Multiple Actions of the Chemokine CXCL12 on Epithelial Tumor Cells in Human Ovarian Cancer

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ABSTRACT

Of 14 chemokine receptors investigated, only CXCR4 was expressed on ovarian cancer cells [C. J. Scotton et al., Cancer Res., 61: 4961–4965, 2001]. To further understand the role of this chemokine receptor in ovarian tumor biology, we studied the action of its ligand, CXCL12 (stromal cell-derived factor 1), on the CXCR4-expressing ovarian cancer cell lines IGROV. Ligand stimulation of the CXCR4 receptor resulted in sustained activation of Akt/protein kinase B and biphasic phosphorylation of p44/42 mitogen-activated protein kinase in IGROV. When IGROV cells were cultured under suboptimal conditions, CXCL12 stimulated their in vitro growth, an effect that was abrogated by neutralizing antibodies to CXCR4. This increase in cell number was attributable to stimulation of DNA synthesis, not protection from apoptosis. CXCL12 treatment of IGROV cells also induced mRNA and protein for tumor necrosis factor α, a cytokine that is expressed by tumor cells in ovarian cancer biopsies. IGROV cells invaded through Matrigel toward a CXCL12 gradient. Invasion was abrogated by the broad spectrum matrix metalloproteinase and TNFα converting enzyme inhibitor Marimastat and was partially inhibited by neutralizing antitumor necrosis factor α antibodies. These effects were not limited to the IGROV cell line. They could also be demonstrated in the CAOV-3 ovarian cancer cell line and primary ovarian tumor cells isolated from ovarian ascites. These biological effects of CXCL12 on IGROV cells were also inhibited by the small molecular weight CXCR4 antagonist AMD3100. Finally, we found abundant intracellular CXCL12 protein in tumor cells in 15 of 18 ovarian cancer biopsies but not in epithelial cells from normal ovary or borderline disease. The chemokine CXCL12 may have multiple biological effects in ovarian cancer, stimulating cell migration and invasion through extracellular matrix, as well as DNA synthesis and establishment of a cytokine network in situations that are suboptimal for tumor cell growth.

INTRODUCTION

Chemokines are small, secreted peptides that control adhesion and transendothelial migration of leukocytes, especially during immune and inflammatory reactions (1). They are divided into four subfamilies: CC, CXC, C, and CX3C based on the position of their NH2-terminal cysteine residues and bind to seven transmembrane domain G protein-coupled receptors, the two major subfamilies that are designated CCR and CXCR. There is strong evidence that infiltration of cancers by host cells is regulated by tumor-derived chemokines (2, 3). For instance, we have found that a complex chemokine network exists in both solid and ascitic tumors of human epithelial ovarian cancer, with a range of CC and CXC chemokines detected (4–7). Chemokine receptor expression is more restricted in the solid tumor microenvironment than ascites (4). Expression of CCL2 (monocyte chemoattractant protein 1) by tumor and stromal cells correlated with the extent of the leukocyte infiltrate in ovarian cancer biopsies as did stromal cell expression of CCL5 (regulated on activation, normal T-cell expressed and secreted) and its receptor CCR1 (4, 5). Tumor-infiltrating leukocytes may contribute to tumor progression and spread by providing tumor-promoting cytokines, angiogenic factors, and matrix metalloproteases and subverting useful host responses to the tumor (6).

However, chemokines may play other roles in cancer. Some are potent angiogenic factors, whereas others can be angiostatic; alterations in the chemokine balance may contribute to the development of the tumor vasculature (8, 9).

In addition, the restricted expression of chemokine receptors, especially CXCR4 and CCR7, by tumor cells, may be one important step in the development of site-specific metastasis (10–17). Tumor cells from breast, prostate, pancreatic and ovarian carcinomas, neuroblastoma, glioblastoma, and some leukemias express chemokine receptors. In breast, prostate and ovarian cancer, neuroblastoma, and leukemia, the respective ligand is expressed at sites of tumor spread. When B16 melanoma cells were transduced with a retroviral vector containing cDNA for the chemokine receptor CCR7, metastasis to lymph nodes was increased; these results provided experimental proof that cancer cells may co-opt normal mechanisms of leukocyte homing to lymph nodes (18).

As described above, we recently reported that CXCR4 was the only one of 14 chemokine receptors investigated that was expressed on a subset of tumor cells in ovarian neoplasms (10). Stimulation of CXCR4 induced a calcium flux and directed migration of the tumor cells. Moreover, CXCR4 was expressed on a subset of cells in primary ovarian tumors and its ligand CXCL12 (stromal cell-derived factor 1) was found in nanogram quantities in ascitic fluid from ovarian cancer patients. In this report, we have further studied the role of the CXCR4 receptor on ovarian tumor cell behavior. We report that stimulation of this chemokine receptor with CXCL12 also promotes invasion of cells through extracellular matrix, production of the proinflammatory cytokine TNF-α (19), and stimulates cell growth. These actions may allow tumor cells to grow in suboptimal conditions and initiate a cytokine network in the surrounding stroma. The relevance of this is shown by the abundant and tumor-specific expression of CXCL12 in ovarian epithelium. Taken together with recent observations on the role of tumor cell CXCL12 in attracting tumor-promoting pre-DC2 cells into the microenvironment of human ovarian cancer (19), our data suggest that CXCR4/CXCL12 may provide important paracrine and autocrine signals that promote malignant progression in ovarian cancer.

MATERIALS AND METHODS

Samples. Access to human samples satisfied the requirements of the East London and City Health Authority Research Ethics Subcommittee (LREC no. ELCHA T/01/010).

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2 To whom requests for reprints should be addressed, Phone: 44-0-20-7882-6106; Fax: 44-0-20-7882-6105; E-mail: frances.balkwill@can.cancer.org.uk.

3 The abbreviations used are: TNF-α, tumor necrosis factor α; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; RT-PCR, reverse transcription-PCR; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of MMP; uPA, urokinase plasminogen activator; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKB, protein kinase B; PI3k, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase.
Immunohistochemistry. Using a standard avidin-biotin complex technique, a rabbit polyclonal mouse anti-CXCL12 antibody (Peprotech, London, United Kingdom) was used at a dilution of 1 in 250 and localized with 3,3'-diaminobenzidine, followed by counterstaining with Toluidine blue dye. Sections were scored ± if staining was equivocal, exhibiting either pale, heterogeneous, or barely above background and + where there was definite, consistent, and appropriate immunoreactivity. Appropriate controls were performed with omission of the primary antibody and also using preimmune serum before secondary reagents. No nonspecific staining was detected.

Cell Lines. The ovarian cancer cell line IGROV (20) was cultured in pyrogen-free conditions in RPMI 1640 supplemented with 10% FCS, and the CAOV-3 ovarian cancer cell line (American Type Culture Collection) was cultured in DMEM supplemented with 10% FCS. To allow recovery of cells after treatment with 100 ng/ml CXCL12, cells were cultured as single cell suspensions in Teflon-coated pots (Tuf Tainer; Perbio Science, United Kingdom Ltd., United Kingdom) at 0.5–1 × 10^6 cells/ml. IGROV cells were usually treated with 100 ng/ml CXCL12 (Peprotech). This concentration was previously shown to stimulate calcium flux and migration of these cells (10). In some experiments, concentrations of 10 and 50 ng/ml CXCL12 were also used.

Isolation of Primary Ovarian Tumor Cells from Ascitic Fluid. Samples of ascitic fluid were collected from patients with ovarian carcinoma at the time of surgery or by paracentesis for palliative/diagnostic purposes. Each sample was spun down, and the cell isolate was treated with erythrocyte lysis buffer to remove RBCs. Tumor cells expressing the human epithelial antigen were isolated using MACS HEA Microbeads (Miltenyi Biotec, Bisley, Surrey, United Kingdom) according to the manufacturer’s instructions.

Proliferation Assays. Cells (2 × 10^4) were plated in 24-well plates and cultured for 18 h before replacing the media with RPMI 1640 supplemented with 1% BSA (Sigma, Poole, United Kingdom) ± 100 ng/ml CXCL12 (Peprotech). In some experiments, 1 μg/ml anti-TNF-α mAb (R&D Systems, Abingdon, United Kingdom), 20 ng/ml CCL2 (Peprotech, Peprotech), or 20 μg/ml anti-CXCR4 mAb (R&D Systems) were added. Cells were harvested by trypsinization and counted using trypan blue exclusion with a hemocytometer. Cells were cultured for 40 h in the presence of 1 μCi/ml [14C]thymidine (TRA61; Amersham Pharma Biotech, Amersham, United Kingdom). The radioactivity incorporated into the acid insoluble material was measured using liquid scintillation counting.

Apoptosis Assay. IGROV cells (2 × 10^4) were cultured in Teflon pots overnight before stimulation with 100 ng/ml CXCL12. Apoptosis was measured using the Cell Death Detection ELISA (Roche Molecular Biologicals, Mannheim, Germany) according to the manufacturer’s instructions.

Western Blotting. Cells (3 × 10^6) were cultured overnight in Teflon pots before stimulation with CXCL12. Cell lysates were prepared by adding ice-cold lysis buffer [50 mM Tris (pH 8.0), 1% Triton X-100, 100 mM NaCl, 1 mM MgCl₂, 1 mM Na Van, 20 mM NaF, 25 μg/ml Aprotinin, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, and 1 mM β-glycerophosphate; all from Sigma]. A total of 10 μg of protein was treated with 100 ng/ml CXCL12-stimulated cells extract run on a SDS 10% SDS-polyacrylamide gel and transferred to a Hybond-ECL nylon membrane (Amersham). The membrane was probed using an antiphospho-AKT antibody and anti-AKT antibody (New England Biolabs, Beverly, MA) or anti-p44/42 MAPK antibody and antiphospho-p44/42 MAPK antibody (New England Biolabs) according to the manufacturer’s instructions. Western Blot Chemiluminescence Reagent Plus Kit (NEN Life Science Products, Boston, MA) was used for detection. Protein concentration equivalence was confirmed after probing by amido black staining and β-actin antibody.

RNA Extraction and Real-Time Quantitative RT-PCR Analysis. RNA was extracted using Tri Reagent (Sigma) and treated with 10 units of DNase (Pharmacia, Milton Keynes, United Kingdom) following the manufacturers’ instructions. DNase-treated RNA (2 μg) was reverse transcribed with Moleney murine leukemia virus reverse transcriptase (Promega, Southampton, United Kingdom) according to the manufacturer’s instructions and diluted to 100 μl with distilled water. Multiplex real-time analysis was performed using premade TNF-α (FAM), and 18 s rRNA (VIC) specific primers and probes with the ABI Prism 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Warrington, United Kingdom). RT-PCR was carried out with the TaqMan Universal RT-PCR Master Mix (PE Applied Biosystems) following 2.5 μl of cDNA in a 25-μl final reaction mixture. Cycling conditions were incubation at 50°C for 2 min, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Experiments were performed in triplicate for each sample. TNF-α was normalized (ΔCt) to 18 s RNA by subtracting the cycle threshold (Ct) value of 18 s RNA from the Ct value of TNF-α. Fold difference compared with control was calculated.

ELISA for TNF-α in IGROV Cell Culture Supernatants. TNF-α was measured using the Quantikine TNF-α ELISA kit (R&D Systems). The sensitivity of the assay was 4.4 pg/ml.

Invasion. Invasion was assayed using Matrigel Invasion Chambers (24-well format, 8-μm pore; BD PharMingen). Medium (0.5 ml) containing 5 × 10⁵ cells was added to the upper chamber, and 0.5 ml of either medium alone or media supplemented with 100 ng/ml CXCL12 was added to the lower chamber. Chambers were incubated overnight at 37°C and 5% CO₂. Cells on the upper surface of the filter were removed using a cotton wool swab. Migrated cells on the lower surface were stained using DiffQuik (Dade Behring, Didingen, Switzerland) before microscopic analysis. The number of migrated cells in 10 medium power fields (×20) was counted. In some experiments 1 μg/ml of a neutralizing anti-TNF-α mAb (R&D Systems) was added to the cells in the upper chamber.

RT-PCR Screen for MMPs. Total RNA and cDNA was prepared from all samples using TRI Reagent as described above. For RT-PCR, total RNA was DNase-treated to remove contaminating genomic DNA using RNase-free DNase I (Pharmacia Biotech). cDNA was synthesized from DNase-treated total RNA using the Ready-to-Go T-primed First Strand kit (Pharmacia Biotech). The primers for GAPDH were designed from sequences submitted to GenBank using Primer 3.0. The primers for MMPs, membrane-type MMPs, TIMPs, uPA, uPA receptor, and plasminogen activator inhibitor were designed by Thomas Leber, Stephen Robinson, and Kate Scott in our laboratory. The primer sequences and product sizes of the proteases and inhibitors expressed by IGROV cells are as follows in Table 1.

In a previous paper, we reported that CXCR4 was the only chemo- kinase receptor of 14 assayed that was expressed on ovarian cancer cells.
and human tumor samples (10). The CXCR4 ligand, CXCL12, induced a calcium flux, stimulated chemotaxis of ovarian cancer cells, and was present at nanogram levels in ascitic fluid from ovarian cancer. The ovarian cancer cell line IGROV was one of the lines that strongly expressed CXCR4. We used this line to further study the biological activity of CXCL12 on tumor cells.

**Activation of Akt/PKB in IGROV Cells after CXCL12 Stimulation.**
PKB is a downstream effector of PI3k and has been implicated in several signal transduction pathways, many of which promote cell survival (22). It is also important in the chemotactic response (23). IGROV cells were stimulated with 100 ng/ml CXCL12, and total cell lysates were prepared at various time points. Western blots were performed using 10 μg of total protein, and these were probed for phospho-Akt and Akt. Activation of Akt was seen in IGROV cells, with a 3–4-fold increase in phospho-Akt (relative to Akt) within 10 min. This induction of phospho-Akt was still maintained at 2 h (Fig. 1). Phospho-Akt (100 ng/ml) was used as this was the concentration shown in our previous publication to induce calcium flux within the cells. CXCL12 (50 ng/ml) also induced activation of Akt, whereas low concentrations of CXCL12 (10 ng/ml) did not induce detectable signaling (data not shown).

**Activation of ERK1/ERK2 in IGROV Cells.** The MAPK pathway is thought to be activated via PI3kγ (24) and can lead to a variety of cell-type specific effects and gene induction. p44 and p42 MAPK (ERK1 and ERK2, respectively) are important components of this MAPK cascade. IGROV cells were stimulated with 100 ng/ml CXCL12, and total cell lysates were prepared at various time points. Western blots were performed using 10 μg of total protein, and these were probed for phospho-p44/42 MAPK, nonphosphorylated p44/42 MAPK, and β-actin. In IGROV cells, there was approximately a 3-fold increase in the ratio of phospho-p44/42 MAPK to p44/42 MAPK after 15 min (Fig. 2). The ratio then decreased back to control levels after 30 min before increasing to 4–5-fold of control levels after 2 h. These results suggest that there is biphasic MAPK signaling in IGROV cells in response to CXCL12, as has been shown for other cell types (25).

Activation of these intracellular signaling pathways suggested that CXCL12 might affect growth, survival, and stimulate the production of inflammatory mediators, as well as cell migration.

**CXCL12 Causes Proliferation of IGROV Cells.** CXCL12 was added to IGROV cells maintained in serum-free suboptimal growth conditions. There was a significant increase in IGROV cell numbers after stimulation with 100 ng/ml CXCL12 at 4 days (Fig. 3; \( P < 0.005 \)). There was also significant proliferation of IGROV cells to 50 ng/ml CXCL12 (\( P < 0.05 \)), whereas lower concentrations of CXCL12 (10 ng/ml) did not significantly increase cell number (data not shown). Proliferation assays performed in Teflon pots with non-adherent cells showed a similar increase in cell number. There was no increase in cell number when IGROV cells were stimulated with 50 ng/ml of the chemokine CCL2 (data not shown). Addition of anti-
CXCR4 mAb at 10 μg/ml inhibited the CXCL12-dependent increase in cell number; the anti-CXCR4 mAb had no effect on the growth of unstimulated cells. Thus, CXCR4 was necessary for the CXCL12-dependent increase in cell number, but there was no evidence for an autocrine growth loop because addition of anti-CXCR4 mAb did not decrease proliferation of IGROV cells below control levels. We found no evidence that CXCL12 was acting as a survival factor for IGROV cells. When we used the Cell Death Detection ELISA, no evidence of apoptosis was detected either in control or CXCL12-stimulated cultures (data not shown). However, CXCL12 stimulated uptake of [3H]thymidine in IGROV cells (Fig. 4), demonstrating that CXCL12 stimulates DNA synthesis.

**Induction of TNF-α after CXCL12 Stimulation of IGROV Cells.** We have previously shown that the proinflammatory cytokine TNF-α is an important mediator of tumor:stromal cell communication in the tumor microenvironment of epithelial ovarian cancer (26). We have also found that chemokine (CCL1, CCL2, or CCL5) treatment of mononuclear cells and macrophages induces a pulse of de novo TNF-α synthesis (27). IGROV cells were stimulated with 100 ng/ml CXCL12 and TNF-α protein, and mRNA levels were measured at various time points using ELISA and real-time quantitative TaqMan RT-PCR. TNF-α mRNA was up-regulated after CXCL12 stimulation, reaching a 4-fold increase over control levels after 3 h (Fig. 5A). TNF-α protein was not detected in control cultures, but 15 pg/ml TNF-α was found in the medium 8 h after CXCL12 stimulation, increasing to 32 pg/ml at 24 h (Fig. 5B). This difference in detection may reflect the extreme sensitivity of quantitative real time RT-PCR in comparison to ELISA or may indicate a role for the membrane bound form of TNF.

As TNF-α can be a growth factor for some tumor cell lines, we also added 1 μg/ml neutralizing antibody to TNF-α when IGROV cells were growing in the presence of CXCL12. This concentration of anti-TNF-α was used as it had been previously demonstrated in this lab to neutralize chemokine-induced TNF-α and MMP-9 production by monocytes (27). However, neutralization of TNF-α release had no effect on the CXCL12 stimulation of [3H]thymidine uptake (Fig. 4).

**CXCL12 Stimulates Ovarian Cancer Cell Invasion.** IGROV cells were also tested for their ability to invade through growth...
factor-reduced Matrigel invasion chambers. In response to a gradient generated by 100 ng/ml CXCL12, IGROV cells invaded through Matrigel (Fig. 6A). Two and 20 μM of the metallo-enzyme inhibitor Marimastat inhibited invasion toward CXCL12 (Fig. 6A). These doses of Marimastat had no effect on IGROV cell growth (data not shown). IGROV cells do not invade through Matrigel in response to a gradient of 20 ng/ml CCL2, a chemokine widely expressed in ovarian cancer (data not shown).

We conducted a RT-PCR screen for MMP and TIMP expression in IGROV cells. These cells did not express MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, MMP-13, MT1-MMP, MT3-MMP, or TIMP-3 (data not shown). However, IGROV cells expressed mRNA for MMP-7, MMP-11, MMP-15, uPA, TIMP-1, and TIMP-2 (Fig. 7). Treatment of IGROV cells with CXCL12 for 8 or 24 h did not induce the expression of additional MMPs (Fig. 7). Real-time RT-PCR was used to look for increase in abundance of MMP-7, MMP-11, and MMP-15 after CXCL12 treatment. CXCL12 does not appear to alter the expression of the MMPs that we have investigated, however, there are many more MMPs that could be affected by stimulation with CXCL12. Alternatively a cytokine downstream of CXCL12 could affect MMP expression. The induction of TNF-α by CXCL12 appeared to be involved in the process of invasion. As shown in Fig. 6B, addition of a neutralizing anti-TNF-α mAb significantly inhibited CXCL12-stimulated Matrigel invasion (P < 0.002). However, IGROV and CAOV-3 cells do not migrate or invade toward 30, 50, or 100 ng/ml TNF-α, and addition of these concentrations of TNF-α to tumor cells in the Matrigel assay did not enhance the invasive capacity of these cells (data not shown).

**Biological Actions on Other Ovarian Cancer Cells.** Many of the experiments described above were also performed on the CXCR4 expressing cell line CAOV-3. In CAOV-3 cells, there was a 4-fold increase in the ratio of phospho-p44/42 MAPK to p44/42 MAPK after 15 min of stimulation with CXCL12 and this was still sustained after 2 h (Fig. 8, A and B).

There was a significant increase in cell number after stimulation of CXCL12 in suboptimal growth conditions (Fig. 8C), and CAOV-3 cells were able to invade through growth factor-reduced Matrigel invasion chambers toward a CXCL12 gradient (Fig. 8D). CAOV-3 cells also produced TNF-α after stimulation with 100 ng/ml CXCL12; there was a 2-fold increase in TNF-α mRNA after 3 h of stimulation with CXCL12 (data not shown).

Primary ovarian tumor cells isolated from ascites were able to produce TNF-α mRNA in response to stimulation with CXCL12 (Fig. 5C).

**AMD3100, a CXCR4 Antagonist, May Have Therapeutic Potential in Ovarian Cancer.** AMD3100 is a specific antagonist for CXCR4 that blocks HIV infection of T-tropic X4-using virus in vitro and in vivo and inhibits migration of monocytic cells toward CXCL12 (21). AMD3100 has been evaluated in a Phase I clinical trial in healthy volunteers and subsequently in HIV-positive patients. Treatment of the ovarian cancer cell lines with AMD3100 abrogated the increase in cell number after CXCL12 stimulation (Fig. 9A). AMD3100 treatment alone did not affect cell proliferation compared with the unstimulated control, again suggesting that no autocrine growth stimulatory loops exist, at least in this cell line. These results demonstrate that CXCR4 is necessary and sufficient for CXCL12-induced growth stimulation. AMD3100 also inhibited the invasion of IGROV cells toward CXCL12 (Fig. 9B).

**CXCL12 Is Present in Solid Ovarian Tumor Biopsies.** Because of the multiple effects of CXCL12 on ovarian cancer cells in vitro, we wished to see whether this chemokine was present in the solid tumor microenvironment and whether its expression was tumor specific. Ovarian surface epithelium and inclusions from 19 normal ovaries were negative for CXCL12 (Table 2, Fig. 10A), including 5 samples from women with a family history of ovarian cancer. There was weak staining of Fallopian tube epithelium and rete ovarii. Seven benign specimens were studied (2 serous, 4 mucinous, and 1 teratoma dess.) including 5 samples from women with a family history of ovarian cancer. There was weak staining of Fallopian tube epithelium and rete ovarii. Seven benign specimens were studied (2 serous, 4 mucinous, and 1 teratoma dess.)

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Fig. 8. Biological effects of CXCL12 in the CAOV-3 cell line. Total cell lysates were prepared at various time points after stimulation with 100 ng/ml CXCL12. Western blots were performed with 10 μg of total protein from CAOV-3 and probed for p44/42 MAPK and the active form, phospho-p44/42 MAPK (A). Densitometry was also performed on the blots, and the ratio of phospho-p44 MAPK or phospho-p44 MAPK to the non-phosphorylated form was calculated (B). One representative experiment of three is shown. CAOV-3 cells were grown in serum-free medium ± 100 ng/ml CXCL12. The number of cells was counted after 2, 4, and 6 days of culture. CXCL12 stimulation resulted in a significant increase in cell number of CAOV-3 (A) above control cultures. Values are the mean ± SE of eight determinations. * denotes P < 0.05; ** denotes P < 0.01. One representative experiment of three is shown (C). CAOV-3 cells showed significant invasion through Matrigel toward 100 ng/ml CXCL12 (P < 0.0001). This invasion was inhibited by treating the cells with 2μM Marimastat (D). Values are the mean ± SD of 15 determinations, and the results are representative of three experiments.

Fig. 9. AMD3100 can inhibit proliferation and invasion of IGROV cells in response to CXCL12. IGROV cells were grown in serum-free medium ± 100 ng/ml CXCL12 ± 1 μg/ml AMD3100. The number of cells was counted after 2, 4, and 6 days of culture. CXCL12 stimulation resulted in a significant increase in cell number of IGROV cells (A) compared with control cultures (B), but this increase was inhibited by AMD3100 (C); AMD3100 alone had no effect on cell number (D). Values are the mean ± SE of eight determinations. * denotes P < 0.05; ** denotes P < 0.01. IGROV cells showed significant invasion through Matrigel toward 100 ng/ml CXCL12, and this invasion was inhibited by treating the cells with 1 μg/ml AMD3100 (B).

negative, 3 of 7 were weakly positive (Fig. 10b), and 1 of 7 was positive.

Of the 18 malignant tumors studied, the 11 serous tumors were all positive, 1 of 11 weakly positive and 10 of 11 positive (Table 2, Fig. 10, c and d). Both endometrioid ovarian cancers were positive. Finally of the five clear cell cancers, 3 of 5 were positive and 2 of 5 were negative. It may be significant that, in keeping with the results on invasion in vitro, enhanced staining was observed in epithelial cells lining cystic spaces or in areas of invasion (Fig. 10c) and in more pleomorphic neoplastic cells. There was also a trend toward stronger expression in the higher grade tumors. Another consistent observation was increased staining for CXCL12 in tumor-associated myofibroblasts in areas of desmoplasia (Fig. 10d). Fibroblast or stromal cell staining was not seen in normal ovaries.

These immunohistochemistry data suggest that CXCL12 could be acting locally within the ovarian solid tumor microenvironment, especially in papillary serous carcinomas and that expression of CXCL12 may increase with malignant progression.

DISCUSSION

The expression of the chemokine CXCL12 was related to malignant transformation of the ovarian surface epithelium. All normal samples of ovarian epithelium were negative for this chemokine, as was the normal epithelium from ovaries of women with a family history of ovarian cancer. In contrast, all papillary serous and endometrioid ovarian tumors stained strongly for CXCL12. The benign tumors were

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<tr>
<th>Sample description</th>
<th>CXCL12 expression</th>
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<tr>
<td>Normal ovaries (n = 19)</td>
<td>+</td>
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<tr>
<td>Control women (n = 14)</td>
<td>+</td>
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<tr>
<td>Family history (n = 5)</td>
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<td>Benign tumors (n = 7)</td>
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<td>Serous (n = 2)</td>
<td>+</td>
</tr>
<tr>
<td>Mucinous (n = 4)</td>
<td>+</td>
</tr>
<tr>
<td>Dermoid (n = 1)</td>
<td>+</td>
</tr>
<tr>
<td>Borderline tumors (n = 8)</td>
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<tr>
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</tr>
<tr>
<td>Mucinous (n = 1)</td>
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</tr>
<tr>
<td>Malignant tumors (n = 18)</td>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>Endometroid (n = 2)</td>
<td>+</td>
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<tr>
<td>Clear cell (n = 5)</td>
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</table>

Table 2 CXCL12 expression in epithelial cells from normal ovary, borderline ovarian cancer, and ovarian cancer
Fig. 10. CXCL12 protein expression in ovarian cancer. Immunohistochemical staining (original magnification ×400) showing normal ovarian surface epithelium which is negative for CXCL12 (a). A papillary process from a borderline serous tumor with equivocal weak staining of the epithelial cell cytoplasm (b). Grade 1 papillary serous cystadenocarcinoma with weak CXCL12 immunostaining in the papillary intracyctic component (left of field, center) and in the invasive areas of the tumor (right of field, c). Grade 2 serous carcinoma with psammoma bodies with strong immunoreactivity in the neoplastic cells, but also weak expression in stromal fibroblastic cells (d).

interesting in that the mucinous tumors were all negative, but the serous benign tumors, perhaps being related to serous cancers, were positive. We have previously found strong expression of the CXCL12 receptor CXCR4 in a subset of epithelial tumor cells from ovarian cancers (10). In more recent experiments (data not shown), we failed to detect CXCR4 expression in normal ovarian epithelium. Thus, we suggest that this chemokine might have other roles in the solid tumor microenvironment, stimulating DNA synthesis and inflammatory cytokine production as well as invasion through Matrigel. These actions may be mediated by well-defined MAPK and PI3K signaling pathways. CXCL12 may not only stimulate a subset of CXCR4-expressing tumors cells to migrate but may act in an autocrine or paracrine manner to permit tumor cells to grow in suboptimal conditions.

These actions of CXCL12 were not exclusive to IGROV cells. CXCL12 stimulated cell proliferation in another CXCR4-expressing ovarian cancer cell line CAOV-3, with a 2–3-fold increase in cell numbers after 6 days. CAOV-3 also stimulated biphasic activation of the MAPK pathway in CAOV-3 cells, their invasion through Matrigel and induced TNF-α mRNA in CAOV-3 cells and primary tumor cells.

In our previous study, we detected CXCL12 in 63 ovarian cancer ascitic fluid samples (range, 0.61–9.33 ng/ml; median, 6.02 ng/ml; Ref. 10). The concentration of CXCL12 that was active on ovarian cancer cell lines in vitro was generally one log higher than this, but it is entirely possible that local concentrations of the chemokine are much higher in vivo but we have no way of measuring this. Indeed, immunohistochemistry showed very strong staining for CXCL12. It would also be interesting to know if other cytokines/growth factors present in the solid ovarian tumors and ascites synergize with the action of CXCL12 on the tumor cells. In this respect, we note that CXCL12 was not generally secreted by ovarian cancer cells in vitro but cells in vivo stained strongly for this chemokine.

In IGROV and CAOV-3 cells, CXCL12 stimulation resulted in the phosphorylation of p44/42 MAPK (ERK1/ERK2) and stimulation of IGROV cells also resulted in the phosphorylation of Akt/PKB. Phosphorylation of Akt/PKB was maintained for at least 2 h. Activation of Akt/PKB has been shown to have roles in chemotaxis and survival (22) and is recruited to the leading edge of the cell during neutrophil chemotaxis (28). In fibroblasts, Akt/PKB can promote survival by blocking caspase 3 activity (29), whereas in hematopoietic cells, Akt/PKB can promote the induction of Bcl-2 and thereby inhibit apoptosis. Cheng et al. (30) demonstrated that Akt/PKB is overexpressed in a small proportion of solid ovarian tumors. In animal models of prostate cancer, increased Akt/PKB activity has also been shown to contribute to tumor progression by accelerating tumor growth (31).

In IGROV cells, phosphorylation of p44/42 MAPK was biphasic. There was an initial increase in the ratio of phospho-p44/42 MAPK to p44/42 MAPK after 15 min. This ratio then decreased back to control levels after 30 min before increasing again after 1 h. Recent work by Han et al. (25) showed biphasic activation of p44/42 MAPK in astrocytes in response to CXCL12. Early activation of p44/42 MAPK was directly attributable to CXCL12 stimulation; late activation was indirectly mediated by CXCL12-induced TNF-α. A similar mechanism may be responsible for the biphasic response seen in IGROV cells.

The invasion of IGROV and CAOV-3 cells through Matrigel may be dependent on the presence of MMPs and other matrix-degrading enzymes. *In vivo*, metastasizing cancer cells must degrade extracellular matrix and cross basement membranes to reach the lymphatic system and other sites of metastasis. IGROV expressed mRNA for MMP-7 and MMP-11, which are secreted enzymes, and MMP-15, which is a transmembrane MMP that can activate pro-MMP-2. IGROV also expressed mRNA for TIMP-1 and TIMP-2, which can inhibit the activity of various MMPs, and uPA (32). None of these molecules appeared to be regulated by CXCL12. Therefore, it is probable that IGROV cells produce a variety of matrix-degrading enzymes and that CXCL12 merely gives them a direction in which to move. Invasion was inhibited by...
Marimastat and also anti-TNF-α mAb. Apart from being a broad-spectrum MMP inhibitor, Marimastat is also a TNFα converting enzyme inhibitor and as such blocks TNF-α release from cells (33). The doses of Marimastat used in these experiments inhibited TNF-α production by THP-1 cells in our laboratory (27). There are some reports that TNF-α stimulates cancer cell invasion and modulates adhesion molecule expression and activity (34–36). We previously showed that CXCL12 up-regulated β1 integrin on IGROV and CAOV-3 cells (10). The role of TNF-α in this integrin up-regulation is now being investigated.

The induction of TNF-α in IGROV and CAOV-3 cells and primary tumor cells after CXCL12 stimulation may have additional roles in the tumor microenvironment. When chronically produced, this cytokine may act as an endogenous tumor promoter, contributing to tissue remodeling and stromal development necessary for tumor growth and spread. TNF-α mRNA is abundant in malignant ovarian epithelium, and a series of experiments has implicated TNF-α in stromal/tumor communication in ovarian cancer (reviewed in Refs. 2, 37). However, apart from TNF-α itself, this is the first factor that we have found that induces TNF-α in ovarian cancer cells. A pulse of TNF-α induced by CXCL12 could therefore stimulate production of other cytokines and proteases in the microenvironment around the tumor cell.

In this paper, we have focused on the action of CXCL12 on the epithelial ovarian tumor cells. However, a recent paper defines a paracrine role for this chemokine in the ovarian cancer microenvironment. In agreement with our data, Zou et al. (19) reported that ovarian epithelial tumor cells express high levels of CXCL12. They demonstrated that this CXCL12 induces DC2 precursor chemotaxis and adhesion/transmigration, up-regulates preDC2 VLA-5, and protects pre-DC2s from tumor macrophage IL-10-induced apoptosis. As these pre-DC2 are poor inducers of T-cell responses, tumor immunity may be modulated/dysregulated by CXCL12.

The biological actions of CXCL12 on ovarian cancer cells were inhibited by the bicyclam inhibitor of CXCR4, AMD3100. AMD3100 is a specific antagonist for CXCR4, blocks HIV infection of T-tropic X4-using virus in vitro and in vivo, and inhibits migration of monocytic cells toward CXCL12 (21, 38). AMD3100 has been evaluated in a Phase I clinical trial in healthy volunteers and subsequently in HIV-positive patients (39). The leukocytosis observed in the Phase I trial of AMD3100 led to the initiation of an additional Phase I study in healthy volunteers in which it was demonstrated that AMD3100 was able to mobilize pluripotent hematopoietic stem cells (40). IGROV cells grow as i.p. xenografts in nude mice, and AM3100 is active in murine models of HIV/AIDS and rheumatoid arthritis (41, 42). Hence, we will be able to carry out preclinical studies of the potential of AMD3100 alone and in combination with biological and chemotherapeutic agents in ovarian cancer.

In conclusion, the selective expression of the chemokine receptor CXCR4 and its ligand CXCL12 by malignant ovarian epithelium is a number of important consequences to tumor development and spread. This chemokine/ligand receptor pair may stimulate directed migration and invasion of tumor cells, as well as promoting their growth and the establishment of a tumor-promoting cytokine network in suboptimal conditions and subverting tumor immunity. CXCR4 or its ligand is a target for therapeutic intervention.

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Multiple Actions of the Chemokine CXCL12 on Epithelial Tumor Cells in Human Ovarian Cancer

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