

Epithelial Cancer Cell Migration: A Role for Chemokine Receptors?

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ABSTRACT

We investigated the possibility that chemokine gradients influence migration of human ovarian epithelial tumor cells. Of 14 chemokine receptors investigated, only CXCR4 was expressed on ovarian cancer cells. CXCR4 mRNA localized to a subpopulation of tumor cells in ovarian cancer biopsies. Ovarian cancer cell lines and cells freshly isolated from ascites expressed CXCR4 protein. The CXCR4 ligand, CXCL12, was found in ascites from 63 patients. CXCL12 elicited intracellular calcium flux and directed migration and changes in integrin expression in ovarian cancer cells. CXCR4 may influence cell migration in the peritoneum, a major route for ovarian cancer spread, and could be a therapeutic target.

INTRODUCTION

Chemokines are small, secreted peptides that control the migration of leukocytes, especially during immune and inflammatory reactions (1, 2). They are divided into two major subfamilies, CC and CXC, based on the position of their NH₂-terminal cysteine residues, and bind to G protein-coupled receptors, whose two major subfamilies are designated CCR and CXCR. Chemokines are also produced by most, if not all, cancers. There is strong evidence that they are major determinants of the macrophage and lymphocyte infiltrate found in melanomas; in carcinomas of the ovary, breast, and cervix; and in sarcomas and gliomas (3–5). However, chemokines may play other roles in cancer. Some are potent angiogenic factors, whereas others can be angiostatic (1, 6). Alterations in the balance between these chemokines may contribute to the development of the tumor vasculature.

Using human epithelial ovarian cancer as an example, we have investigated another role for chemokines in cancer: the possibility that the malignant cells in epithelial tumors may use chemokine gradients as part of the process of metastatic spread. Only 1 of 14 chemokine receptors investigated was expressed on the ovarian tumor cells. We present evidence that interaction of this chemokine receptor, CXCR4, with its ligand, CXCL12, may influence the spread of epithelial ovarian cancer. This observation has implications for the development of new biological therapies for cancer.

MATERIALS AND METHODS

Samples. Human ovarian tumor biopsies were frozen in liquid nitrogen. Sections for *in situ* hybridization were mounted on baked glass slides coated with 3-aminopropyl-triethoxy-silane, air-dried, and stored at –70°C. Red cells were removed from ascites with ACK buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂ EDTA).

Cell Lines. Ovarian cancer cell lines PEO1 and PEO14 (from S. Langdon, Imperial Cancer Research Fund Oncology Unit, Edinburgh, United Kingdom), OVCAR-3 (purchased from American Type Culture Collection), and IGROV (from J. Bénard) were grown in RPMI 1640 supplemented with 10% FCS and 10 μg/ml insulin (for PEO1 and PEO14); SKOV-3 and CAOV-3 (purchased

from American Type Culture Collection) were grown in DMEM supplemented with 10% FCS. Cell lines were cultured in pyrogen-free conditions. To allow recovery of cells after trypsinization, cells were cultured as single cell suspensions in Teflon-coated pots (Tuf Tainer, Perbio Science UK Limited) at 0.5–1 × 10⁶ cells/ml.

RNA Extraction and RPA.² Total RNA was prepared with Tri Reagent (Sigma Chemical Co., Poole, United Kingdom). Tumor biopsies were homogenized in Tri Reagent using an Ultra-turrax T25 (Janke & Kunkel, Staufen, Germany). Total RNA was treated with RNase-free DNase I (Pharmacia Biotech, St Albans, United Kingdom). RPA using Riboquant hCR5 and hCR6 template sets (BD PharMingen, Oxford, United Kingdom) was carried out using α-³⁵S-labeled UTP (Amersham International plc, Aylesbury, United Kingdom). RNase-protected fragments were run on an acrylamide-urea sequencing gel (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom), adsorbed to filter paper, and dried under vacuum. Autoradiography was performed using Kodak Biomax MS film with a Transcreen LE intensifying screen (Sigma Chemical Co.).

In Situ Hybridization. Antisense and sense riboprobes labeled with α-³⁵S-labeled UTP were generated from 1100-bp fragments of CXCR4 cDNA cloned in pcDNA3 (Stratagene, Cambridge, United Kingdom) using Sp6 and T7 RNA polymerases (Promega Ltd., Southampton, United Kingdom). The cDNA was a gift from Antonio Sica (Mario Negri Institute, Milan, Italy). Antisense β-actin was used as a positive control in all experiments. *In situ* hybridization was carried out using the method described in Ref. 5. Image capture was performed using Image Grabber PCI (Neotech Ltd., London, United Kingdom).

mAbs and Flow Cytometry. PE-labeled mAbs against CXCR4 (12G5; R&D Systems, United Kingdom), FITC-labeled Her2/Neu (Neu 24.7; BD PharMingen), and isotype-matched labeled controls were used. Unconjugated β₁ antibodies were a gift from Fiona Watt (Imperial Cancer Research Fund, London, United Kingdom). Cells were resuspended in PBS supplemented with 1% heat-inactivated FCS and 0.01% NaN₃. Antibodies diluted in this buffer were used at concentrations between 2 and 20 μg/ml and incubated for 30 min on ice. For unconjugated antibodies, cells were incubated with a 1:200 dilution of PE-conjugated polyclonal secondary antibody (BD PharMingen). After a final wash, labeled cells were fixed with 1% paraformaldehyde solution, and 10,000 cells were analyzed by flow cytometry on a FACScan flow cytometer using Cellquest software (BD PharMingen).

Calcium Flux. Five × 10⁵ cells/ml were incubated with 5 μM Fluo-3 (Molecular Probes, Cambridge Biosciences, Cambridge, United Kingdom) in HBSS plus 0.5% BSA, 1 mM Ca²⁺, 1 mM Mg²⁺, and 10 μM Zn²⁺ for 30 min at 37°C and then washed. Fluorescence was measured in a PTI fluorometer (excitation wavelength, 485 nm; emission wavelength, 530 nm).

Migration. Chemotaxis was assayed using Falcon Transwells (24-well format, 8-μm pore; BD PharMingen). Media (0.5 ml) containing 5 × 10⁵ cells were added to the upper chamber, and 0.5 ml of medium alone or media supplemented with CXCL12 were added to the lower chamber. After overnight incubation 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, Düringen, Switzerland). For each transwell, the number of migrated cells in 10 medium-power fields (×20) was counted.

ELISA for CXCL12 in Ascitic Fluid. Concentration of the chemokine CXCL12 (SDF-1α) in ascitic fluid was measured using Quantikine ELISA kits (R&D Systems, Abingdon, United Kingdom) according to the manufacturer's protocol. The sensitivity of the assay was 18 pg/ml.

Received 3/15/01; accepted 5/9/01.

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² The abbreviations used are: RPA, RNase protection assay; mAb, monoclonal antibody; PE, phycoerythrin.

Statistical Analysis. The results of the migration assays were evaluated using Welch's approximate *t* test (which is a more stringent analysis than Student's *t* test).

RESULTS

Ovarian Tumor Cells Express CXCR4 mRNA. mRNA extracted from six ovarian cancer cell lines (SKOV-3, IGROV, OVCAR-3, CAOV-3, PEO1, and PEO14) was screened for chemokine receptor expression by RPA. The cells did not express CCR1, CCR2a, CCR2b, CCR3, CCR4, CCR5, CCR7, CCR8, CXCR1, CXCR2, CXCR3, CXCR5, or CX₃CR1, but two of the six cell lines (IGROV and CAOV-3) strongly expressed CXCR4 mRNA (Fig. 1, A and B). PEO1 and PEO14 also had detectable levels of CXCR4 mRNA (Fig. 1A). This chemokine receptor was also expressed in biopsies from 8 of 10 primary ovarian tumors and 19 of 20 samples of ovarian cancer ascites (Fig. 1C).

CXCR4 Localizes to Tumor and Stromal Cells in Tumor Biopsies. *In situ* hybridization localized CXCR4 mRNA to a proportion of neoplastic cells in 10 of 10 ovarian cancer biopsies studied (Fig. 2, A–C). Expression of CXCR4 mRNA was not uniform throughout the tumor. In five of the biopsies, between 5% and 20% of tumor cells were labeled, and in the other five biopsies, 1–5% of tumor cells were positive for CXCR4. There was no correlation between CXCR4 positivity and areas of necrosis or hot spots of angiogenesis. CXCR4 mRNA was also detected in mononuclear and endothelial cells. Tumors with a strong lymphoid infiltrate in the stroma showed highest mononuclear cell labeling for CXCR4 (Fig. 3, A and B). The presence and distribution of tumor-infiltrating lymphocytes were assessed by H&E staining and CD8 immunostaining, and the CXCR4 labeling pattern was clearly in excess of these populations in every sample. CD8 cells also showed a different distribution from CXCR4-labeled tumor cells. In addition, it was possible to distinguish between neo-

plastic and tumor-infiltrating lymphocyte cell nuclei. Some endothelial cells within the tumor were also labeled in each biopsy (Fig. 3, C–F). CXCR4-expressing endothelial cells were detected in fibrovascular cores of papillary tumors, adjacent to the epithelial tumor cells, or within stromal invaginations (Fig. 3, C and D). Endothelial cells in tissue adjacent to the tumors were not positive for CXCR4.

CXCR4 Protein Can Be Detected on Ovarian Cancer Cells. As shown in Fig. 4A, CXCR4 protein could be detected on IGROV and CAOV-3 cells using flow cytometry. Two-color flow cytometric analysis of cells freshly isolated from ovarian cancer ascites revealed CXCR4 surface expression on cells that were also positive for the tumor marker HER2/neu (Fig. 4B).

The CXCR4 Receptor Is Functional on Ovarian Cancer Cells. CXCL12 (100 ng/ml) elicited an intracellular calcium flux in both IGROV and CAOV-3 cells (Fig. 4C). No release of intracellular calcium was detectable when cells were incubated with 10 ng/ml CXCL12. Both IGROV and CAOV-3 cells demonstrated chemotaxis toward CXCL12 (Fig. 4D).

Binding of CXCL12 to its receptor caused internalization of CXCR4 on IGROV cells after approximately 15 min, as determined by flow cytometry (Fig. 5A). The receptor was recycled to the cell surface within 30 min. CXCL12 induced significant migration of IGROV and CAOV-3 cells at concentrations of 100 ng/ml (IGROV, *P* = 0.0001; CAOV-3, *P* = 0.0002) and 300 ng/ml (IGROV, *P* = 0.0001; CAOV-3, *P* = 0.0003). Stimulation of CAOV-3 and IGROV cells with CXCL12 at 100 ng/ml for 24 h increased cell surface expression of the β_1 integrin (Fig. 5B).

Ascitic Fluid from Ovarian Cancer Contains High Levels of CXCL12. CXCL12 levels were assayed by ELISA in 63 samples of ascitic fluid from patients with ovarian cancer. High concentrations of this chemokine (range, 613–9333 pg/ml; median, 6021 pg/ml) were detected in all of the samples.

DISCUSSION

We provide evidence that chemokine gradients may influence routes of ovarian tumor cell migration. Response to chemokine gradients may be an important component of the multistep process of metastasis of epithelial tumors. Binding of ligand to the only chemokine receptor that was reproducibly expressed by human ovarian cancer cells (CXCR4) induces actions that would permit a role in tumor cell migration.

Only a proportion (between 1% and 20%) of the tumor cells in the ovarian cancer biopsies expressed CXCR4 mRNA. Receptor levels could be regulated by cytokines in the tumor microenvironment. For example, in T cells, CXCR4 can be up-regulated by interleukin 4 (7). We have preliminary evidence that ovarian cancer cell CXCR4 protein is up-regulated by transforming growth factor β , a cytokine that is found in ovarian cancer, but down-regulated by IFN- γ , which is absent from this tumor microenvironment (8). This could explain why a proportion of the tumor cells was positive at any one time. Alternatively, some cells may have acquired constitutive CXCR4 expression during malignant progression. In contrast, all of the HER2/neu-positive tumor cells that had spread into ascites were CXCR4 positive.

The ability of the ovarian cancer cell lines to elicit an intracellular calcium flux and migrate in response to CXCL12 indicates the functionality of the CXCR4 cell surface receptors. It is possible that the high levels of CXCL12 found in ascites could create a chemokine gradient for migration of tumor cells into the peritoneum. Treatment of the ovarian cancer cell lines with CXCL12 significantly increased cell surface expression of β_1 integrin, which may affect peritoneal adhesion of cells. The significance of this remains to be determined.

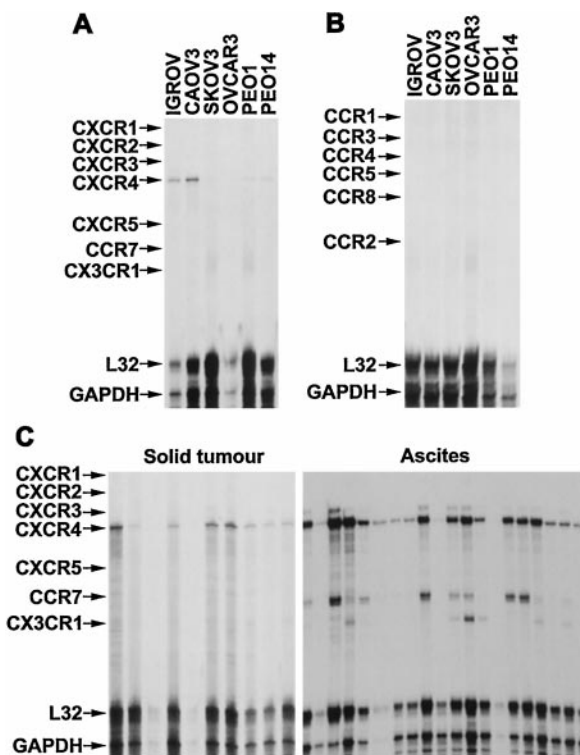


Fig. 1. Chemokine receptor mRNA expression in ovarian cancer. mRNA expression was measured by RPA. GAPDH and L32 are housekeeping genes. A and B, chemokine receptor expression by ovarian cancer cell lines. C, chemokine receptor expression in primary solid tumor biopsies and cells isolated from ovarian cancer ascites.

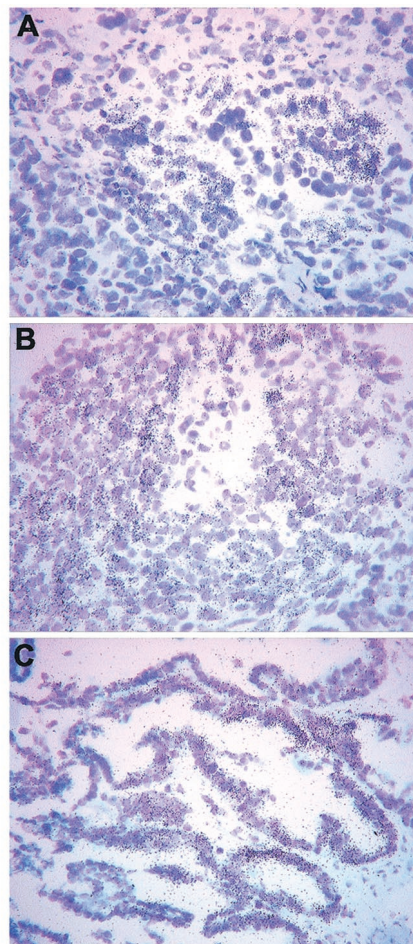


Fig. 2. CXCR4 mRNA expression by epithelial tumor cells in biopsies of human ovarian cancer. *A*, variable degree of labeling of two tumor epithelial cell groups in a serous carcinoma separated by a cellular stroma that is largely unlabeled. *B*, grade 3 serous carcinoma with heterogeneous labeling of tumor epithelial cells and focal weak labeling of some cells in the stroma. *C*, grade 1 serous carcinoma with neoplastic epithelial cells clearly labeled for CXCR4.

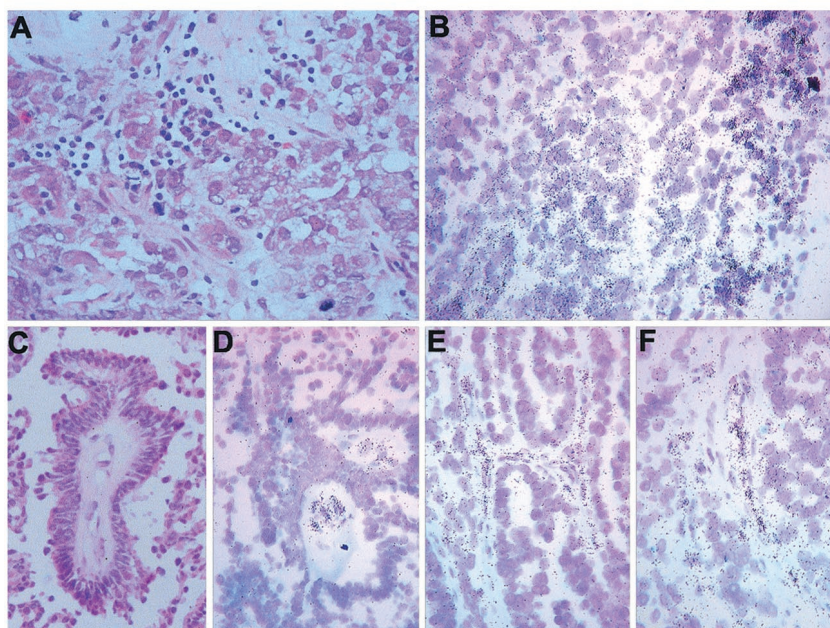


Fig. 3. CXCR4 mRNA expression by tumor and stromal cells in biopsies of human ovarian cancer. *A*, H&E-stained section of grade 3 serous carcinoma with stromal lymphoid infiltrate. *B*, same serous carcinomas in *A* with CXCR4 labeling of tumor epithelial cells (*left of field*). The lymphoid cells within the stroma (*right of field*) show a denser pattern of label. *C*, endothelial cells in the papillary core of a grade 1 serous carcinoma (stained with H&E) are clearly labeled for CXCR4. *D*, there is focal weak labeling of occasional tumor cells. *E*, stromal endothelial cell labeling in a grade 2 serous carcinoma with equivocal epithelial cell labeling. *F*, tumor epithelial cells show a low index of CXCR4 labeling together with a linear pattern of CXCR4-labeled endothelial cells in the adjacent stroma.

However, previous studies have shown that CXCL12 induced adhesion of most circulating lymphocytes and CD34+ progenitor cells (9).

Analysis of mRNA by RPA showed expression of other chemokine receptors in the tumor and ascites samples, which are a mixture of tumor cells, leukocytes, and connective tissue cells. In solid tumors we also detected expression of the CC chemokine receptor CCR1, but

this localized to infiltrating leukocytes.³ More chemokine receptor mRNAs were expressed in cells from ascites, but apart from CXCR4,

³ C. Scotten, D. Milliken, J. Wilson, S. Raju, and F. R. Balkwill. Chemokine and chemokine receptor expression in solid ovarian tumors, submitted for publication.

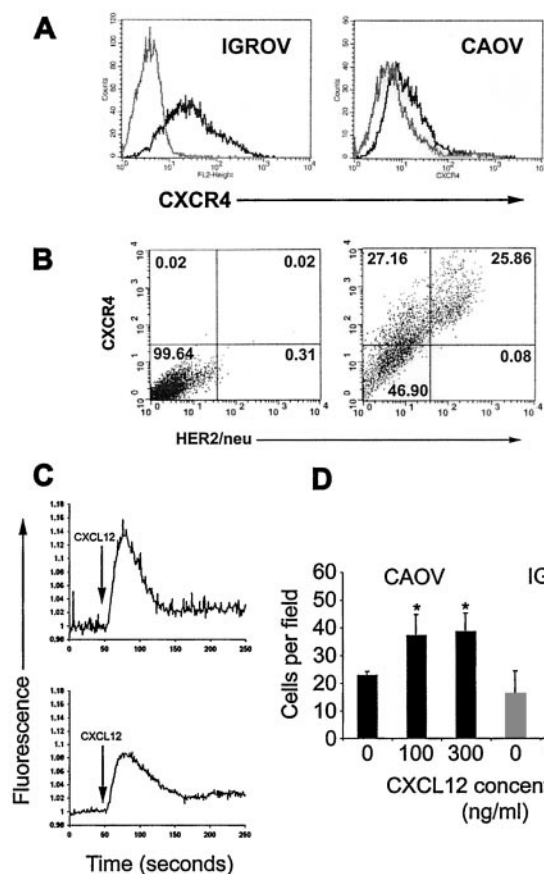


Fig. 4. Functional CXCR4 protein on ovarian cancer cells. *A*, flow cytometry histograms for cell surface expression of CXCR4 on the ovarian cancer cell lines IGROV and CAOV-3. Cells were incubated with IgG2a isotype control mAb (gray line) or CXCR4 mAb (black line). Results are expressed as mean fluorescence intensity and show one representative experiment of five experiments performed. *B*, two-color flow cytometric analysis of CXCR4 versus HER2/neu expression on cells from ovarian ascites. Representative plots are shown with isotype control panel (left) and percentages of HER2/neu-positive CXCR4-positive cells (right). Cells from five different patients gave similar results. *C*, Ca^{2+} flux in IGROV (top panel) and CAOV-3 (bottom panel) tumor cell lines in response to CXCL12 stimulation. Arrows indicate the point at which the chemokine was added. Results shown are representative of five experiments. *D*, migration of CAOV-3 (■) and IGROV (□) cell lines to CXCL12. Values are the mean \pm SD of 10 determinations from 2 experiments. *, statistically significant results, $P < 0.0005$

these were also detected on leukocyte but not on tumor cell populations.⁴

Thus the only chemokine receptor we have found on ovarian cancer cells is CXCR4. Two recent studies (10, 11) described tumor and endothelial cell CXCR4 expression in human pancreatic cancer and glioblastoma, but it is not clear whether these tumor cells expressed additional chemokine receptors. In brain tumors, the receptor and its CXCL12 ligand were primarily expressed in regions of angiogenesis and degeneration and were associated with high-grade tumors (11).

Most persuasively, Muller *et al.* (12) recently reported high expression of CXCR4 and CCR7 on human breast cancer cells. As measured by real-time PCR, mRNA levels of the respective ligands CXCL12 and CCL21 were highest at sites of breast cancer metastasis. As we have also shown in this study, the chemokines induced migration of tumor cells. Furthermore, neutralizing antibodies to these chemokine receptors reduced experimental metastasis in a xenograft model of breast cancer. Thus it seems that malignant cells from common tumors express restricted and specific patterns of chemokine receptors.

There is increasing evidence that the inflammatory cells, cytokines,

⁴ D. Milliken, C., Scotten, S. Raju, F. Balkwill, and J. Wilson. Analysis of chemokines and chemokine receptor expression in ovarian cancer ascites, manuscript in preparation.

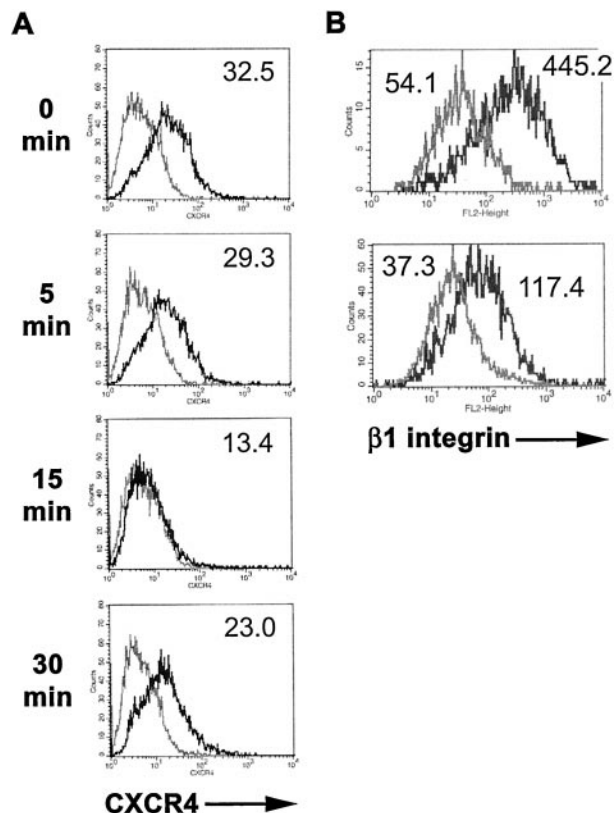


Fig. 5. CXCL12 induced CXCR4 internalization, integrin up-regulation, and CXCL12 expression in ascitic fluid. *A*, the expression/internalization of CXCR4 by IGROV cells at various time points after stimulation with CXCL12 (isotype control, gray line; CXCR4, black line). Numbers on figures represent the mean fluorescent intensity of CXCR4. *B*, up-regulation of the $\beta 1$ integrin on IGROV (left panel) and CAOV-3 cells (right panel) after stimulation with CXCL12. Unstimulated control, gray line; CXCL12-stimulated cells, black line.

and chemokines found in human tumors are more likely to contribute to tumor growth, progression, and immunosuppression than they are to mount an effective antitumor response (reviewed in Ref. 13). Over the past 10 years, study of the cytokine and chemokine network has led to the development of a range of antagonists for the treatment of inflammation and allergy. We suggest that such agents may also be of benefit in the treatment of malignant disease. The chemokine receptor CXCR4, which is now known to be functional on both breast and ovarian cancer cells, is of particular interest. It is the coreceptor for macrophage trophic HIV virus (14), and CXCR4 antagonists are currently in Phase I clinical trial for the treatment of HIV/AIDS. In view of the expression of this receptor on both tumor and endothelial cells in human ovarian cancers, there is a rationale for the use of CXCR4 antagonists as part of a biological approach to the treatment of ovarian cancer. IGROV cells express functional CXCR4. These cells form i.p. tumors in nude mice and may provide a useful preclinical model to study the action of CXCR4 antagonists in tumor growth and spread (15).

ACKNOWLEDGMENTS

We thank Sue Hoare for performing the CXCL12 ELISA analysis and surgeons at the Department of Obstetrics and Gynecology, Guy's and St Thomas' Hospital for providing patient material.

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Cancer Res 2001;61:4961-4965.

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