Cancer Receptor-1 Silencing Inhibits Androgen-Independent Prostate Cancer

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Abstract
The CXC receptor-1 (CXCR1) is a coreceptor for interleukin-8 (IL-8) and is expressed on both normal and tumor cells. The function of CXCR1 in prostate cancer was investigated by silencing its expression, using RNA interference. We established stable cell colonies of PC-3 cells, depleted of CXCR1, using lentiviral plasmids (pLK0.1puro) generating small hairpin RNA (shRNA) against CXCR1 mRNA. Stable shRNA transfectants (PLK1–PLK5) that express significantly reduced CXCR1 mRNA (≥90% down) and protein (≥43% down) or vector-only transfectants (PC-3V) were characterized. PLK cells showed reduced cell proliferation (down, ≥66%), due to cell cycle arrest at G1-S phase, decreases in Cyclin D1, CDK4, phosphorylated Rb, and extracellular signal-regulated kinase 1/2 levels compared with those in PC-3V cells. CXCR1 depletion lead to increases in spontaneous apoptosis by mitochondria-mediated intrinsic mechanism and increases in proapoptotic proteins (BAD, 40%; BAX, 12%), but decreases in antiapoptotic proteins (BCL2, down 38%; BCL-X1, 20%). PLK2 cells grew as slow-growing tumors (decrease of 54%), compared with that of PC3V tumors in athymic mice. Ex vivo analyses of PLK2 tumor tissues showed reduced expression of Cyclin D1 and vascular endothelial growth factor, and increased apoptosis activity. Other IL-8–expressing prostate cancer cell lines also exhibited similar phenotypes when CXCR1 was depleted by CXCR1 shRNA transfection. In contrast to these cells, CXCR1 depletion had little effect on IL-8 ligand–deficient LNCaP cells. RNA interference rescue using mutated CXCR1 plasmids reversed the silencing effect of PLK2, thus demonstrating the specificity of phenotypic alteration by CXCR1 shRNA. These studies establish that CXCR1 promotes IL-8–mediated tumor growth. [Cancer Res 2009;69(21):8265–74]

Introduction
Cancer of the prostate (CaP) affects one in six men in the United States (1). Localized CaP is curable but it recurs in 30% of patients after primary therapy (2). Recurrent disease is treated by single or combination of radiation, androgen blockade, or chemotherapy, all of which are palliative. The median life span of patients who are unresponsive to androgen blockade, termed androgen-independent CaP (AIPC), is 2 to 8 years (3). Several factors may contribute to the transition of localized, androgen-responsive CaP to AIPC (4, 5). The proinflammatory cytokines, chemokines, and enzymes that produce prostaglandins are implicated as important factors in this process. Interleukin-8 (IL-8), an 8-kDa polypeptide with Cysteine-X-cysteine domain, belongs to this group of potential mediators of hormone-refractory cancer progression (6–8).

IL-8–mediated activities in these diseases are multidimensional, including angiogenesis, cell proliferation, motility, stimulation of invasive enzymes, and interaction with other multifunctional mediators such as NF-κB (9–11). Most activities of IL-8 are initiated by its binding to two cell surface receptors, CXCR1 and CXCR2, which are GTP-binding protein–coupled receptors, expressed in both epithelial and mesenchymal cells (12–14).

CXCR1 binds only IL-8, but CXCR2-2 is more promiscuous, which interacts with other protein ligands, such as GRO-α, β, γ, neutrophil-activating protein-2, and GCP-2 (12–15). CXCR1 and CXCR2 function distinctly when IL-8 binds and dimerizes the receptors. CXCR-1 stimulates chemotaxis, calcium influx, activation of phosholipase D and the release of neutrophil granules in nontransformed cells, as well as proliferation, and motility in tumor cells (10, 11, 16). CXCR2 triggers a distinct signaling pathway, which stimulates expression of angiogenesis factors, such as vascular endothelial growth factor (VEGF) and metalloproteinases (17, 18).

IL-8 is secreted by AIPC cells (e.g., PC-3 and DU145). It is absent in androgen-responsive CaP cells such as LNCaP and LAPC-4 cells (6–8, 11). However, both types of CaP cells express functional CXCR1 and CXCR2 and respond to IL-8 (19). It has been reported that although the expressions of CXCR1 and CXCR2 are low, undetectable in normal, or benign prostate tissues, their levels are significantly elevated in high-grade prostatic intraepithelial neoplasia and in CaP with increasing intensity with Gleason grade (20–22). Murphy and colleagues (23) reported that although cells from normal prostate or benign prostatic hyperplasia tissues show apical expression of these two receptors, the expression shifts to cytoplasm with an association with Gleason score. The significance of CXCR1 and CXCR2 in CaP cells with or without IL-8 expression is not well characterized, especially the relative roles of CXCR1 versus that of CXCR2 in AIPC (24). We showed previously that neutralizing antibodies to CXCR1, but not that for CXCR2, inhibits cell proliferation in IL-8–expressing cells (11). The objective of the present study is to determine the role of CXCR1 on modulating cellular phenotypes associated with prostate cell proliferation, survival, and angiogenesis. We tested the hypothesis that down modulation of one or both receptors for IL-8 should decrease growth and survival of CaP cells in vitro and tumor growth in vivo. Our results show that depletion of CXCR-1 by RNA interference significantly reduces CaP cell proliferation, tumor growth, and angiogenesis, and increases spontaneous apoptosis.

Materials and Methods
Chemicals. We obtained five lentiviral constructs (pLK0.1puro) containing custom synthesized, 21-mer short hairpin RNA (shRNA) directed...
to various coding regions of CXCR1 cDNA, but nonoverlapping with CXCR2, and with puromycin resistance locus (Mission shRNA, plKO.1 puro) from Sigma-Aldrich, Inc. Custom-synthesized siRNA for CXCR2, non-target siRNA control, and siRNA transfection reagent, DharmaFect-2, were from Dharmacon, Inc. We purchased IL-8RA (NM_000634) human cDNA ORF in Myc-DDK Tagged pCMV6-entry vector from Origene, Inc.

**Cells and culture conditions.** All cell lines used in this study were obtained from American Type Culture Collection and other sources. They were maintained in the laboratory in RPMI (Media Tech, Inc.) with supplements as described before (11).

**Generation of stable shRNA-expressing cell lines.** We transfected PC-3 cells with all five plasmids or the empty vector (without the cDNA insert (control)), individually with 1 μg DNA/10^5 cells using Fugene-6 transfection reagent as per the suppliers’ instructions (Roche, Inc.). The transfection efficiency of Fugene F6 was 84 ± 4% at 72 h after transfection, as determined by cotransfection with a plasmid containing enhanced green fluorescent protein (11). Stable colonies of transfectants were clonally selected in medium with 2 μg/mL puromycin for 10 d, and cell colonies were screened for CXCR1 silencing by Real-time reverse transscriptase PCR (Q-RTPCR; ref. 11). Cell colonies that showed ≥90% reduction in CXCR1 mRNA compared with vector-only transfectant were further characterized. These isolates are named PLK1 through PLK5 and the empty vector-transfected isolate as PC-3V.

**Real-time reverse-transcriptase PCR.** Total RNA was subjected to RT-PCR using the Bio-Rad (Cycler iQ) real-time-PCR instrument, as described before (11), using SYBR green fluorescence detector to determine DNA saturation curves (Bio-Rad). The primer sets used for amplification were as described before (11).

The expression of the target mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from each total RNA sample. The threshold cycle (Ct) of each sample was determined, and the relative level of a transcript as fold difference (FD = 2^(-ΔΔCt)) was calculated by obtaining a ΔCt value (test Ct - GAPDH Ct) expressed as arbitrary units [(1/2^ΔΔCt)] × 100) = fold difference], as described before (11).

**Immunoprecipitation and immunoblot analysis.** We used the semi-quantitative determination of CXCR1 protein expression in various cell lysates (0.2 mg/100 μL cell lysate) with a combination of antigen pull-down and immunoblotting techniques, using two separate anti-CXCR1 antibodies, raised in two different species, as reported previously (25). Antibody bound to CXCR1 was made visible on X-ray film using the electrochemiluminescence technique (ECL Plus kit, GE Life Sciences). The same blot was bound to CXCR1 was made visible on X-ray film using the electrochemiluminescence technique (ECL Plus kit, GE Life Sciences). The same blot was bound to CXCR1 was made visible on X-ray film using the electrochemiluminescence technique (ECL Plus kit, GE Life Sciences). The same blot was bound to CXCR1 was made visible on X-ray film using the electrochemiluminescence technique (ECL Plus kit, GE Life Sciences).

**Cell proliferation assay.** We compared the growth rate of all transfectants against that of PC-3V by estimating cell numbers after 2, 3, 5, and 7 d of cultures in replicate 35-mm dishes. In addition, we determined the viability of cells using a colorimetric (MTT) assay as described previously (26).

**Cell cycle phase fractionation.** Estimation of fraction (percent of total) of cells in various phases of cell cycle was determined based on their DNA content, as described before (27).

**Tumor induction and growth measurement.** PC-3V and PLK cells, suspended in 50% basement membrane extract (Path-Clear BME, Trevigen Inc.) at 1 x 10^6 cells/mL, were implanted s.c. into the right and left dorsal flanks of 6-wk-old athymic mice (1 x 10^6 cells/site, 5 animals x 2 sites/group). Group size was determined using a power analysis program (28) so that the difference between the test groups (PLK2 tumor versus PC-3V tumor) with the power of lower and upper confidence interval of ≥95% is <2 x SD of tumor volume. All tumor-bearing mice were euthanized 42 d post tumor cell injection, and tumor tissues were harvested at necropsy. Excised tissues were part frozen and the remainder fixed in buffered formalin. Fresh frozen tissues were homogenized and extracted in SDS-gel sample buffer. These samples were analyzed by Western blotting to determine the levels of antigens of interests. Tissues fixed in formalin were embedded in paraffin blocks, 3-μm sections were cut for histology.

**Apoptosis activity in tumor tissues.** We used the terminal deoxynucleotidyl transferase, dUTP nick-end labeling (TUNEL) assay to determine the extent of apoptosis in tumor tissues derived from PC-3V and PLK2 xenografts. TUNEL assay was performed using a kit (In situ Cell Death detection kit, Roche Inc.). We followed the protocol of Negoescu and colleagues (29) that relies on fluorescent labeling of DNA strand breaks, which was slightly modified as described before (27).

**Multisite-directed mutagenesis for CXCR1 shRNA rescue experiment.** We performed this test to corroborate specificity of phenotypic changes associated with shRNA-induced CXCR1-depletion. Primers were designed using QuikChange Primer Design software (Stratagene) to introduce six or seven nucleotide substitutions within the CXCR1 shRNA (PLK2) hybridizing sequence (5’ GCCACTGAGCTGG TAGTCTC 3’), while retaining the amino acid identity of the wild-type protein (Supplementary Table S1). We introduced these mutations in a DDK-Myc-tagged CXCR1 cDNA clone (Origene). Mutagenesis reaction was carried out using the Stratagene MultiSite-Directed Mutagenesis kit (Agilent Technologies). Details of the procedures are included in the Supplementary Table S2. DNAs from five colonies were isolated using Wizard Plus SV DNA Miniprep kit (Promega) and sequenced to verify the presence of the designed mutations.

**Statistical analysis.** All in vitro experiments in which quantitative data are presented were performed at least twice, each in triplicate samples. Western blots and immunohistochemistry were repeated once. All data are presented as mean ± SD, with significance of each result determined with Student’s t test or ANOVA.

**Results**

**shRNA constructs specifically reduce CXCR-1 expression in PC-3 cells.** As shown in Fig. 1A, transfection with all the CXCR1 shRNA plasmids (PLK1-PLK5) reduced the levels of CXCR1 mRNA. Specifically, colonies derived from single cell isolates of PLK1, PLK2, and PLK4 transfectants expressed significantly reduced levels of CXCR-1 mRNA (10–17% of that of PC-3V); colonies of PLK1 and PLK4 had similar level of decreased CXCR1 expression (17%). We choose PLK2 (lowest level of CXCR1 mRNA) and PLK4 for further characterization. CXCR1 protein expression in control and CXCR1 shRNA transfectants showed significant decreases in CXCR1 protein expression in PLK2 and PLK4 (~43%), but to a lesser extent than that of mRNA levels (Fig. 1A, inset). As shown in Fig. 1B, cell surface expression of CXCR1 in PLK2 cells was significantly lower (46%) than that of the vector-only transfectant (PC-3V), as determined by flow cytometry (Fig. 1B, inset). We next investigated the biological consequence of CXCR1 silencing by examining cell proliferation, cell cycle progression, spontaneous apoptosis, and tumorigenic potential of the PC-3V and PLK cells.

**PLK cells show decreased proliferation.** The proliferation activity, as measured by increase in cell number over time, was significantly low in each of the three PLK isolates, PLK1, PLK2, and PLK4 when compared with that of PC-3V (Fig. 1C). Compared with PC-3V and PLK4, the proliferation activity of PLK2 was the lowest (66 ± 4.95%). The PLK1 and PLK4 cell proliferation rates were also reduced by 41 ± 8.0% and approximately 30 ± 9.85%, respectively, significantly lower than the proliferation rate of PC-3V but less pronounced than that of PLK2. Because PLK2 transfectants had the most inhibition of cell proliferation and lowest level of CXCR1 mRNA expression, we further investigated the cellular physiologic consequence of this inhibition in PLK2 cells. To investigate and establish a more general occurrence of IL-8-CXCR1 mitogenic signaling in CaP, we transfected cells of two other common CaP cell lines, DU145 and LAPC-4IL-8. Cells of both of these lines express IL-8 and show IL-8-dependent growth (11, 25). We determined the...
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Figure 1. Characterization of CXCR1-depleted CaP cells. A, CXCR1 in vector-control (PC-3V) and CXCR1 shRNA–transfected PC-3 cells (PLK). Q-RT PCR analysis of CXCR1 expression in PC-3 cell clones selected after plk0.1-CXCR1shRNA (PLK1–5) or plk0.1 vector alone DNA transfection (PC-3V). Data presented are normalized against GAPDH mRNA. Inset, CXCR1 protein expression in PC-3V and PLK cells. Protein expression of CXCR1 in PC-3V, PLK2, and PLK4 cells were identified by immunoprecipitation followed by immunoblotting. β-Actin bands were used to normalize loading variation. Band intensities, in arbitrary units, are shown, normalized to that of control, PC-3V cells. B, cell surface expression of CXCR1 in PC-3V and PLK2 clones was determined by flow cytometry. Fresh cells were incubated with anti-CXCR1 antibody (2 μg/mL; BioLegend) followed by labeling with Q-dot antimouse IgG (20 nm Q-dot, Invitrogen). Relative fluorescence intensities were analyzed on the Beckman-Coulter EPICS XL flow cytometer with Log FL1 (FITC channels) filter set, as described before (26). Inset, median fluorescence intensities of PC-3V-CXCR1Ab and PLK2-CXCR1Ab cells. C, growth curves of CXCR1-depleted PC-3 cells and PC-3V cells. Growth of PC-3V, PLK1, PLK2, and PLK4 cells over a 7-d period determined by direct cell counting. D, cell proliferation inhibition by CXCR1 depletion in other CaP cells. Two CaP cell lines, DU 145 and LAPC-4IL-8, both constitutive IL-8 producers, were transfected with PLK2, and cell proliferation activity was determined 72 h after transfection by MTT assay (11). PLK2 transfection, but not that of empty vector, significantly reduced the proliferation activity in both cell lines (P ≤ 0.05; n = 3).

As shown in Fig. 1D, both these cells also exhibited decreased cell proliferation (34 ± 4% in DU145PLK2 and 42 ± 2.8 in LAPC4-IL8-PLK2) and decrease in cell cycle–regulated protein Cyclin D1 (Supplementary Fig. 1A and B).

CXCR-1 knockdown causes cell cycle arrest at G1-S phase. Since we observed a 66% decrease in growth rate of CXCR1-silenced PC-3 cells, we examined whether this is due to the inhibition of cell cycle progression, increase in spontaneous apoptosis, or both. We analyzed the cell cycle–phase distribution in PLK2 cells by flow cytometry. We found a significant increase in the G1 phase and a greater decrease in PLK2 cells entering S phase, which also reduced the fraction of cells entering G2–M phase. As shown in Fig. 2A, we observed ~51% increase in the fraction of cells in G1 phase, decreases of 78% of cells in S phase and 54% in G2–M phase, respectively, in PLK2 cells compared with that of PC-3V cells.

CXCR1 depletion causes alteration in cell cycle–regulated proteins. As shown in Fig. 2B, we found a significant decrease in the expression of Cyclin D1, CDK4 (Fig. 2C), and phosphorylated Rb in PLK2 cells compared with those of PC-3V cells (Fig. 2B). In addition, we found the level of Cyclin D1 expression increases in PC-3V cells, but only marginally in PLK2 cells after exogenous addition of IL-8 (25 ng/ml) or endothelial growth factor receptor (EGF; 20 nmol/L; Fig. 2B). The levels of phosphorylated Rb increased (20%) in PLK2 cells after EGF stimulation, but it was still significantly lower than that of PC-3V cells (50%). Further, we observed the upregulation of two inhibitors of G1-S transition, P21 and P27kip1, in CXCR1-depleted PLK2 cells (Fig. 2C). These results
confirm that in the absence of IL-8-CXCR1 signaling, cell cycle progression can be arrested in AIPC, regardless of the presence or absence of other growth factors (e.g., EGF).

**CXCR1 depletion causes decrease in extracellular signal-regulated kinase 1/2 phosphorylation.** The two known pathways through which GPCR (e.g., CXCR1) proteins regulate the mitogenic response in tumor cells are Ras/Raf/mitogen-activated protein kinase (extracellular signal-regulated kinase 1/2, ERK1/2) and the phosphoinositide 3-kinase/AKT pathways (25, 30). We determined the level of phosphorylated ERK1/2 levels in PC-3V and PLK2 cells, following stimulation with EGF (20 nmol/L). We found a time-dependent increase in phospho-ERK following EGF addition in both PC-3V and PLK2 cells, following stimulation with EGF (20 nmol/L). We found a time-dependent increase in phospho-ERK following EGF addition in both PC-3V and PLK2 cells (Fig. 2D). However, the level of phospho-ERK was significantly lower by ∼2-fold in PLK2 cell lysates compared with that in PC-3V cells. More importantly, there was a 5-fold decrease in the level of p-ERK in PLK2 cells without growth factor stimulation, indicating this is likely due to G1-S arrest in PLK2 cells.

**CXCR1 depletion increases spontaneous apoptosis in AIPC cells.** We reasoned that because depletion of CXCR1 causes increase in population doubling time (Fig. 1C), increased spontaneous apoptosis could contribute to this slow doubling time. Because cleavage of poly ADP ribose polymerase (PARP) by caspase-3 leads to irreversible phase of apoptosis, it is a determinant of apoptotic activity in tumor cells (31, 32). We used the levels of cleaved PARP as a read out for spontaneous apoptosis activity in PC-3V and PLK2 cells. The level of cleaved PARP was significantly elevated in PLK2 cells compared with that of PC-3V cells (Fig. 3A). Further, although addition of IL-8 decreased even the basal levels of cleaved PARP in PC-3V cells, it did not affect the cleaved PARP levels in PLK-2 cells, suggesting IL-8-CXCR1 interaction may prevent spontaneous apoptosis.

We next inquired whether this increase in apoptosis is due to the mitochondria-mediated intrinsic process (33, 34). To determine the integrity of mitochondria, we used the uptake and J-aggregate formation of the mitochondrial potential-sensitive dye JC-1 in PC-3V and PLK2 cells, as described before (26). As shown in Fig. 3B, in PLK2 cells, the level of intracellular monomeric JC-1 (green fluorescence) was significantly higher when compared with that of PC-3V cells, indicating increased uptake of JC-1 by depolarized mitochondria in PLK2 cells plus decrease in J-aggregate formation. This level of depolarization was comparable with that induced by incubation with 100 nmol/L Stauroporine (Sts in Fig. 3B), a known inducer of apoptosis. Increased green fluorescence
suggested that CXCR1 depletion causes alteration in mitochondrial integrity that may contribute to increased apoptosis (26). Involvement of mitochondrial apoptosis machinery in PLK2 cells was further confirmed by measuring the relative levels of proapoptotic and antiapoptotic mitochondrial proteins, (BCL2, BCL-XL, BAX, and BAD) in PLK2 and PC-3V cells (35). Indeed, a comparison of the levels of BCL-2, BCL-XL, BAX, and BAD proteins in the immunoblots of PLK2 and PC-3V cells showed a significant shift toward apoptosis in PLK2 cells. For example, when compared with PC-3V cell lysates, we found a decrease in the BCL-2 protein level (38%) and an increase in BAD levels (40%) in PLK2 cells (Fig. 3C). Furthermore, we observed an increase in the levels of cleaved (activated) caspase-9 in PLK2 cell lysates compared with that in PC-3V cells. These results establish that IL-8-CXCR1 axis plays an integral role in the survival of AIPC cells.

**CXCR-1 depletion reduced angiogenic potential.** We investigated whether CXCR1 depletion affects VEGF levels in PC-3 cells, as several previous studies implicate IL-8 in VEGF secretion and angiogenesis (7). We found a significant decrease in VEGF mRNA in PLK2 cells (86% decrease) compared with that of PC-3V cells

**Figure 3.** Increased apoptosis activity in CXCR1-depleted PC-3 (PLK2) cells. A, levels of cleaved PARP in PC-3V and PLK2 cells specific antibody that recognizes only cleaved PARP (Cell Signaling, Inc.) was used as the primary antibody. B, decrease in mitochondrial membrane potential in PLK2 versus PC-3V cells. Relative accumulation of monomeric JC-1 dye in PC-3V, PLK2, and in PC-3V cells treated with 100 nmol/L Staurosporine (Sts; Cell Signaling, Inc.) for 48 h was determined using flow cytometry (27). C, levels of apoptosis-related mitochondrial proteins in PLK2 and PC-3V cells. Levels of BCL-2, BAX and BCL-XL, and cleaved caspase-9 were determined by Western blotting from 72-h culture of untreated PC-3V and PLK2 cells. Comparable band density of β-actin from PC-3V and PLK2 samples assured equal loading of total proteins. D, reduction in VEGF mRNA and CXCR2 mRNA in PC-3V and PLK2 cells. Levels of VEGF mRNA was determined by Q-RTPCR as described before (11).
Determination of secreted level of VEGF in PLK2 cells by an ELISA (R&D Systems, Inc.) showed that VEGF protein level was also significantly lower (≤43%; data not shown), indicating a potential effect of CXCR1 in angiogenesis activity of CaP cells.

CXCR1 depletion in the absence of IL-8 has no effect on cell proliferation in androgen-responsive CaP cells. Previous reports (11, 36) have shown that IL-8–nonproducing CaP cells, such as LNCaP and LAPC-4, express CXCR1 and respond to IL-8 if provided externally (11, 36). However, it is not known whether CXCR1 can function in these cells in the absence of IL-8. We transiently transfected LNCaP and LAPC-4 cells with PLK2 shRNA plasmid to deplete them of CXCR1 and determined whether it has

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any physiologic effect. The level of CXCR1 mRNA in these cells was reduced by ~90% at 72 hours following PLK2 DNA transfection (data not shown). However, we found no significant change in the cell cycle phase distribution in transfected CXCR1 LNCaP cells when compared with the vector DNA-only–transfected LNCaP cells (see Supplementary Fig. S2A). Similarly, LAPC-4 cells showed no changes in mitogenic activity and, following PLK-2 transfection, failed to respond to IL-8 added to the culture medium (Supplementary Fig. S2B).

**CXCR1 regulates CXCR2 expression.** Previous reports show that CXCR1 and CXCR2 are coregulated as well as transregulated by each other at the protein level (37, 38). We examined whether depletion of CXCR1 leads to an alteration in CXCR2 levels. As shown in Fig. 4A, we observed ~80% decrease in CXCR2 mRNA levels in PLK2 cells compared with that in PC-3V cells, showing that depletion of CXCR1 leads to downregulation of CXCR2. To determine whether the reciprocal is true, we used CXCR2 siRNA (Smart pool siRNA, Dharmacon) in PC-3V and PLK2 cells and investigated phenotypic changes. As shown in Fig. 4B(i), silencing CXCR2 alone in PC-3V cells did not inhibit cell proliferation or changes in Cyclin D1 levels. However, the suppression of CXCR1 and CXCR2 together, by RNA interference, caused 33% decrease in Cyclin D1 level. In addition to siRNA approach, we used CXCR2 shRNA to silence CXCR2 by >90% as monitored by Q-RTPCR. Furthermore, Cyclin D1 level did not change in any of the transfected cultures with four separate shRNA constructs (Fig. 4B, ii). We also tested a small molecular inhibitor of CXCR2 (SB225002, Alexis Biochemicals; IC₅₀ ~8 nmol/L) to inhibit cell proliferation (39). PC-3 cell cultures treated with SB225002 for 72 hours showed <17% inhibition of cell proliferation at the highest concentration tested (Supplementary Fig. S3A). These results show that IL-8–mediated cell proliferation in PC-3 cells is executed by CXCR1, but not CXCR2.

**Demonstration of specificity of CXCR1 shRNA.** Although PLK2 shRNA construct did not share sequence homology with CXCR2, we reasoned that the observed silencing effect on CXCR2 might be due to some off-target effect of PLK2 plasmid, or indeed, silencing CXCR1 affects CXCR2 by cross-regulation. We used RNA interference rescue (40), to normalize shRNA-induced depletion of CXCR1. We used multisite-directed mutagenesis and introduced six to seven nucleotide substitutions within the PLK2 shRNA hybridizing sequence, while retaining the amino acid identity of wild-type protein (Supplementary Table S1). As shown in Fig. 4C, introduction of the mutated CXCR1 cDNA in PLK2 cells not only restored the shRNA induced CXCR1 depletion but also increased CXCR1 mRNA levels, as multiple copies of the mutant cDNA is likely incorporated in the cells. Furthermore, cDNA transfection for shRNA rescue also increased CXCR2 mRNA levels, indicating coregulation of CXCR1 and CXCR2 at the level of transcription, or before translation. As shown in Fig. 4D (i), PLK2 cells transfected with mutant CXCR1 cDNA expressed functional CXCR1 protein and rescued CXCR1 function (Fig. 4D, ii). Further, characterization of mutant 6 and mutant 7 clones of PLK2+CXCR1-DDK-Myc transfectants showed increase in VEGF mRNA comparable with that of PC-3V (Fig. 5A) and abolition of spontaneous apoptosis, as indicated by lack of cleaved PARP (Fig. 5B). These results show that PLK2 shRNA–mediated mRNA depletion leads to downregulation of both CXCR1 and CXCR2, and further, this downregulation has significant physiologic effect in tumor cells.

**Decreased tumor growth and decreased angiogenic activity in CXCR1-depleted PC-3 tumors.** We generated tumors in athymic mice by injecting PC-3V and PLK2 cells. As shown in Fig. 6A, we observed a significant reduction of tumor growth (54% reduction in tumor volume) in mice injected with PLK2 cells, compared with the tumors in mice injected with PC-3V cells. We found that along with CXCR1, VEGF mRNA levels were significantly reduced in PLK2 tumors compared with that of PC-3V tumors (Fig. 6B). Furthermore, the extent of apoptotic activity, as measured by the TUNEL assay, was increased in PLK2 tumors. (Fig. 6C). Furthermore, the levels of cleaved PARP in PLK-2 tumor tissues determined by Western blotting was significantly increased (63%) in PLK2 tumors (Fig. 6D) with a decrease in Cyclin D1 (32%).

**Discussion**

We provide a comprehensive data to establish the significant contribution of CXCR1, in CaP cell growth, survival, and tumorigenic potential. The CXCR1 was active in AIPC cells where the cells produce its ligand, IL-8. It was not active in an IL-8–deficient androgen-responsive cell line, such as LNCaP. Using both positive
(overexpression) and negative (shRNA-mediated knockdown) expression strategies, we showed the distinct role of CXCR1, and the lack of such function by CXCR2 in cell proliferation and survival (inhibition of apoptosis) in CaP cells.

Growth inhibition induced by CXCR1 depletion was causally associated with alteration in the expression of critical cell cycle regulatory proteins. Cell proliferation involves activation and suppression of a large repertoire of both cytoplasmic and nuclear proteins. However, we could show a link between IL-8-CXCR1 signaling and cell cycle progression. A depletion of CXCR1 led to both cell cycle arrest and decreased expression of molecules that promote the transit of cells in G0-G1 phase to S phase. This included decreased expression of Cyclin D1, increased expression of P27, but reduction in the levels of phosphorylated ERK1/2 and Rb proteins (Fig. 1B and C). These results show that IL-8-CXCR1 signaling is upstream in the mitogenic pathway, and controls several critical molecules during cell cycle progression. These finding increase the potential to inhibit critical cell proliferation pathways using inhibitors of IL-8 and/or CXCR1.

MacManus and colleagues (25) reported that external addition of IL-8 can also regulate the expression of Cyclin D1 in AIPC cells, such as PC-3 and DU145. We show here that blocking one of its receptor by mRNA depletion is sufficient to produce not only cell proliferation inhibition and cyclinD1 synthesis but also induction of apoptosis and angiogenic molecules. Because IL-8 is constitutively produced by AIPC cells, an autocrine growth stimulation is likely to be present in these cells, and probably in patients with AIPC, as increased IL-8 levels have been reported in patients with AIPC (22, 41). Considering multiple publications have reported the differential expression of IL-8Rs (CXCR1 and CXCR2) in aggressive prostate tumors and the lack of them in benign tissues or normal epithelial cells (23–25), our results clearly establish the critical and functional role of such increased expression of IL-8Rs in aggressive tumors. The present finding in concert with earlier reports establishes that external and internal stimulation of CXCR1 by IL-8 mediate autocrine growth stimulation. Furthermore, our experiments show active growth signaling by autocrine mechanism by IL-8 produced by the tumor cells alone, not the stromal cells, a condition necessitated by the earlier results by MacManus and colleagues (25). However, our observation that external IL-8 (paracrine stimulation) can induce mitogenic response in LAPC-4 cells, which lack autocrine IL-8 stimulation (Supplementary Fig. S2C), suggests that

Figure 6. CXCR1 depletion inhibits tumor growth. A, growth of PC-3V and PLK2 tumors in athymic mice. Tumor volume was determined twice weekly. Points, mean of tumor volume measured posttumor cell injection; bars, SD. Tumor growth was significantly different in two groups (\( P < 0.05 \), Student’s t-test). B, ex vivo analysis of CXCR1 and VEGF mRNA expression. Total RNA was prepared from three tumors from each group to quantitative VEGF and CXCR1 mRNA levels by Q-RTPCR. Columns, mean from three separate Q-PCR reactions from each RNA sample; bars, SD. C, increased apoptosis in PC-3 shRNA CXCR-1 tumors. Apoptotic cells were identified by TUNEL assay. Cells undergoing apoptosis (green, DNA fragmentation; blue, nuclear staining. A, PC-3V tumors; B, CXCR-1 shRNA (PLK2) tumors. D, expression of Cyclin D1 in tumor tissues. The levels of Cyclin D1 were lower in PLK2 tumors compared with that of PC-3V tumors. A significant increase in cleaved PARP (Cell Signaling; binds both PARP and cleaved PARP) was found in PLK2 tumor tissue.
controlling CXCR1 may have a greater consequence than that produced by controlling IL-8 levels for tumor growth inhibition. Although we observed an increase in Cyclin D1 expression by addition of other growth factor, such as EGF, in PC-3V cells, we found a significantly diminished response to EGF signaling in CXCR1-depleted cells (e.g., PLK2: Fig. 2D), indicating that CXCR1-IL8 interaction may mediate EGF–epidermal growth factor receptor mitogenic signaling, as shown in lung cancer cells (42).

The depletion of CXCR1 in PC-3 cells, thus depletion of IL-8–CXCR1 signaling pathway, caused decrease in cell survival via increase in apoptosis (Fig. 3A and B) and it was through mitochondria-mediated (intrinsic) mechanism. To our knowledge, this is the first report of control of mitochondrial integrity by IL-8–CXCR1 signaling. Lack of IL-8–CXCR1 signaling resulted in not only depolarization of mitochondria but also significant decreases in antiapoptotic proteins, BCL2 and BCLXL. Furthermore, CXCR1 depletion caused an increase in proapoptotic proteins, BAX and BAD, and caspase-9 activation. It was reported previously that increased levels of IL-8 in the serum of cancer patients might increase tissue BCL2, and thus increase the survival of tumor cells (43). More over, Nor and colleagues (44) noted that increased IL-8 is associated with increased endothelial cell survival in tumor vasculature, which may cause increased tumor cell survival. This is also evident in PLK2 tumors in vivo, as a steep increase in apoptosis was observed in the tumor tissue, demonstrating potential clinical application of CXCR1 (and therefore, CXCR2 downmodulation) antagonists for clinical application.

Our results show a coregulation of CXCR1 and CXCR2 at the transcriptional level. We show that (Fig. 4C) a concordant downregulation of CXCR2 mRNA when we downregulated CXCR1 by shRNA, and an upregulation of CXCR2 when shRNA knockdown is rescued by shRNA incompatible mutant CXCR1 cDNA. However, downregulation of CXCR2 by siRNA did not alter CXCR1 mRNA, indicating a hierarchical regulation, most likely in the posttranscriptional level of CXCR2 mRNA. Although this may provide a unique opportunity to regulate CXCR2 function, we reserve this investigation for a later study. However, our observation of a hierarchical regulation of CXCR1 and CXCR2 does bear significance in the regulation of tumor angiogenesis, largely controlled by tumor cells’ angiogenic activity largely mediated by CXCR2 (24, 45).

The expression of angiogenic CXC chemokines is high in AIPC cells (7). CXCR2 activation does not contribute to tumorigenesis but it stimulates angiogenesis (24, 45). We corroborate this finding with our results, as we found no change in Cyclin D1 level by depleting CXCR2 mRNA (Fig. 4B, i), and further, a CXCR2-specific inhibitor did not inhibit cell proliferation (Supplementary Fig. S3). Our finding of decreased VEGF in PLK2 cells may be due to the downregulation of both CXCRs, as noted above.

In summary, the data presented in this report clearly establish that IL-8–CXCR1 autocrine activity is essential for AIPC growth and survival. Because AIPC is the most difficult form of CaP to treat, patients typically survive <2 years at this stage. Our study provides a novel avenue to control, potentially, the growth of AIPC, by using inhibitors of IL-8–CXCR1 signaling, or by direct antagonists of IL-8 or CXCR1. Overall, these studies establish a novel strategy to control AIPC.

Disclosure of Potential Conflicts of Interest

We disclose no assumption of potential conflict of interest.

Acknowledgments

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