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

Hagen Kulbe, Thorsten Hagemann, Piotr W. Szlosarek, Frances R. Balkwill, and Julia L. Wilson

Cancer Research UK, Translational Oncology Laboratory, Bart’s and The London, Queen Mary’s School of Medicine and Dentistry, Charterhouse Square, London, United Kingdom

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). 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 (IkB), 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.

1 P.W. Szlosarek et al., submitted for publication.
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% human AB serum (Sigma, Poole, United Kingdom) until the cells took on a typical macrophage morphology (7-14 days; ref. 13).

Macrophage/tumor cell cocultures. Tumor cells (2.5 × 10^5) were seeded in transwell inserts (pore size, 0.4 μm; Nunc, Wiesbaden, Germany), which are permeable for membrane TNF-α/C2 (300 nmol/L). Macrophages were cultured in AIM-V medium (Life Technologies, Paisley, United Kingdom) supplemented with 2% R&D Systems, Abingdon, United Kingdom) until the cells took on a typical macrophage morphology (7-14 days; ref. 13).

Macrophage/tumor cell cocultures. Tumor cells (2.5 × 10^5) 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% CO2. 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'-TCCTCTCTAGAC-TATTCGCCAGCT-3' (800 nmol/L); reverse, 5'-GGGTTAGAGCGGTACACAGATATC-3' (200 nmol/L); probe, 5'-TCTATGCTACAGGTTGGCAA-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 dianimobenzidine, followed by counterstaining with Hematoxylin. 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 pB-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, pB-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. 1A). Expression of TNF-α mRNA was related to TNF-α protein production (Fig. 1B). 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. 1B). 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. 1C).

Four cultures of primary ovarian cancer cancer cells derived from ascites (AS1–AS4) also expressed mRNA for endogenous TNF-α (Fig. 1D).

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. 2A). 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. 2B). 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. 2C). 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. 3A and B. This elevation was observed 1 hour...
after stimulation and was sustained for the 24-hour period of observation (Fig. 3A 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. 3C 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 or 6 hours, IGROV-1 cells had augmented migration (P < 0.0001 and P < 0.0001, respectively; Fig. 3E). TOV12G cells also had enhanced levels of migration after 3 hours of stimulation (P < 0.0001) and after 6 hours of stimulation (P < 0.0001; Fig. 3F). 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
As tumor-associated macrophages are also a source of TNF-\(\alpha\) 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 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-\(\alpha\) production within the coculture system, because a neutralizing antibody to TNF-\(\alpha\) partially inhibited the up-regulation of CXCR4 mRNA (Fig. 3G and H).

**Effect of anti-TNF-\(\alpha\) antibody or TNF-\(\alpha\) RNAi on CXCR4 expression.** To investigate the role of endogenous TNF-\(\alpha\) production on cellular levels of CXCR4, ovarian cancer cell lines that produced TNF-\(\alpha\) protein, namely IGROV-1 and TOV21G, were treated with anti-TNF-\(\alpha\) antibodies. Treatment of both cell lines with Infliximab, a neutralizing antibody to TNF-\(\alpha\), 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-\(\alpha\) (Fig. 4A). Likewise, a reduction in CXCR4 mRNA was observed in the TOV21G cell lines at 6, 24, and 48 hours after treatment with anti-TNF-\(\alpha\) (Fig. 4B). In both cell lines, this reduction was maximal between 6 and 24 hours of treatment with anti-TNF-\(\alpha\), 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. 4C 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-\(\alpha\) (5).

Further proof for the role of TNF-\(\alpha\) 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-\(\alpha\). Two independently isolated clones of IGROV-RNAi TNF-\(\alpha\)-transfected cells exhibited down-regulation of total CXCR4 protein (Fig. 4F), demonstrating that inhibition of endogenous TNF-\(\alpha\) results in down-regulation of CXCR4.

**mRNA stability.** In all cell lines, TNF-\(\alpha\) 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-\(\alpha\) for 1 hour before the addition of actinomycin D and the mRNA decay was compared with cells treated singly with either TNF-\(\alpha\) or actinomycin D. The decay of mRNA from cells pretreated with TNF-\(\alpha\) was comparable to those treated with actinomycin D alone (data not shown). This suggests that TNF-\(\alpha\) stimulation of ovarian cancer cell lines may lead to de novo synthesis of CXCR4 mRNA but does not exclude the possibility that TNF-\(\alpha\) is also involved in other processes of CXCR4 regulation.
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. 4E and F, respectively).

Correlation between TNF-α and CXCR4 expression in clinical samples. CXCR4 and TNF-α levels varied in ovarian cancer biopsies (2). 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. 6A). 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. 6B-G).

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 epithelial intestinal 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 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).](image-url)
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 coinoculation 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.

Acknowledgments

Received 3/24/2005; revised 7/21/2005; accepted 8/16/2005.

Grant support: Cancer Research UK, Barts and The London Cancer Research Committee and Joint Research Board, Deutsche Forschungsgemeinschaft grant no. HA 3565/1-1 (T. Hagemann) and the Association of International Cancer Research (J.L. Wilson).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Anja Müller for help with the CXCR4 immunohistochemistry.

References


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22-24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society.

Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if \( M = +0.27 \) and \( L = -0.16 \) and the normal differential is 65 per cent M and 35 per cent L, then

\[
0.65 \times (+0.27) + 0.35 \times (-0.16) = +0.12
\]

a figure identical to the observed +0.12 for normal leukocytes.

308
The Inflammatory Cytokine Tumor Necrosis Factor-α Regulates Chemokine Receptor Expression on Ovarian Cancer Cells

Hagen Kulbe, Thorsten Hagemann, Piotr W. Szlosarek, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/22/10355

Cited articles
This article cites 24 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/22/10355.full#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/22/10355.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.