The Inflammatory Cytokine Tumor Necrosis Factor-α Regulates Chemokine Receptor Expression on Ovarian Cancer Cells

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Abstract

Epithelial ovarian cancer cells express the chemokine receptor, CXCR4, which may be associated with increased survival and metastatic potential, but the regulation of this receptor is not understood. The inflammatory cytokine tumor necrosis factor-α (TNF-α) is found in ovarian cancer biopsies and is associated with increased tumor grade. In this report, we show that CXCR4 expression on human epithelial ovarian cancer cells is associated with, and can be modulated by, TNF-α. Ovarian cancer cells with high endogenous expression of TNF-α expressed higher levels of CXCR4 mRNA and protein than cells with low TNF-α expression. Stimulation of ovarian cancer cell lines and primary epithelial cancer cells with TNF-α resulted in increased CXCR4 mRNA and protein. The TNF-α–stimulated increase in CXCR4 mRNA was due partly to *de novo* synthesis, and up-regulation of CXCR4 cell surface protein increased migration to the CXCR4 ligand CXCL12. CXCR4 mRNA and protein was down-regulated by anti-TNF-α antibody or by targeting TNF-α mRNA using RNAi. TNF-α stimulation activated components of the nuclear factor κB pathway, and overexpression of the inhibitor of κB also reduced CXCR4 expression. Coculture of macrophages with ovarian cancer cells also resulted in cancer cell up-regulation of CXCR4 mRNA in a TNF-α–dependent manner. Finally, there was a correlation between the levels of TNF-α and CXCR4 mRNA in clinical biopsies of ovarian cancer, and TNF-α protein was expressed in CXCR4-positive tumor cells. TNF-α is a critical mediator of tumor promotion in a number of experimental cancers. Our data suggest that one mechanism may be through nuclear factor κB–dependent induction of CXCR4. (Cancer Res 2005; 65(22): 10355-62)

Introduction

The directed migration of tumor cells to distant organs, via lymphatics and blood, resembles chemokine-directed lymphocyte migration. Recent studies suggest that chemokine receptor expression on tumor cells may have similar functions as on leukocytes, controlling migration, homing, and survival (1, 2). Tumor cells may express restricted and specific patterns of chemokine receptors, and response to chemokine gradients may contribute to disease progression (3). The chemokine receptor most commonly expressed on cancer cells is CXCR4; expression has been reported on at least 23 different tumor cell types (1, 4). We have been studying chemokine receptor expression in epithelial ovarian cancer, and have previously reported that of 14 chemokine receptors investigated, only CXCR4 was expressed by ovarian cancer cells (2). Stimulation of CXCR4-expressing ovarian cancer cells with the CXCR4 chemokine ligand, CXCL12, not only increased cell migration and invasion, but promoted proliferation under suboptimal conditions, phosphorylation of p44/42 mitogen-activated protein kinase and Akt/PKB, and induced tumor necrosis factor-α (TNF-α) mRNA and protein (5).

Regulation of chemokine receptors on cancer cells may be genetic or microenvironmental. In alveolar rhabdomyosarcoma, CXCR4 expression is activated by the fusion of *PAX3* and *PAX7-FKHR* genes (6). In renal cell carcinoma, acquisition of CXCR4 expression may involve mutations in the von Hippel-Lindau factor tumor suppressor gene (*VHL*; ref. 7). Her2 expression is associated with CXCR4 in human breast cancer (8). Hypoxia in the tumor microenvironment may contribute to this CXCR4 up-regulation (9). Additional studies suggest that there may be other factors controlling chemokine receptor expression on tumor cells such as transcription and growth factors. Examples include nuclear factor κB (NF-κB; ref. 10) and vascular endothelial growth factor (VEGF; ref. 11), which increase CXCR4 expression on breast cancer cell lines.

Earlier work by this laboratory has shown that the proinflammatory cytokine TNF-α is overexpressed in ovarian tumors compared to normal ovarian tissue and that expression is related to increasing grade. TNF-α has been implicated in tumor/stromal communication and tumor progression (12).[1] Furthermore, low doses of endogenous TNF-α produced by epithelial or stromal cells can act as a tumor promoter (13–15).

We previously showed that stimulation of CXCR4 on ovarian cancer cells results in TNF-α production, and that neutralizing antibody to TNF-α inhibits ovarian cancer cell migration to CXCL12 (5). In this article, we show that this mechanism works by increased CXCR4 expression in the tumor cells. We report that TNF-α acts as an autocrine or paracrine regulator of functional CXCR4 expression on ovarian cancer cells in an NF-κB–dependent manner. Antibodies to TNF-α, RNAi directed towards TNF-α or overexpression of inhibitor of κB (IκB), all decrease CXCR4 expression on ovarian cancer cells. Furthermore, we show that interaction of tumor cells with macrophages enhances CXCR4 expression in the ovarian cancer cells in a TNF-α–dependent manner. Finally, we show a correlation with CXCR4 and TNF-α mRNA levels and colocalization of CXCR4 and TNF-α protein in biopsies of epithelial ovarian cancer.

One reason why TNF-α may act as a tumor promoter in ovarian cancer is through increasing tumor cell invasiveness via up-regulation of CXCR4. Targeting TNF-α and ultimately CXCR4 could be a therapeutic strategy in ovarian cancer.

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[1] P.W. Szlosarek et al., submitted for publication.

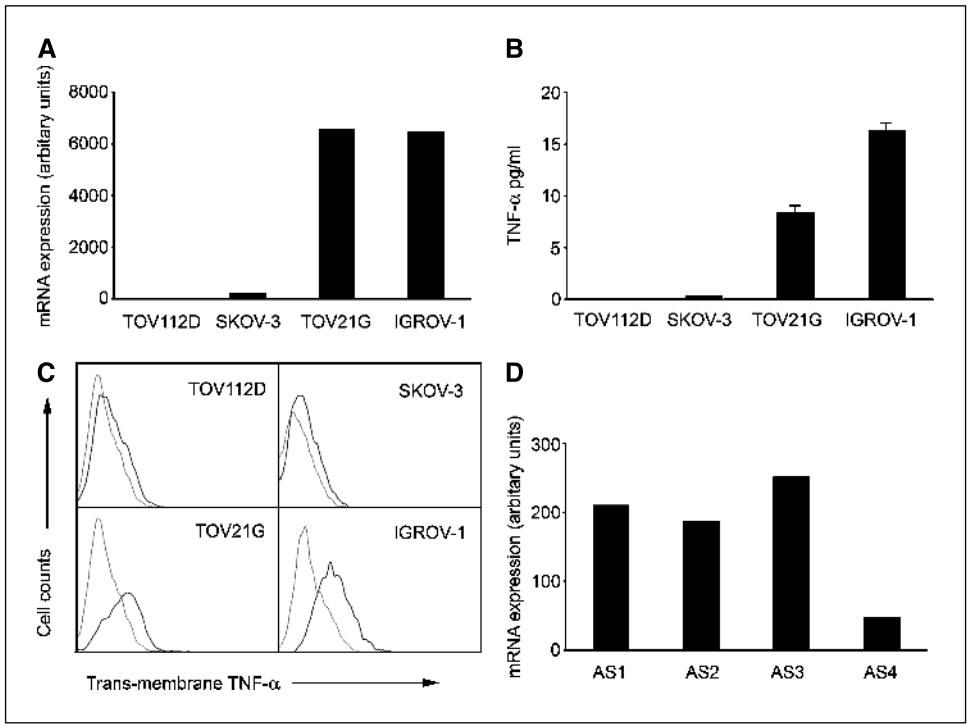

Figure 1. TNF-α expression by ovarian cancer cells. *A,* TNF-α mRNA expression by ovarian cancer cell lines measured by real-time reverse transcription-PCR. *B,* TNF-α protein secreted by ovarian cancer cell lines measured by ELISA. *Columns,* mean; *bars,* ±SD. *C,* expression of trans-membrane TNF-α measured by flow cytometry. *D,* TNF-α mRNA expression by primary ovarian cancer cell cultures. Representative of three experiments.

Materials and Methods

Ovarian cancer cells. The ovarian cancer cell lines SKOV-3, TOV112D, TOV21G (all from American Type Culture Collection, Rockville, MD) and IGROV-1 (12) were cultured in DMEM supplemented with 10% FCS. Cells were passaged using enzyme-free cell dissociation buffer (Life Technologies, Paisley, United Kingdom). In experiments where endogenous TNF-α was neutralized with specific antibody, cells were cultured in DMEM supplemented with 1% FCS and 1 μg/mL anti-TNF-α (Infliximab) or control antibody (M003; R&D Systems, Abingdon, United Kingdom). Antibodies were replaced daily.

Culture of primary ovarian epithelial cells and extraction of mRNA from tumor biopsies was approved by the East London and City Health Authority Research Ethics Committee, informed consent was obtained from patients attending the gynecologic oncology unit at St. Bartholomew's Hospital, London. Primary ovarian cancer cells from ascites were obtained from patients undergoing surgery for ovarian cancer and cultured in RPMI medium supplemented with 10% FCS.

Macrophage isolation and culture. Peripheral blood mononuclear cells (PBMC) were isolated from whole human blood using a Ficoll-Hypaque gradient (Amersham Pharmacia Biotech, Bucks, United Kingdom). $CD14^+$ monocytes were isolated from the PBMC using MACS super-paramagnetic microbeads (Miltenyi Biotech, Bisley, United Kingdom). Isolated cells were cultured in AIM-V medium (Life Technologies) supplemented with 2% human AB serum (Sigma, Poole, United Kingdom) in Teflon bags (Süd-Laborbedarf GmbH, Gauting, Germany) until the cells took on a typical macrophage morphology (7-14 days; ref. 13).

Macrophage/tumor cell cocultures. Tumor cells (2.5×10^5/well in 2 mL DMEM) were seeded into the upper well of the chamber. For coculture experiments, macrophages (5×10^5 cells) were seeded in transwell inserts (pore size, 0.4 μm; Nunc, Wiesbaden, Germany), which are permeable for liquids, but not for cells, and were inserted into the upper well of the Boyden chamber (13). At the indicated time points, cells were isolated and RNA was extracted for analysis.

Flow cytometry. Monoclonal antibodies against CXCR4 (MAB173), membrane TNF-α (6401), and isotype-matched control (11711.11) were used (all R&D Systems). Antibodies were used between 2 and 20 μg/mL. Cells were counterstained with FITC-conjugated secondary antibody (Sigma) and analyzed on a FACScan flow cytometer using CellQuest software (BD PharMingen, Oxford, United Kingdom).

Migration. Chemotaxis was assayed using Falcon transwells (24-well format, 8 μm pore; BD PharMingen). Cells (5×10^5) were added to the upper chamber and medium alone or supplemented with CXCL12 was added to the lower chamber. For some experiments, cells were prestimulated for 3 or 6 hours with 10 ng/mL TNF-α, or 1 μg/mL anti-TNF-α was added to the upper chamber. Migration assays were incubated for 18 hours at 37°C and 5% CO_2. Migrated cells on the lower surface were stained using DiffQuik (Dade Behring, Düdingen, Switzerland). For each transwell, the number of migrated cells in 10 medium power fields (×20) was counted.

RNA extraction and real-time quantitative reverse transcription-PCR analysis. RNA was extracted from primary cells and cell lines using Tri Reagent (Sigma) and treated with 10 units of DNase (Pharmacia, Milton Keynes, United Kingdom) following the manufacturer's instructions. DNase-treated RNA (2 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Southampton, United Kingdom) according to the manufacturer's instructions. Multiplex real-time reverse transcription-PCR analysis was done using premade TNF-α (FAM) and 18s rRNA (VIC) specific primers and probes with the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Warrington, United Kingdom). Primers and probe for human CXCR4 were designed using Primer Express 1.5 (PE Applied Biosystems) from sequences submitted to the Genbank. The sequences and concentrations of primers and probe are as follows: CXCR4 forward, 5′-TCCTGCTGACTATTCCCGACTT-3′ (800 nmol/L); reverse, 5′-GGGTAGAAGCGGTCACAGATATATC-3′ (200 nmol/L); probe, 5′-TCATCTGCCTCACTGACGTTGGCAA-3′ (300 nmol/L).

Expression values were normalized (ΔCt) to 18s rRNA by subtracting the cycle threshold (Ct) value of 18s rRNA from the Ct value of the experimental value. The fold differences compared with controls were calculated.

mRNA stability. Ovarian cancer cell lines were stimulated with either 10 ng/mL TNF-α, 5 μg/mL actinomycin D or 10 ng/mL TNF-α for 1 hour before the addition of actinomycin D (5 μg/mL) to the cultures to prevent mRNA synthesis. Thereafter, RNA was extracted at different time points as indicated and analyzed using real-time reverse transcription-PCR for CXCR4 mRNA expression.

Western blotting. Cell extract (10 μg) was run on an SDS 12% acrylamide gel and transferred to a nylon membrane. The membrane was blocked overnight (4°C in PBS with 0.1% Tween and 10% milk powder) and probed using anti-CXCR4 antibody (Abcam, Cambridge, United Kingdom). A horseradish peroxidase–conjugated secondary antibody was used for detection (1:5,000) dilution at room temperature for 1 hour. The secondary antibody was detected using the Western Lighting Chemiluminescence kit (Perkin-Elmer Life Sciences, Beaconsfield, United Kingdom). Protein concentration equivalence was confirmed after probing by amido black staining and β-actin antibody.

ELISA for TNF-α in cell culture supernatants. Cell culture supernatants were removed after 24 or 48 hours of culture and TNF-α concentration was measured using the Quantikine TNF-α ELISA kit (R&D Systems). The sensitivity of the assay was 4.4 pg/mL.

Transcription factor analysis. Transcription factors were measured using the TransFactor Profiling kits (Inflammation 1 and Inflammation 2 from BD Biosciences) following the manufacturer's instructions. Briefly, subconfluent cultures of TOV21G and IGROV-1 were treated with 10 ng/mL TNF-α for 15 minutes before extraction of nuclear proteins for analysis.

Immunohistochemistry. Paraffin-embedded sections were stained for TNF-α and CXCR4. TNF-α expression (MAB610; R&D Systems) was localized with diaminobenzidine, followed by counterstaining with Hemotoxylin. CXCR4 expression was assessed with anti-CXCR4 monoclonal antibody MAB173 (R&D Systems). Expression was localized with AEC and counterstained with hematoxylin. Positive controls were obtained by staining sections of human skin. Control antibodies were used to provide negative controls.

Transfection of IGROV-1 cells. IGROV-1 cells were transfected with the pIκB-enhanced green fluorescent protein (EGFP) vector (BD Clontech, San Diego, CA), SUPER RNAi plasmids for TNF-α, or the empty retroviral vector (IGROV-Mock) and isolated according to the protocols described (14). Cells were transfected using Lipofectamine 2000 (Invitrogen, Paisley, United Kingdom) following the manufacturer's instructions. Antibiotic selection for stable cell lines started after 48 to 72 hours, pIκB-EGFP clones in 500 μg/mL G418 (Invitrogen) and SUPER RNAi plasmid–expressing cells in 4 μg/mL puromycin (Sigma) for 30 days.

Transfection efficacy, luciferase, and β-galactosidase assays. To monitor NF-κB activation, we used the pNF-κB-Luc vector (BD Clontech). When endogenous NF-κB proteins bind to the κ enhancer element (κB4), transcription is induced and the reporter gene is activated. Luciferase reporter gene activity was determined by the luciferase reporter assay (BD Clontech) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was evaluated using Student's *t* test or one-way ANOVA (Instat software, San Diego, CA).

Results

CXCR4 expression on ovarian cancer cells is related to endogenous levels of TNF-α. In this report, we used four different ovarian cancer cell lines as well as primary cancer cells isolated from ovarian cancer ascites. The ovarian cancer cell lines expressed variable levels of endogenous TNF-α; the TOV112D and SKOV-3 lines expressed low levels of mRNA for TNF-α, whereas the TOV21G and IGROV-1 lines expressed higher levels (up to 6,000-fold more) of mRNA for TNF-α (Fig. 1*A*). Expression of TNF-α mRNA was related to TNF-α protein production (Fig. 1*B*). No detectable TNF-α protein was produced by the TOV112D cell line and the SKOV-3 cell line secreted levels of TNF-α below the sensitivity of the ELISA (0.3 pg/mL). The TOV21G and IGROV-1 lines secreted significantly more TNF-α protein into the supernatant (8.4 and 16.4 pg/mL, respectively; Fig. 1*B*). The expression of membrane-bound TNF-α was determined by flow cytometry; low expression was detected on the surface of the TOV112D and SKOV-3 cell lines than the TOV21G and IGROV-1 cell surface (Fig. 1*C*). Four cultures of primary ovarian cancer cells derived from ascites (AS1-AS4) also expressed mRNA for endogenous TNF-α (Fig. 1*D*).

TNF-α production by ovarian cancer cells was directly related to CXCR4 expression. The SKOV-3 and TOV112D cell lines expressed low levels of CXCR4 mRNA, whereas the TOV21G and IGROV-1 cell lines had significantly higher expression of CXCR4 mRNA (Fig. 2*A*). Furthermore, the SKOV-3 and TOV112D cell lines expressed little CXCR4 cell surface protein, whereas the TOV21G and IGROV-1 cell lines were highly positive for this receptor (Fig. 2*B*). CXCR4 mRNA was also detected in primary ovarian cancer epithelial cells (AS1-AS4); the primary ovarian culture with the lowest expression levels of TNF-α (AS4) also had the lowest expression of CXCR4 mRNA (Fig. 2*C*). To further investigate the relationship between CXCR4 expression and TNF-α, we stimulated ovarian cancer cell lines with exogenous TNF-α.

TNF-α induces CXCR4. In all four ovarian cancer cell lines, treatment with 1, 10, or 100 ng/mL TNF-α resulted in a significant increase in the expression CXCR4 mRNA. Maximal up-regulation was observed in all cell lines when stimulated with 10 ng/mL TNF-α. Stimulation with 1 or 100 ng/mL TNF-α resulted in a 2- to 7-fold increase in CXCR4 mRNA, whereas 10 ng/mL induced up to 15-fold increase. Data from the IGROV-1 and TOV21G cell lines are shown in Fig. 3*A* and *B*. This elevation was observed 1 hour

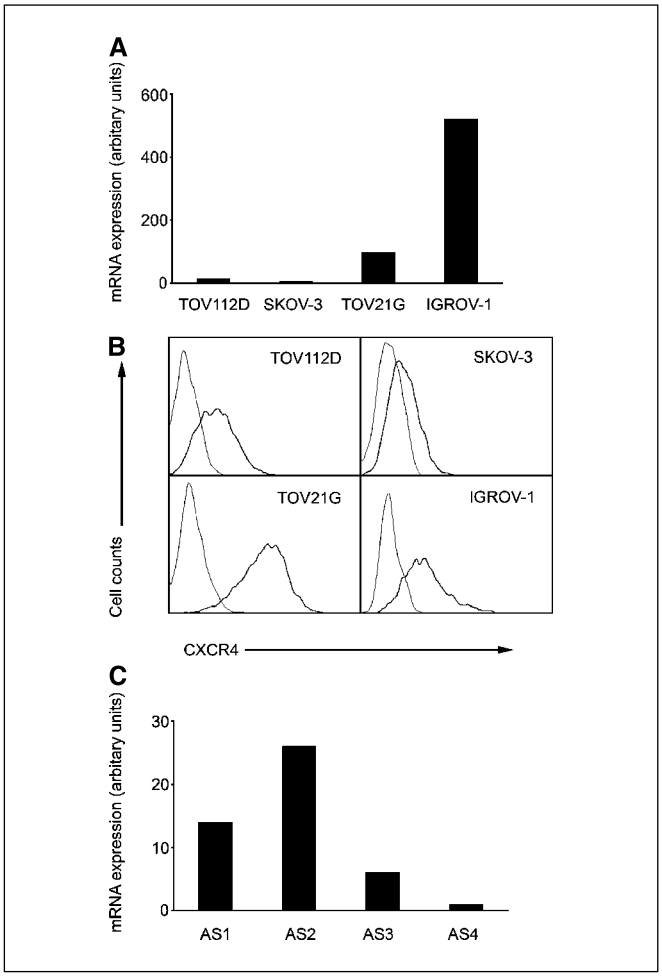

Figure 2. CXCR4 expression by ovarian cancer cells relates to TNF-α expression. *A,* CXCR4 mRNA expression in ovarian cancer cell lines; *B,* CXCR4 cell surface protein expression in ovarian cancer cell lines; *C,* CXCR4 mRNA expression in primary ovarian cancer cell cultures (AS1-AS4). Representative of three experiments.

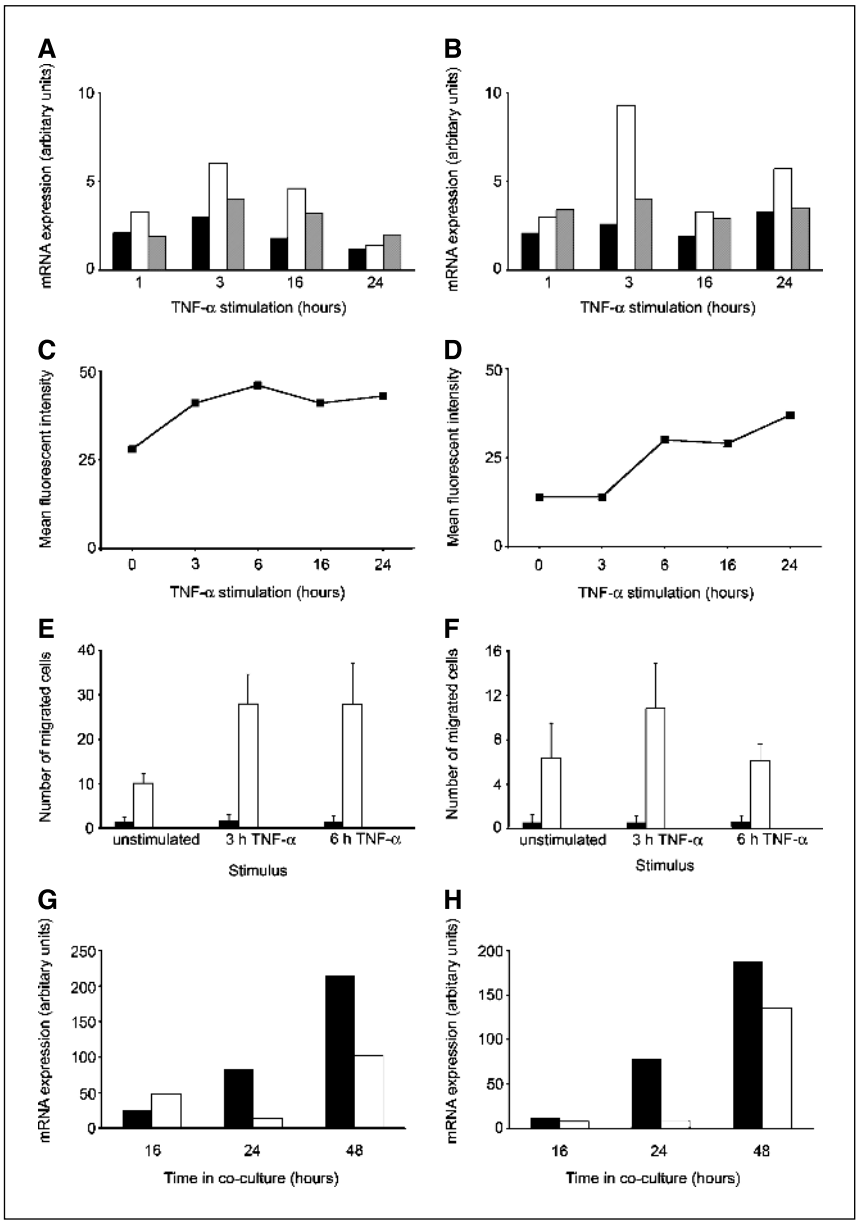

Figure 3. CXCR4 mRNA expression is up-regulated in ovarian cancer cell lines following stimulation with TNF-α. RNA was extracted from ovarian cancer cell lines TOV21G (*A*) and IGROV-1 (*B*) treated with 1, 10, and 100 ng/mL TNF-α (*black, white*, and *hatched columns*, respectively) at the time points indicated and real-time reverse transcription-PCR was done. The values represent fold difference in relation to the unstimulated control and the results are representative of three experiments. CXCR4 protein, measured by flow cytometry, is up-regulated on the cell surface of ovarian cancer cell lines IGROV-1 (*C*) and TOV21G (*D*) following stimulation with 10 ng/mL TNF-α. One of five representative experiments. *E* and *F,* enhanced migration of IGROV-1 (*E*) and TOV21G (*F*) ovarian cancer cells following stimulation with 10 ng/mL TNF-α. Ovarian cancer cells have low basal migration (*black columns*) but migrate towards CXCL12 (*white columns*). This is augmented by stimulating ovarian cancer cells with TNF-α for 3 or 6 hours before the migration assay. *Columns,* mean; *bars,* ± SD of 15 determinations and the results are representative of three experiments. CXCR4 mRNA expression is up-regulated in the ovarian cancer cell lines TOV21G (*G*) and IGROV-1 (*H*) following culture with macrophages (*black columns*), up-regulation was partially inhibited by addition of anti-TNF-α antibodies (*white columns*). Ovarian cancer cells were harvested from cocultures at the time points indicated and real-time reverse transcription-PCR was done. Values represent fold differences in relation to unstimulated controls at each time point and the results are representative of four experiments done.

after stimulation and was sustained for the 24-hour period of observation (Fig. 3*A* and *B*; data not shown). Stimulation of the primary ovarian cancer cells derived from ascites (AS1-AS4) with 10 ng/mL TNF-α also resulted in a significant up-regulation of CXCR4 mRNA expression. Maximal expression of CXCR4 was observed between 6 and 24 hours of stimulation with TNF-α (data not shown).

Following stimulation with 1, 10, or 100 ng/mL TNF-α, cell surface expression of CXCR4 protein was increased in the IGROV-1 and TOV21G cell lines but remained unchanged in the SKOV-3 and TOV112D lines (data not shown). Up-regulation of cell surface expression of CXCR4 protein in the IGROV-1 and TOV21G cell lines was strongest following stimulation with 10 ng/mL TNF-α and is shown in Fig. 3*C* and *D*. All ovarian cancer cell lines and primary ovarian cancer cells derived from ascites express TNF-RI but do not express TNF-RII. There were no differences in expression of TNF-RI between primary cancer cells and established cancer cell lines or between cells with low or high endogenous expression of TNF-α (data not shown).[1]

Stimulation of ovarian cancer cell lines with TNF-α enhances migration towards CXCL12. To determine whether the TNF-α-induced increase in cell surface CXCR4 expression was functional, simple transwell migration assays towards CXCL12, the only known ligand for CXCR4, were done. After stimulation with TNF-α for 3 and 6 hours, IGROV-1 cells had augmented migration towards CXCL12 ($P < 0.0001$ and $P < 0.0001$, respectively; Fig. 3*E*). TOV12G cells also had enhanced levels of migration after 3 hours of stimulation with TNF-α ($P < 0.0001$) and after 6 hours of stimulation ($P < 0.0001$; Fig. 3*F*). Stimulation of the IGROV-1 and TOV21G cell lines with TNF-α (10 ng/mL) for 3 and 6 hours did not alter the basal levels of migration.

Coculture of tumor cells with macrophages up-regulates CXCR4. Coculture of epithelial breast cancer cells with macrophages results in TNF-α–dependent enhanced tumor cell migration

(13). As tumor-associated macrophages are also a source of TNF-α in the ovarian cancer microenvironment, we investigated CXCR4 expression within a coculture system. Following coculture with macrophages, all four ovarian cancer cell lines up-regulated CXCR4 mRNA expression. Data for the ovarian cancer cell lines IGROV-1 and TOV21G are shown in Fig. 3*G* and *H*. Up-regulation of CXCR4 mRNA was observed after 16 hours of coculture, was further increased after 24 hours, and continued to increase after 48 hours of coculture. This increase in CXCR4 mRNA expression was due, in part, to TNF-α production within the coculture system, because a neutralizing antibody to TNF-α partially inhibited the up-regulation of CXCR4 mRNA (Fig. 3*G* and *H*).

Effect of anti-TNF-α antibody or TNF-α RNAi on CXCR4 expression. To investigate the role of endogenous TNF-α production on cellular levels of CXCR4, ovarian cancer cell lines that produced TNF-α protein, namely IGROV-1 and TOV21G, were treated with anti-TNF-α antibodies. Treatment of both cell lines with Infliximab, a neutralizing antibody to TNF-α, resulted in reduced mRNA and cell surface expression of CXCR4 (Fig. 4).

In IGROV-1 cells, a reduction in CXCR4 mRNA was observed at 6, 24, 48, and 72 hours after treatment with anti-TNF-α (Fig. 4*A*). Likewise, a reduction in CXCR4 mRNA was observed in the TOV21G cell lines at 6, 24, and 48 hours after treatment with anti-TNF-α (Fig. 4*B*). In both cell lines, this reduction was maximal between 6 and 24 hours of treatment with anti-TNF-α, where there was, on average, a reduction of 50% in CXCR4 mRNA expression. Cell surface protein expression was also down-regulated in both the IGROV-1 and TOV21G cell lines. This was observed as early as 3 hours after stimulation and was reduced for the duration of the experiment (Fig. 4*C* and *D*). This explains why, in a previous report, we observed significant inhibition of migration of CXCR4-expressing ovarian cancer cell lines to CXCL12 in the presence of anti-TNF-α (5).

Further proof for the role of TNF-α in maintaining and up-regulating expression of CXCR4 on ovarian cancer cell lines was provided from IGROV-1 cells, which were stably transfected with RNAi for TNF-α. Two independently isolated clones of IGROV-RNAi TNF-α-transfected cells exhibited down-regulation of total CXCR4 protein (Fig. 4*F*), demonstrating that inhibition of endogenous TNF-α results in down-regulation of CXCR4.

mRNA stability. In all cell lines, TNF-α stimulation resulted in an increase in CXCR4 mRNA, cells treated with actinomycin D exhibited a time-dependent decay in CXCR4 mRNA levels. In some experiments, the ovarian cancer cell lines were stimulated with TNF-α for 1 hour before the addition of actinomycin D and the mRNA decay was compared with cells treated singly with either TNF-α or actinomycin D. The decay of mRNA from cells pretreated with TNF-α was comparable to those treated with actinomycin D alone (data not shown). This suggests that TNF-α stimulation of ovarian cancer cell lines may lead to *de novo* synthesis of CXCR4 mRNA but does not exclude the possibility that TNF-α is also involved in other processes of CXCR4 regulation.

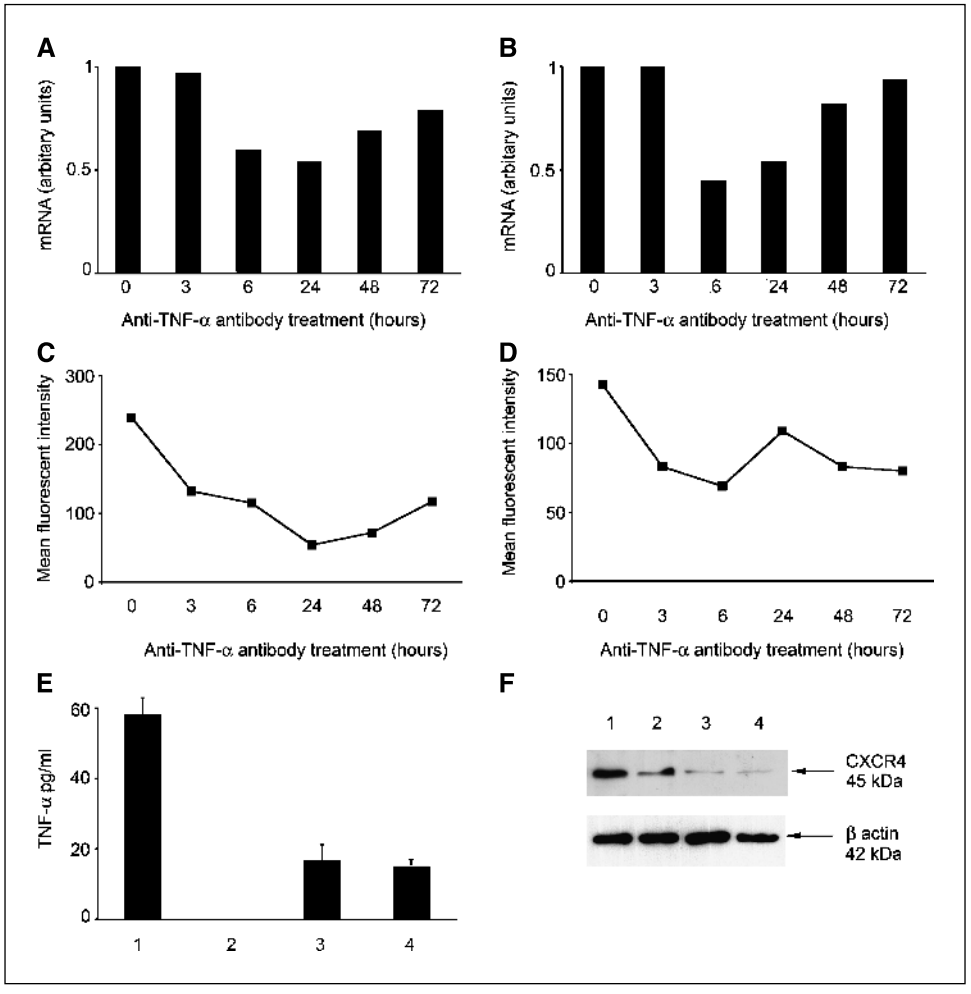

Figure 4. CXCR4 mRNA and protein expression is down-regulated in ovarian cancer cell lines following treatment with anti-TNF-α antibodies. RNA was extracted from the ovarian cancer cell lines IGROV-1 (*A*) and TOV21G (*B*) treated with anti-TNF-α antibody at the time points indicated and real-time reverse transcription-PCR was done. The values represent fold difference in relation to the untreated control and the results are representative of four experiments. Cell surface CXCR4 protein, measured by flow cytometry, is down-regulated on IGROV-1 (*C*) and TOV21G (*D*) following treatment with anti-TNF-α antibodies. One experiment of five done. *E* and *F,* down-regulation of secreted TNF-α and total CXCR4 protein in IGROV-1 cells after overexpression of the IκB or transfection with RNAi to TNF-α: (1) IGROV-Mock–transfected, (2) IGROV-IκB, (3 and 4) two independently isolated clones IGROV-TNF-αRNAi. *E,* TNF-α protein secreted by transfected cells as measured by ELISA; *columns,* mean; *bars,* ±SD. *F,* total CXCR4 protein expression in transfected cells measured by Western blot. One of three experiments done.

Transcription factors expressed following TNF-α stimulation. To identify transcription factors induced in ovarian cancer cells following stimulation with TNF-α, TransFactor Profiling inflammation kits were used. After 15 minutes of stimulation with TNF-α, the transcription factors NF-κB p65, NF-κB p50, c-Rel, FosB, JunD, and c-*Jun* were up-regulated in both the IGROV-1 and TOV21G lines (Fig. 5). In the TOV21G cell line, the SP1 transcription factor was also up-regulated. No up-regulation of the transcription factors cAMP-responsive element binding protein-1, activating transcription factor-2, or signal transducers and activators of transcription-1 was detected under these conditions.

IκB overexpression and TNF-α RNAi down-regulates CXCR4 expression. Components of the NF-κB pathway were activated following TNF-α stimulation of ovarian cancer cell lines. To investigate the influence of the NF-κB pathway on CXCR4 regulation in ovarian cancer cell lines, we overexpressed IκB in the IGROV-1 cell line to inhibit this pathway. Overexpression of IκB resulted in down-regulation of both TNF-α secretion and total CXCR4 protein (Fig. 4*E* and *F*, respectively).

Correlation between TNF-α and CXCR4 expression in clinical samples. CXCR4 and TNF-α levels varied in ovarian cancer biopsies (2).[1] To assess whether CXCR4 and TNF-α expression were linked, quantitative real-time reverse transcription-PCR was used to assess expression of these molecules in 15 isolates of mRNA from patient tumor samples. We divided the samples into groups with low and high expression of TNF-α mRNA. Seven samples had low expression whereas eight samples had high expression of TNF-α mRNA. We then assessed expression of CXCR4 mRNA in these isolates and found a significant correlation between levels of expression of TNF-α and levels of expression of CXCR4 (P = 0.002; Fig. 6*A*). Immunohistochemical staining of serial sections of paraffin-embedded ovarian cancer tissue revealed that epithelial cancer cells that expressed CXCR4 protein were also positive for TNF-α staining (Fig. 6*B-G*).

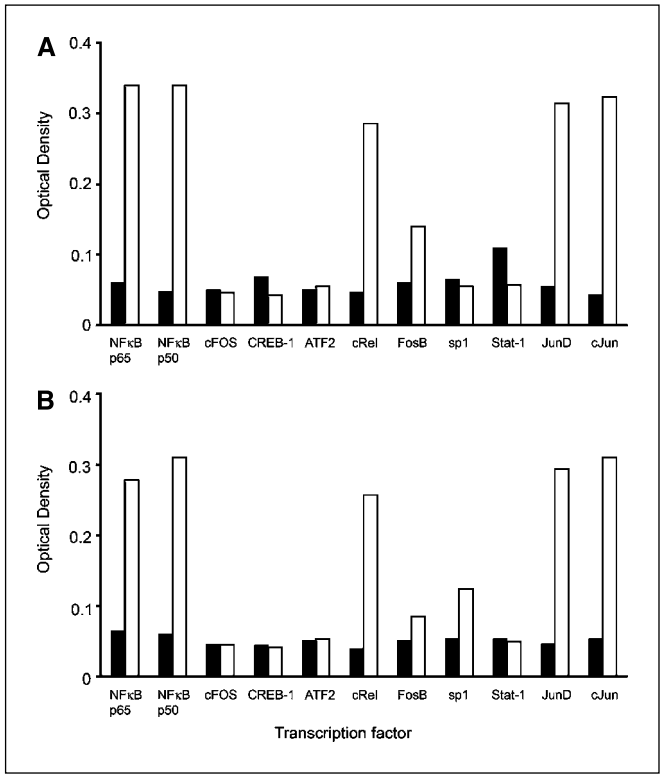

Figure 5. Transcription factors induced in IGROV-1 (*A*) and TOV21G (*B*) following stimulation with TNF-α. Nuclear cell extracts were prepared from control cells (*black columns*) or cells stimulated with 10 ng/mL TNF-α for 15 minutes (*white columns*).

Discussion

In this report, we provide evidence that CXCR4 expression in human ovarian cancer is related to the expression of the inflammatory cytokine, TNF-α. Using ovarian cancer cell lines and primary epithelial ovarian cancer cells, we have shown that expression of endogenous TNF-α correlates with expression of CXCR4. Stimulation of ovarian cancer cells with exogenous TNF-α can further increase CXCR4 mRNA expression, and in cells which up-regulate CXCR4 protein in response to TNF-α stimulation, there is a corresponding increase in migration towards the CXCR4 ligand, CXCL12. Furthermore, we have established that treatments which target TNF-α, such as neutralizing antibodies or RNAi, decrease expression of both CXCR4 mRNA and protein. TNF-α stimulation results in *de novo* synthesis of CXCR4 mRNA in a pathway that involves the transcription factor NF-κB.

CXCR4 expression has been reported in many types of cancer (4), although few studies address the mechanism(s) by which cancer cells acquire or modulate such expression. CXCR4 expression can be induced by overexpression of the transcription factor NF-κB in breast cancer cell lines (10), or in thyroid epithelial cells by overexpression of the RET/PTC oncogene (15). CXCR4 protein expression can be further up-regulated in cancer cells by treatment with VEGF (11), or culture under hypoxic conditions (9). Acquisition of CXCR4 in renal cell carcinoma has been related to mutations in the *VHL* gene (7). Renal carcinoma cells which lack VHL protein have elevated TNF-α expression; as this functions normally to repress TNF-α translation (16), this may provide an additional mechanism for the up-regulation of CXCR4 via increased TNF-α expression.

The molecular mechanisms linking inflammation and cancer are being elucidated. There is increasing evidence that TNF-α is produced by cancer cells and can act as an endogenous tumor promoter (17, 18). Stromal production of TNF-α may also influence tumor behavior as inhibition of stromal TNF-α decreases the incidence of inflammation-induced liver tumors (19). Recent studies have shown the role of NF-κB in malignant progression in inflammation-induced colon cancer (20) and liver cancer (19); where activation of NF-κB in premalignant cells by TNF-α and other inflammatory cytokines can result in transformation. NF-κB activation can promote a pro-tumor microenvironment as expression of interleukin-6, interleukin-8, urokinase-type plasminogen activator, matrix metalloproteinase-9, and VEGF are up-regulated via NF-κB–dependent pathways (10). Furthermore, selective deletion of IκB kinase in intestinal epithelial cells reduced subsequent development of intestinal tumors (20). In this study, three rel family members that make up the NF-κB transcription complex, p65, p50, and cRel, were activated in ovarian cancer cells within 15 minutes of stimulation with TNF-α. The NF-κB complex is normally confined to the cytosol through its interaction with the IκB protein; upon stimulation, IκB is degraded and NF-κB is activated. In ovarian cancer cells where IκB was overexpressed, we observed a down-regulation of CXCR4 mRNA and protein providing further evidence of the link between TNF-α/NF-κB and CXCR4 expression. The activator protein (AP-1) transcription factor forming family members, JunD, c-*Jun*, and FosB were also activated in ovarian cancer cell lines following stimulation with

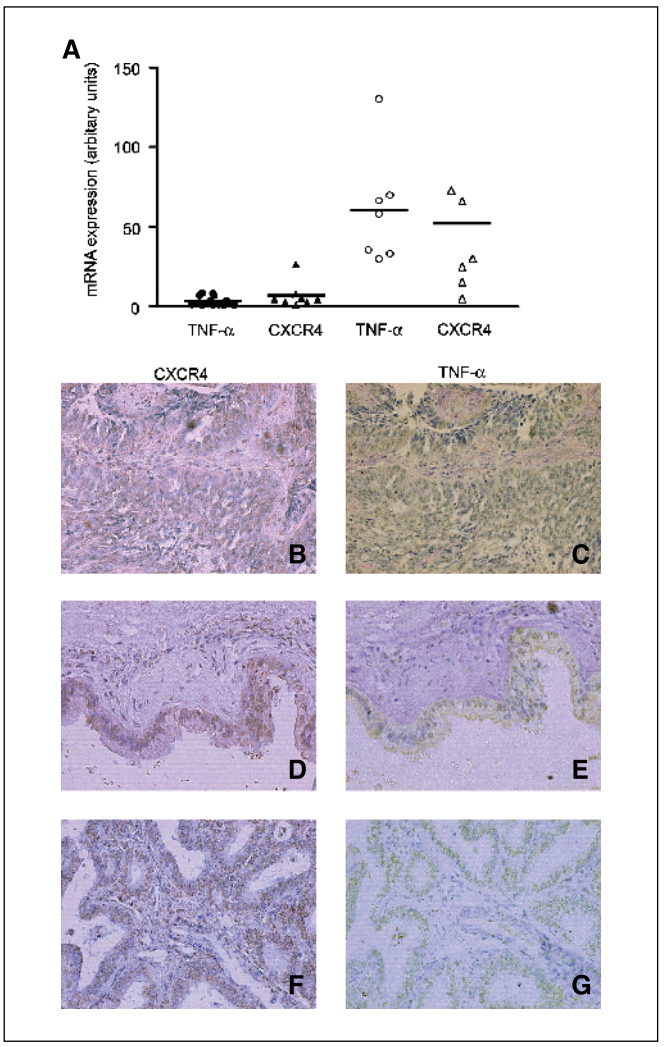

Figure 6. CXCR4 and TNF-α expression in clinical isolates of epithelial ovarian cancer. *A,* RNA was extracted from 15 primary tumor samples and real-time reverse transcription-PCR for TNF-α was done, samples were divided into those with low (●) or high (○) expression of TNF-α. CXCR4 mRNA was then measured in these grouped samples (▲ and △, respectively). *B-G,* expression of CXCR4 (*red stain*) and TNF-α (*gray/brown stain*) protein in serial sections of human ovarian cancer. Serous adenocarcinoma stage IV (*B* and *C*), mucinous adenocarcinoma stage IA (*D* and *E*), and endometrioid adenocarcinoma stage IA (*F* and *G*).

TNF-α. These data show that two pathways involved in inflammation are initiated after TNF-α stimulation, i.e., AP-1 and NF-κB. Furthermore, in our coculture system, TNF-α-mediated activation of NF-κB and AP-1 in both macrophages and ovarian cancer cells has been observed (21). NF-κB and AP-1 are also activated by liver-infiltrating inflammatory cells (19).

Tumor-associated macrophages may influence tumor growth and progression (22). We reported previously that coincubation of breast cancer cells with macrophages resulted in enhanced tumor cell invasiveness via pathways that were dependent on macrophage production of TNF-α and matrix metalloproteases (13). In this study, we show that coculture of ovarian cancer cell lines results in cancer cell up-regulation of CXCR4. This increase is partially dependent on TNF-α production as neutralizing antibodies to TNF-α inhibited tumor cell up-regulation of CXCR4. Within the tumor microenvironment, both macrophages and tumor cells produce TNF-α. This interaction between macrophages and tumor cells, and the resulting up-regulation of CXCR4 is physiologically relevant and may partially explain the up-regulation of CXCR4 observed when cancer cell lines are grown in experimental murine models *in vivo* (23).

Our experiments suggest that stimulation of ovarian cancer cells with TNF-α induces *de novo* transcription of CXCR4 mRNA. This agrees with a previous report where it was shown that the p65 and p50 subunits of NF-κB bind to sequences within the CXCR4 promoter and activate CXCR4 transcription (10). Another study suggested that the hypoxia-induced increase in CXCR4 expression was due to both increased transcription and stability of CXCR4 mRNA (9). Increased mRNA stability of TNF-α, CXCL8, and VEGF was reported in malignant glioma following stimulation with exogenous TNF-α (24).

We reported previously that stimulation of ovarian cancer cells with CXCL12 induces expression of TNF-α mRNA and protein (5), and several studies have shown that tumor and stromal TNF-α have tumor-promoting activities (13–15). One mechanism may be that the TNF-α induced by CXCL12 stimulation can act back on the ovarian cancer cells to increase CXCR4 expression and tumor cell invasiveness. In effect, TNF-α "amplifies" the CXCL12 signal and is central to the induction of an inflammatory tumor–promoting milieu. TNF-α can then initiate a chemokine/cytokine cascade which may be beneficial for tumor growth in ovarian cancer (25).

We have shown an association of CXCR4 and TNF-α expression in clinical isolates of epithelial ovarian cancer, therefore, it may be possible to target NF-κB or TNF-α in this disease which would ultimately have an effect on the cancer cell expression of CXCR4.

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References

1. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature 2001;410:50–6.
2. Scotton CJ, Wilson JL, Milliken D, Stamp G, Balkwill FR. Epithelial cancer cell migration: a role for chemokine receptors? Cancer Res 2001;61:4961–5.
3. Balkwill F. Cancer and the chemokine network. Nat Rev Cancer 2004;4:540–50.
4. Balkwill F. The significance of cancer cell expression of the chemokine receptor CXCR4. Semin Cancer Biol 2004;14:171–9.
5. Scotton CJ, Wilson JL, Scott K, et al. Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. Cancer Res 2002;62:5930–8.
6. Libura J, Drukala J, Majka M, et al. CXCR4-SDF-1 signaling is active in rhabdomyosarcoma cells and regulates locomotion, chemotaxis, and adhesion. Blood 2002;100:2597–606.
7. Staller P, Sulitkova J, Lisztwan J, et al. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature 2003;425:307–11.
8. Li YM, Pan Y, Wei Y, et al. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. Cancer Cell 2004;6:459–69.
9. Schioppa T, Uranchimeg B, Saccani A, et al. Regulation of the chemokine receptor CXCR4 by hypoxia. J Exp Med 2003;198:1391–402.
10. Helbig G, Christopherson KW II, Bhat-Nakshatri P,

et al. NF-κB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. J Biol Chem 2003;278:21631–8.
11. Bachelder RE, Wendt MA, Mercurio AM. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. Cancer Res 2002;62:7203–6.
12. Benard J, Da Silva J, De Blois MC, et al. Characterization of a human ovarian adenocarcinoma line, IGROV1, in tissue culture and in nude mice. Cancer Res 1985;45:4970–9.
13. Hagemann T, Robinson SC, Schulz M, et al. Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-α dependent up-regulation of matrix metalloproteases. Carcinogenesis 2004;25:1543–9.
14. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002;296:550–3.
15. Castellone MD, Guarino V, De Falco V, et al. Functional expression of the CXCR4 chemokine receptor is induced by RET/PTC oncogenes and is a common event in human papillary thyroid carcinomas. Oncogene 2004;23:5958–67.
16. Galban S, Fan J, Martindale JL, et al. von Hippel-Lindau protein-mediated repression of tumor necrosis factor α translation revealed through use of cDNA arrays. Mol Cell Biol 2003;23:2316–28.
17. Moore RJ, Owens DM, Stamp G, et al. Mice deficient in tumor necrosis factor-α are resistant to skin carcinogenesis. Nat Med 1999;5:828–31.
18. Szlosarek PW, Balkwill FR. Tumour necrosis factor α: a potential target for the therapy of solid tumours. Lancet Oncol 2003;4:565–73.
19. Pikarsky E, Porat RM, Stein I, et al. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–6.
20. Greten FR, Eckmann L, Greten TF, et al. IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 2004;118:285–96.
21. Hagemann T, Wilson J, Kulbe H, et al. Macrophages induce invasiveness of epithelial cancer cells via NF-{kappa}B and JNK. J Immunol 2005;175:1197–205.
22. Mantovani A, Allavena P, Sica A. Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. Eur J Cancer 2004;40:1660–7.
23. Zeelenberg IS, Ruuls-Van Stalle L, Roos E. The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. Cancer Res 2003; 63:3833–9.
24. Nabors LB, Suswam E, Huang Y, et al. Tumor necrosis factor α induces angiogenic factor up-regulation in malignant glioma cells: a role for RNA stabilization and HuR. Cancer Res 2003;63:4181–7.
25. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001;357:539–45.