

CXCL16 Functions as a Novel Chemotactic Factor for Prostate Cancer Cells *In vitro*

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Abstract

A variety of tumor cells produce chemokines that promote tumor cell proliferation and chemotaxis. We previously reported that CXCL16 production is increased in aggressive prostate cancer cells compared with the less aggressive tumor cells and benign cells as identified in a cytokine antibody array. The functional contribution of CXCL16 in prostate cancer development has not yet been evaluated. Accordingly, mRNA expression of CXCL16 and its receptor, CXCR6, were determined by real-time reverse transcription-PCR in various cancer cell lines, including prostate cancer and tissues obtained from localized and metastatic prostate cancer. Consistent with our finding on CXCL16 protein production by prostate cancer cells, aggressive prostate cancer C4-2B and PC3 cells, as well as bone and liver metastatic tissues, expressed higher levels of both CXCL16 and CXCR6 mRNA compared with the less aggressive prostate cancer LNCaP cells, nonneoplastic PrEC and RWPE-1 cells, and benign prostate tissues, respectively. Furthermore, CXCR6 and CXCL16 protein expressions were examined in tissue specimens by immunohistochemistry. Immunohistochemical examination of CXCR6 expression showed strong epithelial staining that correlated with Gleason score, whereas CXCL16 staining was not. Finally, we found that both interleukin-1 β and tumor necrosis factor α significantly induced CXCL16 production by prostate epithelial cells, thereby indicating that inflammatory cytokines may play a role in the CXCL16 induction. CXCL16 was found to promote prostate cancer cell

migration and invasion *in vitro*. Therefore, we concluded that CXCL16 functions, through CXCR6, as a novel chemotactic factor for prostate cancer cells. (Mol Cancer Res 2008;6(4):546–54)

Introduction

The majority of men with progressive prostate cancer develop skeletal metastases. The tumor cells survive and progress in the skeleton due to cross-talk between tumor cells and the bone microenvironment (1-3), the latter of which is composed of stromal cells, osteoblasts, osteoclasts, endothelial cells, fibroblasts, adipocytes, bone marrow precursor cells, cells of the immune system, and the extracellular matrix. The unique growth factors and chemotactic factors (chemokines), produced by the bone microenvironment, enhances the growth of prostate cancer cells and tumor progression in bone.

Chemokines are classified based on the relative position of cysteine residues near the NH₂ terminus into four major families: CC, CXC, C, and CX3C. Chemokines activate receptors, members of a large family of seven-transmembrane G-coupled proteins, and play primary roles in controlling the trafficking of leukocytes during inflammation. There is emerging evidence that pairs of chemokines and their receptors play critical roles in cancer development such as tumor cell adhesion, migration, invasion, and metastasis (4-6). For example, the expression of certain chemokines, such as SDF-1/CXCL12; fractalkine/CX3CL1; interleukin-8 (IL-8)/CXCL8; and monocyte chemoattractant protein-1 (MCP-1/CCL2); and their corresponding receptors, CXCR4, CXCR1, CXCR2, and CCR2, respectively, have been reported in cancer cells and/or other cell types in the bone microenvironment (5-14). These cancers include various types of cancer, such as prostate cancer and breast cancer, that preferentially metastasize to the bone (4-6, 10, 11, 13).

We have previously reported that MCP-1/CCL2, via binding to its receptor CCR2, acts as an autocrine and paracrine factor for prostate cancer proliferation and invasion *in vitro* (6). In that study, we observed that blocking CCR2 with a potent antagonist only partially diminished MCP-1-induced cell proliferation and invasion, suggesting that other chemokines may be involved in this action. Additionally, we have previously noted that interfering with signals generated by SDF-1(CXCL12) binding to its receptor CXCR4 only partially inhibited proliferation or invasive activities (5). Together, these data suggest that other functional chemokines plus chemokine receptor pairs may play an important role in tumor development.

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CXCL16, a newly discovered CXC chemokine, exists both in a transmembrane and a soluble form (15). Membrane-bound CXCL16 is expressed by antigen-presenting cells such as monocytes, macrophages, B cells, and dendritic cells in the T-cell zone of lymph nodes. Soluble CXCL16 can be generated by constitutive cleavage from the cell membrane and further enhanced by cell stimulation with phorbol esters (16). Soluble CXCL16 has been shown to induce chemotaxis of Th1, Tc1, and natural killer T cells, which express the functional CXCR6 receptor (15, 17-19). CXCL16 has also been reported as a novel angiogenic factor for human umbilical vein endothelial cells (20). Moreover, CXCL16 cDNA was shown to be identical to a novel scavenger receptor that binds phosphatidylserine and oxidized lipoprotein (SRP5OX; ref. 21). Recently, expression of CXCL16 and/or CXCR6 was shown in nasopharyngeal carcinomas (22), gliomas (23), and rectal cancer (24). Through a cytokine antibody array, we reported that CXCL16 protein production was increased in aggressive prostate cancer cells compared with the less aggressive prostate cancer cells or benign prostate cells (25). The functional contribution of this CXCL16 induction in prostate cancer development, however, has not been evaluated.

Accordingly, we first determined CXCL16 mRNA and CXCR6 mRNA expression by real-time reverse transcription-PCR (RT-PCR) in a variety of cell lines from different cancer types. We further assessed their mRNA and protein expression in tumor specimens collected from patients with localized or metastatic prostate cancer compared with benign prostate tissues. Finally, we investigated the role of CXCL16 in prostate cancer cell migration and invasion *in vitro*.

Results

CXCL16 mRNA and CXCR6 mRNA Are Expressed in Prostate Cancer Cell Lines

To determine whether CXCL16 mRNA and CXCR6 mRNA are expressed in cancer cells, RT-PCR was done on RNA isolated from the prostate cancer cell lines LNCaP, PC3, C4-2B, and DU145; normal (PrEC) or immortalized (RWPE-1) prostate epithelial cells; breast cancer cell lines (estrogen-sensitive MCF-7 and estrogen-insensitive MDA-MB-231); lung cancer cell lines (A549 and H1299); an immortalized human bronchial epithelial cell line (BEAS2B); and an immortalized human osteoblast (hFOB) and a marrow endothelial (HBME) cell line. We found that CXCL16 mRNA and CXCR6 mRNA were expressed in all prostate cancer cell lines as well as benign PrEC and RWPE-1 cells (Fig. 1A). When expression of CXCL16 and CXCR6 mRNA were quantified by real-time RT-PCR, all prostate cancer cell lines expressed greater levels of CXCR6 and CXCL16 mRNA compared with PrEC and RWPE-1 (Fig. 1B and C). In addition, aggressive prostate cancer C4-2B and PC3 cell lines expressed higher levels of both CXCL16 mRNA and CXCR6 mRNA compared with the less aggressive prostate cancer LNCaP cells. To extend this study to other types of cancer and the cells in the bone microenvironment, the expression of CXCL16 mRNA and CXCR6 mRNA was also determined in human non-small cell lung carcinoma A549 and H1299 cells and compared with BEAS2B cells; breast cancer MDA-MB-231 and MCF-7 cells; and hFOB and HBME cells. All cells expressed CXCL16 and CXCR6 mRNA

to varying degrees. Similar to the findings on prostate cancer, lung cancer cell lines A549 and H1299 expressed greater levels of CXCR6 and CXCL16 mRNA compared with the non-neoplastic bronchial epithelial BEAS2B cells. Breast cancer MDA-MB-231 cells (hormone-insensitive and highly invasive) expressed greater levels of both CXCR6 and CXCL16 mRNA compared with MCF-7 (hormone-responsive and low-invasive) cells. Both hFOB and HBME cells expressed CXCR6 mRNA and CXCL16 mRNA.

CXCL16 mRNA and CXCR6 mRNA Are Expressed in Prostate Cancer Tissues

To determine whether CXCL16 mRNA and CXCR6 mRNA are expressed in prostate cancer, real-time RT-PCR was done with total RNA collected from tissues from localized prostate

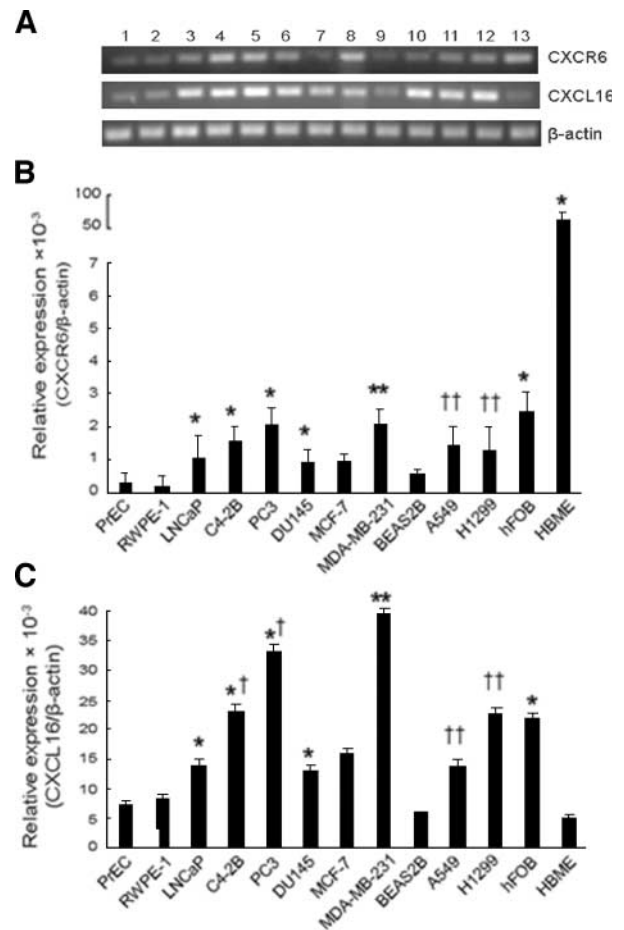


FIGURE 1. CXCR6 mRNA and CXCL16 mRNA are expressed in prostate cancer cells. The expression of CXCR6 and CXCL16 mRNA in different cell lines was determined by RT-PCR and real-time PCR. **A.** Total RNA was extracted from the indicated cells and then subjected to RT-PCR. Lane 1, PrEC; lane 2, RWPE-1; lane 3, LNCaP; lane 4, C4-2B; lane 5, PC3; lane 6, DU145; lane 7, MCF-7; lane 8, MDA-MB-231; lane 9, BEAS2B; lane 10, A549; lane 11, H1299; lane 12, hFOB; lane 13, HBME. CXCR6 mRNA (**B**) and CXCL16 mRNA (**C**) expressions were measured by real time RT-PCR. Data are from three separate experiments. Columns, mean of triplicates; bars, SE. β -Actin was used as the internal control. *, $P < 0.001$ compared with PrEC or RWPE-1. †, $P < 0.001$ compared with LNCaP. **, $P < 0.001$ compared with MCF-7. ††, $P < 0.001$ compared with BEAS2B.

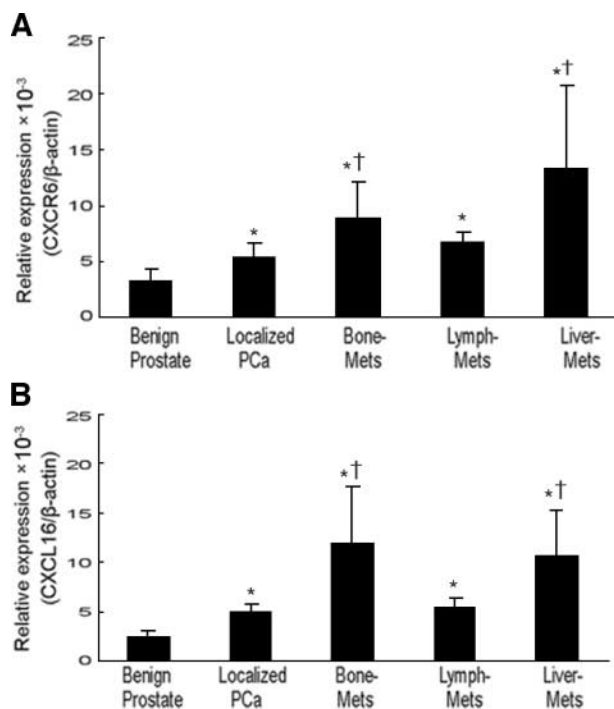


FIGURE 2. CXCR6 and CXCL16 mRNA are highly expressed in metastatic prostate cancer tissues. Total RNA was extracted from benign prostate tissue, localized prostate cancer, prostate cancer with bone metastases, prostate cancer with lymph node metastases, and prostate cancer with liver metastases. Real-time RT-PCR was done, and relative CXCR6 mRNA and CXCL16 mRNA expressions were calculated. **A.** CXCR6 mRNA expression. **B.** CXCL16 mRNA expression. The data are from three separate experiments. Columns, mean of triplicates; bars, SE. β -Actin was used as the internal control. *, $P < 0.001$ compared with benign tissues. †, $P < 0.001$ compared with localized prostate cancer.

cancer ($n = 6$), metastatic prostate cancer ($n = 6$, bone-metastases; $n = 3$, lymph node metastases; and $n = 6$, liver-metastases), and normal prostate ($n = 6$). All prostate cancer tissues expressed greater levels of CXCR6 and CXCL16 mRNA compared with normal prostate tissues (Fig. 2A and B). The tissues from metastatic prostate cancer, including bone metastases and liver metastases, but not lymph node metastases, expressed greater levels of CXCR6 and CXCL16 mRNA compared with the tissues from localized prostate cancer.

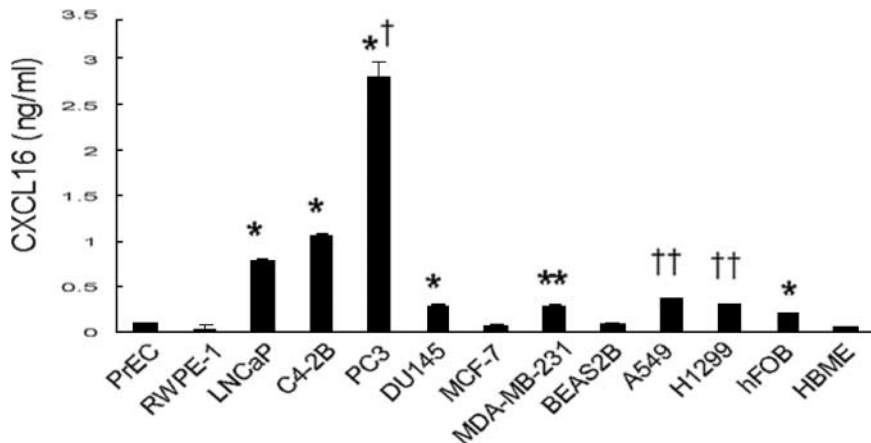


FIGURE 3. CXCL16 is produced by prostate cancer cells. To determine whether CXCL16 is produced by prostate cancer cells, ELISAs were done using the conditioned medium from prostate cancer cell lines LNCaP, C4-2B, PC3, and DU145 cells; nonneoplastic PrEC and RWPE-1 cells; breast cancer MCF-7 and MDA-MB-231 cells; lung cancer A549 and H1299 cells; nonneoplastic BEAS2B cells; and bone hFOB and HBME cells. The data include two separate experiments. Columns, mean from triplicates; bars, SE. *, $P < 0.001$ compared with PrEC or RWPE-1. †, $P < 0.001$ compared with LNCaP. **, $P < 0.001$ compared with MCF-7. ††, $P < 0.001$ compared with BEAS2B.

CXCL16 Is Produced by a Variety of Cancer Cells

To determine whether CXCL16 protein is produced by the cancer cells, ELISA was done on the conditioned medium derived from cancer cells that express CXCL16 mRNA (Fig. 3). Consistent with the observation that prostate cancer cells expressed greater levels of CXCR6 and CXCL16 mRNA compared with nonneoplastic PrEC and RWPE-1 cells, CXCL16 protein production was greater in the prostate cancer cell lines LNCaP, C4-2B, PC3, and DU145 cells compared with PrEC and RWPE-1 cells. In addition, CXCL16 production was greater in PC3 cells compared with LNCaP cells. Similar to the findings in prostate cancer, the production of CXCL16 protein was greater in lung cancer A549 and H1299 cells compared with BEAS2B cells. The production of CXCL16 protein was greater in breast cancer cell line MDA-MB-231 compared with MCF-7. Both hFOB and HBME produced minimal amounts of CXCL16 compared with prostate cancer cells.

Immunohistochemical Staining of CXCR6 Protein Expression in Human Prostate Cancer

Because CXCR6 is the functional receptor for the only known ligand CXCL16, we examined the CXCR6 expression by immunohistochemistry in a wider range of prostate cancer specimens. The high-density tissue microarrays were obtained from clinical samples obtained from a cohort of 80 patients. Tumors were graded using the Gleason grading system and examined to identify areas of benign prostate, prostate cancer, and bone metastases. Positive immunostaining using a monoclonal antibody to CXCR6 was primarily detected in the epithelial cells. The levels of CXCR6 protein expression in the malignant epithelia were greater than in the benign epithelia, and CXCR6 expression correlated with tumor Gleason score (Fig. 4A and B). Furthermore, immunohistochemical staining revealed weak or no staining of CXCL16 in normal prostate, whereas CXCL16 was positively stained, at different levels, on tumor epithelial cells (data not shown). However, levels of CXCL16 expression were not correlated to tumor grade.

CXCL16 Production by Prostate Epithelial Cells Is Induced by Inflammatory Cytokines Tumor Necrosis Factor α and IL-1 β In vitro

As noted previously that CXCL16 expression was abundant in prostate cancer cell lines as determined by real-time RT-PCR

and ELISA, and the positive staining of CXCL16 was largely restricted to the tumor epithelial cells as determined by immunohistochemical analysis, we explored the regulation of CXCL16 by inflammatory cytokines in prostate cancer and prostate epithelial cells. Accordingly, we evaluated whether tumor necrosis factor α (TNF α) and IL-1 β induced CXCL16 production in prostate cancer and RWPE-1 cells. LNCaP, PC3, DU145, and RWPE-1 cells were treated with either TNF α (as positive control) or IL-1 β *in vitro*. CXCL16 protein production was measured by ELISA. Both TNF α and IL-1 β significantly induced CXCL16 production in LNCaP, PC3, DU145, and RWPE-1 cells (Fig. 5). IL-1 β induced CXCL16 production in a dose-dependent manner. Of interest, we further observed that both TNF α and IL-1 β induced CXCR6 mRNA expression determined by real-time RT-PCR (data not shown).

CXCL16 Induces Prostate Cancer Cell Migration and Invasion *In vitro*

To determine whether CXCL16 induces prostate cancer cell migration and invasion, we used BD chambers to assess the ability of prostate cancer cells to cross the polycarbonate membrane in the presence of exogenous CXCL16 *in vitro*. Cell migration was determined by the number of cells that penetrated from the top to the bottom through a porous membrane, whereas in the cell invasion assay, the cells had to invade through the barrier of the reconstituted Matrigel layer on the membrane. We found that CXCL16 induced LNCaP, PC3, and DU145 cell migration (Fig. 6A) and invasion (Fig. 6B) in a dose-dependent manner. However, we failed to observe the

induction of cell migration and invasion in RWPE-1 cells supporting the notion of CXCR6 differential expression in the tumor cells versus nonneoplastic cells. When the CXCR6-neutralizing antibody was added to the upper chamber, the CXCL16-induced migration and invasion were significantly diminished in these tumor cells. In this experiment, SDF-1 (100 ng/mL) was added to the wells of the lower chamber and used as a positive control for the induction of migration and invasion. The efficacy of 100 ng/mL CXCL16-induced migration was less than the SDF-1-induced invasion (~40% in both cell types, data not shown).

Discussion

Most chemokines are small soluble peptides, but there are two exceptions that are expressed as transmembrane multidomain molecules—CX3CL1 (26) and CXCL16 (15, 17). The expression of CX3CL1 and its receptor CXCR1 has been reported to regulate cellular mechanisms involved in adhesion, migration, and survival of human prostate cancer cells (10). However, there was no report on the expression and function of CXCL16 and its receptor CXCR6 in prostate cancer. In previous reports, we explored the regulation of tumor progression by the chemokines SDF-1/CXCL12 (5) and MCP-1/CCL2 (6). We noted that not all of the invasive activities could be neutralized by inhibiting the activity of these molecules. Accordingly, we explored whether other members of the chemokine family may participate in prostate cancer development. The data from the current study show that CXCL16 and its receptor CXCR6 are likely to contribute to prostate cancer progression.

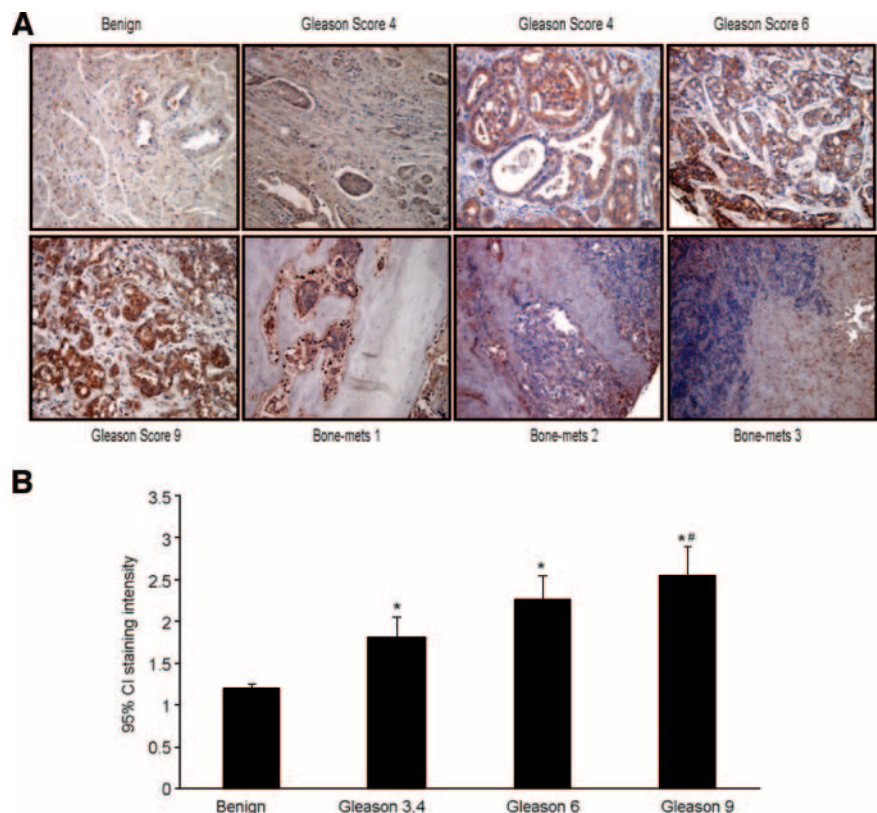


FIGURE 4. The expression of CXCR6 in human prostate cancer. Formalin-fixed, paraffin-embedded tissues were dewaxed and placed in a pressure cooker containing 0.01 mol/L buffered sodium citrate solution (pH 6.0), boiled, and chilled to room temperature for antigen retrieval. The slides were incubated overnight with anti-human CXCR6 antibody. MBA171 (IgG2a) was used as a negative control. **A.** Representative micrographs were taken at original magnification ($\times 100$) and were labeled as indicated. **B.** Quantitative evaluation of CXCR6 expression in prostate cancer. Immunostaining was scored and analyzed as described in Materials and Methods. Columns, mean expression scores for all prostate cancer and nonneoplastic tissues examined; bars, SD. Cases are presented in a graphical format using error bars with 95% confidence intervals. *, $P < 0.001$ compared with benign tissues. #, $P < 0.01$ compared with Gleason score 3/4 and six specimens.

CXCL16 is a type I membrane protein with a non-ELR motif-containing CXC chemokine domain in its extracellular region. The gene for human CXCL16 predicts a 273-amino-acid residue precursor protein with a putative signal peptide, a CXC chemokine domain, a mucin-like spacer region, a transmembrane domain, and a cytoplasmic domain with a potential site for tyrosine phosphorylation and SH2 protein binding. It is a unique molecule that not only attracts T cells and natural killer T cells toward dendritic cells but also supports their firm adhesion to dendritic cells (27). The functional receptor for CXCL16 has been identified as CXCR6 (also known as Bonzo, STRL33, or TYMSTR), a receptor previously shown to be a coreceptor for HIV entry (15, 17). CXCL16 has also been independently cloned and named SRP5OX (21). Moreover, CXCL16/CXCR6 signaling has been associated with liver-specific homing (19, 28-30) and lung-specific homing (31, 32) in the events of inflammation. To our knowledge, this is the first report presenting evidence for the expression and function of both CXCL16 and CXCR6 in prostate cancer.

In this study, mRNA expression of CXCL16 and CXCR6 were further examined on various cancer cell lines and tissues from localized and metastatic prostate cancer and normal prostate tissues. The mRNA expression levels of CXCR6 and CXCL16 were greater in prostate cancer tissues compared with normal prostate tissues. This is consistent with our observations of mRNA alterations in prostate cancer cell lines compared with normal PrEC cells. This result suggests a positive correlation between CXCR6 and CXCL16 mRNA expression and prostate cancer aggressiveness. As determined by immunohistochemistry, we also found that levels of CXCR6 protein expression in the malignant epithelia were greater than in the benign epithelia and that CXCR6 protein expression correlated with the prostate cancer tumor progression. In support of these findings, a similar correlation has been shown in patients with nasopharyngeal carcinoma (22). In that study, Ou et al., combined laser capture microdissection with real-time RT-PCR to quantify CXCR6 mRNA expression in primary and metastatic tumor specimens. They confirmed a positive correlation between higher CXCR6 mRNA levels and the existence of metastases. However, in contrast to the differential expression of CXCR6 mRNA in prostate cancer cell lines, there were no significant differences

among the levels of CXCR6 mRNA expression in various nasopharyngeal carcinoma cell lines, indicating that CXCR6 mRNA alteration may be cell type specific (22). In our current report, we presented that the tissues from metastatic prostate cancer, including bone metastases and liver metastases, but not lymph node metastases, expressed greater levels of CXCR6 and CXCL16 mRNA compared with the tissues from localized prostate cancer.

We speculate that differential expression of CXCR6 between primary and metastatic tumors may be due to environmental factors in the tumor growth microenvironment that up-regulate CXCR6 expression in the metastatic cells. This possibility has been supported by several studies using *in vitro* cell systems, which revealed cytokine-mediated induction of CXCR6 expression (4, 22, 33, 34).

To determine CXCL16 protein production in prostate cancer and the possible regulation by inflammatory cytokines, we measured CXCL16 protein in the conditioned medium collected from LNCaP, C4-2B, PC3, DU145, and PrEC cells. We found that the production of CXCL16 protein was greater in the prostate cancer cell lines LNCaP, C4-2B, PC3, and DU145 compared with normal PrEC or RWPE-1 cells. The production of CXCL16 was greater in the aggressive PC3 cells compared with the less aggressive LNCaP cells. In addition, we found that breast cancer MDA-MB-231 cells (hormone-insensitive and highly invasive) expressed greater levels of both CXCR6 and CXCL16 mRNA/protein compared with MCF-7 (hormone-responsive and low-invasive) cells. These results suggest that CXCL16/CXCR6 expression correlate with tumor aggressiveness in various tumor types. We further observed that both TNF α and IL-1 β significantly induced CXCL16 production in LNCaP, PC3, DU145, and RWPE-1 cells. This result is in accordance with a prior report on a study of human gliomas (23). In that study, CXCL16 mRNA and protein was constitutively expressed by human glioma cells and further up-regulated by TNF α . However, there was a controversial report on human rectal cancer (24) in which CXCL16 protein expression was found, by immunohistochemistry, to be lower in tumor-associated macrophages than in macrophages in normal tissues. Western blot analysis showed a suppression of CXCL16 protein in rectal cancer compared with adjacent

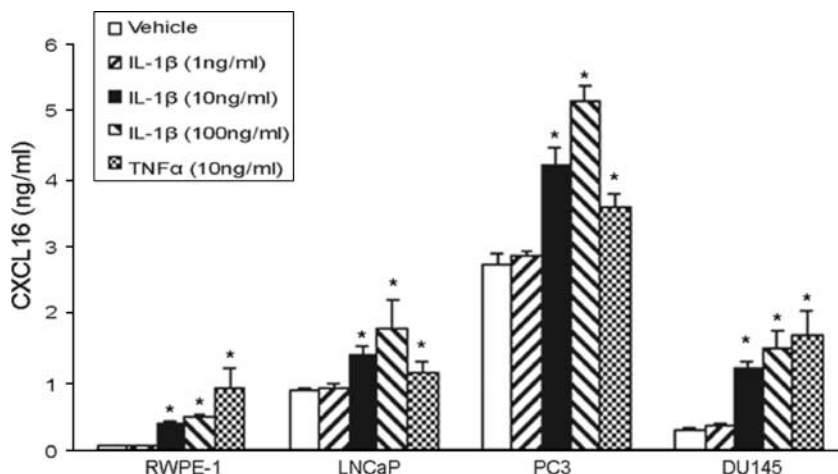
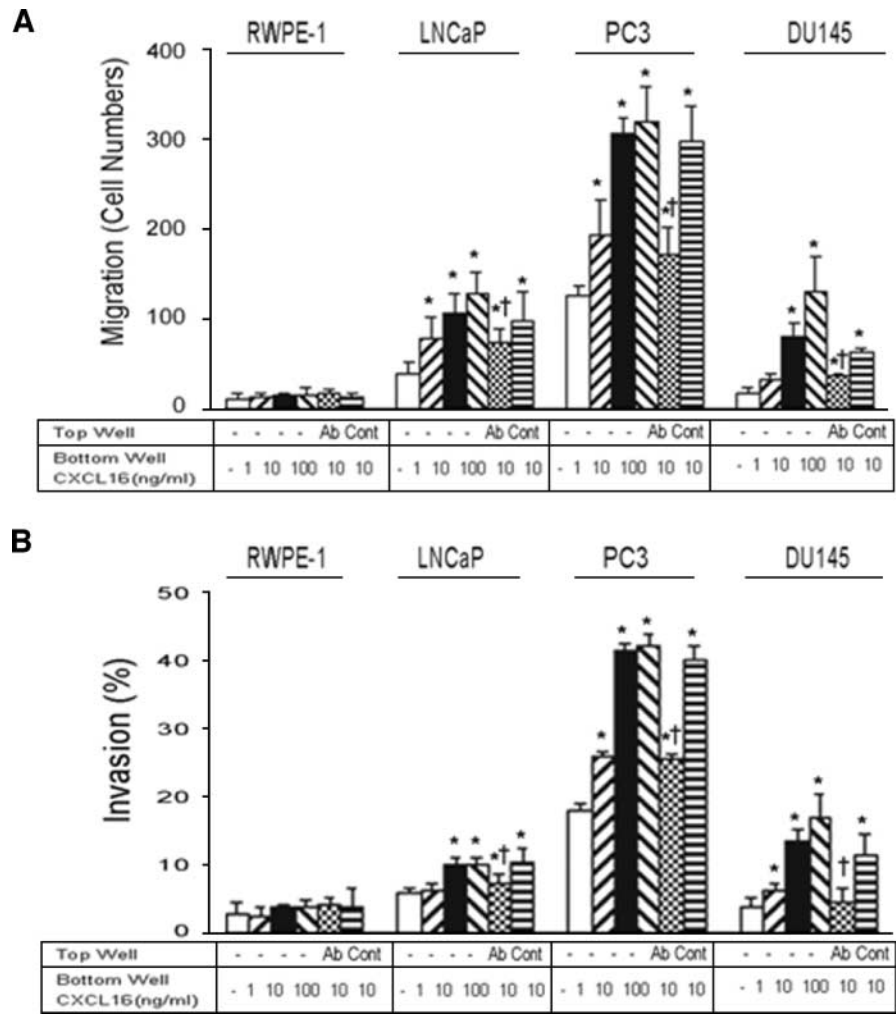


FIGURE 5. CXCL16 production in prostate cancer cells is induced by inflammatory cytokines TNF α and IL-1 β *in vitro*. LNCaP, PC3, DU145, and RWPE-1 cells were seeded into 60-mm culture dishes at 8×10^5 per dish and treated with the indicated treatments (vehicle; IL-1 β 1 ng/mL, 10 ng/mL, 100 ng/mL; or TNF α 10 ng/mL) for 48 h. The supernatant was collected and CXCL16 levels were measured by ELISA. The data are from two separate experiments. Columns, mean of triplicates; bars, SE. *, $P < 0.01$ compared with the vehicle-treated cells.

FIGURE 6. CXCL16 induces prostate cancer cell migration and invasion *in vitro*. A reconstituted Matrigel-coated and noncoated membrane BD chamber was used to determine the migration and invasion of LNCaP, PC3, DU145, and RWPE-1 cells. Briefly, cells were placed into either a porous 8- μ m membrane insert (for the migration assay) or a Matrigel-coated membrane insert (for the invasion assay). The indicated concentration of rhCXCL16 (0-100 ng/mL) was added into the lower chambers. CXCR6 blocking antibody (Ab) or isotype control (Cont) was used to evaluate the CXCL16 specificity. After 24 h of incubation, for cell migration (A), cells that migrated to the lower chambers were counted; for cell invasion (B), cell penetration through the membrane with Matrigel was quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at $\times 200$ magnification) per filter. Invasive ability (%) was defined as the proportion of cells that penetrated the Matrigel-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration). Columns, mean of triplicate assays; bars, SE. *, $P < 0.001$ compared with vehicle-treated cells. †, $P < 0.01$ compared with rhCXCL16 (10 ng/mL)-treated cells.



noncancer tissue. In that report, the CXCL16 mRNA expression determined by real-time RT-PCR was not altered in cancer tissues compared with normal tissues, nor was there any correlation between the CXCL16 expression level and tumor stage (24). Unfortunately, they did not measure CXCL16 production by any tumor cell lines. In our studies on CXCL16 expression on prostate cancer specimens from tissue microarrays, we observed weak or no staining of CXCL16 in normal prostate, whereas CXCL16 was positively stained, at different levels, on tumor epithelial cells. However, levels of CXCL16 expression were not correlated to tumor grade.⁶ This result supports the idea that CXCL16 acts as a secreted protein in prostate cancer development.

In our current study, we observed that CXCL16 induced prostate cancer cell migration and invasion in a dose-dependent manner. Both the CXCL16-induced migration and invasion were significantly diminished by neutralizing antibody against CXCR6. These results suggest that CXCL16 may function as a

chemoattractant in its soluble form (35). Chemokines are major determinants of macrophage and lymphocyte infiltration in human carcinomas (36). Monocytes recruited into the tumor from the peripheral blood, in response to chemoattractants, differentiate into tumor-associated macrophages. It has been reported that the majority of tumors, including prostate cancer, contain numerous tumor-associated macrophages compared with adjacent nonneoplastic tissues (37-39). Tumor-associated macrophages may also produce a variety of factors that affect tumor progression depending on which cytokines are produced (36). It is well known that inflammatory cells in cancer tissues interact with tumor cells (40); in certain cases, tumor-associated macrophages seem to produce growth stimulators and angiogenic factors that favor invasion and metastasis (40, 41). We postulate that the sources of CXCL16 in prostate cancer skeletal metastases are from tumor-associated macrophages, tumor cells, and vascular endothelial cells, although this needs to be further examined.

In summary, this is the first report exploring the expression of CXCL16 and its receptor CXCR6 in prostate cancer tissues and cells. We show that CXCL16 induces prostate cancer cell migration and invasion *in vitro*. Our data suggest that

⁶ Unpublished data.

CXCL16/CXCR6 may form a network together with other pairs of chemokines and their receptors, such as SDF-1/CXCR4, CX3CL1/CXCR1, IL-8/CXCR2, and MCP-1/CCL2, and mediate tumor development in targeted organs.

Materials and Methods

Reagents and Antibodies

Recombinant human CXCL16 and mouse anti-human CXCR6 monoclonal antibody and isotype control antibody (mouse IgG) were purchased from R&D Systems. All chemical reagents were purchased from Sigma.

Cell Culture

Prostate cancer cell lines LNCaP, PC3, and DU145; and nontumorigenic human prostate epithelial cells RWPE-1, hFOB (SV40 large T antigen transfected and immortalized human osteoblasts), and HBME (human bone marrow endothelial cells) were obtained from the American Type Culture Collection. C4-2B cells, derived from the parental cell line LNCaP but with characteristics of skeletal metastasis, were obtained from UroCor. A549, H1299 (human non-small-cell lung carcinoma cell lines), and BEAS2B (nonneoplastic bronchi epithelial cells) were kindly provided by Dr. Xue Wang (University of Pittsburgh, Pittsburgh, PA). MDA-MB-231 and MCF-7 (breast cancer cells) were kindly provided by Dr. Shiyuan Cheng (University of Pittsburgh). PrEC cells were purchased from Cambrex. LNCaP, PC3, DU145, and H1299 cells were cultured in RPMI 1640 (Invitrogen). Human FOB cells were grown in medium with 50% DMEM and 50% Ham's F12 medium (Invitrogen) at 34°C. At this temperature, the cells exhibit rapid cell division due to the establishment of this cell line by transfection of a temperature-sensitive expression vector pUCSVtsA58. C4-2B cells were maintained in T medium [80% DMEM, 20% Ham's F12 medium (Invitrogen), 5 µg/mL insulin, 13.6 pg/mL triiodothyronine, 5 µg/mL transferrin, 0.25 µg/mL biotin, and 25 µg/mL adenine]. HBME, A549, BEAS2B, and MDA-MB231 cells were cultured in DMEM medium. MCF-7 cells were cultured in DMEM medium supplemented with 5 mg/mL insulin. All cell cultures were supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (HyClone). PrEC were maintained in PrEGM BulletKit medium (Cambrex). The RWPE-1 cells were grown in keratinocyte-serum free medium supplemented with 5 ng/mL human recombinant epidermal growth factor and 0.05 mg/mL bovine pituitary extract (Invitrogen). All cells, except hFOB, were maintained in 10-cm tissue culture dishes in a 37°C incubator equilibrated with 5% CO₂ in humidified air.

Reverse Transcription-PCR

Total RNA was extracted from LNCaP, C4-2B, DU145, PC3, PrEC, BEAS2B, A549, H1299, MDA-MB-231, MCF-7, HBME, and hFOB cells; and from benign prostate tissue (six cases), localized prostate cancer (six cases), prostate cancer with bone metastases (six cases), prostate cancer with lymph node-metastases (three cases), and prostate cancer with liver metastases (six cases), using TRIzol reagent (Life Technologies). The extracted total RNA was then subjected to RT-PCR

for detection of CXCL16 and CXCR6 mRNA expression. Cases of localized prostate cancer were identified from a radical prostatectomy series at the University of Michigan, and cases with prostate cancer metastases were obtained from a Rapid Autopsy Program through the Michigan Prostate Specialized Programs of Research Excellence Tissue Core. PCR primers for CXCL16 consists of sense 5'-CTGACTCAGCCAGGCAA-TGG-3' and antisense 5'-TGAGTGGACTGCAAGGTGGA-3' (42). PCR primers for CXCR6 consisted of sense 5'-ATGG-CAATGTCTTTAATCTCGACAA-3' and antisense 5'-TGAA-AGCTGGTCATGGCATAGTATT-3' (43). PCR primers for β-actin consists of sense 5'-GTCCTCTCCAAGTCCACACA-3' and antisense 5'-CTGGTCTCAAGTCAGTGACAGGTAA-3' (42). RT-PCR was done with 1 µg of total RNA using the Access RT-PCR system (Promega Corp.) in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems) under the following conditions: first strand cDNA was synthesized at 48°C for 45 min and then denatured at 94°C for 2 min for the first cycle and at 30 s for an additional 35 cycles; annealing was done at 55°C for 30 s, and extension was done at 72°C for 60 s. The final extension was at 72°C for 7 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Real-time RT-PCR

Total RNA was extracted as in RT-PCR. Real-time RT-PCR was done in an iCycler iQ multicolor real-time PCR detection system (Bio-Rad) using iScript one-step RT-PCR kit with SYBR Green (Bio-Rad). Primers for CXCL16 were as follows: sense 5'-GGCCCACCAGAAGCATTAC-3' and antisense 5'-CTGAAGATGCCCCCTCTGAG-3'. Primers for CXCR6 were as follows: sense 5'-ATGCCATGACCAGCTTCACT-3' and antisense 5'-TTAAGGCAGGCCCTCAGGTA-3'. PCR primers for β-actin were as follows: sense 5'-CTGGCTGCT-GACCGAGG-3' and antisense 5'-GAAGTCTCAAACAT-GATCTGGGT-3' (44). Synthesis of cDNA was done at 50°C for 10 min; then, denaturation was done at 95°C for 5 min, followed by 45 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 15 s, and elongation at 72°C for 1 min. The fluorescence intensity of the double strand-specific SYBR Green, reflecting the amount of formed PCR product, was monitored at the end of each elongation step. Melting curve analysis was done to confirm the purity of the PCR products. Relative expression of CXCL16 or CXCR6 was normalized by β-actin.

Conditioned Medium

LNCaP, C4-2B, DU145, PC3, PrEC, BEAS2B, A549, H1299, MDA-MB-231, MCF-7, HBME, and hFOB cells at 2×10^6 were grown in 100-mm tissue culture dishes overnight in cell culture medium and washed twice with PBS. Then, the medium was changed to 1% FBS in RPMI 1640. After 48 h, the conditioned medium was collected. To investigate the regulation of CXCL16 expression, LNCaP, PC3, DU145, and RWPE-1 cells were seeded in their maintenance medium into 60-mm culture dishes at 8×10^5 per dish overnight. Then, the medium was changed to 1% FBS in RPMI 1640 with indicated treatments (vehicle, IL-1β 1 ng/mL, 10 ng/mL, 100 ng/mL, or TNFα 10 ng/mL) for 48 h. The supernatant was collected and CXCL16

levels were measured by ELISA. To normalize for differences in cell density because of proliferation during the culture period, cells from each plate were collected and the total DNA content per plate was determined (spectrophotometric absorbance, 260 nm). The conditioned medium was then normalized for DNA content between samples by adding RPMI.

ELISA

Quantikine human CXCL16 ELISA kits were purchased from R&D Systems. ELISAs were done according to the manufacturer's instructions.

Tissue Microarray and Immunohistochemical Staining

High-density tissue microarrays were constructed by Folio Biosciences with clinical samples obtained from a cohort of 80 patients. Tumors were graded using the Gleason grading system and examined to identify areas of benign prostate, prostate cancer, and bone metastases. The formalin-fixed, paraffin-embedded tissues were dewaxed and placed in a pressure cooker containing 0.01 mol/L buffered sodium citrate solution (pH 6.0), boiled, and chilled to room temperature for antigen retrieval. The slides were then incubated overnight at 4°C with anti-human CXCR6 antibody (Novus Biologicals). MBA171 (IgG2a; Clone 44708.111, R&D Systems) was used as a negative control. Vectastain ABC-AP substrate kit (Vector Laboratories) was used for signal detection and Harris hematoxylin was used as a counterstain. Quantitative analysis of the CXCR6 expression was determined by scoring protein expression as negative (score, 1), weak (score, 2), moderate (score, 3), or strong (score, 4), which was previously used (45). Differences in CXCR6 expression were evaluated statistically using the mean scores from each case. Scoring was done (by Dr. Jianhua Wang at the University of Michigan) without knowledge of overall Gleason score (e.g., tumor grade), tumor size, or clinical outcome.

Cell Migration and Invasion Assay

The invasiveness of LNCaP, PC3, DU145, and RWPE-1 cells was evaluated in a 24-well BD chamber (BD Biosciences), as directed by the manufacturer. Briefly, the upper and lower culture compartments of each well are separated by polycarbonate membranes (8- μ m pore size). The membranes in some wells were precoated with 100 mg/cm² of Matrigel that was reconstituted by adding 0.5 mL of serum-free RPMI medium to the well for 2 h. To assess the ability of the cells to cross the polycarbonate membrane (i.e., baseline migration), 5 \times 10⁴ cells in 0.5 mL of RPMI medium containing 5% FBS were placed into the upper compartment of the wells that did not contain Matrigel, and 0.75 mL of RPMI medium containing 10% FBS was placed into the lower compartment. In parallel, the ability of the same cells to penetrate the Matrigel was assessed by placing 5 \times 10⁴ cells in 0.5 mL of RPMI medium containing 5% FBS in the upper compartment of wells that were coated with the reconstituted Matrigel and 0.75 mL of RPMI medium containing 10% FBS in the lower compartment. In the lower compartments, either vehicle or recombinant human CXCL16 (1-100 ng/mL) was added. The effect of 1 μ g/mL CXCR6 blocking antibody (R&D Systems) added to the upper chamber of the transwell was used to provide additional

verification that observed responses are dependent on CXCR6 receptor binding. The transwell chambers were incubated for 24 h at 37°C in 95% air and 5% CO₂. For cell migration, cells that migrated to the lower chambers were counted. For cell invasion, cell penetration through the membrane with Matrigel was detected by staining the cells on the porous membrane with a Diff-Quik stain kit (Dade Behring) and quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at \times 200 magnification) per filter. Invasive ability (%) was defined as the proportion of cells that penetrated the Matrigel-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration).

Statistical Analysis

Statistical analysis was done using Statview software (Abacus Concepts). ANOVA was used for initial analyses, followed by Fisher's protected least significant difference for post hoc analyses. Differences with a $P < 0.05$ were determined as statistically significant.

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