria, CA, USA), the mouse anti-human bcl-2 (prediluted form; DAKO) and the rabbit anti-mouse CD34 monoclonal antibody (1:100; BD Pharmingen, San Jose, CA, USA) in a xenograft tissue study. In brief, formalin-fixed, paraffin-embedded 4 μm specimens were deparaffinized and rehydrated. The sections were treated with 2% hydrogen peroxide to inactivate endogenous peroxidase, and nonspecific binding was blocked by treatment with the blocking reagent. The primary antibody was applied to each section for 1hr at 37°C, and the appropriate HRP-conjugated secondary antibody was applied at a dilution of 1:100 for 1 hr at room temperature. Immunoreactivity was subsequently detected using a 3,3'-diaminobenzidine system (Vector Laboratories, Burlingame, CA, USA). The nuclei were counterstained using Meyer's hematoxylin.

7. Data analysis

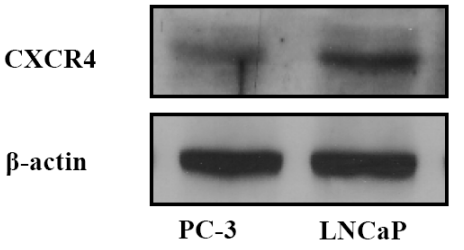
For data from the *in vitro* experiments, we performed statistical comparisons among groups using Student's t-test or the Mann-Whitney U test. Differences between groups were considered statistically significant at $P < 0.05$. The Statistical Package for Social Sciences (SPSS) for Windows, version 12.0, was used for statistical analysis. Data are expressed as mean ± standard deviation. All *in vitro* experiments were repeated with triplicate or quadruplicate samples and similar results were obtained across trials.

III. RESULTS

1. Expression of CXCR4 in prostate cancer cells

The expression of CXCR4 in two prostate cancer cell lines (PC-3, LNCaP) was examined. Western blot analyses demonstrated definite expression of CXCR4 protein in prostate cancer cell lines. The level of CXCR4 expression was higher in LNCaP cells than in PC-3 cells (Fig. 1).

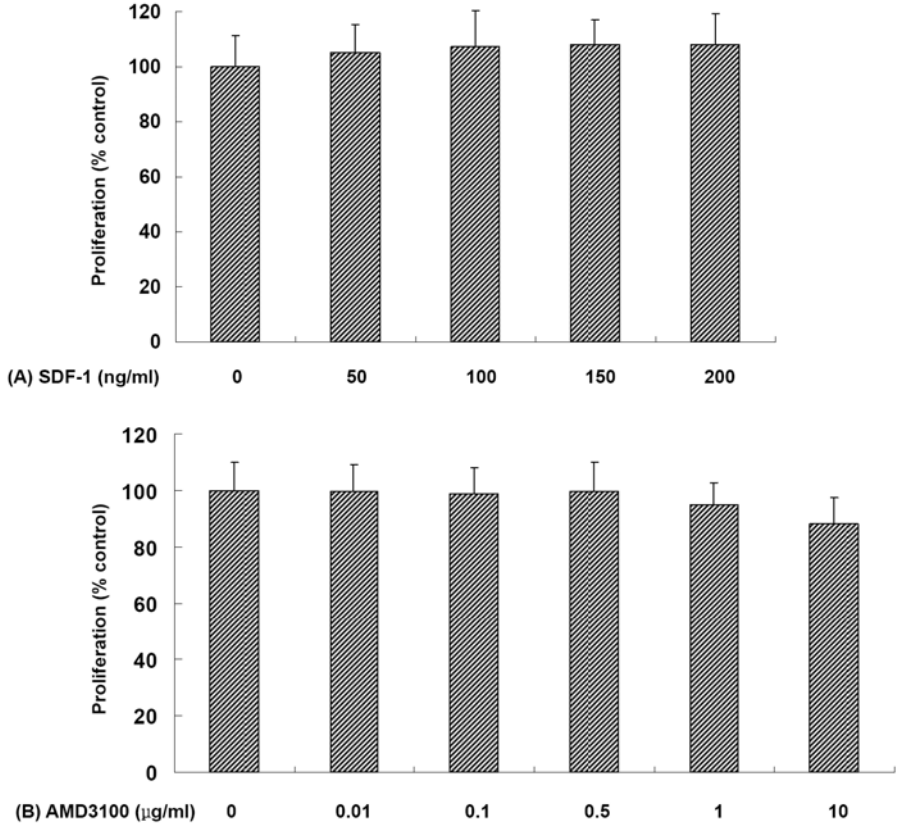
Fig. 1. Immunoreactivity of CXCR4 protein in prostate cancer cells.



2. Effect of SDF-1 and CXCR4 antagonist on PC-3 cell proliferation

The effects of SDF-1 and a specific CXCR4 antagonist, AMD3100 on cell proliferation were examined in PC-3 cells. After incubation for 72 hr, cell proliferation was not significantly affected by SDF-1 at concentrations ranging from 50 to 200 ng/ml (Fig. 2). The effects of AMD3100 at concentrations up to 10 μg/ml were tested in terms of proliferation of PC-3 cells. AMD3100 showed no inhibitory effect on cell proliferation at concentrations up to 0.5 μg/ml. Although the cell proliferation was slightly suppressed at 1 and 10 μg/ml, the effect was not statistically significant (Fig. 2).

Fig. 2. Effects of SDF-1 (A) and a CXCR4 antagonist (B) on PC-3 cell proliferation. Points, mean; bar, standard deviation; there is no statistical difference among groups.

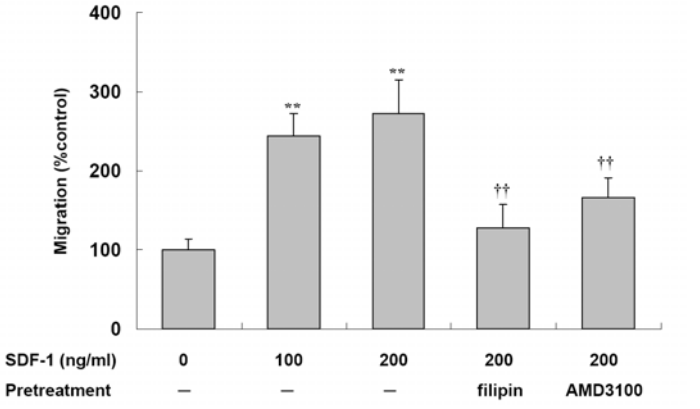


3. Effect of CXCR4-specific antagonist and cholesterol depleting agent on SDF-1-induced migration of PC-3 cells

SDF-1 stimulated the migration of PC-3 cells. Migration at a SDF-1 concentration of 200 ng/ml was slightly higher than that at a concentration of 100 ng/ml, but these differences were not significant. The inhibitory effect of the CXCR4–specific antagonist, AMD3100, on SDF-1-induced migration was tested. The migration induced by SDF-1 at

200 ng/ml was decreased by 39.1% after treatment with AMD3100 at a concentration of 1 μg/ml in PC-3 cell lines (Fig. 3). Next, SDF-1 induced migration was tested after treatment with the cholesterol depleting agent, filipin. Migration of cholesterol-depleted PC-3 cells was significantly reduced by approximately 53% following filipin treatment.

Fig. 3. Effects of a CXCR4 antagonist and cholesterol depleting agent on SDF-1-induced migration of PC-3 cells. Migratory cells in the lower chambers were evaluated using a cell proliferation assay kit and a multiwall scanning spectrometer at 490 nm. Points, mean; bar, standard deviation; **, $P < 0.01$ compared to control group; ††, $P < 0.01$ compared to a 200 ng/ml of SDF-1 treated group.



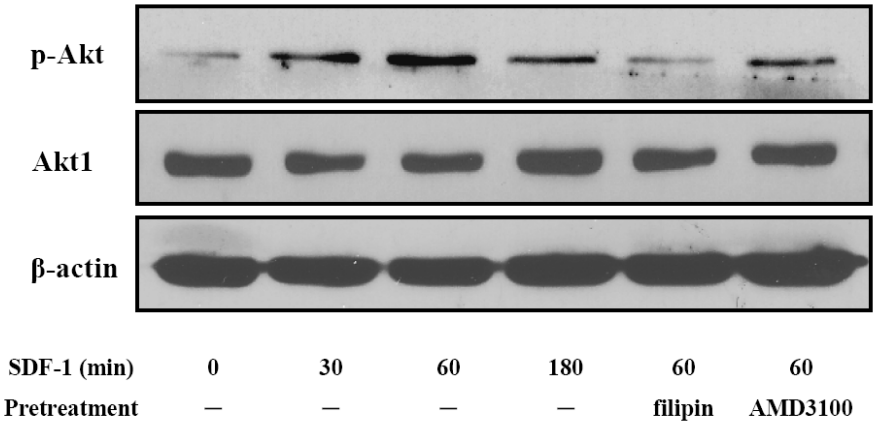
4. Effect of CXCR4 antagonist and cholesterol depleting agent on SDF-1/CXCR4-mediated Akt signaling in PC-3 cells

We next explored the effect of the CXCR4 antagonist and cholesterol depleting agent on SDF-1/CXCR4-mediated Akt signaling in prostate cancer cells. The Akt pathway is an important signaling pathway in prostate cancer.[40, 41] Akt is a serine–threonine kinase and its phosphorylation is linked to mitogenic signals. In addition to its role in

survival, Akt participates in many intracellular pathways including the integration of proliferation and differentiation signals that mediate migration and angiogenesis.

Following SDF-1 stimulation, the expression of phosphorylated Akt was enhanced in PC-3 cells at 30, 60 and 180 min compared to control. The highest levels of Akt phosphorylation after SDF-1 stimulation were observed between 30 and 60 min. The expression of phosphorylated Akt induced by SDF-1 was slightly increased in AMD3100-treated PC-3 cells compared to controls, but the levels did not reach those in PC-3 cells stimulated by SDF-1 alone. Meanwhile, the SDF-1-induced expression of phosphorylated Akt was not increased in cholesterol depleted PC-3 cells. Examination of the total Akt levels demonstrated that roughly all of the conditions resulted in similar levels of Akt. These results indicate that this axis is inhibited by the CXCR4-specific antagonist, AMD3100, and depletion of the membrane cholesterol abrogates SDF-1-induced pAkt activation.

Fig. 4. Effects of a CXCR4 antagonist and cholesterol depleting agent on SDF-1/CXCR4-mediated Akt signaling in PC-3 cells. Serum-starved PC-3 cells were stimulated by 200 ng/ml of SDF-1 for 0, 30, 60 and 180 min. In some cases, serum starved PC-3 cells were pretreated with filipin or AMD3100 before SDF-1 stimulation.



5. Blocking CXCR4 suppresses tumor growth of prostate cancer in the xenograft mouse model

To assess the effects of CXCR4 inhibition on prostate cancer *in vivo*, we used a xenograft animal model for prostate cancer in which PC-3 cells were implanted into the right dorsal region of nude mice. When tumors measured 40 mm3, mice were randomized to receive AMD3100 or vehicle. In Fig. 5, we show representative gross images of tumors harvested from each group after 28 days. Our data reveal that the CXCR4 antagonist significantly suppressed growth of PC-3 tumors. We tracked changes in tumor volume using caliper measurements and body weight measurements on the 7th, 14th, 21st and 28th days of treatment for both CXCR4 antagonist-treated mice and control groups. The tumor volumes of the AMD3100-treated and control groups were 49.0 ± 16.7 mm3 and

102.5 ± 20.9 mm3 on day 7, 54.9 ± 19.4 mm3 and 234.0 ± 92.9 mm3 on day 14, 56.3 ± 18.5 mm3 and 559.9 ± 167.8 mm3 on day 21, and 42.9 ± 18.4 mm3 and 751.9 ± 276.4 mm3 on day 28 after the beginning of treatment, respectively (Fig. 5). These data indicate that the CXCR4 antagonist delayed tumor growth at an early stage of tumor development.

Meanwhile, the body weights of the AMD3100-treated and control groups were 21.1 ± 0.6 gm and 21.1 ± 0.5 gm on day 7, 21.6 ± 0.7 gm and 21.4 ± 0.6 gm on day 14, 22.1 ± 0.7 gm and 21.3 ± 0.7 gm on day 21, and 22.4 ± 0.7 gm and 21.1 ± 0.7 gm on day 28, respectively (Fig. 5). The difference on day 28 was statistically significant.

H&E staining revealed a definite histological change in the PC-3 xenograft tumors after treatment with the CXCR4 antagonist. CXCR4 antagonist-treated tumors were characterized by their spindle cell shapes as compared with control tumors, and by their enlarged, pleomorphic and hyperchromatic nuclei. The treatment effects of CXCR4 antagonism on xenografts were also evaluated by immunohistochemistry, using the proliferation marker Ki-67 and anti-apoptotic marker bcl-2 on tumor tissue sections. Immunohistochemistry for bcl-2 expression showed brownish cytoplasmic staining in both groups, but bcl-2 immunostaining was more predominant and more frequently found in control tumors compared to CXCR4 antagonist-treated tumors (Fig. 6). Ki-67 was used as an estimator of tumor aggressiveness, with dark red-brown nuclear staining regarded as positivity for Ki-67. The Ki-67 staining index was calculated as the percentage of positively stained nuclei. Approximately 28.5% of PC-3 cells in the control tumor were Ki-67 positive. Treatment with CXCR4 antagonist significantly reduced the number of proliferating cells (10.2 ± 1.9% vs. 28.5 ± 4.7%, $P < 0.05$) (Fig. 7). To determine whether the suppression of primary tumor growth was a result of an antiangiogenic effect of the CXCR4 antagonist, i.e., inhibition of

microvessel formation, we examined the immunohistochemistry for CD34 on primary tumor tissue sections. As expected, we observed an obvious reduction in microvessel formation in the tumors of CXCR4 antagonist-treated mice compared with those of the control group (Fig. 6).

Fig. 5. Effects of a CXCR4 antagonist on PC-3 tumors *in vivo*. The body weights (A) and tumor volumes (B) of the vehicle (n = 6) and CXCR4 antagonist-treated (n = 6) nude mice are shown. The body weights and tumor volumes of the mice were measured at days 7, 14, 21 and 28. Points, mean; bar, standard deviation; *, $P < 0.05$; **, $P < 0.01$. Tumors were excised and photographed, and the representative examples of PC-3 tumors are shown (C).

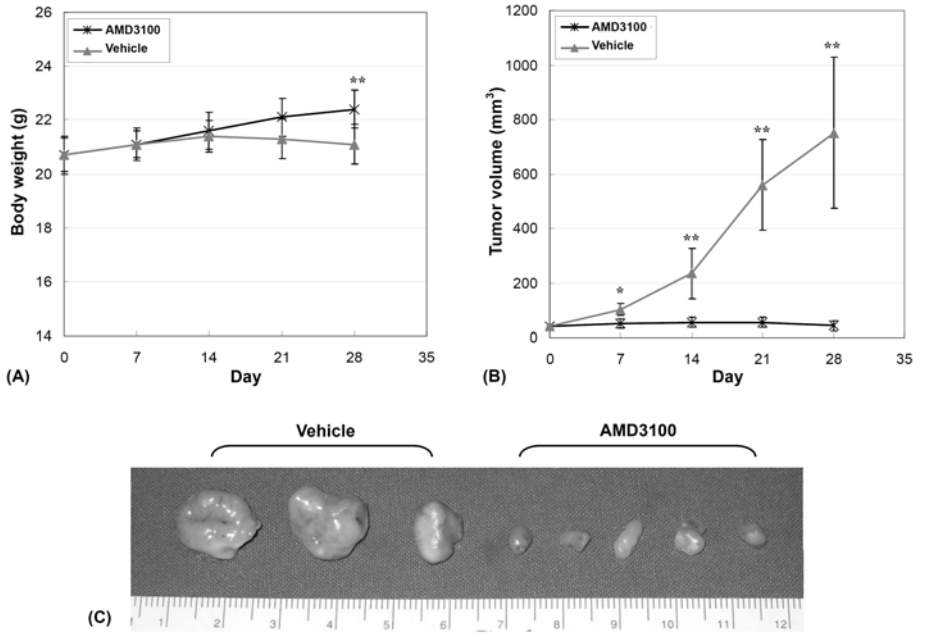
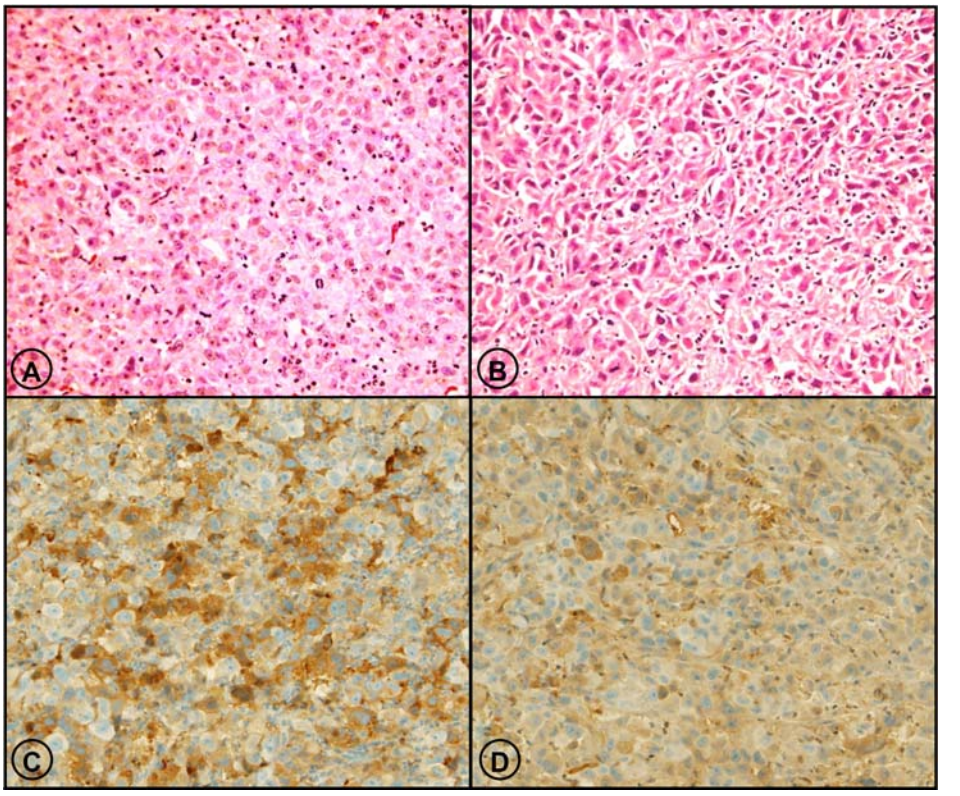


Fig. 6. Histopathologic examination of PC-3 xenograft tumors after treatment with vehicle or CXCR4 antagonist. H&E staining in vehicle (A) and CXCR4 antagonist (B)–treated tumors. Immunohistochemistry of bcl-2 in vehicle (C) and CXCR4 antagonist (D)-treated tumors, Ki-67 in vehicle (E) and CXCR4 antagonist (F)-treated tumors, and CD 34 in vehicle (G) and CXCR4 antagonist (H)-treated tumors. Immunohistochemistry for bcl-2 expression showed brownish cytoplasmic staining (C, D). For Ki-67, dark red-brownish nuclear staining was regarded as positivity (E, F). There was an obvious reduction in microvessel formation in the tumors of CXCR4 antagonist-treated mice compared with those of the control group (G, H). A – F, original magnifications, × 200; G & H, × 100.



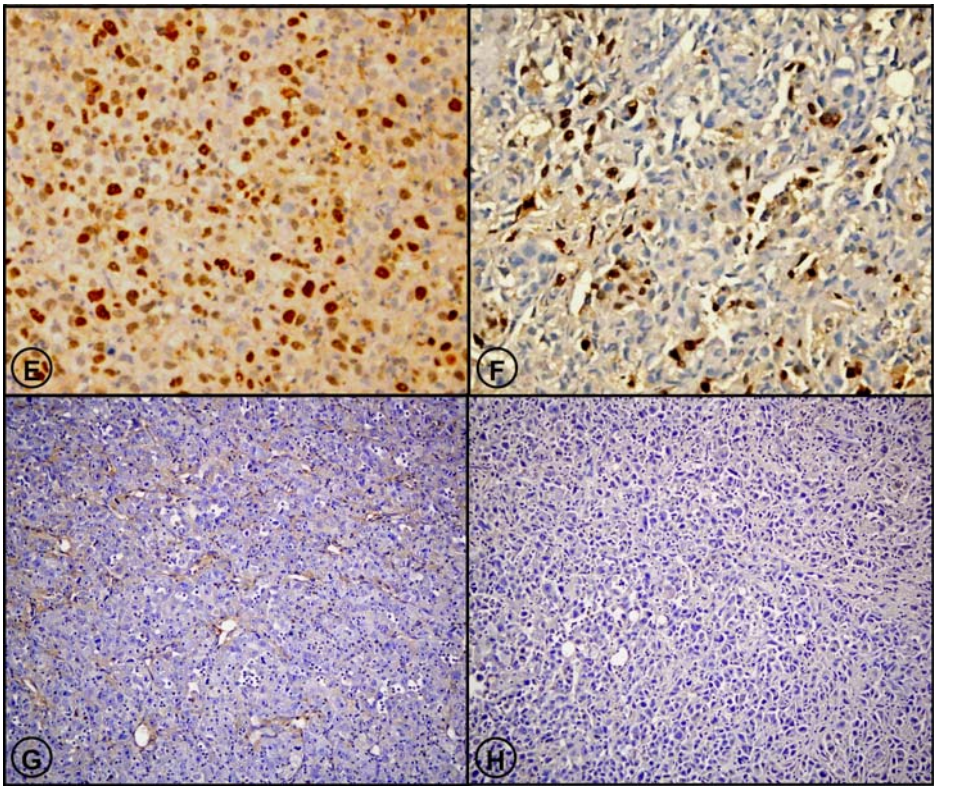
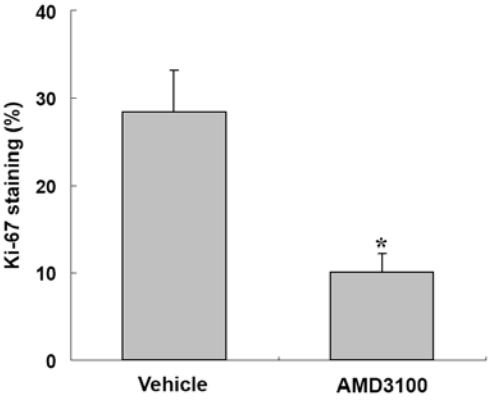


Fig. 7. Ki-67 staining index of PC-3 xenograft tumors after treatment with vehicle or CXCR4 antagonist. Points, mean; bar, standard deviation; *, $P < 0.05$.



IV. DISCUSSION

We found that a CXCR4-specific antagonist effectively inhibits SDF-1-induced migration and CXCR4/Akt signal transduction in PC-3 cells, and that this CXCR4 antagonist also effectively inhibits tumor growth in nude mice inoculated with PC-3 cells. We suggest that CXCR4 targeting might represent a novel, potentially effective strategy for the treatment of human prostate cancer.

It is well known that the binding of chemokines to their G protein-linked receptors on target cells leads to a series of signal transduction events involving the generation of inositol 1, 4, 5-triphosphate, cyclic adenosine monophosphate-dependent protein kinase, activation of phosphatidylinositol 3-kinase (PI3K), phosphorylation of protein kinase B (Akt), phosphorylation of extracellular signal-regulated kinase (ERK), components of focal adhesion complexes and activation of protein kinase C.[42] SDF-1 binding to CXCR4 generates various signaling mechanisms affecting regulation of angiogenesis, activation of cell invasion, promotion of cell growth, inhibition of apoptosis and, notably, plays an important role in organ-specific metastasis. In a previous study of prostate cancers, differential activation of the ERK and PI3K/Akt pathways resulted in differential secretion of interleukin-6, interleukin IL-8, tissue inhibitors of metalloproteinase-2 and vascular endothelial cell growth factor, which affected the ability of the cancer cells to induce angiogenesis.[43] Exogenous SDF-1 induces Akt phosphorylation in PC-3 cells, which is independent of PI3K, and indispensable for matrix metalloproteinase (MMP)-9 secretion, migration and invasion.[35] SDF-1 induction enhances various MMPs (MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-13, and MMP14) in PC-3 cells.[44] It has also been reported that SDF-1-induced expression of CXCR4 in PC-3 cells is dependent on the MEK/ERK signaling cascade and NF-κB activation,

which enhances endothelial adhesion and transendothelial migration.[45]

We have shown that SDF-1 has no direct effects on proliferation of PC-3 cells. Our findings are in accordance with results from recent studies of other types of cells, showing that SDF-1 has no proliferative effect on glioma cells (U251n), cholangiocarcinoma cells (RMCCAI, KKU100), testicular germ cell tumor cells (TCAM2), rhabdomyosarcoma or pancreatic cancer.[29, 46-49] Our findings are also consistent with a report demonstrating that SDF-1 lacks a proliferative effect on colony-forming unit megakaryocytes (CFU-MK) or lymphohematopoietic cells.[50, 51] Interestingly, Sun et al. also showed that recombinant SDF-1 does not alter the growth rate of PC-3 cells under various conditions.[30] However, an antibody to SDF-1 significantly decreased the number of PC-3 cells, which suggests that SDF-1 derived from the PC-3 cells themselves acts in an autocrine fashion to stimulate growth. Meanwhile, recent reports demonstrated that SDF-1 stimulates the proliferation of small cell lung cancer cells (NCI-H69) in the presence of serum and colorectal cancer cells (SW480) and epithelial ovarian cancer cells (ES-2) in the absence of serum.[52-54] These reports also demonstrate that SDF-1 acts together with thrombopoietin to enhance the development of megakaryocytic progenitor cells,[55] and that SDF-1 at low doses enhances the proliferation of peripheral blood CD34+ cells.[56] In addition, antisense CXCR4 overexpression in glioblastoma cells caused inhibition of cell proliferation, suggesting that the SDF-1/CXCR4 system is involved in cell proliferation in glioblastoma cell lines as well.[57, 58] We suggest that these differences may be due to different culture systems or to different target cells.

The effects of AMD3100 on the viability of tumor cells are controversial. In this study, AMD3100 did not significantly influence the viability of PC-3 cells. Glioma cells (U251n), testicular germ cell tumor

cells (TCAM2), epithelial ovarian cancer cells (ES-2) and oral squamous carcinoma cells (B88-SDF-1) were also insensitive to AMD3100.[48, 49, 54, 59] The enhancing effect of SDF-1 on cell proliferation was strongly inhibited by AMD3100 treatment in colorectal cancer cells (SW480), but AMD3100 alone did not significantly affect cell proliferation compared with the SDF-1 unstimulated group, suggesting that there is no autocrine growth stimulatory loop in the cell line.[53]

Although AMD3100 did not affect the viability of PC-3 cells, it could inhibit SDF-1-induced migration and Akt activation. These results are similar to those of previous studies, which also revealed that migration stimulated by SDF-1 was inhibited by AMD3100 treatment in CXCR4-expressing cervix cancer cells (HeLa), lung cancer cells (A459), oral squamous carcinoma cells (B88-SDF-1), epithelial ovarian cancer cells (ES-2), hepatocellular carcinoma cells (PLC/PRF5), testicular germ cell tumor cells (TCAM2), colorectal cancer cells (SW480) and cholangiocarcinoma cells (RMCCAI, KKU100).[46, 49, 53, 54, 59-62] To determine whether a CXCR4-specific antagonist inhibits SDF-1/CXCR4-mediated Akt phosphorylation, cells were pre-treated with AMD3100. The phosphorylation of Akt in AMD3100 pre-treated cells was significantly lower than in untreated cells, similar to that observed in studies of cholangiocarcinoma cells.[46] It has also been reported that in SDF-1-stimulated activation of ERK1/2 and Akt, the rapid responses to SDF-1 are attenuated by AMD3100 in medulloblastoma and glioblastoma cells (Daoy and U87).[63]

Our results clearly demonstrate the inhibitory effects of a CXCR4-specific antagonist on PC-3 tumor growth in nude mice, although AMD3100 treatment had no direct effect on the proliferation of PC-3 cells *in vitro*. The effects of CXCR4 antagonism on inhibition of tumor growth and prevention of metastasis in other xenograft models have been

investigated during recent years. AMD3100 effectively inhibits anaplastic thyroid carcinoma tumor growth.[64] In xenograft brain tumors, treatment of animals with AMD3100 resulted in decreased activation of the MAPK and Akt pathways, which are pathways downstream of CXCR4 that promote survival, proliferation and migration.[63] Intraperitoneal treatment with AMD3100 resulted in reduced dissemination in nude mice inoculated with epithelial ovarian cancer cells (ES-2).[54] In an oral squamous cell carcinoma xenograft model, AMD3100 significantly inhibited lung metastasis of the SDF-1 transfectant, ameliorated body weight loss and improved the survival rates of tumor-bearing nude mice.[59] Another anti-CXCR4 treatment, TN14003, has been shown to suppress primary tumor growth by inhibiting tumor angiogenesis, and to prevent lung metastasis of squamous cell carcinoma of the head and neck in animal models.[65] We have also shown that there is an obvious reduction in microvessel formation in CXCR4 antagonist-treated tumors compared to those of the control group. In our study, the dramatic differences in the biological effects of CXCR4 inhibition observed in animals and in cell culture can be explained by the fact that SDF-1 can act at multiple levels in the tumor microenvironment. Indeed, tumoral stromal cells, such as fibroblasts and bone marrow-derived cells, express high levels of SDF-1, which can directly enhance the growth of epithelial tumor cells and can recruit endothelial progenitors, thus favoring angiogenesis.[66] It is believed that that chronic treatment with AMD3100 efficiently blocks SDF-1-mediated vasculogenesis. Accordingly, the suppression of tumor growth in treated mice could be explained by the inhibition of CXCR4+ tumor cell proliferation and diminishing recruitment of CXCR4+ angiogenic cells. We observed a strong inhibition of PC-3 tumor growth after AMD3100 treatment, but AMD3100 did not induce a complete

regression of tumors. Therefore, we believe that combined treatment with AMD3100 and antineoplastic agents, such as platinum or taxanes, may be a promising strategy.

We did not detect any toxic effects of AMD3100 in our animal model, and single doses of AMD3100 have proven useful for the mobilization of bone marrow stem cells before autologous bone marrow transplant.[67, 68] But the appropriate therapeutic approach for antagonizing CXCR4 remains unclear, because long-term sustained dosing of AMD3100 results in some toxicity.[69] Sustained dosing of AMD3100 over a 10-day period was associated with mild toxicities. Reflecting its effects on bone marrow function, an elevation in white blood cell count was evident throughout an 18-day follow-up period after cessation of AMD3100.[69] For these reasons, further studies, aimed at understanding the effects of long-term administration of CXCR4 inhibitors, must be pursued. Despite these considerations, our data, together with data from several other reports, strongly indicate that the inhibition of this pathway should be actively evaluated as a novel anticancer therapy. We have identified CXCR4 as another potential target for prostate cancer therapy and suggest that AMD3100 or other CXCR4-specific inhibitors should be developed and tested as therapies for human prostate cancer.

Meanwhile, epidemiological studies suggest a possible correlation between prostate cancer risk and cholesterol levels.[70] Recently, a large-scale prospective study showed that the use of cholesterol lowering medications, statins, is associated with a reduced risk of advanced prostate cancer.[71] There has been great interest in lipid rafts as a theoretical basis for these epidemiologic and clinical observations. Lipid raft microdomains are characterized by light buoyant density, insolubility in cold nonionic detergents, and relatively high levels of glycosphingolipids and cholesterol, which contribute to their detergent-

resistant, liquid-ordered structure. Lipid rafts appear to act as a means of assembling components of specific pathways in ways that provide a regulatory architecture for transmission of signals such as Src-family kinases, heterotrimeric G protein subunits and receptor tyrosine kinases.[72] Lipid rafts have been shown to regulate signal transduction by activating or suppressing phosphorylation cascades.[73] The cholesterol content of lipid rafts affect survival mechanisms and Akt signaling in prostate cancer cells,[74] and disruption of lipid rafts by dispersion or extraction of membrane cholesterol results in inhibition of raft-dependent signaling events.[75-77] Our previous work also showed that depletion of cholesterol in lipid raft microdomains inhibits both epidermal growth factor receptor (EGFR)/Akt and EGFR/ERK pathways in LNCaP cells.[78]

The relationship between lipid rafts and CXCR4 has been investigated by some researchers. Lipid raft inhibitor disrupts the interaction between CD45 and CXCR4 in T-cells,[79] and blocks SDF-1 induced chemotaxis in T-cells, hematopoietic stem/progenitor cells and pro-B cells.[79-81] In pro-B cells, SDF-1 induced mitogen activated protein kinase (MAPK) activation is dependent on lipid rafts.[81] Nguyen et al. established that cholesterol directly participates in CXCR4 function by preserving the functional conformation of the receptor.[82] Published results have shown that the SDF-1/CXCR4 axis activates Akt in various cell lines including prostate cancer cells.[35, 42] Recently, Chinni et al. showed that CXCR4 and HER2 coexist in lipid rafts of prostate cancer cells and the SDF-1/CXCR4 axis produces transactivation of HER2, which is inhibited by lipid raft disrupting agents.[83] However, the effects of lipid rafts on CXCR4 induced chemotaxis and Akt signaling in prostate cancer cells are still poorly understood. Thus, we tested whether SDF-1/CXCR4-induced Akt signaling and cell migration were affected by membrane cholesterol in PC-3 cells. Our studies showed that SDF-1

induced expression of phosphorylated Akt was not increased, and cell migration was significantly reduced in cholesterol-depleted PC-3 cells. These observations suggest that membrane cholesterol is important for the proper function of the SDF-1/CXCR4 axis in PC-3 cells. These results also provide verification of the results from the aforementioned clinical studies, which demonstrated a relationship between cholesterol and prostate cancer aggressiveness. It can also be inferred that cholesterol-lowering drugs such as statins can be a beneficial adjuvant to CXCR4 targeting in the treatment of prostate cancer. Further experimental studies are needed to elucidate the additive or synergistic effects of this challenging combination.

V. CONCLUSIONS

The SDF-1/CXCR4 axis plays an important role in chemotactic migration and the Akt signal pathway in PC-3 cells. A CXCR4-specific antagonist, AMD3100, also effectively inhibits SDF-1 induced PC-3 cell migration and CXCR4/Akt signal transduction. Moreover, AMD3100 obviously suppresses tumor growth in nude mice inoculated with PC-3 cells, and AMD3100 treated PC-3 tumors show less microvessel formation and less immunoreactivity for the proliferation marker, Ki-67 and anti-apoptotic marker bcl-2, when compared to control tumors *in vivo*. Thus, we suggest that CXCR4 targeting might represent a potentially effective strategy for the treatment of human prostate cancer.

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ABSTRACT (in Korean)

전립선암 이종이식 모델에서 새로운 치료 전략으로써 케모카인 수용체 CXCR4의 억제 효과

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목적: 전립선암은 전 세계에서 세 번째로 흔한 남성암이며, 우리나라의 전립선암은 인구 고령화와 식생활 서구화의 영향으로 남성암 중에서 가장 빠른 증가율을 보이고 있다. 국소 전립선암은 수술적 치료 또는 방사선 치료를 통해 완치를 기대할 수 있으나, 현재 진행성 또는 전이성 전립선암에 대한 효과적인 치료법은 전무한 실정이다. 최근에 암의 진행 및 전이와 관련하여 케모카인 및 케모카인 수용체의 역할에 많은 관심이 집중되고 있다. 이 중 stromal derived factor-1 (SDF-1)/CXCR4 신호전달축은 다양한 암에서 세포의 이동과 침윤을 자극시키는 것으로 알려져 있으며, 전립선암에서는 CXCR4의 발현 정도가 전이 전립선암 환자에서 암특이 생존률과 관련이 있으며, 몇몇 실험적 모델을 통해 은 SDF-1/CXCR4 신호전달 축이 전립선암의 골전이 기전에 관여함이 알려진 바 있다. 본 연구에서는 특이적 CXCR4 길항제가 SDF-1/CXCR4 신호전달 축의 기능 및 하위 신호전달 경로에 미치는 영향을 확인하고자 하였다. 또한, 전립선암 이종이식모델에서 CXCR4 길항제의 종양 억제 효과를 확인함으로써, 궁극적으로 전립선암의 잠재적 치료 표적으로써 CXCR4의 가능성을 규명하고자 하였다.

대상 및 방법: *In vitro* 및 *in vivo* 실험에서 대표적인 전이 전립선암 세

포주인 PC-3를 이용하였다. 첫째, proliferation assay를 이용하여 SDF-1 및 특이적 CXCR4 길항제인 AMD3100이 PC-3 세포의 증식에 미치는 효과를 평가하였다. 둘째, AMD3100이 SDF-1에 의한 PC-3 세포의 이동에 미치는 영향을 migration assay를 이용하여 알아보고자 하였다. 셋째, AMD3100이 SDF-1/CXCR4 신호전달 축의 하위 경로인 Akt 신호 전달에 미치는 영향을 Western blot을 이용하여 확인하였다. 끝으로 PC-3 이종이식모델에서 AMD3100이 종양의 성장에 미치는 영향을 평가하고, AMD3100 치료군과 대조군의 조직학적 차이를 확인하기 위해서 H&E 염색과 bcl-2, Ki-67 및 CD34에 대한 면역조직화학염색을 시행하였다.

결과: 전이 전립선암 세포주인 PC-3에서 CXCR4 단백질의 발현을 명확히 확인할 수 있었다. Proliferation assay에서 SDF-1 및 AMD3100는 세포 증식의 의미있는 변화를 유발하지 않았다. Migration assay에서 SDF-1/CXCR4 신호전달 축은 PC-3 세포의 이동에 중요한 역할을 한다, 그러나 AMD3100을 처치하였을 때, SDF-1 (200 ng/ml)에 의한 PC-3 세포의 이동이 39% 가량 감소하는 것을 알 수 있었다 ($P < 0.01$). 전립선암 세포에서 SDF-1/CXCR4 신호전달 축의 하위 경로로 Akt 신호 경로가 알려져 있으며, 본 연구에서 PC-3 세포에 SDF-1을 200 ng/ml의 농도로 자극하였을 때, phosphorylated Akt의 발현이 30분, 60분 후 확연히 증가하고, 이 후로 감소하는 경향을 확인할 수 있었다, 그러나 AMD3100을 처치한 PC-3 세포에서는 phosphorylated Akt 발현의 증가가 억제되는 것으로 나타났다. 누드 마우스에 PC-3 세포를 주입한 이종 이식 모델에서 종양의 크기가 40 mm^3로 형성된 시점에서부터 치료군에는 AMD3100 (3 mg/kg)을, 그리고 대조군에는 식염수를 주 5회 총 4주간 복강내 주사하였다. 종양의 크기는 치료 시작 1주일 후부터 의미있는 차이를 보이기 시작하여, 치료 종결 시점에서는 치료군과 대조군에서 각각 42.9 ± 18.4 mm^3 및 751.9 ± 276.4 mm^3으로 통계학적으로 유의한 차이를 확인할 수 있었다 ($P < 0.01$). 한편 체중은 치료군(22.4 ± 0.7 gm)이 대조군 (21.1 ± 0.7 gm)에 비하여 높

게 나타났으며, 이는 통계학적으로 의미있는 차이였다 ($P < 0.01$). 적출한 조직에 H&E 염색을 시행하였을 때, AMD3100 치료군의 세포 모양은 대조군과 달리 방추형으로 관찰되었고, 핵은 대조군에 비하여 크기가 보다 크고, 다형성이며, 과염색성으로 나타나는 차이를 보였다. 면역조직화학염색에서 AMD3100 치료군은 대조군에 비하여 증식 표지자인 Ki-67, 항 고사 표지자인 bcl-2의 발현이 감소하였다. 또한 미세혈관형성이 현저히 억제됨을 알 수 있었다.

결론: PC-3 세포주에서 SDF-1/CXCR4 신호전달 축은 화학주성에 의한 세포 이동 및 Akt 신호 전달에 중요한 역할을 하는데, 특이적 CXCR4 길항제인 AMD3100은 SDF-1에 의한 PC-3세포의 이동과 SDF-1/CXCR4 신호전달 축의 하위 경로인 Akt 신호 전달을 효과적으로 억제한다. 또한 PC-3 이종이식모델에서 AMD3100 처치는 종양의 성장을 의미있게 억제하며, 미세혈관형성을 저해시킨다. 이러한 결과는 전립선암의 치료에서 CXCR4가 새로운 잠재적 치료 표적이 될 수 있는 가능성을 제시한다. 본 연구에서 CXCR4 길항제의 종양 억제 효과는 *in vitro* 실험 및 *in vivo* 실험에서 상충되게 관찰되고 있는데, 이는 CXCR4 길항제가 종양 세포에 직접적인 세포 독성을 가지기 보다는 주로 종양 성장에 필수 불가결한 미세환경의 형성을 방해하는 데 작용하기 때문으로 풀이된다. 또한 AMD3100의 강력한 종양 성장 억제 효과에도 불구하고 종양의 완전 관해를 확인할 수 없었다. 따라서 특이적인 CXCR4 길항제와 함께 세포독성 항암제의 병용치료가 향후 전립선암의 유망한 치료 전략으로 검토될 수 있을 것으로 생각한다.

핵심되는 말: 전립선암, SDF-1/CXCR4 신호전달 축, CXCR4 길항제, 이종이식모델