Androgen receptor negatively influences the expression of chemokine receptors (CXCR4, CCR1) and ligand-mediated migration in prostate cancer DU-145

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Abstract. We previously reported that androgen receptor (AR) plays a role in the regulation of adhesion to the extracellular matrix and invasion of human prostate cancer cells by influencing the expression of specific integrin subunits. It is now considered that chemokines play a significant role in organ-selective cancer metastasis. In this study, we hypothesized that AR may influence the expression of these chemokine receptors and cell function. The mRNA expression of chemokine receptors in human prostate cancer cell line DU-145 and DU-145 cells expressing AR (DU-145/AR) was investigated by RT-PCR. DU-145 cells selectively expressed CXCR4 and CCR1 mRNA at high levels compared with DU-145/AR cells. DU-145 showed vigorous migratory responses to its ligand CXCL12 (also called stromal-derived factor-1α, SDF-1α) and CCL3 (also called macrophage inflammatory protein-1, MIP-1α). In contrast, neither CXCL12 nor CCL3 affected the migration of DU-145/AR cells. These results indicate that expression of AR down-regulates the migratory responses of human prostate cancer cells via chemokine and its receptor systems.

Introduction

Metastasis is one of the major causes of mortality in cancer. The precise cellular and molecular mechanisms used by cancer cells for metastasis are not fully understood; however, the metastatic spread of neoplastic cells is probably related to the ability of these cells to migrate, invade, home and survive locally. Despite the obvious importance of metastasis, this process remains incompletely characterized at both the cellular and molecular levels (1). Many factors have been implicated in the process of metastasis, but the precise mechanisms for the directional migration of malignant cells into different organs are unknown (2-4). Chemokines, a superfamily of small cytokine-like proteins, induce cytoskeletal rearrangement through binding corresponding G-protein-coupled receptors, adhesion to endothelial cells, and directional migration (5-7). For example, it has been shown that SDF-1α-CXCR4 interactions may play a significant role in the metastasis of prostate cancer to bone (8). It has also been demonstrated that normal breast tissue expresses low amounts of CXCR4, whereas neoplastic breast tissue expresses higher levels of CXCR4 (9). CXCL12 and CXCR4 have been implicated in the pathogenesis and progression of breast cancer, Burkitt's lymphoma, leukemias and neuroblastomas (10-12). It has been shown that CXCR4 is expressed by prostate cancer cell lines (PC3, DU-145 and LNCaP) (13).

Moreover, one report discusses the estrogen receptor and down-regulation of mRNA in view of the relationship between chemokine receptor and hormone receptor. For example, estrogen and Tamoxifen (competitive inhibitors of estrogen receptor) significantly decreased the expression of CCR2 and CXCR3 on murine monocytes, and estrogen decreased the chemotaxis of monocytes towards monocyte chemoattractant protein-1 (MCP-1) (14). As mentioned above, there remain many uncertain points in prostate cancer regarding AR and its relationship to the metastasis mechanism. Moreover, in prostate cancer, little is known about the relationship between AR and the 'hormone non-dependency' of cancer cells.

We have investigated whether or not AR can influence the expression of these chemokine receptors and cell function. It was difficult to analyze the function of cells that purely related to AR because their origins were different, although there were some prostate cancer cell lines. Moreover, there are no reports of experiments related to whether AR is present in cells of the same cell line. However, we have already established a clonal DU-145 prostate cancer cell line (DU-145/AR) stably transfected with AR cDNA, (15) and analyzed its function (16). In the present study we examined the influence of AR on the expression of 18 different chemokine receptors and cell migration.

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Table I. Primers used for RT-PCR.

Genes		Primer sequences
CXCR1	Forward	5'-GGCTGCTGGGGACTGTCTATGAAT-3'
	Reverse	5'-GCCCGGCCGATGTTGTTG-3'
CXCR2	Forward	5'-CCGCCCCATGTGAACCAGAA-3'
	Reverse	5'-AGGGCCAGGAGCAAGGACAGAC-3'
CXCR3	Forward	5'-CAACGCCACCCACTGCCAATACAA-3'
	Reverse	5'-CAGGCGCAAGAGCAGCATCCACA-3'
CXCR4	Forward	5'-ATCTTCCTGCCCACCATCTACTCCATCATC-3
	Reverse	5'-ATCCAGACGCCAACATAGACCACCTTTTCA-3'
CXCR5	Forward	5'-AACTACCCGCTAACGCTGGAAATGGAC-3'
	Reverse	5'-CACGGCAAAGGGCAAGATGAAGACC-3'
CXCR6	Forward	5'-ATGGCAATGTCTTTAATCTCGACAA-3'
	Reverse	5'-TGAAAGCTGGTCATGGCATAGTATT-3'
CCR1	Forward	5'-CAACTCCGTGCCAGAAGGTGAA-3'
	Reverse	5'-GCCAGGGCCCAAATGATGAT-3'
CCR2	Forward	5'-CCAACGAGAGCGGTGAAGAAGTC-3'
	Reverse	5'-TCCGCCAAAATAACCGATGTGAT-3'
CCR3	Forward	5'-GAGCCCGGACTGTCACTTTTG-3'
	Reverse	5'-CAGATGCTTGCTCCGCTCACAG-3'
CCR4	Forward	5'-AAGAAGAACAAGGCGGTGAAGATG-3'
	Reverse	5'-AGGCCCCTGCAGGTTTTGAAG-3'
CCR5	Forward	5'-CTGGCCATCTCTGACCTGTTTTTC-3'
	Reverse	5'-CAGCCCTGTGCCTCTTCTTCTCAT-3'
CCR6	Forward	5'-CCTGGGGAATATTCTGGTGGTGA-3'
	Reverse	5'-CATCGCTGCCTTGGGTGTTGTAT-3'
CCR7	Forward	5'-GTGCCCGCGTCCTTCTCATCAG-3'
	Reverse	5'-GGCCAGGACCACCCCATTGTAG-3'
CCR8	Forward	5'-GGCCCTGTCTGACCTGCTTTTT-3'
	Reverse	5'-ATGGCCTTGGTCTTGTTGTGGTT-3'
CCR9	Forward	5'-CACTGTCCTGACCGTCTTTGTCT-3'
	Reverse	5'-CTTCAAGCTTCCCTCTCTCCTTG-3'
CCR10	Forward	5'-TGCTGGATACTGCCGATCTACTG-3'
	Reverse	5'-TCTAGATTCGCAGCCCTAGTTGTC-3'
CX3CR1	Forward	5'-TGGCCTTGTCTGATCTGCTGTTTG-3'
	Reverse	5'-ATGGCTTTGGCTTTCTTGTGGTTC-3'
XCR1	Forward	5'-TGACCATCCACCGCTACC-3'
	Reverse	5'-ATCTGGGTCCGAAACAGC-3'
GAPDH	Forward	5'-GCCAAGGTCATCCATGACAACTTTGG-3'
	Reverse	5'-GCCTGCTTCACCACCTTCTTGATGTC-3'

Materials and methods

Cells and cell culture. DU-145 prostate cancer cells were kindly provided by Dr T. Tsukamoto (Sapporo Medical College). DU-145/AR prostate cancer cells were described previously (16). Transfections were performed using the calcium phosphate precipitation method. In short, DU-145 cells ($1x10^6$) were plated on a 10-cm dish, 24 h before adding

10 μg pSG5-AR (17) or empty vector (DU-145/Neo cells). Forty-eight hours after transfection, the cells received fresh medium with G418 at a concentration of 1.0 mg/ml. The resulting G-418 resistant clones were seeded onto 96-well plates and selected for AR expression by Western blotting technique. These cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), L-glutamine, 2-mercapto-ethanol, 10^2 U/ml penicillin and 0.1 mg/ml streptomycin.

Reagents. CXCL12 (also called stromal-derived factor-1α, SDF-1α) and CCL3 (also called macrophage inflammatory protein-1, MIP-1α) used in these experiments were purchased from R&D Systems (Minneapolis, MN, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR). The expression levels of chemokine receptors were determined by RT-PCR analysis. Total-RNA was isolated from prostate cancer cell lines (DU-145, DU-145/AR and DU-145/Neo).

First-strand complementary DNA (cDNA) was prepared from RNA template (1 μg) using oligo$(dT)_{18}$ primer and SuperScript II reverse transcriptase (Invitrogen Corp.).

The RT-reaction profile was 42˚C for 50 min, followed by 70˚C for 15 min. PCR amplification was performed by denaturation at 94˚C for 30 sec, annealing at 57˚C or 60˚C for 1 min, and extension at 72˚C for 1 min and 30 sec, using temperature cDNA and a Takara 'Ex Taq', HS PCR kit (Takara Shuzo Co., Ltd.).

All primers of 18 different chemokine receptors (CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CX3CR1 and XCR1) (Table I) were confirmed to yield the expected products under these conditions. The PCR products were electrophoresed on 1.5% agarose gels and detected by ethidium bromide staining.

Haptotactic migration assay. Cell migration along a gradient of substratum-bound fibronectin was assayed in transwell cell culture chambers as reported previously (18). The lower surface of the filters was precoated with 4 μg of fibronectin. Prostate cancer cells ($1x10^5$) were pretreated with various concentrations of chemokines (SDF-1α, MIP-1α) for 30 min on ice, then added to the upper compartment, and incubated at 38˚C for 3 h. Chemokines were added in the lower compartments. The filters were fixed with methanol and stained with 0.5% crystal violet in 20% methanol for 30 min. After gentle rinsing with water, the cells on the upper surface of the filters were removed by wiping with a cotton swab. The filters containing the stained cells that had migrated to their lower surfaces were removed from the transwell chambers and individually transferred to a separate well in a 96-well culture plate. The crystal violet dye retained on the filters was extracted with 30% acetic acid and the absorbance was colorimetrically measured at 590 nm.

Statistical analysis. The statistical significance of differences between the groups was determined by applying Student's t-test and one-way analysis of variance (ANOVA). A p-value <0.05 was considered significant.

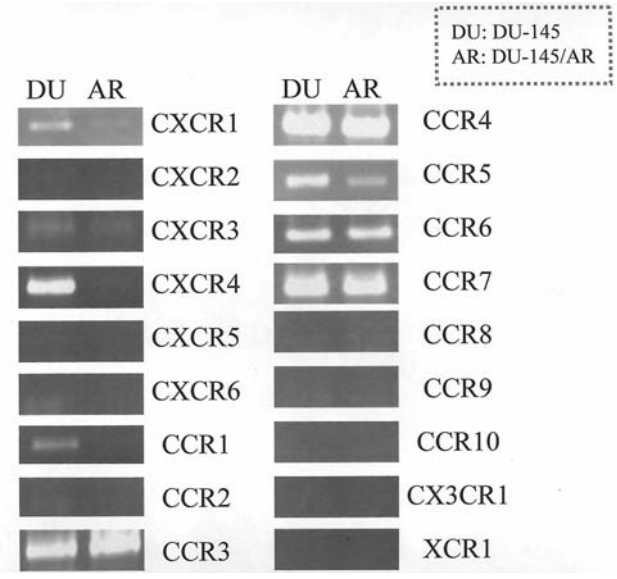

Figure 1. Expression of 18 different chemokine receptors was studied by RT-PCR in DU-145 and DU-145/AR cells.

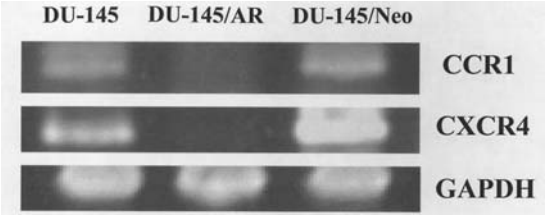

Figure 2. Expression of CXCR4 and CCR1 was analyzed by RT-PCR in prostate cell lines: DU-145, DU-145/AR, DU-145/Neo. GAPDH expression was used as an internal standard.

Results

Expression of 18 chemokine receptors in DU-145 and DU-145/AR prostate cancer cells. Human cell lines can serve as a model for the behavior of prostate cancer cells. To study whether AR may influence the expression of chemokine receptors, we initially performed a comprehensive analysis of mRNA expression of 18 different chemokine receptors in DU-145 and DU-145/AR human prostate cancer cells by RT-PCR (Fig. 1). The 16 chemokine receptors showed almost the same mRNA level in DU-145 and DU-145/AR. Both DU-145 and DU-145/AR cells constitutively expressed almost the same mRNA level of CXCR1, CXCR3, CCR3, CCR4, CCR5, CCR6 and CCR7, and both DU-145 and DU-145/AR cells hardly expressed the mRNA level of CXCR2, CXCR5, CXCR6, CCR2, CCR8, CCR9, CCR10, CX3CR1 and XCR1; however, in the other two chemokine receptors (CXCR4 and CCR1), mRNA expression was markedly more prevalent in DU-145 than in DU-145/AR. DU-145 and DU-145/Neo (empty vector-transfected) expressed the three mRNA (Fig. 2).

The low levels of these two chemokine receptor (CXCR4, and CCR1) mRNA found in the DU-145/AR prostate cancer cell line provided a model system that enabled us to study the effects of down-regulation of these receptors on the function of prostate tumor.

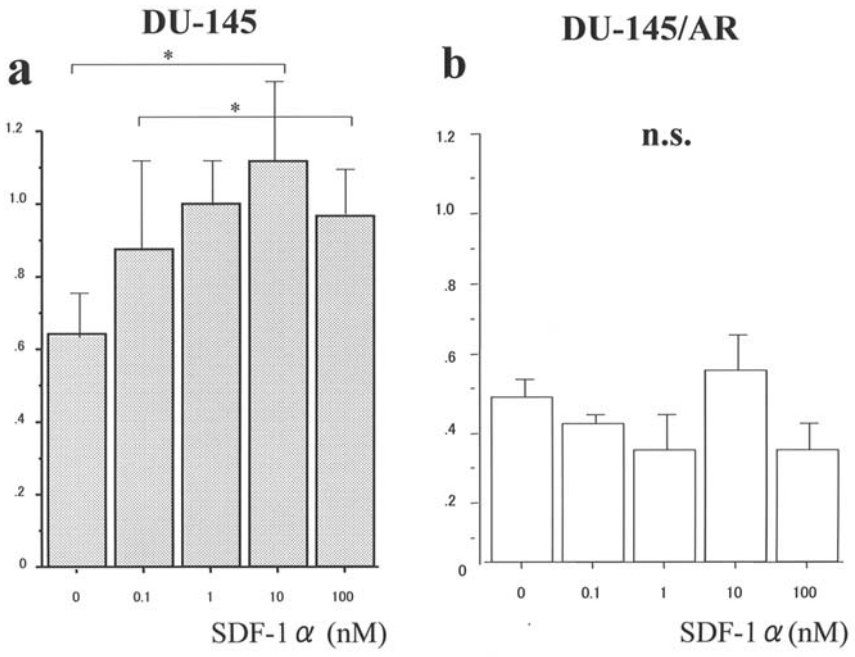

Figure 3. CXCR4-mediated cell migration. DU-145 (a) and DU-145/AR (b) cell responsiveness to various concentrations (0-100 μM) of chemokine SDF-1α was tested in a transwell migration assay. *P<0.05.

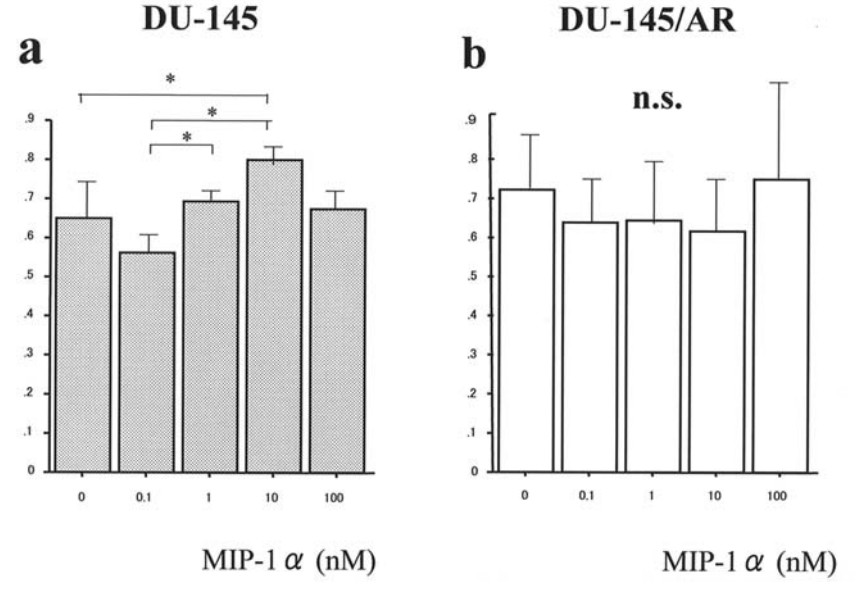

Figure 4. CCR1-mediated cell migration. DU-145 (a) and DU-145/AR (b) cell responsiveness to various concentrations (0-100 μM) of chemokine MIP-1α was tested in a transwell migration assay. *P<0.05. The result is the average of triplicates ± SD and shows 1 representative experiment out of 3.

Haptotactic migration of prostate cancer cells. For cells to metastasize, they must first firmly attach to the vascular endothelium and then transmigrate into the tissue. We next investigated the haptotactic migration of DU-145 and DU-145/AR cells to the two chemokine receptors (CXCR4 and CCR1). Fig. 3 shows that during *in vitro* migration assays, pretreatment of the DU-145 cells with SDF-1α or MIP-1α significantly ($p<0.05$) increased their migration in a dose-dependent manner. On the other hand, DU-145/AR cells did not increase migration (Fig. 4). These results indicate that AR may influence the expression of these chemokine receptors and the cell function.

Discussion

Most prostate cancer-related deaths are not the result of primary tumor growth but are rather caused by the spread of cancer to other organs. AR, a member of the steroid hormone superfamily, is a master switch that controls prostate cell gene expression, growth and differentiation. Despite our molecular understanding of the roles of AR regulation, the downstream target gene transcription, the direct or indirect (stromally mediated) actions of the androgen in controlling prostate cell gene expression, growth and differentiation are still unclear.

Studies performed using available prostate cancer cell lines show that the expression of AR in androgen-independent prostate cancer cell lines or overexpression in ARCaP (19,20) cells leads to decreased invasion and adhesion properties *in vitro* (21,23). Overall, these results suggest that the expression of functional AR shows a more differentiated and less invasive cell phenotype. During the last few years, evidence has emerged that these effects may be due to regulating the expression of molecules involved in these processes.

We have already established a clonal DU-145 prostate cancer cell line (DU-145/AR) stably transfected with AR cDNA, (15) and analyzed its function (16). In RT-PCR, DU-145/AR cells constitutively expressed both type 1 vasoactive intestinal peptide (VIP) receptor (VIP1R) and type 2 VIP receptor (VIP2R) mRNA at higher levels than DU-145 cells. VIP did not enhance the migration of DU-145 cells to fibronectin but did enhance DU-145/AR cell migration (15). We also investigated the expression of integrin subunits, adhesion to extracellular matrices, and the invasion of DU-145/AR cells. DU-145/AR cells exhibited a lower expression of alpha6 and beta4 integrin subunits and a higher expression of $\alpha 2$ and $\alpha 5$ than DU-145 cells. Haptoinvasion of DU-145/AR cells into Matrigel/fibronectin-coated filter was significantly reduced compared with DU-145 cells. These results indicate that AR may play a role in the down-regulation of cell motility including the migration of prostate cancer cells. Migration of cancer cells as well as leucocytes is regulated partly by chemokine receptor-ligand interactions (9). It has been shown that some chemokine-ligand interactions play a significant role in the metastasis of cancer (24). Therefore, we hypothesized that AR may influence the expression of these chemokine receptors.

We first examined the mRNA expression of 18 different chemokine receptors in both DU-145 and DU-145/AR by using RT-PCR. As shown in Figs. 1 and 2, DU-145 cells selectively expressed CXCR4 and CCR1 mRNA at high levels compared with DU-145/AR cells. Consistently, DU-145 also showed significant migratory responses to its ligand CXCL12 and CCL3 (Figs. 3 and 4).

The metastasis of prostate cancer to bone is the most significant cause of morbidity and mortality in this disease. Of the 220,000 men with prostate cancer in 2003, approximately 28,900 died from secondary bone metastasis (25).

CXCR4 has been shown to participate in the development of cancer metastasis, including bone metastatic prostate cancer (8).

In addition to CXCR4, there are a few reports about the expression of CCR1 in cancer or normal tissues. CCR1 is expressed by hepatoma, ovarian cancer, multiple myeloma, lymphocytes, monocytes, basophils and bone marrow progenitor cells (26-30). CCR1 is known as a chemokine receptor shared by various chemokine ligands, CCL3, CCL5 (also called regulated upon stimulation, normal T cells expressed and secreted, RANTES), CCL7 (also called monocyte chemotactic protein-3, MIP-3) and CCL9 (also called macrophage inflammatory protein-1γ).

Previously, CCL9 was shown to be produced by differentiating osteoclasts, and CCL9 played an important role in the differentiation and survival of osteoclasts, most likely via a CCL9-CCR1 autocrine pathway (31).

Therefore, our data about the expression of CXCR4 and CCR1 in DU-145 cells implies that it is closely associated with the development of bone metastasis of prostate cancer.

However, why was the expression of both CXCR4 and CCR1 mRNA in DU-145 cells down-regulated by the influence of AR expression? We are now investigating the association of the expression of CXCR4, CCR1 and AR with bone metastasis of prostate cancer.

Estrogen has been shown to regulate the expression of many cytokines and their receptors in various cell systems (32-35). Janis *et al* reported that estrogen and tamoxifen (antiestrogen) significantly decreased the expression of CCR2 and CXCR3 on murine monocytes. In addition to down-regulating the expression of chemokine receptors by treatment with estrogen, both basal migration and the chemokine-mediated chemotaxis of murine monocytes were decreased (14). In the present study, we reported that some chemokine receptors were down-regulated by transfecting AR into DU-145 cells, shedding some light on the relationship between AR existence and chemokine receptor expression. Since estrogen can decrease chemokine receptor expression as well as migration or chemotaxis, androgen may also influence the receptor-ligand pathways. Further study will be needed to examine steroid hormone-mediated regulation of the migratory responses of prostate cancer.

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