EXPERIMENTAL STUDY

Regulation of UT-OC-3 ovarian carcinoma cells by cytokines: inhibitory effects on cell proliferation and activation of transcription factors AP-1 and NF-κB

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Abstract

The present study was designed to investigate the growth regulatory effects of cytokines in UT-OC-3 ovarian cystadenocarcinoma cells in vitro. The effects of interleukin-6 (IL-6), interferons α (IFN-α) and γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor α (TNF-α), and transforming growth factor β1 (TGF-β1) were investigated by 125I-deoxyuridine (125IuDR) incorporation assay. In order to understand better the molecular mechanisms of the observed effects, the activation of DNA-binding proteins was studied by electrophoretic mobility shift assay. In addition, cellular DNA was tested by fragmentation analysis to determine if the most growth inhibitory cytokines are able to induce programmed cell death (apoptosis). After 48 h in culture, TGF-β1, TNF-α, IFN-α and IL-6 showed a clear inhibitory effect on 125IuDR incorporation (P<0.005), and IFN-γ and GM-CSF caused even more significant inhibition (P<0.001). IFN-α and IFN-γ were both growth inhibitory after 72 h in culture (P<0.005). Similarly, GM-CSF induced a slight inhibition (P<0.05), whereas TGF-β1 and TNF-α almost blocked DNA synthesis (P<0.001) after 72 h. IL-6 had no statistically significant effect on cell proliferation after 72 h. Transcription factors AP-1 and NF-κB were both constitutively expressed in UT-OC-3 cells. The binding activity of AP-1 was found to be stimulated by the growth inhibitory cytokines, TGF-β1 and TNF-α, and the binding of NF-κB was stimulated by TNF-α. Apoptosis does not seem to be induced by any of these cytokines in the UT-OC-3 ovarian cancer cell model.

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Introduction

The aetiology of ovarian carcinoma is unknown, but numerous studies suggest that the number of ovulations during the lifespan of a woman is critical; nulliparity, early menarche, and late menopause are all considered as risk factors (1, 2). The most commonly used classification of ovarian tumours was defined by the World Health Organization in 1973 (3). This classification divides the primary tumours into epithelial, sex cord–gonadal and germ cell type. Most ovarian tumours are of the epithelial type, and serous cystadenocarcinoma is the most common histological type (4).

Cytokines have a wide range of growth regulatory effects on different types of cells. Specific cytokines can directly or indirectly inhibit tumour growth and have been used in cancer therapy (5, 6). Furthermore, leukocytes and cytokines have been reported to have an influence on both normal and pathological ovary (7, 8). Leukocytes and macrophages are associated with ovarian cancer cells within solid tumour implants and ascites fluid (9–11). Better understanding of the interaction between malignant ovarian cells and immunological secretory products may help in developing novel strategies for cancer therapy.

The purpose of this study was to investigate in more detail the effects of characterized cytokines on ovarian serous cystadenocarcinoma cells in vitro.

Materials and methods

Cytokines

Recombinant human interferon α (IFN-α; 0.3–30 ng/ml), IFN-γ (0.3–30 ng/ml), and granulocyte-macrophage colony-stimulating factor (GM-CSF; 1–100 ng/ml;
Leukomax) were all from Schering–Plough Research Institute (Kenilworth, NJ, USA). Tumour necrosis factor α (TNF-α; 1–100 U/ml), transforming growth factor β1 (TGF-β1; 0.3–30 ng/ml) and interleukin-6 (IL-6; 3–300 U/ml) were purchased from R&D systems (Minneapolis, MN, USA).

**Cell line**

The UT-OC-3 cell line has been established at the University of Turku from a primary tumour of a 60-year-old patient with well differentiated stage III ovarian serous cystadenocarcinoma (12, 13). Low-passage cells were used throughout all experiments.

**Cell culture**

Cells were cultured in DMEM/Ham’s F12 medium (Life Technologies, Gaithersburg, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Paisley, Renfrewshire, Scotland), penicillin and streptomycin (2000 U/l and 2 mg/l respectively; both from Life Technologies), l-glutamine (2 mmol/l; Life Technologies) and 1% non-essential amino acids (Life Technologies) in 250 ml tissue culture plates (Greiner, Frickenhausen, Germany). This supplemented medium was referred to as basal medium throughout this article.

**DNA labelling**

Before the experiments, the cells were trypsinized and 20–50 000 cells/well were plated on the wells of microtitre plates (Nunc, Roskilde, Denmark), and were allowed to adhere for 16 h, followed by a 24 or 48 h preincubation in the presence or absence of cytokine. Subsequently, the cells were labelled with 5-(125I-iodo)-2-deoxyuridine (125IIdUrd; Amersham, Amersham, Bucks, UK) for 24 h (14). Finally, the cells were trypsinized and harvested. Incorporation of the radioactivity into cellular DNA was measured with a 5–125I-labeled consensus oligonucleotide probe (AP-1: 5’-GCC TTG ATG AGT CAG CCG GAA-3′; NF-κB: 5′-AGT TGA GGG GAC TTT CCA AGG C-3′). Binding reactions were performed at room temperature for 30 min. The anti-p50, anti-p65, anti-c-jun and anti-'c-fos antibodies were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA) and used according to the manufacturer's instructions. Samples were finally analysed by electrophoresis on 4% PAG in 0.25% TBE–borate containing 22 mmol/l boric acid and 0.5 mmol/l EDTA. Gels were dried and visualized by autoradiography. The band density was quantified by a Personal Densitometer (Molecular Dynamics Inc. Sunnyvale, USA). In densitometric

was added and the incubation at 50°C was continued for an additional 1 h. Samples were heated to 70°C, and 10 μl 10 mmol/l EDTA (pH 8.0) containing 1% (w/v) low-gelling-temperature agarose, 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose were mixed with each sample before loading onto the wells of agarose gel (1%) containing 0.1 mg/ml ethidium bromide. Electrophoresis was carried out in 800 mmol/l Tris–phosphate buffer (pH 7.8) containing 2 mmol/l EDTA at 80 V for 4 h. All experiments were carried out in quadruplicate (15).

**Extraction of nuclear proteins**

Nuclear proteins were isolated from UT-OC-3 cells essentially as originally described by Andrews & Faller (16). Briefly, 3 × 10^6 cells were cultured in the presence or absence of cytokine for 30, 60, 120 and 240 min; control cells were cultured in basal conditions. After culture, the cells were washed, pelleted and resuspended in 400 μl cold (0°C) buffer A (10 mmol/l HEPES–KOH (pH 7.9) containing 1.5 mmol/l MgCl_2, 10 mmol/l KCl, 0.5 mmol/l dithiothreitol (DTT), 0.2 mmol/l phenylmethylsulfonyl fluoride (PMSF) and 10 mmol/l aprotinin). The cells were allowed to swell on ice for 10 min and vortexed for 10 s. Samples were centrifuged (30 s at 15 000 × g) and the supernatant was discarded. The pellet was resuspended in 30 μl buffer C (20 mmol/l HEPES–KOH (pH 7.9) containing 25% glycerol, 420 mmol/l NaCl, 1.5 mmol/l MgCl_2, 0.2 mmol/l EDTA, 10 mmol/l DTT, 10 mmol/l PMSF and 10 mmol/l aprotinin) and incubated on ice for 20 min. Cellular debris was removed by centrifugation (2 min at 15 000 × g) at +4°C and the supernatant fraction (containing DNA binding proteins) was stored at −70°C.

**Electrophoretic mobility shift assay (EMSA)**

Nuclear protein–DNA binding reactions were carried out in a volume of 20 μl containing 5 μg nuclear extract protein, 10 mmol/l Tris–HCl buffer (pH 7.5), 40 mmol/l KCl, 1 mmol/l EDTA, 1 mmol/l DTT, 10% glycerol, 2 μg poly(dI-dC) as a non-specific competitor, and 10 fmol 32P-labelled consensus oligonucleotide probe (AP-1: 5’-GCCTTGATAGAGTCTGCTGGCCGAAGCT-3′; NF-κB: 5’-AGTAGGGGACTTTCAAGGCCCAGG-3′). Binding reactions were performed at room temperature for 30 min. The anti-p50, anti-p65, anti-c-jun and anti-'c-fos antibodies were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA) and used according to the manufacturer’s instructions. Samples were finally analysed by electrophoresis on 4% PAGE in 0.25% TBE–borate containing 22 mmol/l boric acid and 0.5 mmol/l EDTA. Gels were dried and visualized by autoradiography. The band density was quantified by a Personal Densitometer (Molecular Dynamics Inc. Sunnyvale, USA). In densitometric
analyses, the arbitrary value (ADU) chosen for basal activity was the integral (surface area) of the specific band in the control after background calibration. All experiments were carried out in quadruplicate.

Data expression and statistical analyses

The effects of different factors on DNA synthesis of the cells cultured in the presence of cytokine were compared with the control. The results are expressed as disintegrations per minute (d.p.m.). Statistical analyses of the results were performed by paired Student’s t-test. A P value less than 0.05 was considered statistically significant.

Results

All studied cytokines had a clear growth inhibitory effect on UT-OC-3 cells, but the duration and the degree of growth inhibition varied between cytokines.

The effects of TGF-β1 (5 ng/ml) and TNF-α (100 U/ml) on UT-OC-3 cells after 48 h in culture are shown in Fig. 1a. TGF-β1 induced a 2.0-fold and TNF-α a 1.8-fold inhibition of the incorporation of 125I-UdR (P < 0.005). IFN-α (10 ng/ml) and IFN-γ (20 ng/ml) both decreased the incorporation of 125I-UdR. IFN-α inhibited DNA synthesis 2.1-fold (P < 0.005), and IFN-γ 3.6-fold (P < 0.001) after 48 h, respectively (Fig. 1b). A dose-dependent inhibitory effect of TNF-α was seen after 48 h in culture (Fig. 1c) at concentrations 1–100 U/ml.

Figure 2 shows the effect of IFN-α (10 ng/ml), IFN-γ (20 ng/ml) TGF-β (5 ng/ml) and TNF-α (100 U/ml) after 72 h in culture. IFN-α and IFN-γ decreased DNA synthesis 1.6-fold and 1.9-fold (P < 0.005) respectively, whereas TGF-β1 and TNF-α almost blocked the incorporation of 125I-UdR (P < 0.001) at this time point. The inhibitory effect of IFN-α was dose dependent at 72 h (Fig. 2b) at concentrations of 0.3–30 ng/ml. A dose–response analysis was also made for TGF-β and TNF-α after 72 h, but we were not able to see a clear dose-responsiveness at this time point.

GM-CSF exerted an inhibitory time-dependent effect on 125I-UdR uptake by UT-OC-3 cells: we found a 10.0-fold inhibition after 48 h (P < 0.001) and a 1.2-fold inhibition after 72 h (P < 0.05) (Fig. 3a). Similarly, IL-6 (Fig. 3b) was able to decrease DNA synthesis by UT-OC-3 cells after 48 h (a 1.8-fold inhibition; P < 0.005) in a dose-dependent way (Fig. 3c) at concentrations of 3–300 U/ml, but no statistically significant effect was seen after 72 h.

In order to detect apoptosis, cellular DNA was extracted after UT-OC-3 cells were cultured in the presence of TGF-β1 and TNF-α for 48 h (data not shown) or 72 h (Fig. 4). The control cells were cultured in basal medium. In all experiments, the electrophoretic analyses showed intact DNA: no signs of DNA fragmentation – a feature typical of programmed cell death.
death – were observed in any of these analyses. DNA extract- ed from cultured mouse thymocytes served as a positive control for apoptosis throughout these studies.

As shown in Fig. 5a, the transcription factor, AP-1, was constitutively activated in UT-OC-3 ovarian carcinoma cells under basal conditions. Its binding activity was further increased by incubating the cells for 30, 60, 120 and 240 min in the presence of the growth inhibitory cytokines, TGF-β1 and TNF-α. Densitometric analyses of these data indicated that the apparent amounts of the specific complexes were increased by both TGF-β1 and TNF-α when compared with control (Fig. 5b).

Figure 2 (a) IFN-α (10 ng/ml), IFN-γ (20 ng/ml), TGF-β1 (5 ng/ml) and TNF-α (100 U/ml) all inhibited DNA synthesis by UT-OC-3 cells in vitro. Cells were cultured for 48 h in the presence or absence of cytokine and thereafter labelled with 125I UdR for 24 h. Control cells (CTRL) were cultured in medium supplemented with serum. DNA synthesis is expressed as disintegrations per minute (dpm; means ± S.E.M.). Significant differences compared with control: ***P < 0.001, **P < 0.005. (b) Dose–response curve showing the inhibitory effect of IFN-α, after 72 h, on DNA synthesis by UT-OC-3 cells. The concentrations of the cytokine are on a log scale.

Figure 3 GM-CSF (50 ng/ml) (a) and IL-6 (100 U/ml) (b) inhibited DNA synthesis in UT-OC-3 ovarian carcinoma cells. Cells were cultured for 24 h (open columns) or 48 h (hatched columns) in the presence or absence of cytokine and thereafter labelled with 125I UdR for 24 h. Control cells (CTRL) were cultured in medium supplemented with serum. DNA synthesis is expressed as disintegrations per minute (dpm; means ± S.E.M.). Significant differences compared with control: ***P < 0.001, **P < 0.005, *P < 0.05. (c) The dose–dependent inhibitory effect of IL-6, after 48 h, on DNA synthesis by UT-OC-3 cells. The concentrations of the cytokine are on a log scale.

TNF-α at 30, 120 and 240 min. Densitometric analyses of these data indicated that the amounts of the specific complexes were constantly increased only by TNF-α when compared with control (Fig. 6b).
To analyse the proteins binding to the DNA, the nuclear extracts were treated with anti-p50 or anti-p65 (NF-κB) and anti-c-jun or anti-c-fos antibodies (AP-1) before EMSA, in order to identify components of the transcription factor–DNA-complex. Figure 7a demonstrates that NF-κB complexes present in UT-OC-3 cells were predominantly composed of p50/p65 heterodimers, as NF-κB–DNA complexes could mostly be supershifted by the antibodies. Figure 7b shows the appearance of new bands associated with preincubation with AP-1-specific antibodies, but the AP-1–DNA-complex could not be supershifted completely, suggesting that AP-1 is composed only partially by c-jun/c-fos proteins; the nature of other AP-1 complexes remains to be determined in UT-OC-3 cells.

In addition to supershift analyses, the specificity of the AP-1–DNA or NF-κB–DNA complex was confirmed by competition analyses using a 50-fold molar excess of unlabelled AP-1 or NF-κB oligonucleotide (data not shown).

Discussion

Cytokines are multifunctional signalling peptide molecules that have been found to interact with malignant cell growth either by direct inhibition of tumour cells or by inducing defence mechanisms of the immune system (5, 6). Beneficial growth inhibitory effects of cytokines have also been observed in a series of ovarian cancer cell lines (5, 17, 18). In the present study, the growth regulatory effects of a battery of
cytokines has been studied, using a newly established ovarian cystadenocarcinoma cell line. TNF-α has been shown to exert anti-proliferative effects on ovarian cancer cells by inducing cytolysis (18) and enhancing the anti-tumour activity of macrophages against malignant ovarian cells (7). TNF-α also has therapeutic potential in combination with IFN-γ in experimental human ovarian cancer (19). In this study, we have shown that TNF-α has a clear growth inhibitory effect on UT-OC-3 ovarian carcinoma cells. NF-κB plays an important regulatory role in the transcription of genes encoding proteins involved with immune responses and cell growth. NF-κB activity can be induced in a wide variety of cell types in response to treatment with such agents as TNF-α (20, 21). The inducible NF-κB activity is attributable to the release of cytoplasmic NF-κB from the inhibitor, IκB. In this manner, NF-κB can serve as an important second messenger in signal transduction (22, 23). The significant activation of NF-κB by TNF-α suggests that this transcription factor might mediate the anti-proliferative effect in UT-OC-3 cells. TNF-α has been reported to inhibit cell proliferation and induce NF-κB in human myelomonoblastic leukaemia cells (20), and we have reported similar phenomena in vulvar (unpublished data) and ovarian carcinoma cells (21). The biological effects of TNF-α may be attributed to its ability to induce the transcription of several genes. The induction of IL-6 and GM-CSF genes in response to TNF-α is known to be mediated through NF-κB (22).

In the present study, TGF-β1 was a growth inhibitory factor in UT-OC-3 cells. Similar results in ovarian cancer cell lines have been obtained by Jozan et al. (17) and by Grunt et al. (24). It has also been reported that TGF-β1 is able to induce apoptosis in epithelial ovarian carcinoma cells (25). Although TGF-β1 significantly inhibited proliferation of UT-OC-3 carcinoma cells, we were unable to demonstrate that these cells undergo apoptosis in response to TGF-β1. However, the possibility cannot be excluded that apoptosis could have been induced in a small fraction of cells, but that this was below the detection threshold of the DNA fragmentation assay. Interestingly, we have been able to show cytokine-regulated induction of apoptosis in UT-OC-2 ovarian carcinoma cells by applying the method described in this study (unpublished observations). Programmed cell death is regulated by a number of genes, including the tumour suppressor
gene, p53. In some cell lines, the loss of p53 function has been associated with induction of resistance to TGF-β-induced programmed cell death (26, 27). It is possible that the inability of UT-OC-3 cells to undergo apoptosis may be related to the p53 status of our cell model. In fact, p53 mutations have been described recently in human ovarian (28) and other gynecological cancers (29).

Transcription factor NF-κB plays a significant part in the regulation of apoptosis. According to Mayo et al. (30), the activation of NF-κB is able to suppress a p53-independent apoptotic response. In the case of ovarian and breast cancer, NF-κB has been found to be constitutively activated (31–33). Inhibition of NF-κB activity in human breast cancer cell lines leads to apoptosis (33). Accordingly, the finding of constitutively active NF-κB in ovarian carcinoma cells further suggests that this transcription factor may be associated with growth inhibitory effects and the events ultimately leading to programmed cell death.

The binding activity of AP-1 was increased after TGF-β1 treatment in the present study. We have previously shown a similar result in endometrial and ovarian carcinoma cells (21). These findings suggest that the stimulation of AP-1 activity might be one of the mechanisms by which TGF-β1 inhibits the growth of both ovarian and endometrial carcinoma cells. In fact, AP-1 is believed to play a central role in the control of the proliferation of many different kinds of cells (34). In addition, it has been found to be involved in the autoinduction of TGF-β1 (35). Furthermore, there is evidence of an association between the anti-mitogenic action of TNF-α and AP-1 (36). In the present study, growth inhibitory TNF-α was also able to increase the binding activity of AP-1.

Both in vitro and in vivo studies have shown that human ovarian cancer is sensitive to the anti-proliferative effects of recombinant human IFN-α and IFN-γ (19, 37, 38). Similarly, the results of this study indicate that both IFNs inhibit the proliferation of UT-OC-3 ovarian carcinoma cells in vitro. It has been observed that, under appropriate conditions, the anti-cancer activity of IFNs is mediated by cytolysis in ovarian and cervical cancer cells (37–39). We have also previously found that both IFNs are growth inhibitory in endometrial carcinoma cells (21), and that IFN-γ exerts a growth inhibitory effect on vulvar carcinoma cells (40). All these findings indicate that IFNs are able to inhibit the proliferative activity of several types of neoplastic cells originating from female reproductive organs.

In the present study, the cytokines were tested only as single agents. However, it has been shown that, for example, IFN-γ is able to enhance the anti-proliferative effect of TNF-α in cervical carcinoma cells (41), and that the combination therapy of TNF-α and IFN-γ used together significantly inhibits ovarian cancer cell growth (19). Interestingly, in colorectal carcinoma cells, the initiation of apoptosis was achieved by the combination of TNF-α and IFN-γ (42). In contrast to these studies, the combination of IFN-γ and TNF-α produced no further growth inhibitory response.
compared with immunomodulatory factors used alone in lung carcinoma cell models (43). It would be very interesting to test different combinations of cytokines in UT-OC-3 ovarian carcinoma cells.

A growth inhibitory effect of GM-CSF on human ovarian carcinoma cells was observed in the present study. This result is in accordance with those reported by Salmon & Liu (44). In addition, we have previously found that DNA synthesis was inhibited by GM-CSF in endometrial (21) and vulvar (40) carcinoma cells. It has also been observed that irradiated tumour cells secreting GM-CSF show specific anti-tumour immunity in a number of tumour models (45). In contrast to the growth inhibitory effects observed in the present studies, GM-CSF has been believed to have only a modest (if any) growth modulatory effect on most non-haematopoietic neoplasms (39). In clinical practice, at present, the primary indication for GM-CSF treatment of patients with malignant disease is to improve the recovery of the bone marrow after chemotherapy-induced myelosuppression (46).

In the present study, the effect of IL-6 on DNA synthesis was found to be highly time-dependent and observed only after 48 h of exposure. Anti-tumour effects of IL-6 against sarcoma and adenocarcinoma of the colon have been demonstrated in vivo (47), and Chen et al. (48) have observed that IL-6 inhibits the growth of human breast and ovarian carcinoma cells in vitro. However, IL-6 has also been reported to stimulate the growth of ovarian carcinoma cells in vitro (46). It seems likely that IL-6 is able either to inhibit or to stimulate cell growth, depending on the cell type used and the different culture conditions. In summary, our results indicate that IFN-α, IFN-γ, TGF-β1, TNF-α, IL-6 and GM-CSF have a growth inhibitory effect on UT-OC-3 ovarian carcinoma cells. Transcription factors AP-1 and NF-xB may be involved in mediating these growth-inhibitory effects of cytokines. Programmed cell death could not be demonstrated after exposure to these cytokines, but constitutive active NF-xB might protect cells from induction of apoptosis by cytokines. Further studies of cytokine-mediated growth inhibition and the role of transcription factors AP-1 and NF-xB in the regulation of cell growth in ovarian cancer cells are needed.

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