CXCR4 and CXCL12 (SDF-1) in Prostate Cancer: Inhibitory Effects of Human Single Chain Fv Antibodies

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ABSTRACT

Purpose: Metastasis is a major cause of morbidity in prostate cancer (PCa). Several studies have shown that the chemokine receptor CXCR4 and its ligand, CXCL12 (stromal cell-derived factor-1), regulate tumor cell metastasis to specific organs. Recently, it was demonstrated that CXCL12 enhances PCa cell adhesion, migration, and invasion, implicating CXCR4 in PCa metastasis. In this study, we examined the inhibitory effects of anti-CXCR4 antibodies on CXCL12-mediated PCa cell activities.

Experimental Design: We developed fully human single chain Fv antibodies (scFv), Ab124 and Ab125, against CXCR4 using the yeast two-hybrid system. We performed immunofluorescent staining, flow cytometry, and ELISA-binding assays to measure scFv binding to PCa cells. We also examined the effects of scFv on CXCL12-mediated calcium mobilization, cell migration, and invasion.

Results: Our results confirmed that PCa cell lines express cell-surface CXCR4. Real-time quantitative reverse transcription-PCR and immunohistochemical staining also verified that CXCR4 is expressed in primary cultures of prostate epithelial cells from adenocarcinomas and in human prostate tissues. Ab124 and Ab125 demonstrated specific binding to PCa cell lines by flow cytometry and in binding assays. Preincubation with scFv resulted in significant reduction of CXCL12-induced calcium mobilization in PC-3 and LNCaP cells. Ab124 and Ab125 also inhibited PCa cell migration toward CXCL12, as well as invasion through extracellular matrix gels.

Conclusions: Ab124 and Ab125 inhibit CXCL12-mediated cellular activities by binding the receptor CXCR4. Recombinant scFv are an efficient mode of targeting tumor antigens. Considering the high incidence of PCa, the development of fully human scFv may be a useful therapeutic approach in the prevention and treatment of PCa metastasis.

INTRODUCTION

Prostate cancer (PCa) is the most common noncutaneous malignancy and second leading cause of cancer-related deaths among men in the United States (1). Although early diagnosis of PCa by surveillance improves clinical outcome, metastatic PCa remains a late-stage event with a poor prognosis. Recent work has been aimed at identifying key molecules involved in metastasis as therapeutic targets. Because PCa has a striking tendency to metastasize to bone and local lymph nodes, potential targets for PCa therapy include tumor cell antigens that bind to molecules found at these principal sites of metastasis (2, 3).

Monoclonal antibodies (mAbs) have recently become a new class of cancer therapeutic agents because of their precise target-binding specificity. This is primarily due to the establishment of several technologies to generate human or humanized mAbs. Such methods include humanization of mouse or rat mAbs and transgenic mice engineered to produce human antibodies (4). An alternative strategy is the production of recombinant human single chain variable fragments (scFv) comprised of the antigen-binding variable heavy (VH) and variable light (VL) human immunoglobulin chains, joined with a flexible polypeptide linker. Recombinant scFv have reduced immunogenicity compared with mAb, and their smaller (∼30,000) size facilitates their rapid clearance from the blood and penetration deeper into tumors from the vasculature. The lack of constant regions in scFv also prevents retention in tissues such as liver and kidney. Thus, scFv may be an efficient, versatile mode of targeting specific tumor antigens (4, 5). Single chain antibodies directed against growth factors and their receptors (6, 7), oncogenes (8, 9), or transcription factors (10) have been effective in inhibiting tumor growth. Tumor-specific scFv have also been designed as a delivery vehicle for radioisotopes or toxins to malignant cells (11). The proven efficacy of scFv may constitute a novel approach for targeting molecules to inhibit progression to advanced PCa.

Chemokines have emerged as an important group of mediators of cancer growth and dissemination. On the basis of their prominent role in leukocyte trafficking to select anatomical sites during inflammation and homeostasis, chemokines are thought to mediate similar events of tumor cell adhesion, migration, and invasion during metastasis to specific organs (12). The chemokine CXCL12 (stromal cell-derived factor-1) and its receptor,
CXCR4, were found to induce transendothelial migration by acute myeloblastic leukemia and B-lymphoma cells (13, 14). CXCR4 has also been identified as an essential molecule in human stem cell engraftment into the bone marrow of NOD/SCID mice, underlying the importance of CXCL12/CXCR4 in site-specific homing (15). A recent study described the role of CXCR4 in mediating breast cancer cell homing and metastasis to specific organs, including bone and lymph nodes, which constitutively express CXCL12 (16). Studies on a substantial number of other cancers, including prostate (17), non-small-cell lung cancer (18), and neuroblastoma (19), have recently followed, demonstrating that CXCR4 receptors may be critical in orchestrating tumor cell metastasis to specific organs rich in CXCL12. Taichman et al. (17) showed that CXCL12 enhanced PCa cell adhesion to bone marrow endothelial cells and transendothelial migration and invasion, implicating CXCL12 and CXCR4 as important molecules in advanced PCa and metastasis. It was further demonstrated that CXCR4 and CXCL12 are expressed in human malignant prostate tissue but not in normal prostate tissues (20). Thus, it is evident that the CXCL12/CXCR4 biological axis may be a significant target for PCa therapy.

Herein, we studied the inhibitory effects of anti-CXCR4 scFv on PCa cell responses to CXCL12. Anti-CXCR4 scFv, developed using the yeast-two hybrid system, demonstrated specific binding to PCa cell lines. CXCL12-mediated cell signaling and chemotaxis were inhibited by anti-CXCR4 scFv. The development of CXCR4 antagonists such as scFv may be a useful therapeutic modality in the prevention and treatment of PCa metastasis.

**MATERIALS AND METHODS**

**Single Chain Antibody Library Construction.** Poly A+ RNA from human bone marrow, human fetal liver, human spleen, and human peripheral blood leukocytes were purchased from Clontech Laboratories (Palo Alto, CA). First-strand cDNAs were made from the poly(A)+ RNA using the PowerScript reverse transcriptase kit (Clontech). To amplify variable regions of all heavy chain and light chain genes of human antibodies by PCR, we used a set of oligonucleotides that recognize all functional V genes (21). cDNAs encoding heavy chain variable region (VH) and light chain variable region (VL) were joined by a shorter linker sequence (22). The VH linker–VL cassettes were flanked by sequences homologous to the multiple cloning site flanks of the yeast two-hybrid vector pACT2 (23, 24).

The above-assembled PCR products containing scFv were co-transformed with linearized pACT2 DNA into yeast strains Y187 (MATα: ura3-52, his3-200, ade2-101, trp1-901, leu2-3,112, gal4Δ, gal80Δ, LYS2::GALIuAS-GALI TATA-HIS3, GAL2uAS-GAL2 TATA-ADE2, and URA3::MELIuAS-MELI TATA-lacZ; Clontech), pGBK-CXCR4 was transformed into the yeast strain AH109 (MATα: ura3-52, his3-200, ade2-101, trp1-901, leu2-3,112, gal4Δ, gal80Δ, LYS2::GALIuAS-GALI TATA-HIS3, GAL2uAS-GAL2 TATA-ADE2, and URA3::MELIuAS-MELI TATA-lacZ; Clontech). The transformants were selected on synthetic medium lacking tryptophan (SD/-W). AH109 cells containing pGBK-CXCR4 were mated with MATα-type yeast cells (Y187 strain) containing the scFv library following the protocols from Clontech. Colonies able to grow on the yeast synthetic medium lacking adenine, leucine, histidine, and tryptophan (SD/-ALHW) were picked and assayed for lacZ expression using a β-galactosidase filter-lysis assay, as described by the manufacturer (Invitrogen, Carlsbad, CA). Plasmid DNA of pACT2 containing the scFv fragment was retrieved from the yeast cells. The sequences of scFv fragments were determined with an ABI automatic sequencer. Plasmid pGBKTLam (Clontech), which contains a sequence encoding a fusion protein of the Gal4 DNA-binding domain with human lamin C, was used in the specificity analyses.

**Protein Expression in Escherichia Coli.** Each anti-CXCR4 scFv clone was cloned in frame with the leader sequence of pelB at its NH2 terminus, and with the herpes simplex virus (HSV) tag and His tag at its COOH terminus of the expression vector pET25b (+) (Novagen, Madison, WI). The resulting plasmid was used to transform E. coli BL21/DE3 (Novagen). Protein expression of scFv in BL21/DE3 cells was performed according to the instructions from the manufacturer (Novagen). The scFv protein was purified using Ni-NTA following instructions from the manufacturer (Qiagen, Inc., Valencia, CA). The quality of the purified protein was evaluated by SDS-PAGE and immunoblotting with HSV tag antibody (Novagen).

**Cell Culture.** PC-3, DU 145, and LNCaP PCa cell lines, MDA-MB 231 breast carcinoma cells, and HuT 102 T lymphoma cells were obtained from American Type Culture Collection (Manassas, VA). PC-3 cells were cultured in Ham’s F-12 medium. LNCaP, MDA-MB-231, and HuT 102 cells were cultured in RPMI media. DU 145 cells were grown in DMEM media. All cell cultures were supplemented with 10% fetal bovine serum, l-glutamine (2 mM), and penicillin-streptomycin (1%; Invitrogen-Life Technologies, Inc., Carlsbad, CA) and grown at 37°C and 5% CO2.

**Isolation of Primary Prostate Epithelial Cells.** Primary cultures of prostate epithelial cells were established from radical prostatectomy specimens and maintained in serum-free medium, as described previously (25). Normal cells were obtained from peripheral zone tissues that were histologically confirmed to be free of benign prostatic hyperplasia (BPH) or cancer. BPH cultures were isolated from the transition zone, and tissues of origin were histologically confirmed as BPH. Cancer cultures were derived from tissues that were composed of ≥90% adenocarcinoma. The Gleason grades of the cancers of origin ranged from 3/3 to 4/5.

**Real-Time Quantitative Reverse Transcription-PCR.** Total RNA was isolated from primary cultures passaged twice and grown to ∼80% confluency. Cells were fed fresh medium −24 h before extraction of total RNA using RNAeasy kits (Qiagen, Inc.). RNA was extracted from DU 145, PC-3, and LNCaP using Tri-Reagent according to the manufacturer (Molecular
Research Center, Cincinnati, OH). Reverse transcription of 1 μg of RNA was performed using avian myeloblastosis virus reverse transcriptase in a cDNA synthesis kit (Roche, Indianapolis, IN). CXCR4 expression was analyzed by real-time quantitative PCR using a LightCycler instrument and LightCycler-Fast Start DNA Master SyBr Green I reagents (Roche). The following primers were used: CXCR4 sense, 5'-AGCATGACGGACAAGTA-3'; CXCR4 antisense, 5'-GATGAAAGTCGGGAATAGTCAGC-3'; actin sense, 5'-AGATGCCAGATCGTGGTA-3'; and actin antisense, 5'-CACAGCTTCTCCTAATGC-3'.

A standard curve was made using four different concentrations of CXCR4 cDNA (a gift from Dr. Adit Ben-Baruch, Tel Aviv University, Tel Aviv, Israel). Reactions were performed at 95°C for 5 min, followed by 30 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 15 s, 86°C for 15 s, and a melt curve analysis to confirm the purity of the reaction products. The quantitative information in the PCR reactions was derived from the log-linear portion of the amplification curve, where a designated threshold of fluorescence was reached. Raw data from reactions using CXCR4 primers were normalized to data from corresponding reactions using β-actin primers. Data are expressed as the ratio of relative CXCR4 mRNA and actin mRNA. The mRNA ratios are considered 0 if the calculated value of CXCR4, based on the standard curve, is <3 femtograms/100 ng cDNA.

Immunohistochemical Staining. All reagents for immunohistochemical staining were purchased from Vector Laboratories (Burlingame, CA). Paraffin-embedded human prostate tissues, obtained by radical prostatectomy, were obtained from the pathology files from the Northport Veteran Affairs Medical Center. Tissue sections were deparaffinized and rehydrated, followed by treatment with Antigen Unmasking Solution for antigen retrieval. Immunostaining was performed with the Vectastain ABC kit. After blocking nonspecific sites with normal horse serum, tissue sections were incubated with anti-CXCR4 mAb clone 12G5 (25 μg/ml; R&D Systems) or isotype control mouse IgG2a (Chemicon, Temecula, CA). Next, incubations were done with biotinylated horse antimouse IgG, Vectastain ABC reagent, and 3,3'-diaminobenzidine substrate. Slides were counterstained with hematoxylin. Photographs were taken at ×200 magnification on a Nikon TE2000 microscope with an attached Nikon DXM1200F digital camera.

scFv Cell-Binding ELISA. PC-3, LNCaP, and MDA-MB-231 cells were grown in 96-well tissue culture plates until confluent. Culture medium was aspirated, and cells were fixed with cold 1% gluteraldehyde in PBS for 10 min. All incubations were performed at room temperature, and all washes were performed four times with PBS/0.05% Tween 20 between incubations. Plates were blocked in 1% BSA in PBS for 1 h. Control scFv (Ab16) or anti-CXCR4 scFv (Ab124 or Ab125) were diluted in PBS at various concentrations and incubated in triplicate wells for 2 h. Plates were then incubated with anti-HSV-Tag mAb (0.5 μg/ml in PBS; Novagen) for 1 h, biotinylated goat antimouse IgG (1:1000 in PBS; KPL Laboratories, Gaithersburg, MD) for 1 h, streptavidin-alkaline phosphatase conjugate (1:1000 in PBS; Invitrogen-Life Technologies, Inc.) for 30 min, and p-nitrophenyl phosphate substrate tablets dissolved in 0.1 M glycine/1 mM MgCl₂/1 mM ZnCl₂ (Sigma-Aldrich, St. Louis, MO). Absorbance was read at 405 nm on a microplate reader.

Immunofluorescent Staining. Cells were removed from culture flasks using Cellstripper (Fisher Scientific, Springfield, NJ), a nonenzymatic cell dissociation solution. Cells were washed and resuspended (1 × 10⁶ cells/ml) in PBA buffer (PBS/1% BSA/0.01% sodium azide). All incubations were performed on ice for 30 min, and washes consisted of two washes with PBA buffer. For membrane and intracellular staining with anti-CXCR4 mAb, cells were incubated with mAb clone 44716 (10 μg/ml; R&D Systems) or mouse IgG₂a (Chemicon), followed by Alexa Fluor 488 F(ab')₂ fragment of goat antimouse IgG (Molecular Probes, Eugene, OR). Intracellular staining was performed using fixation medium and permeabilization medium obtained from Calltag Laboratories (Burlingame, CA). Briefly, cells were fixed for 15 min, washed with PBA buffer, then incubated with anti-CXCR4 mAb diluted in permeabilization medium. Flow cytometry was done with a FACSort instrument (Becton Dickinson, San Diego, CA).

A three-step procedure was used for staining with scFv. Cells were incubated with control (Ab16) or anti-CXCR4 (Ab125) scFv (10 μg/ml), followed by anti-HSV-Tag mAb (0.5 μg/ml; Novagen) and Alexa Fluor 488 F(ab')₂ fragment of goat antimouse IgG (10 μg/ml). Cells were analyzed by flow cytometry.

Calcium Flux. Cells were grown in 96-well tissue culture plates until confluent. Calcium flux was measured using a Flex station II scanning fluorometer (Molecular Devices, Sunnyvale, CA). Cells were preincubated with or without scFv (5–15 μg/ml in serum-free media) in triplicate wells for 30 min at 37°C. Assay reagent (excitation wavelength, 485 nm; emission wavelength, 530 nm), in the Calcium Plus Assay kit, was incubated with the cells for 30 min, then medium containing CXCL12 (100–400 ng/ml; Peprotech, Rocky Hill, NJ) was mechanically added to each well. Within 10 s of the addition of CXCL12, a sharp peak in relative fluorescence units (RFU) was observed. Percent inhibition of CXCL12-mediated calcium mobilization by scFv was calculated by determining the ΔRFU from baseline to the peak fluorescence for each sample and averaging the values for each triplicate set. The following calculation was then used to determine percent inhibition of calcium flux, where experimental samples consist of CXCL12 + scFv and control samples consist of CXCL12 only: percent inhibition = 100 − [(experimental ΔRFU value/control ΔRFU value) × 100].

Invasion and Migration Assays. Cell migration or invasion toward CXCL12 was examined using 6.5-mm Transwell chambers (Corning Costar, Cambridge, MA) with 8-μm pores. Filters were coated with human fibronectin (25 μg/ml in PBS; Sigma-Aldrich) for HuT102 cells, laminin (25 μg/ml in PBS; ICN Biomedicals, Aurora, OH) for PC-3 cells, or Matrigel (BD Biosciences, San Diego, CA) for invasion assays with PC-3 and LNCaP cells. Cells were removed from culture flasks using Cellstripper and resuspended (1 × 10⁶ cells/ml) in media containing 0.1% BSA (assay media). CXCL12 (0–400 ng/ml) was placed in the lower wells. Test cells were preincubated with or without scFv (1–10 μg/ml) for 30 min at 37°C, then triplicate sets were placed in the upper chamber (1 × 10⁵ cells/well). HuT102 cells were incubated for 4 h, and migrated cells in the lower
well were counted using a hemacytometer. After overnight incubation, PC-3 cells on the upper surface of the filter were removed using a cotton swab. PC-3 cells on the lower surface of the filter were stained using a Hema 3 staining kit (Fisher Scientific). For each filter, the number of migrated cells in five fields (×200 magnification) was counted. Data were normalized as the migration index: number of migrating cells in experimental group/number of migrating cells in control group without CXCL12. Statistical analysis was performed using a two-tailed t test, where significant values are \( P < 0.05 \).

RESULTS

Expression of Functional CXCR4 on PCa Cell Lines. Recent studies by Taichman et al. (17) have shown that PCa cell lines express functional CXCR4 protein. CXCR4 was additionally shown to be expressed in prostate tissues in vivo. In this study, we first verified the expression of CXCR4 in PCa cell lines using a commercial mAb against CXCR4, clone 44716. This mAb was selected because it recognizes multiple CXCR4 conformations, which can be found on many cell lines and types (26). PC-3, LNCaP, and DU 145 cells were immunostained with anti-CXCR4 mAb 44716. MDA-MB-231 breast carcinoma cells were also included in these experiments as a positive control because CXCR4 has been identified as a potential mediator of breast cancer metastasis (16). To measure the cell-surface and intracellular CXCR4 expression within each cell population, flow cytometry was performed using anti-CXCR4 mAb and a fluorescent secondary antibody. Intracellular staining is indicated as total cellular CXCR4 because the mAb binds to both intracellular and cell-surface CXCR4. As shown in Fig. 1A, intracellular and surface CXCR4 expression was found in PC-3, DU 145, LNCaP, and MDA-MB-231 cells. The percentage of CXCR4-positive cells in each cell population ranged from 53.2 to 93.4% (total cellular) and 45.8 to 64.3% (cell surface).

One of the rapid cellular responses elicited by chemokines is the mobilization of \( Ca^{2+} \). To confirm that prostate cell CXCR4 is functional, we measured \( Ca^{2+} \) mobilization in response to CXCL12. Rapid \( Ca^{2+} \) mobilization was detected in PC-3, DU 145, LNCaP, and MDA-MB-231 cells in response to CXCL12 at all of the concentrations tested (Fig. 1B). Thus, these results verified that PCa cells express functional cell-surface CXCR4.

CXCR4 Expression in Primary Cultures of Prostate Epithelial Cells and Human Prostate Tissues. We measured CXCR4 expression in primary cultures of prostatic epithelial cells isolated from radical prostatectomy specimens. Total RNA was isolated from primary cultures from normal peripheral zone \(( n = 10)\), BPH \(( n = 10)\), or cancer \(( n = 10)\). Reverse transcription reactions were performed, and quantitative real-time PCR was used to compare the levels of CXCR4 expression among peripheral zone, BPH, and cancer cells. Significant differences in patterns of CXCR4 expression were found. Only 1 of 10 normal peripheral zone cultures demonstrated measurable levels of CXCR4 mRNA (Fig. 2). In contrast, CXCR4 mRNA was detected in all of the 10 cancer cell cultures. Five of 10 BPH cultures showed CXCR4 expression. We additionally performed flow cytometry to measure CXCR4 cell-surface expression on cancer and normal cell cultures. The results showed that CXCR4

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**Fig. 1** Expression of functional CXCR4 on PCa cell lines. Immunofluorescent staining and calcium mobilization assays were performed on PC-3, LNCaP, DU 145 PCa cells and MDA-MB-231 breast cancer cells. A, flow cytometric analysis of total cellular (= intracellular + cell surface) and cell surface CXCR4 was performed with anti-CXCR4 mAb (bold line) or control mouse IgG2b (thin line) and fluorescent secondary antibody. B, calcium flux was measured in cells grown in microtiter plates using a scanning fluorometer. CXCL12 was added to the cells at the time point, as indicated by the arrow. Calcium mobilization is depicted as a sharp peak in RFU. Data are representative of three separate experiments.
was marginally higher (10%) on cancer than on normal cells (data not shown).

CXCR4 expression in human PCa was additionally analyzed by immunohistochemical staining of human prostate tissues. Representative photographs taken from 10 human prostate specimens are shown in Fig. 3. Incubation with control mouse IgG2a (Fig. 3A) did not show any background staining. Intense specific staining for CXCR4 was found on PCa cells (Fig. 3, E and F), whereas faint positive staining was detected on normal prostate epithelial cells (Fig. 3, C and D). CXCR4 expression was also found on prostate epithelial cells lining areas of prostatic intraepithelial neoplasia (Fig. 3B). Taken together, these results and those of a previous study (20) demonstrate that CXCR4 is more highly expressed in PCa than in normal prostate tissues.

**Construction of the Human Single Chain Antibody Library.** Considering the potential function of the CXCR4/CXCL12 axis in PCa and a growing number of other cancers (16–19, 27–33), we sought to create inhibitory anti-CXCR4 scFv. A human scFv library was constructed in the yeast two-hybrid vector pACT2 that contains sequences encoding the Gal4 activation domain. The scFv antibody fragments consist of variable regions of heavy (VH) and light chain (VL) of full antibodies that are joined by a linker sequence and flanked by sequences at each end that are homologous to the pACT2 multiple cloning sites. Assembled PCR products were cloned into pACT2 by homologous recombination. Such derived human scFv are fused in-frame with the Gal4 activation domain. A total of $5 \times 10^8$ independent yeast colonies have been generated to date. To check the quality of this human scFv library, we randomly picked 21 colonies. Sequence analyses of the inserts indicated that all of the clones contained VH linker-VL cassettes fused in-frame with the upstream Gal4-activation domain, and all of the scFv sequences analyzed were distinct.

**Library Screening.** To determine whether human antibody specific to CXCR4 extracellular loop could be isolated from our scFv library, we cloned a human CXCR4 fragment encoding an extracellular loop into pGBK7, resulting in

**Fig. 2** CXCR4 expression in primary prostatic epithelial cells. Quantitative real-time reverse transcription-PCR was performed using RNA extracted from primary epithelial cell cultures established from radical prostatectomy specimens. Cell cultures consisted of epithelial cells isolated from normal peripheral zone (PZ), BPH, and cancer (CA) zones. Ten RNA samples from each group were subjected to reverse transcription. Quantitative real-time PCR was performed on resultant cDNA using primers for human CXCR4 and β-actin. Data are presented as the relative ratio of CXCR4 mRNA and β-actin mRNA. Data are representative of two separate experiments.

**Fig. 3** CXCR4 expression in human PCa tissues. Immunohistochemical staining was done using anti-CXCR4 mAb (clone 12G5; 25 μg/ml) for detection of CXCR4 in paraffin-embedded human prostate specimens. Ten prostate tissue samples were stained, and representative photographs taken at ×200 magnification are shown. A, isotype control mouse IgG2a staining, as depicted in an area of prostate adenocarcinoma. B, CXCR4 staining in an area of PIN. C and D, CXCR4 staining in normal prostate epithelium. E and F, CXCR4 staining in an area of prostate adenocarcinoma.
pGBK-CXCR4. The construct was transformed into a MATα-type yeast strain, AH109. To screen the scFv library, the AH109 transformants were mated with MATα-type yeast cells containing the library. The scFv library-harboring vector pACT2 contains the LEU2 gene, whereas pGBK-CXCR4 contains the TRP1 gene. Cells harboring both plasmids can grow in the yeast synthetic medium lacking leucine and tryptophan (SD/-LW). Cells containing scFv/CXCR4 interactions would be able to activate expression of the Gal4-dependent reporter genes ADE2 and HIS3, which are built into the genome of the strains, thus allowing the cells to grow on medium lacking adenine, histidine, leucine, and tryptophan (SD/-AHLW). The mated diploid cells of the library were plated on SD/-AHLW plates. These colonies were assayed for the expression of an additional reporter gene, lacZ, in the β-galactosidase colony-lifting assay.

We additionally analyzed the specificities of these lacZ-positive clones. Plasmid DNAs were rescued from yeast cells. They were cotransformed into AH109 with pGBK-CXCR4, or empty vector pGBK7, or pGBK7-Lam, which encodes an irrelevant antigen, human lamin C. The transformants were grown on selection medium lacking leucine and tryptophan (SD/-LW) and were additionally analyzed by the β-galactosidase colony-lifting assay. Two clones, Ab124 and Ab125, showed specificity to human CXCR4 on the β-galactosidase assay and were selected for these studies. We also used a control scFv, Ab16, which specifically recognizes an intracellular protein, human p53. Such scFvs constructs were cloned into pET25b(+), resulting in a fusion protein with a HSV tag and 6x His at their COOH termini. The fusion proteins were expressed and purified from the E. coli-periplasmic space.

**Binding of Ab125 to PCa Cells.** To determine whether Ab124 and Ab125 bind to PC-3, LNCaP, and MDA-MB-231 cells, we performed ELISA binding assays using cells grown in 96-well microtiter plates. Ab16, Ab124, or Ab125 (1–20 μg/ml) were incubated with the cells, followed by anti-HSV-Tag mAb, which recognizes the HSV-Tag site cloned into each scFv. Biotinylated goat antimouse IgG was then used for subsequent colorimetric detection. As shown in Fig. 4, Ab124 and Ab125 bind with high efficiency to PC-3, LNCaP, and MDA-MB-231 cells starting at 5 μg/ml and continuing to increase until 20 μg/ml. Incubation with Ab16 resulted in lower levels of binding indicative of background binding to the cells. Lactate dehydrogenase cytotoxicity assays indicated that scFv concentrations >10 μg/ml were toxic (<97% viability) to PC-3 cells (data not shown). Thus, we did not use concentrations >10 μg/ml for subsequent experiments.

We further examined the binding of Ab124 and Ab125 to the cell surface by performing immunofluorescent staining after incubation with the scFv. Cells were either analyzed by flow cytometry or by fluorescent microscopy. The flow cytometry results shown in Fig. 5 demonstrate highly efficient binding of Ab124 and Ab125 (10 μg/ml) to the surface of PC-3, LNCaP, DU 145, and MDA-MB-231 cells. Greater than 70% of each cell culture expressed Ab125-detectable CXCR4. In contrast, Ab16 binding was comparable with control cells incubated without scFv.

**Inhibitory Effects of Anti-CXCR4 scFv on CXCL12-Mediated Processes.** The results obtained in Fig. 4 demonstrated that Ab124 and Ab125 bind to CXCR4 on PCa and breast cancer cells. We pursued additional experiments to determine whether these anti-CXCR4 scFv have antifunctional activity. Because it is established that Ca2⁺ flux is a rapid response evoked by CXCL12-CXCR4 binding and we observed such a response in PCa cells (Fig. 1C), we studied the effects of Ab124 and Ab125 on CXCL12-induced Ca2⁺ mobilization. LNCaP and PC-3 cells were pretreated with Ab124 or Ab125 (10 μg/ml), then exposed to CXCL12 (100 ng/ml). The peak of

Fig. 4 Anti-CXCR4 scFv-binding assays. ELISA-binding assays were performed to measure anti-CXCR4 scFv (Ab124; Ab125) binding to PC-3 and LNCaP PCa cells and MDA-MB-231 breast cancer cells. Cells were fixed, then incubated with various concentrations of Ab124 (■), Ab125 (▲), or Ab16 (○). Anti-CXCR4 scFv binding was detected by additional incubation with anti-HSV Tag mAb (0.5 μg/ml), biotinylated goat antimouse IgG, and detection reagents. Absorbance was measured at 405 nm using a microplate reader. Data are representative of two separate experiments.
fluorescence, induced by CXCL12, in scFv-treated cells was compared with that of control (no scFv) and Ab16-treated cells. We found that both Ab124 and Ab125 caused significant reductions (33.3 and 46.7%) in Ca\(^{2+}\) flux in PC-3 cells (Fig. 6).

Interestingly, Ab124 exhibited greater inhibitory effects (41.4%) than Ab125 (15.6%) on calcium flux in LNCaP cells. Ab16 demonstrated lower background levels of inhibition in PC-3 (13.3%) and LNCaP (10.9%) cells.

Migration assays toward CXCL12 were also performed to determine whether Ab124 anti-CXCR4 scFv inhibit CXCL12-induced chemotaxis. Experiments using PC-3 cells showed that Ab124 and Ab125 inhibited CXCL12-mediated chemotaxis (Fig. 7). Positive control experiments were also performed with HuT 102 T lymphoma cells. Both Ab124 and Ab125 inhibited migration toward CXCL12 (Fig. 7). Thus, these scFv demonstrate inhibitory effects on chemotaxis of PCa cells, as well as T cells, toward CXCL12. Ab16 demonstrated lesser but statistically significant inhibition of PC-3 cell chemotaxis toward CXCL12.

Invasion through extracellular matrix (ECM) is a major step in tumor cell metastasis. CXCL12 has been shown to stimulate prostate and breast cancer cell invasion through reconstituted ECM (Matrigel; Ref. 16, 17). We further examined the inhibitory effects of Ab124 and Ab125 in invasion experiments. As shown in Fig. 8, Ab124 and Ab125 inhibited CXCL12-induced invasion of LNCaP cells and PC-3 cancer cells through ECM-coated membranes. Similar to the low level of inhibition seen in chemotaxis assays, Ab16 demonstrated lesser but statistically significant inhibition of PC-3 cell invasion in response to CXCL12. These results demonstrate that anti-CXCR4 scFv Ab124 and Ab125 effectively inhibit CXCL12-induced signaling, as well as CXCL12-regulated cellular movement across ECM.

**DISCUSSION**

The mortality rate of PCa is primarily associated with cancer cell dissemination. It is hypothesized that cancer metastasis is influenced by factors expressed in the metastatic organ that promote cancer cell adhesion and invasion (34, 35). PCa predominantly metastasizes to bone (2). CXCL12, which is constitutively expressed in bone, is known to mediate hematopoietic stem cell migration to bone (15). Similarly, lung, liver, and lymph node, which are common metastatic sites of other cancers, express basal levels of CXCL12 (16). Thus, the CXCR4/CXCL12 axis has recently been investigated in a variety of cancers, including prostate (17), as a potential mechanism for organ-specific cancer cell dissemination.
CXCR4 expression in human PCa tissue was recently demonstrated using high-density tissue microarrays consisting of clinical samples from an extensive group of patients. Sun et al. (20) showed that higher levels of CXCR4 protein and mRNA are expressed in primary and metastatic prostate adenocarcinomas than in benign prostate tissue. The real-time PCR data presented in our study demonstrated CXCR4 mRNA expression in all of the primary epithelial cell cultures derived from prostatic adenocarcinomas (10 of 10), whereas CXCR4 was only detected in 1 of the 10 primary cultures of epithelial cells from normal prostate peripheral zone. In contrast to the striking differences in CXCR4 mRNA expression between cancer and normal samples, flow cytometric analyses indicated only a minor (10%) increase in cell-surface CXCR4 on adenocarcinoma cells versus normal epithelial cells. CXCR4 cell-surface expression has been shown to be regulated by posttranslational events, leading to apparent differences in the levels of existing CXCR4 mRNA and protein (36). Such events, which may be cell type specific, are characteristic of chemokine receptors and other G-protein-coupled receptors. Lower levels of receptor cell-surface expression may be mediated by agonist-induced internalization, as well as by receptor phosphorylation by G-protein-coupled receptor kinases or second messenger kinases (37). It is unclear to us why striking differences in CXCR4 mRNA but not protein levels were found on primary PCa cells and normal prostate epithelial cells. However, it is possible that cell-surface CXCR4 was affected by one of several potential posttranslational regulatory mechanisms. Considering the distinct differences in CXCR4 expression demonstrated by Sun et al. (20) and that our immunohistochemical staining showed similar findings, we believe that CXCR4 may indeed play a role in PCa.

Recent in vitro studies by Taichman et al. (17) showed that CXCL12 induces PCa cell adhesion to osteosarcoma cells and bone marrow endothelium, as well as transendothelial migration and invasion. In line with those findings, our results confirmed that membrane CXCR4 is expressed on PCa cell lines and that such CXCR4 is functional, as demonstrated in calcium mobilization assays (Fig. 1).

In addition to CXCR4 expression found in malignant prostate cells, we also found CXCR4 expressed in higher quantities in primary cultures of epithelial cells from BPH, albeit in fewer cases (5 of 10; Fig. 2). These results correlate with previous findings demonstrating increased CXCR4 mRNA in BPH compared with normal tissues (20). The pathogenesis of BPH, which is still obscure, may involve expression of certain factors or surface antigens that have roles in both BPH and PCa. Indeed, increased expression of interleukin 8, a prototypic chemokine, has been shown to be important both in the progression of BPH and in the tumorigenicity of PCa cells (38, 39). Because basal levels of CXCL12 mRNA are expressed in normal and BPH
tissue (20), it is possible that increased CXCR4 expression in BPH leads to retention of proliferating cells via binding to the chemokine. Considering that BPH is a very common prostatic condition in older men, the role of CXCL12/CXCR4 in BPH disease may be an important focus of future studies.

The characterization of CXCR4 as a coreceptor for HIV entry prompted the production of numerous CXCR4 inhibitors in recent years. Additional characterization of CXCR4 in inflammatory diseases and cancer has contributed to the growing interest in developing CXCR4 inhibitors. Several small-molecule inhibitors of CXCR4 have recently been developed. AMD3100, a bicyclam derivative, has proven to be a highly effective inhibitor of HIV infection (40), as well as inflammation (41). Chemotaxis and invasion of acute lymphoblastic leukemia (42) and ovarian carcinoma cells (43) are also inhibited by AMD3100. T22 and T140, small peptidic antagonists of CXCR4, have been shown to inhibit pancreatic cancer cell invasion (44, 45) and pulmonary metastasis in mouse models of melanoma (46) and breast cancer (47). Silencing of the CXCR4 gene in breast cancer cells through an inducible RNA interference system has shown anti-invasive effects, highlighting another possible method of inhibiting cancer metastasis (48).

Because CXCL12 appears to stimulate cancer cell adhesion, migration, and invasion, which are all important steps in metastasis, CXCR4 inhibitors may be a novel approach to prevent metastasis. In this study, we developed scFv antibodies against CXCR4 to test their ability to inhibit CXCR4-mediated activities in prostate cancer cells. A key advantage of scFv is that they are fully human. Thus, in contrast to mouse monoclonal antibodies, scFv may provide a safer therapeutic agent in human disease. Other advantages of scFv over whole antibody molecules in human therapy include lowered retention in the liver and kidneys and smaller size for more efficient penetration into tumors (4, 5). We showed that two anti-CXCR4 scFv, Ab124 and Ab125, bind efficiently to prostate, as well as breast, cancer cells. We further demonstrated that Ab124 and Ab125 inhibit CXCL12-triggered cellular activities, namely calcium mobilization, cell migration, and invasion through reconstituted ECM. These results suggest that Ab124 and Ab125 inhibit CXCR4-mediated functions and may inhibit activities regulating cancer metastasis.

Several studies have demonstrated diverse methods for using scFv in targeting tumors. Radiolabeled scFv specific against tumor antigens have been effective at inducing tumor regression (11). Similarly, scFv fusion proteins have demonstrated antitumor effects by specifically binding tumor antigens and delivering toxins directly to tumor cells (49). A recent study showed that an anti-HER2/neu scFv-toxin fusion protein was cytotoxic to LNCaP prostate cells (9). Angiogenesis and tumor growth have also been inhibited using scFv made specifically against vascular endothelial growth factor (6). Intracellular expression of scFv has successfully targeted intracellular antigens, including activating transcription factor-1, Ras, and p53 (8, 10). Thus, recombinant scFv technology allows multiple ways of targeting tumors with high affinity and efficiency. Some limitations of scFv antibodies in cancer imaging and therapy include rapid renal elimination, short half-life, and transient interactions with target antigens. Such limitations may be overcome by increasing the scFv-antigen affinity with modifications to the antigen-contact regions, by creating multivalent species, and by increasing the size of the scFv (4). The scFv described in this study can be easily converted to full IgG format for in vivo and clinical applications.

Because PCA cell CXCR4 has been shown to be functional and its expression is higher in metastatic tumors than in primary cancer cells, recombinant anti-CXCR4 scFv may have promise as a therapeutic agent in the prevention of PCA metastasis. Future efforts will be focused on testing the ability of anti-CXCR4 scFv to prevent PCA metastasis in experimental models.

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CXCR4 and CXCL12 (SDF-1) in Prostate Cancer: Inhibitory Effects of Human Single Chain Fv Antibodies

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