Angiogenesis, Metastasis, and the Cellular Microenvironment

Microenvironmental Regulation of Chemokine (C-X-C-Motif) Receptor 4 in Ovarian Carcinoma

Maria V. Barbolina1, Mijung Kim1, Yueying Liu4, Jaclyn Shepard2, Abdelhak Belmadani3, Richard J. Miller3, Lonnie D. Shea2, and M. Sharon Stack4

Abstract

The majority of women diagnosed with epithelial ovarian carcinoma (EOC) succumb due to complications of metastatic disease, suggesting that antimetastatic therapies may improve patient survival. EOC metastasis involves intraperitoneal shedding of cells from the primary tumor, followed by adhesion and localized penetration of the submesothelial matrix to anchor metastatic implants. Accumulation of malignant ascites is also common. Thus, a unique microenvironmental niche is established, which includes malignant cells and a plethora of soluble factors secreted by—or in response to—tumor cells. As cells penetrating the submesothelial surface encounter an interstitial collagen-rich extracellular matrix, we have used three-dimensional type I collagen gels to model early events resulting from intraperitoneal anchoring. In this study, we show a novel pathway of CXCR4 upregulation through β1 integrin—and NFκB-dependent signaling pathways in response to three-dimensional type I collagen. We also show the involvement of CXCR4-SDF1 axis in collagen invasion and proliferation, relevant to the metastatic EOC. Our data show that CXCR4 expression in human EOCs, as well as SDF1 presence in the ascites, is correlated with disease progression and metastasis. These data emphasize the importance of the CXCR4-SDF1 axis in EOC metastasis and suggest that this mechanism should be accounted for when targeting EOC metastasis. Mol Cancer Res; 8(5); 653–64. ©2010 AACR.

Introduction

Epithelial ovarian carcinoma (EOC) is a leading cause of death from gynecologic malignancies largely due to i.p. metastasis (1). Unfortunately, the majority of women are diagnosed with late-stage disease, when metastasis has already occurred. Currently available treatments are not efficient in the prevention or retardation of metastatic spread. Optimization of the existing approaches and development of the new ones is held back by the lack of mechanistic knowledge of the biology of EOC metastasis.

Metastatic dissemination of EOC is largely confined to the peritoneal cavity and often involves malignant ascites (2). Metastasizing cells adhere to and disrupt the peritoneal mesothelium and locally invade into the three-dimensional collagen type I (3DCI)—rich submesothelial extracellular matrix to anchor secondary lesions (3, 4). Importantly, in most cases EOC, metastases are confined to the tissues and organs of the peritoneal cavity, and peritoneal metastases ultimately lead to death due to bowel obstruction. This peritoneally localized pattern of metastatic spread suggests the presence of specific microenvironmental factors that provide homing cues to guide malignant cells to receptive niches permissive for the growth of secondary lesions.

We have previously suggested that EOC cell culture in 3DCI gels can be used as a simplified model of peritoneal metastasis (5). Using this consistently reproducible model system, coupled with cDNA microarray analysis and validation of results using human EOC specimens, we have identified several novel EOC metastasis–associated proteins. In particular, results have shown the upregulation of migration-and invasion-promoting genes, such as Wilms’ tumor gene product 1, actinin α-4, and membrane type-1 MMP (5-7). Furthermore, our previous data strongly suggest the potential involvement of additional migration, proliferation, apoptosis, and gene expression–related genes in EOC metastasis (5). An interesting candidate gene found to be transcriptionally upregulated by 3DCI culture is the chemokine (CXC-motif) receptor 4 (CXCR4).

CXCR4 is a G protein–coupled transmembrane receptor initially identified as a cofactor for HIV entry into CD4+ T cells (8). CXCR4 can bind its specific chemokine-designated stromal-derived factor-1 (SDF1; CXCL12). Binding of SDF1 to CXCR4 triggers G protein signalling and activates a variety of intracellular signal transduction pathways and molecules regulating migration, chemotaxis, cell survival, proliferation, and adhesion (9-11). It has been

Authors’ Affiliations: 1Department of Biopharmaceutical Sciences, University of Illinois at Chicago and Departments of Chemical & Biological Engineering and 2Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, Illinois; 3Department of Pathology and Anatomical Sciences, University of Missouri, Columbia, Missouri

Corresponding Author: Maria V. Barbolina, Department of Biopharmaceutical Sciences, University of Illinois at Chicago, 833 South Wood Street, PHARM 355, Chicago, IL 60612. Phone: 312-355-0670; Fax: 312-996-0098. E-mail: mvb@uic.edu

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extensively shown that the CXCR4-SDF1 axis is responsible for stem cell homing, neural cell migration, and tumor metastasis (12-16). It was previously shown that ovarian carcinoma cell lines Caov-3 and IGROV were able to penetrate through basement membrane–mimicking Matrigel in a CXCL12-dependent manner (17, 18). Retardation of metastasis and cell proliferation in response to a CXCR4 antagonist AMD3100 has been observed in a xenograft murine model of EOC (19). This receptor may be a target for antimetastasis drug therapy; moreover, CXCR4-specific drugs, such as AMD3100 (20), have already been developed. However, the mechanistic details of CXCR4 upregulation in EOC remained unknown. Our initial finding of upregulation of CXCR4 in response to 3DCI suggested a potential mechanism for strengthening of the CXCR4-SDF1 axis in advanced and metastatic EOC through elevated production of CXCR4 (5). The goal of this study was to examine the regulation of CXCR4 expression in EOC cells cultured in organotypic 3DCI gels and to evaluate its potential significance as a target for antimetastatic intervention. Here, we show that 3DCI, relevant microenvironment of metastatic EOC cells, induced the expression of CXCR4 through β1 integrin– and NFκB-dependent mechanism. Moreover, active cell surface–localized CXCR4 was contributing to SDF1-directed cell migration that could be efficiently retarded by blocking CXCR4 function with specific antibodies and AMD3100.

Materials and Methods

Materials. The ovarian carcinoma cell lines DOV13, OVCA433, and OVCA429 were kindly provided by Dr. R. Bast, Jr. (M.D. Anderson Cancer Center, Houston, TX) and maintained as previously described (21). OVCA3 cell line was generously provided by Dr. A. Skubitz (University of Minnesota, Minneapolis, MN). Cell lines SKOV-3, ES2, and Caov-3 were obtained from the American Type Culture Collection and maintained according to the manufacturer’s suggestions. LEAF anti-mouse CD29 antibody (clone TS2/16) with adhesion-activating function was purchased from Biolegend. AMD3100 was obtained from the AIDS Reagent Program, NIH. pNFkB1 was purchased from Biolegend. Human recombinant SDF1 was from Sigma.

Three-dimensional matrix cell culture models. We used several three-dimensional cell culture models composed of various matrix components to characterize their interaction with ovarian carcinoma cells and distinguish the effect of matrix geometry and matrix composition on CXCR4 expression. 3DCI (Sigma) gel at 0.8 mg/mL and synthetic 10% polyethylene glycol (PEG) gels containing 0.3 mmol/L Arg-Gly-Asp-Ser peptide (RGDS) were used to mimic three-dimensional matrix conditions encountered by invading ovarian carcinoma cells. Synthetic 10% four-arm PEG-acryl (SunBio) containing 0.3 mmol/L RGDS (Sigma) was prepared by photocross-linking for 5 minutes using 0.5% 2,2-dimethyl-2-phenyl-acetophenone (Ciba) in polyvinylpyrrolidone (600 mg/mL) as the photoinitiator. Cells were cultured atop three-dimensional matrices for various periods of time as described before (7). Control cells were plated either on 10 μg/mL thin layer collagen I (two-dimensional collagen I or 2DCI) or 10 μg/mL thin layer collagen III (2DCIII), or 0.3 mmol/L RGDS (2D).

RNA extraction, cDNA synthesis, and quantitative real-time reverse transcription–PCR. RNA was extracted and cDNA was synthesized using the SV total RNA isolation system (Promega) and cDNA synthesis kit (Quanta). Real-time PCR was carried out with ABI Prism (Applied Biosystems) according to the manufacturer’s instructions as described before (5). SYBR Green was used for quantitative PCR as a double-stranded DNA-specific fluorophore. Primers for the mRNA detection of genes of interest were constructed according to requirements for oligonucleotide primers for real-time reverse transcription–PCR (RT-PCR) using Primer3 software (Table 1). RPL-19 was used as a housekeeping gene control. Before using RPL-19 as a control, it has been established that its expression correlated well with the total RNA concentration and did not change with the time and treatment used in our studies. Efficiency of amplification was determined using the standard curve method. Relative quantification of gene expression between experimental (3DCI) and control (2DCI) samples was measured by normalization against endogenous RPL-19 using the ΔCt method (23). Fold changes were quantified as 2−ΔΔCt method (23) as previously described (23).

Real-time RT-PCR was also used to detect the levels of CXCR4 and SDF1 mRNAs in samples from tissues of normal ovary and ovarian carcinoma patients commercially available from Origene according to the manufacturer’s suggestions. Cycle threshold (Ct) values above 35 were considered as negative expression. RPL19 and ACTNB were expressed in each sample as reported before (5).

Immunohistochemistry. Immunohistochemical analysis was done retrospectively on tumor tissue microarrays prepared with Institutional Review Board approval by the Pathology Core Facility of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University assembled from tissue originally taken for postoperative diagnostic purposes. Monoclonal antibody against human CXCR4 (R&D Systems) was used at 1:100 dilution. Procedures and scoring were done as described before (5-7) by anatomic pathologist Dr. Brian P. Adley blinded to the research design.

Calcium imaging. The intracellular free calcium concentration was measured using digital video microfluorimetry as previously described (24). DOV13 were plated on collagen I–coated glass coverslips, rinsed with HEPES buffer, followed by the addition of 2 mmol/L fura-2 AM.
siRNA, control siRNA (Santa Cruz Biotechnology), or a specific CXCR4 antagonist blocking antibodies (AnaSpec), nonspecific IgG (Santa Cruz Biotechnology), for 20 minutes and allowed to invade toward untransfected cells seeded in the top chamber were allowed to invade through collagen toward SDF1 expressing plasmid and ovarian carcinoma cells transfected with pBABE constructs cultured on 2DCI and 3DCI using the Dual Luciferase system (Promega) according to the manufacturer’s suggestions.

**ELISA.** Ascites samples from 39 patients with benign (n = 6) and malignant (n = 33) ovarian disease obtained with Institutional Review Board approval by Northwestern University were analyzed for SDF1 using the Human CXCL12/SDF1 α Quantikine ELISA kit (R&D Systems).

**Cell proliferation.** A cell proliferation kit based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases (BioVision) was used according to the manufacturer’s instructions.

**Confocal imaging.** To visualize CXCR4 in DOV13 cells cultured on 2DCI and 3DCI, cells were fixed for 10 minutes on ice using 4% paraformaldehyde solution, permeabilized with 0.01% Triton X-100, and blocked in 5% bovine serum albumin for 1 hour at room temperature followed by incubation with 1:100 dilution of the primary anti-CXCR4 antibody (AnaSpec) for 1 hour, then with a 1:500 dilution of secondary anti-rabbit Alexa488-conjugated IgG. Staining was visualized using the Zeiss Pascal confocal microscope and integrated software using a x60 objective. Exposure times were set according to the manufacturer’s instructions.

**Western blotting.** Procedures were done as described before (5-7). The antibodies were used at the following dilutions: 1:200 for human anti-SDF1 antibodies (Santa Cruz Biotechnology) in 3% bovine serum albumin (Sigma), 1:200 for human anti-CXCR4 polyclonal antibody (Santa Cruz Biotechnology) in 3% bovine serum in Tris-Buffered Saline Tween 20 (TBST), 1:1,000 for anti-β-tubulin monoclonal antibody (Sigma) in 5% skim milk in TBST, 1:2,000 for anti-p65 NFκB polyclonal antibodies (Rockland) in 3% bovine serum albumin, and 1:2,000 for anti-actin monoclonal antibodies (Abcam) in 5% skim milk in TBST. Immunoreactive bands were visualized with an anti-(rabbit-IgG)-peroxidase or anti-(mouse-IgG)-peroxidase (Rockland; 1:1,000 in 5% skim milk in TBST) and enhanced chemiluminescence using the LAS3000 (Fujifilm) and LAS3000 ImageReader software.

**Luminescence measurements.** NFκB activity was detected in cells transfected with Renilla luciferase construct and either pNFκB1 or pNFκB2 reporter luciferase constructs cultured on 2DCI and 3DCI using the Dual Luciferase system (Promega) according to the manufacturer’s suggestions.

(Molecular Probes) for 30 minutes at room temperature. Complete dye deesterification was achieved by removing fura-2 AM, rinsing, and keeping the cells in the dark for 30 minutes. Glass coverslips were mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope equipped for digital fluorescence microscopy. Fluorescence was digitally monitored at 520 nm after excitation at 340 nm (bound Ca2+) and 380 nm (free Ca2+). Ratios of F340/F380 were collected before and during treatment with 20 nmol/L SDF1. 

**Table 1.** Sequences of the primers used in real-time RT-PCR experiments

<table>
<thead>
<tr>
<th>Gene (official name)</th>
<th>GeneBank accession number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4 (CXCR4)</td>
<td>NM_003467</td>
<td>Forw: 5' ACTGGCCATTGTGGGCAA 3'</td>
</tr>
<tr>
<td>SDF1 (CXCL12)</td>
<td>NM_000609</td>
<td>Rev: 5' GTCCGTAGTCCTCTCA 3'</td>
</tr>
<tr>
<td>p65 NFκB (REL)</td>
<td>NM_021975</td>
<td>Forw: 5' GATCCAGAGGACCTTGGAGA 3'</td>
</tr>
<tr>
<td>p50 NFκB (NFκB1)</td>
<td>NM_003998</td>
<td>Rev: 5' CCACTCTGATAGCGACTC 3'</td>
</tr>
<tr>
<td>p52 NFκB (NFκB2)</td>
<td>NM_002502</td>
<td>Forw: 5' CATATTGGAAGGCAGT3 3'</td>
</tr>
</tbody>
</table>

*α* ×60 objective. Exposure times were set according to the following dilution: 1:200 for human anti-SDF1 antibodies (Santa Cruz Biotechnology) in 3% bovine serum albumin (Sigma), 1:200 for human anti-CXCR4 polyclonal antibody (Santa Cruz Biotechnology) in 3% bovine serum in Tris-Buffered Saline Tween 20 (TBST), 1:1,000 for anti-β-tubulin monoclonal antibody (Sigma) in 5% skim milk in TBST, 1:2,000 for anti-p65 NFκB polyclonal antibodies (Rockland) in 3% bovine serum albumin, and 1:2,000 for anti-actin monoclonal antibodies (Abcam) in 5% skim milk in TBST. Immunoreactive bands were visualized with an anti-(rabbit-IgG)-peroxidase or anti-(mouse-IgG)-peroxidase (Rockland; 1:1,000 in 5% skim milk in TBST) and enhanced chemiluminescence using the LAS3000 (Fujifilm) and LAS3000 ImageReader software.

**Transient transfections.** Transient transfections were done using the lipofection method with Lipofectamine 2000 (Invitrogen) as a vehicle. p65 NFκB siRNA, CXCR4 siRNA, control siRNA (Santa Cruz Biotechnology), pNFκB1, pNFκB2, and Renilla luciferase expression constructs were transiently transfected into DOV13 cells according to the manufacturer’s instructions.

**Collagen invasion.** Invasion assays were done using Transwell chambers (0.8 μm, BD Biosciences) and human collagen I (Sigma) as described before (25) with the following modifications. Because ovarian carcinoma cells constitutively express MMPs at elevated levels (7, 25, 26) and SDF1 can be proteolytically degraded by MMPs (27), we modified the experimental setup to have a constant supply of SDF1. Monolayer of COS7 cells cultured on 2DCI and 3DCI, cells were fixed for 10 minutes on ice using 4% paraformaldehyde solution, permeabilized with 0.01% Triton X-100, and blocked in 5% bovine serum albumin for 1 hour at room temperature followed by incubation with 1:100 dilution of the primary anti-CXCR4 antibody (AnaSpec) for 1 hour, then with a 1:500 dilution of secondary anti-rabbit Alexa488-conjugated IgG. Staining was visualized using the Zeiss Pascal confocal microscope and integrated software using a x60 objective. Exposure times were set according to the cells in 2DCI to visualize weak membranous CXCR4 staining. Z-stacks of the entire cell had been acquired.

**Confocal imaging.** To visualize CXCR4 in DOV13 cells cultured on 2DCI and 3DCI, cells were fixed for 10 minutes on ice using 4% paraformaldehyde solution, permeabilized with 0.01% Triton X-100, and blocked in 5% bovine serum albumin for 1 hour at room temperature followed by incubation with 1:100 dilution of the primary anti-CXCR4 antibody (AnaSpec) for 1 hour, then with a 1:500 dilution of secondary anti-rabbit Alexa488-conjugated IgG. Staining was visualized using the Zeiss Pascal confocal microscope and integrated software using a x60 objective. Exposure times were set according to the following dilution: 1:200 for human anti-SDF1 antibodies (Santa Cruz Biotechnology) in 3% bovine serum albumin (Sigma), 1:200 for human anti-CXCR4 polyclonal antibody (Santa Cruz Biotechnology) in 3% bovine serum in Tris-Buffered Saline Tween 20 (TBST), 1:1,000 for anti-β-tubulin monoclonal antibody (Sigma) in 5% skim milk in TBST, 1:2,000 for anti-p65 NFκB polyclonal antibodies (Rockland) in 3% bovine serum albumin, and 1:2,000 for anti-actin monoclonal antibodies (Abcam) in 5% skim milk in TBST. Immunoreactive bands were visualized with an anti-(rabbit-IgG)-peroxidase or anti-(mouse-IgG)-peroxidase (Rockland; 1:1,000 in 5% skim milk in TBST) and enhanced chemiluminescence using the LAS3000 (Fujifilm) and LAS3000 ImageReader software.

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**Western blotting.** Procedures were done as described before (5-7). The antibodies were used at the following dilutions: 1:200 for human anti-SDF1 antibodies (Santa Cruz Biotechnology) in 3% bovine serum albumin (Sigma), 1:200 for human anti-CXCR4 polyclonal antibody (Santa Cruz Biotechnology) in 3% bovine serum in Tris-Buffered Saline Tween 20 (TBST), 1:1,000 for anti-β-tubulin monoclonal antibody (Sigma) in 5% skim milk in TBST, 1:2,000 for anti-p65 NFκB polyclonal antibodies (Rockland) in 3% bovine serum albumin, and 1:2,000 for anti-actin monoclonal antibodies (Abcam) in 5% skim milk in TBST. Immunoreactive bands were visualized with an anti-(rabbit-IgG)-peroxidase or anti-(mouse-IgG)-peroxidase (Rockland; 1:1,000 in 5% skim milk in TBST) and enhanced chemiluminescence using the LAS3000 (Fujifilm) and LAS3000 ImageReader software.
Results

**CXCR4 is upregulated by organotypic 3DCI culture and is associated with advanced and metastatic ovarian carcinoma.** Interaction of ovarian carcinoma cells with 3DCI gels can be used as a simplified model to identify novel genes potentially associated with EOC i.p. metastasis, especially when coupled with cDNA microarray analysis and validation of the *in vitro* findings in human EOC specimens (5, 28). Using this model to elucidate novel targets for antimetastatic intervention has revealed the upregulation of an important migration-related protein receptor, CXCR4 (5). In the current study, EOC cell culture on 3DCI resulted in the significant upregulation of CXCR4 in four of six tested ovarian carcinoma cell lines including DOV13, OVCA433, SKOV-3, and Caov-3 (Fig. 1A). Interestingly, ES2 cells, representative of the clear cell EOC histotype, were characterized by the very low expression of CXCR4 (Ct value of 34 when Ct for housekeeping gene *RPL19* was 20) that was further decreased upon 3DCI stimulation (data not shown). Upregulation of CXCR4 RNA was accompanied by increased levels of CXCR4 protein in lysates of OVCA433, SKOV-3, Caov-3, and DOV13 cells cultured in 3DCI (Fig. 1B). As the membrane-bound CXCR4 participates in SDF1-mediated chemotaxis, the cellular localization of CXCR4 was evaluated in cells grown on thin layer versus 3DCI using immunofluorescent confocal microscopy. Total CXCR4 staining was enhanced in cells cultured on 3DCI, with particular enrichment at the tips of filopodial protrusions (Fig. 1C).

A robust SDF1-induced Ca response signal indicated functional CXCR4 in DOV13 cells (Fig. 1D). Organotypic collagen culture places cells in a constrained three-dimensional milieu, but also engages collagen–binding integrins. To differentiate which of these stimuli was responsible for CXCR4 induction, DOV13 cells were cultured in three-dimensional synthetic PEG gels to provide a three-dimensional milieu in the absence of integrin engagement, or in Matrigel to engage type IV collagen– and laminin-1–binding integrins. Only three-dimensional gels composed of type I collagen induced the strong upregulation of CXCR4, whereas PEG gels and Matrigel did not significantly modulate expression (Fig. 1E). This implies a potential involvement of β1 integrins in the mechanism of CXCR4 upregulation.

Examination of CXCR4 expression in normal ovarian surface epithelium and in EOC specimens from stage I to IV tumors showed a progressive increase in CXCR4 expression from early-stage (I-II) to late-stage (III-IV) tumors (Table 1; Fig. 2A), with a smaller overall percentage of CXCR4-positive nonmalignant normal ovarian tissues (Fig. 2A). This is supported by the immunohistochemical examination of 13 paired specimens of primary tumor and peritoneal metastasis from the same surgical procedure. Although CXCR4 expression was detected in only one primary tumor (8%), positive CXCR4 immunoreactivity was observed in 46% of metastases (Fig. 2B).

**Upregulation of CXCR4 on 3DCI through NFκB is mediated by β1 integrin signaling.** Our observations suggested that CXCR4 was upregulated by the 3DCI but not other three-dimensional matrices and the planar substrata (Fig. 1E). It has been previously shown that 3DCI engages α3β1 and α2β1 integrins on the surface of the EOC cells (25, 26). Based on these findings, we tested whether β1 integrin signaling pathway is regulating expression of NFκB and, subsequently, CXCR4. Cells were preincubated with CD29 antibodies at 1:100 dilution for 20 minutes and then cultured on 2DCI and 3DCI for 30 minutes, 1, 2, and 4 hours. Cells were collected; RNA was extracted; and cDNA was synthesized followed by quantitative real-time RT-PCR detection of p65 (RELA) and CXCR4 RNAs. Our results showed that already after 30 minutes of 3DCI culture, p65 was significantly upregulated in the presence of adhesion-activating antibodies (Fig. 3A). Subsequently, upregulation of CXCR4 was significantly stronger in the presence of CD29 antibodies already after 2 hours of culture (Fig. 3B), indicating that upregulation of p65 (RELA) and CXCR4 on 3DCI is regulated through β1 integrin signaling.

**NFκB participates in CXCR4 regulation by 3DCI.** To elucidate the molecular mechanisms by which 3DCI culture regulates the expression of CXCR4 in EOC cells, experiments were done to examine the involvement of NFκB signaling. The rationale for this approach is based on the data showing NFκB-dependent CXCR4 expression in breast, prostate, and lung cancer (29-31), with particular emphasis on the involvement of the p65 catalytic subunit (RELA) of the NFκB complex. To evaluate whether NFκB signaling is activated in EOC cells cultured on 3DCI, cells were transfected with pNFκB1 and pNFκB2 luciferase reporter constructs containing multiple copies of NFκB binding sequence upstream of a minimal TA promoter and the TATA box from the Herpes simplex virus thymidine kinase promoter–driving expression of the luciferase gene, followed by the culture on 3DCI or thin layer collagen I (2DCI). Increased signal arising from NFκB reporter luciferase constructs was observed in cells cultured in 3DCI compared with 2DCI (Fig. 4A), suggesting the enhanced activation of the NFκB pathway in 3DCI. To further evaluate the potential involvement of NFκB signaling in the upregulation of CXCR4 induced by 3DCI, the expression of the p65 subunit was silenced using specific siRNA, cells were cultured on 3DCI or 2DCI, the and expression of CXCR4 was evaluated using qPCR. Silencing of p65 efficiently eliminated the 3DCI-induced upregulation of CXCR4, whereas high-level expression was maintained in cells transfected with control siRNA and PBS (Fig. 4B and C). The p65 subunit of NFκB can form a complex with either the p50 or p52 subunits to make a fully functional transcriptionally active complex (32). Analysis of human EOC RNAs showed the expression of p65, p50, and p52 in human ovarian tumors (Table 2). Further, of 41 tested human ovarian carcinoma RNAs, 63% (26) expressed either one or both subunit combinations (p65/p50 and p65/p52) that would allow for the assembly of functional NFκB. Comparison of the CXCR4 coexpression with p65/p50
FIGURE 1. CXCR4 is upregulated by 3DCI in EOC cells. A, ovarian carcinoma cell lines DOV13, OVCA433, SKOV-3, Caov-3, OVCA429, and OVCAR3 were cultured on 2DCI and 3DCI for 8 h. Cells were collected; RNA was extracted; cDNA was synthesized; and CXCR4 RNA was detected using real-time RT-PCR as described in Materials and Methods. CXCR4 RNA ratio was found using the $2^{-\Delta\Delta Ct}$ method (23), was averaged ($n=4$), and was plotted. *, $P<0.005$ (Student’s $t$ test; comparisons: Ct values on 2DCI versus those on 3DCI). B, OVCA433, SKOV-3, Caov-3, and DOV13 were cultured on 2DCI and 3DCI for 24 h and subjected to Western blot thereafter. CXCR4-specific antibodies (Santa Cruz Biotechnology) were used at 1:200 dilution; anti-β-tubulin antibody was used at 1:1,000 dilution, as indicated in Materials and Methods. Histogram shows levels of CXCR4 expression relative to the loading control β-tubulin; *, $P < 0.005$ (Student’s $t$ test; comparisons included normalized band intensities on 2DCI versus those on 3DCI). C, DOV13 were cultured on 2DCI and 3DCI for 24 h and subjected to immunofluorescent staining. CXCR4-specific antibodies were used at 1:100 dilution. Confocal images were obtained using a Zeiss confocal microscope at ×60 magnification on the objective. Arrows, accumulation of CXCR4 on the tips of protruding pseudopodia in cells cultured in 3DCI, as indicated. Intensity of the CXCR4 staining was quantified from confocal images using ImageJ (NIH). Lines (white dotted) outlined with a circle were drawn across the tips of the protruding lamellipod (2DCI) and pseudopod (3DCI) to generate the intensity scan. Intensities of CXCR4 expression across the protrusions is plotted on the graph. Light gray line, CXCR4 in protrusions of cells cultured on 2DCI; black line, CXCR4 in protrusions of cells cultured on 3DCI, as indicated. Shown are representative images of three independent experiments. D, SDF-1 induces $[Ca^{2+}]_i$ changes in DOV13. Cells were imaged with digital video microfluorimetry as described in Materials and Methods for 15 min as indicated. DOV13 responded to SDF-1 and ATP. A total of 46 cells (96%) from three independent experiments of 48 total (all cells in the field of view) showed $[Ca^{2+}]_i$ increase when stimulated by SDF1. ATP-induced $Ca$ responses were present in all cells tested. E, DOV13 was cultured on 3DCI, 2DCI, 3DPEG, RGD-coated plates, 3DMatrigel and thin-layer Matrigel for 8 h. Cells were collected; RNA was extracted; cDNA was synthesized; and CXCR4 RNA was detected using real-time RT-PCR as described in Materials and Methods. CXCR4 RNA ratio was found using the $2^{-\Delta\Delta Ct}$ method (23), was averaged ($n=3$), and was plotted. Black column, CXCR4 expression on 3DCI is from A and is shown here for comparison; *, $P < 0.05$ (comparisons: Ct values on two dimension versus those on three dimension for all matrices).
or p65/p52 revealed that 72% of CXCR4-positive ovarian carcinoma specimens also express either or both p65/p50 and p65/p52, suggesting that the NFκB-mediated upregulation of CXCR4 may occur in a significant number of human disease specimens (Table 2).

**CXCR4-SDF1 axis in invasion of 3DCI gels and proliferation.** To examine the potential functional consequences of CXCR4 activation by 3DCI, the penetration of EOC cells through 3DCI gels was evaluated. This assay used a modified Boyden chamber 3DCI invasion assay (25) containing SDF1 as a chemoattractant. Our previous data showed that expression of SDF1 in EOC cells is not regulated by 3DCI (5). As ovarian carcinoma cells express MMPs that are able to enzymatically inactivate SDF1 (27), instead of creating a solution containing SDF1, a constant source of SDF1 was introduced into the system. Monolayer COS-7 cells transfected with pBABE/SDF-1 were plated on the bottom of the invasion chamber receptacle, providing constant SDF1 in the system (Fig. 5A). Transfection efficiency was monitored using green fluorescence emitting from cotransfected pEGFP-N1. COS-7 transfected with a vector control did not express SDF1 in contrast to the cells transfected with pBABE-SDF1 (Fig. 5B). Ovarian carcinoma cells were seeded atop 3DCI and allowed to invade through collagen toward the lower chamber containing SDF1. Collagen invasion was significantly enhanced by the presence of SDF1 in the outer chamber compared with the control conditions containing only vector-transfected COS-7 cells (Fig. 5C). Downregulation of CXCR4 expression with specific siRNAs markedly decreased cell invasion toward SDF1. SDF1-mediated invasion was dramatically reduced when DOV13 cells were pretreated with CXCR4-blocking antibodies, whereas the addition of nonspecific IgG did not retard SDF1-mediated invasion (Fig. 5C). Addition of a specific antagonist of CXCR4, AMD3100, also dramatically reduced SDF1-mediated invasion (Fig. 5C). AMD3100 is a small-molecule inhibitor of CXCR4, binding of which to the receptor prevents SDF1 binding (20). In control experiments with cell invading toward COS7 not overexpressing SDF1, the addition of either AMD3100 function– or CXCR4 function–blocking antibodies only slightly reduced the levels of invasion. On the other hand, addition of the β1 integrin–activating antibody CD29 (33) increased invasion (Fig. 5C). These data show that the CXCR4-SDF1 axis is activated through β1 integrin–dependent signaling and it can regulate ovarian carcinoma invasive activity.

It has been shown that SDF1 is produced by many cell types, including stromal fibroblasts, macrophages, and cancer cells themselves (18, 34-36). Evaluation of SDF1 expression by human ovarian carcinoma specimens indicated that expression increased with disease progression, as only 16% of early-stage (I-II) specimens were SDF1 positive compared with 41% SDF positive for advanced stage (III-IV) specimens (Fig. 5D). Soluble factors in ascites fluid provide an important contribution to the ovarian cancer microenvironment. Analysis of SDF1 levels in ascites using ELISA showed that SDF1 levels were significantly elevated (>3-fold, P < 0.05) in ascites from women with late-stage (III and IV) EOC compared with early-stage I and benign disease (Fig. 5E). However, 3DCI did not affect SDF1 expression in EOC cells (5).

Our findings (Fig. 5F) agree with the previously published data showing the effect of SDF1 on EOC cell proliferation (37). Interestingly, low levels of SDF1 (2.5 nmol/L) found in nonmalignant ascites did not induce a noticeable
cell proliferation, whereas higher SDF1 levels (25 nmol/L) characteristic of malignant ascites resulted in a significant increase in cell proliferation (Fig. 5F).

Discussion

EOC is the most common gynecologic malignancy with the highest rate of mortality. Metastatic disease leads to death in the majority of patients, whereas early-stage EOC can be effectively treated with a combination of chemotherapy and surgery. The lack of reliable early detection markers coupled with the anatomic location of the ovaries results in the prevalence of patients diagnosed with late-stage disease, with a concomitant likelihood that micrometastasis and macrometastasis have already occurred. Metastatic ovarian carcinoma cells are empowered by a multitude of largely unknown mechanisms that support invasion, migration, and metastasis. Currently used treatments against EOC metastasis are ineffective and the development of new treatments is limited by the lack of knowledge about the biology of metastasis.

The peritoneum is the primary site for metastatic anchoring and the size of metastases can exceed that of the primary tumor, suggesting the presence of a specific microenvironmental factor(s) that attracts disseminating cells to the peritoneum and supports proliferation. Collagen-rich matrix is a host tissue for EOC i.p. metastasis. According to our data, interaction of EOC cells with three-dimensional collagen leads to a dramatic remodeling of the gene expression machinery (5) and leads to the engagement of integrins (25, 38). It is impossible to either eliminate collagenous matrix from the metastatic sites or restrict EOC cell proliferation whereas higher SDF1 levels (25 nmol/L) characteristic of malignant ascites resulted in a significant increase in cell proliferation (Fig. 5F).
Table 2. RNA expression of CXCR4, p65, p50, and p52 subunits of NFκB in EOC and normal ovary human specimens

| Diagnosis*                          | Tumor grade*                           | Stage†        | CXCR4‡       | p65 NFκB‡     | p50 NFκB‡     | p52 NFκB‡     | CXCR4/ NFκB§  |
|-------------------------------------|----------------------------------------|---------------|--------------|---------------|---------------|---------------|---------------|---------------|
| Adenocarcinoma of endometrium, papillary serous | FIGO G3: Poorly differentiated | 0             | No           | Yes           | Yes           | Yes           |               |
| Carcinoma of cervix, squamous cell  | FIGO G3: poorly differentiated        | 0             | Yes          | No            | Yes           | Yes           |               |
| Abscess of tissue                   | Not reported                           | 0             | No           | Yes           | Yes           | Yes           |               |
| Endometriosis                       | Not reported                           | 0             | No           | Yes           | Yes           | No            |               |
| Endometriosis                       | Not reported                           | 0             | No           | Yes           | Yes           | Yes           |               |
| Endometriosis                       | Not reported                           | 0             | No           | No            | No            | No            |               |
| Carcinoma of ovary, endometrioid    | FIGO G2: moderately differentiated     | I             | No           | No            | No            | Yes           |               |
| Adenocarcinoma of ovary, papillary serous | FIGO G2: moderately differentiated     | IA            | Yes          | Yes           | Yes           | No            |               |
| Tumor of ovary, papillary serous, borderline | AJCC GB: borderline malignancy        | IA            | No           | No            | No            | No            |               |
| Tumor of ovary, papillary serous, borderline | AJCC GB: borderline malignancy        | IA            | No           | No            | Yes           | No            |               |
| Carcinoma of ovary, endometrioid    | FIGO G1: well differentiated           | IA            | Yes          | Yes           | No            | Yes           |               |
| Tumor of ovary, serous, borderline  | AJCC GB: borderline malignancy        | IA            | Yes          | Yes           | No            | Yes           |               |
| Tumor of ovary, borderline          | AJCC GB: borderline malignancy        | IA            | No           | Yes           | Yes           | Yes           |               |
| Tumor of ovary, mucinous, borderline | AJCC GB: borderline malignancy        | IA            | Yes          | Yes           | Yes           | Yes           |               |
| Adenocarcinoma of ovary, mucinous   | FIGO G3: poorly differentiated         | IB            | No           | Yes           | Yes           | Yes           |               |
| Adenocarcinoma of ovary, endometrioid | FIGO G3: poorly differentiated         | IB            | Yes          | No            | Yes           | No            |               |
| Tumor of ovary, borderline          | Not reported                           | IB            | Yes          | Yes           | Yes           | Yes           |               |
| Tumor of ovary, mucinous, borderline | AJCC GB: borderline malignancy        | IC            | No           | Yes           | Yes           | Yes           |               |
| Tumor of ovary, serous, borderline  | AJCC GB: Borderline malignancy        | IC            | No           | Yes           | Yes           | Yes           |               |
| Tumor of ovary, serous, borderline  | AJCC GB: borderline malignancy        | IC            | No           | No            | No            | No            |               |
| Adenocarcinoma of ovary, mucinous   | FIGO G2: moderately differentiated     | IC            | No           | Yes           | No            | No            |               |
| Adenocarcinoma of ovary, endometrioid, squamous features | FIGO G2: moderately differentiated     | IC            | Yes          | Yes           | No            | Yes           |               |
| Adenocarcinoma of ovary, serous     | FIGO G3: poorly differentiated         | IIB           | Yes          | Yes           | No            | Yes           |               |
| Adenocarcinoma of ovary, endometrioid | FIGO G3: poorly differentiated         | IIB           | Yes          | Yes           | No            | No            |               |
| Adenocarcinoma of ovary, endometrioid | FIGO G1: well differentiated          | IIC           | Yes          | Yes           | No            | Yes           |               |
| Adenocarcinoma of ovary, papillary serous | FIGO G2: moderately differentiated     | III           | Yes          | Yes           | Yes           | Yes           |               |

(Continued on the following page)
interaction with this matrix when the metastasis had occurred. However, comprehensive investigation into the possible gene expression change is important to approach the treatment of metastasis rationally.

Studies of many cancer types suggest the importance of the CXCR4-SDF1 axis in metastatic spread (39, 40). In breast carcinoma, CXCR4-SDF1 interaction results in the development of lung, liver, and bone metastasis, due to the high level of SDF1 in these organs (41). The CXCR4-SDF1 axis plays a key role in the peritoneal seeding of gastric carcinoma as well (42). Our findings support a model for functional cooperation between malignant EOC cells and their

<table>
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<th>Diagnosis*</th>
<th>Tumor grade*</th>
<th>Stage†</th>
<th>CXCR4‡</th>
<th>p65 NFκB‡</th>
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<tr>
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<td>Yes</td>
<td>Yes</td>
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</tr>
</tbody>
</table>

*Information obtained from the manufacturer (OriGene) regarding Ovarian Carcinoma Panel I.
†Information on stage is related to ovarian carcinoma.
‡Positive expression defined by Ct value below 35 is indicated by “yes”; negative expression defined by the absence of a detectible signal or Ct value equal or higher that 35 is indicated by “no.”
§Cases that simultaneously express CXCR4 and p65/p50 or p65/p52 or both combinations of subunits combined into the NFκB complex (marked √).
FIGURE 5. The CXCR4-SDF1 axis in ovarian carcinoma cell invasion. A, a schematic representation of an invasion chamber setup used in the experiments. DOV13 cells were seeded into the inner well of a Boyden chamber overlaid with 3DCI in the presence or absence of additives (antibodies, small-molecule inhibitors). The well was placed in an outer chamber containing a layer of COS7 cells transfected with control or SDF-1 expression vector. The number of cells penetrating the 3DCI gel and adherent to the lower surface of the filter was enumerated and is shown in C. B, cells were transiently transfected with pBABE-SDF1 and a control vector containing no SDF1-specific sequence insert, were collected, and were lysed. Twenty micrograms of the total protein lysates was subjected to Western blot and probed with SDF1-specific antibodies and β-actin-specific antibodies (loading control). C, DOV13 cells were transiently transfected with control or CXCR4-specific siRNAs or preincubated in the presence or absence of CXCR4-blocking antibodies (0.2 μg/mL), nonspecific IgG (0.2 μg/mL), AMD3100 (10 μmol/L), or adhesion-activating CD29 clone TS2/16 antibodies (1:100 dilution) and allowed to invade for 18 h toward COS7-expressing SDF1, or control vector, as indicated. Experiments were done four times, each in triplicate, were averaged, and were plotted on a graph as a percent of invading cells; columns, mean of 10%; bars, SEM. DOV13 without additives, invading toward COS7 monolayer (white open columns) was arbitrarily set as 100% of invasion; *, P < 0.05; **, P > 0.05. D, expression of SDF1 in human ovarian carcinoma specimens was detected using real-time RT-PCR. Black columns, the values of the cycle numbers at which the accumulation of fluorescent signal from SYBR Green bound to the gene-specific double-stranded DNA PCR product was above the background. Absence of the black columns, no accumulation of the specific PCR product. A total of 48 samples were tested. Results for samples from 1 to 48 are plotted from left to right on the X axis and separated by small ticks. O, samples from normal ovary; I, II, III, and IV, those belonging to ovarian carcinoma FIGO stages I, II, III, and IV, and are separated by long ticks. E, malignant and nonmalignant ascites samples were obtained from women diagnosed with benign ovarian cysts (n = 6), stage I (n = 2), stage III (n = 21), and stage IV (n = 10) EOC. Patients ascites samples were analyzed using ELISA according to the manufacturer’s specifications to determine the levels of CXCL12/SDF1 α; *, P < 0.05. Average for each group is shown with a vertical bar line across the individual values. F, cell proliferation assay. DOV13 were plated on 48-well plates, allowed to adhere followed by o/n serum starvation. Proliferation was initiated by addition of complete media [fetal bovine serum (FBS); open column], serum-free media (no fetal bovine serum; light gray column), 25 nmol/L SDF1 in serum-free media (black column), and 2.5 nmol/L SDF1 in serum-free media (dark gray column) and by the incubation for 24 h. Proliferation was measured by addition of WST-1 to the growing cells followed by 1 h of incubation and measurement of OD440. Experiments were repeated four times, each in triplicate, were averaged, and were plotted; columns, mean; bars, SEM; *, P < 0.05.
microenvironment that results in the retention in the peri-
toneum through the engagement of CXCR4-SDF1 axis.
EOC cells anchor secondary lesions in the collagen-rich
submesothelial matrix of peritoneal organs. The current
data and previous reports show that advanced and meta-
static human ovarian carcinoma tissues express elevated
CXCR4 (37, 43, 44). Furthermore, interaction of multiple
EOC cell types with organotypic three-dimensional-collag-
gen gels induces the robust upregulation of CXCR4 in a
β1 integrin– and NFκB-dependent fashion, providing a
mechanism for the enhanced activation of the CXCR4-
SDF1 axis. This is further supported by ELISA data show-
ing that peritoneal ascites from women with ovarian cancer
are rich in SDF1 that may be produced by a variety of cells
including stromal macrophages. The host tissue is thereby
primed to receive invading cells that can successfully colonize
and proliferate, enhancing morbidity. Thus, the novel
pathway of upregulation of CXCR4 by 3DCI through
β1 integrins and NFκB described in this report is particularly
relevant to the EOC metastasis.

The abundance of CXCR4-positive advanced primary
and metastatic tumor tissues combined with widespread
SDF1 expression in malignant ascites makes CXCR4-
SDF1 interaction an attractive target for antimetastatic in-
tervention. Moreover, elevated levels of SDF1 in malignant
ascites indicate a possible strengthening of this axis in the
metastatic stages of EOC.

Our studies, as well as those of others, show efficient in-
hibition of cell invasion upon addition of a specific CXCR4
antagonist, AMD3100, both in vitro (17, 18) and in vivo
(19). Interestingly, the manipulation of the CXCR4 expres-
sion or activity through specific siRNAs, function-blocking
antibodies, and AMD3100 led to the decrease in cell inva-
sion below the control levels (Fig. 5C), suggesting that
CXCR4 may play a role in other signaling pathways regu-
lating cell migration and invasion. As our invasion assay is
designed to measure both collagenolysis and cell migra-
tion, it is likely that expression of collagenolytic MT1-MMP
and/or cytoskeletal proteins was concomitantly deregulated
as a result of manipulation of CXCR4. MT1-MMP could
be partially regulated through CXCR4-SDF1, as previously
suggested (45, 46). The studies suggested that CXCR4 and
MT1-MMP coordinate their actions during the process of
melanoma metastasis in a way that supports the require-
ment of CXCR4 in early stages, when cells arrive at sites
of metastasis, and necessity of MT1-MMP in late stages,
when metastatic cells invade and disseminate (45).

Furthermore, G protein–coupled receptor CXCR4 regulates
the signaling of multiple pathways, including Akt pathway
(47), which, in its turn, regulates the expression of MT1-
MMP (48-50). G protein–coupled receptors are upstream
regulations of the Rho pathway (51), regulating cell migra-
tion (52). In light of these findings, it is interesting to
speculate that reduced invasion of cells with blocked or si-
cenced CXCR4 may also be due to perturbation of signal-
pathways downstream of CXCR4.

Moreover, SDF1 is a known inducer of EOC cell prolif-
eration (37). In our experiments, only the high levels of
SDF1 found in malignant ascites were able to induce
EOC cell proliferation. Our data show that the presence
of CXCR4 on cell surface as well as the presence of elevat-
ed levels of SDF1 in late-stage EOC leads to the enhance-
ment of at least two crucial properties necessary for
metastatic cell survival, invasion, and proliferation. These
data suggest that disruption of CXCR4-SDF1 may retard
the peritoneal dissemination and proliferation of metastatic
ovarian cancer, resulting in improved outcomes for women
with ovarian cancer. Furthermore, therapeutic targeting of
the intermediate players in this signaling cascade, such as
NFκB and β1 integrin will provide additional options for
directed exclusion of pathways that determine the well-
being of the metastatic cell.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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