

Tumor Necrosis Factor α as an Autocrine and Paracrine Growth Factor for Ovarian Cancer: Monokine Induction of Tumor Cell Proliferation and Tumor Necrosis Factor α Expression¹

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ABSTRACT

Ovarian tumor cells produce macrophage colony stimulating factor, a potent chemoattractant for monocytes. Monocytes and macrophages produce tumor necrosis factor α (TNF- α) and interleukin 1 α or interleukin 1 β (IL-1 β) that can stimulate ovarian tumor cell growth. The present study has explored whether paracrine stimulation by monocyte derived cytokines might induce autocrine growth stimulation of normal and malignant ovarian epithelial cells. Endogenous expression of TNF- α mRNA was detected in ascites ovarian cancer cells isolated directly from patients, but not in established cultures of normal or malignant ovarian epithelial cells. When ascites tumor cells were cultured for 7 days, TNF- α expression ceased but could be reinduced by treatment with TNF- α or IL-1 β . Ascites fluid contained concentrations of the cytokines that could mediate these effects. Similarly, treatment of normal or malignant ovarian epithelial cells with purified recombinant IL-1 β or TNF- α induced transcription of TNF- α mRNA within 1 h. TNF- α protein could be detected by enzyme-linked immunosorbent assay in conditioned medium from IL-1 β treated ovarian cancer cells. [³H]thymidine incorporation by normal or malignant ovarian epithelial cells was stimulated by a 24-h incubation with IL-1 β or TNF- α . Stimulation of proliferation by IL-1 β could be partially blocked by an antibody against TNF- α or by soluble TNF- α -receptor. Thus, TNF- α may function as both an autocrine and a paracrine growth factor in ovarian cancer.

INTRODUCTION

TNF- α ³ and IL-1 are cytokines produced primarily by monocytes and macrophages. TNF- α and IL-1 share several biological activities, despite the fact that they are structurally unrelated and bind to different cell surface receptors (1, 2). Both TNF- α and IL-1 can inhibit the growth of certain tumor cells (3-5), stimulate human fibroblast proliferation, promote bone reabsorption by osteoclasts, down-regulate lipogenic gene expression, and stimulate the production of collagenase in synovial cells (6). Conversely, TNF- α and IL-1 can also stimulate the growth of normal cells as well as certain cancer cells in culture (6, 7). Since TNF- α can be constitutively produced by malignant cells (8), its expression could contribute to tumor progression and spread (9, 10) as well as indicate resistance to the cytotoxicity of exogenous TNF- α (11).

Ovarian cancer is the leading cause of death from gynecological malignancy. Over 60% of patients present with disease that has spread beyond the pelvis and into the peritoneal cavity (12). Although metastatic disease can be treated with cytoreductive surgery and combination chemotherapy, the survival of these patients is poor. Recent studies have focused on factors that regulate the growth of malignant ovarian epithelium (13), with the expectation that a more fundamental

understanding of these factors might suggest novel and potentially more effective approaches to treatment.

Macrophages are found in solid tumor implants of ovarian cancer as well as in ascites fluid (14, 15). Previous studies have indicated that ovarian cancer cells produce macrophage colony stimulating factor which is a potent chemoattractant for monocytes (16-18). Monocyte derived cytokines including TNF- α , IL-1, and interleukin 6 can stimulate proliferation of ovarian cancer cells (7). Other investigators have shown that ovarian and other cancer cells express TNF- α *in vitro* and *in vivo* (8, 19, 20). Whether or not endogenous expression of TNF- α is important for growth of ovarian cancer cells remains to be resolved. In this article, we have explored the possibility that endogenous expression of TNF- α is maintained *in vivo* by exogenous TNF- α or IL-1 and that endogenous production of TNF- α may trigger tumor cell proliferation.

MATERIALS AND METHODS

Cytokines, Anti-Cytokine Antibodies, and DNA Probes. Human recombinant TNF- α (2×10^7 units/mg) was purchased from Genzyme (Boston, MA). Human recombinant IL-1 β (5×10^8 units/mg) and human recombinant IL-1 α (5×10^8 units/mg) were purchased from R&D Systems (Minneapolis, MN). Murine anti-human TNF- α monoclonal antibody was purchased from Endogen, Inc. (Boston, MA). Soluble TNF- α receptor (TNF-BP I) was purchased from R&D Systems. A cDNA probe for human TNF- α in plasmid pcDV1 was purchased from the American Type Culture Collection (Bethesda, MD).

Cell Cultures. Four ovarian cancer cell lines, OVCA 420, OVCA 429, OVCA 432, and OVCA 433, were established from ascites tumor cells of ovarian cancer patients (21). The cells were grown in Eagle's minimal essential medium that was supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, 1 mM sodium pyruvate, and 1% nonessential amino acid mixture. The ovarian cancer cell line SKOV3 was obtained from the American Type Culture Collection. SKOV3 cells were cultured in McCoy's medium containing 15% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. The OVCAR-3 cell line was obtained from Dr. Thomas C. Hamilton and was cultured in RPMI 1640 containing 15% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Four cultures of normal surface ovarian epithelial cells N-OSE 006R, N-OSE 007L, N-OSE 038L, and N-OSE 039R were established in our laboratory as described previously (22). The epithelial origin of the cultured cells was confirmed by immunocytochemical detection of cytokeratins and the identification of characteristic structures was confirmed by electron microscopy. Cells were grown in medium containing equal volumes of MCDB 105 and medium 199 supplemented with 15% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Medium was changed every 3 days and the cells were subcultured once a week. For subculture and experiments, monolayers were detached with 0.25% trypsin-0.02% EDTA.

Separation of Tumor Cells from Ascites. Ascites fluid was obtained from ovarian cancer patients at the time of surgery. The cells were pelleted by centrifugation, frozen in 10% dimethyl sulfoxide, and stored in the vapor phase of liquid nitrogen. For each experiment, cells were thawed, resuspended in HBSS, and separated on Percoll (Pharmacia, Piscataway, NJ) density gradients. After centrifugation at 1500 rpm for 20 min at room temperature, fractions were collected and washed 3 times with HBSS. Cells were incubated with mouse monoclonal antibodies reactive with ovarian tumor associated antigens including 317G5, 260F9, BT8FF1, and BT4Z4 for 1 h at 4°C. Cells were washed 3 times with HBSS and incubated with goat anti-mouse immunoglo-

Received 9/9/92; accepted 2/8/93.

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¹ Supported in part by Grant R01 CA39930 from the NIH, Department of Health and Human Services.

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³ The abbreviations used are: TNF- α , tumor necrosis factor α ; IL-1, interleukin-1; IL-1 α , interleukin-1 α ; IL-1 β , interleukin-1 β ; FBS, fetal bovine serum; cDNA, complementary DNA; HBSS, Hanks' balanced salt solution; SDS, sodium dodecyl sulfate; SSC, standard saline-citrate; ELISA, enzyme linked immunosorbent assay.

bulin coated beads (Dynabeads; Dynal A.S., Oslo, Norway) for 1 h. Rosetted cells were collected with a magnet. More than 99% of the cells bound OC125 and AE1 detected by immunocytochemistry with avidin-biotin-complex immunoperoxidase, whereas <1% of cells reacted with antibodies against IL-1 α . Total cellular RNA was isolated as described. For cell culture and treatment, 10⁶ purified tumor cells were cultured in 100-mm dishes in medium 199/105 as described above at 37°C in 5% CO₂ and 95% humidified air. On day 7, cells were treated with TNF- α or IL-1 (1 ng/ml) for 1 or 2 h, and total cellular RNA was isolated.

RNA Isolation and Northern Transfers. Total cellular RNA was isolated using guanidinium isothiocyanate/cesium chloride (23) from 4 normal ovarian epithelial cell cultures, 5 ovarian cancer cell lines, and 5 samples of ascites tumor cells from different patients. Purified RNA (10–20 μ g) was analyzed by electrophoresis in 1% agarose/6% formaldehyde gel followed by blot transfer to Genescreen Plus membranes (Dupont, Boston, MA). Human TNF- α and τ -actin cDNA probes were labeled with [³²P]dCTP using a multiprime DNA labeling kit (Amersham, Arlington Heights, IL). Labeled probes were purified on Sepharose-G50 columns (Pharmacia). Transfer membranes were prehybridized in 50% formamide, 10% dextran sulfate, 1% SDS, and 1 μ g/ml denatured salmon sperm DNA at 42°C for 1 h. Membranes were then hybridized at 42°C for approximately 16 h in the same buffer containing 10⁶ cpm/ml heat-denatured TNF- α cDNA probe. Membranes were washed once for 30 min at room temperature in 2 \times SSC with 0.1% SDS, once for 30 min in 0.5 \times SSC with 0.1% SDS, and once for 30 min at 65°C in 0.1 \times SSC with 0.1% SDS prior to autoradiography. Autoradiograms were exposed at –70°C over 2–10 days in the presence of an intensifying screen. After autoradiography the membranes were stripped and rehybridized with a τ -actin cDNA probe.

Nuclear Run-on Assay. OVCA 432 cells were grown in tissue culture flasks to near confluence and the cells (5 \times 10⁷) were treated with 1 ng/ml of TNF- α or IL-1 β for 10 min or 1 h. Cells were washed 3 times with ice-cold phosphate buffered saline, scraped with a rubber policeman, and centrifuged at 500 g for 5 min. The cell pellet was resuspended in lysis buffer [10 mM Tris-HCl, pH 7.4–10 mM NaCl–3 mM MgCl₂–0.5% (v/v) NP-40], incubated for 5 min on ice, and centrifuged at 500 g for 5 min. The pellet of nuclei was washed with lysis buffer, resuspended in 50 mM Tris-HCl (pH 8.3), 40% (v/v) glycerol, 5 mM MgCl₂, and 0.1 mM EDTA, and stored at –70°C. Thawed nuclei were resuspended in transcription buffer containing 0.25 mM each of ATP, GTP, and CTP, 5 mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, and 150 mM KCl, in the presence of 200 μ Ci [³²P]UTP for 30 min at 30°C. Following this incubation, nuclei were digested with RNase free DNase (0.25 mg/ml) and proteinase K (0.3 mg/ml), extracted twice with phenol:chloroform (1:1), and precipitated with 3 M sodium acetate and ethanol as described in Ref. 23. The unincorporated nucleotides were removed from radiolabeled RNA by centrifugation through Sephadex G-50 columns (Pharmacia). Labeled mRNA transcripts were hybridized at 65°C for periods of 2 days with Genescreen Plus membranes that had been slot blotted with 2 μ g/slot of TNF- α or τ -actin DNA. TNF- α and τ -actin DNAs were purified from plasmid vectors by enzyme digestion, aga-

rose gel electrophoresis, and GeneClean (BIO 101, Inc., LaJolla, CA). Membranes were then washed and the bound activity was visualized by autoradiography at –70°C in the presence of 2 intensifying screens. Intensity of bands was measured by densitometry. The intensity ratio was determined by dividing the intensity of the TNF- α band by the intensity of the τ -actin band.

Assay of TNF- α , IL-1 α , and IL-1 β . Supernatant from ovarian cancer cell cultures or ascites fluid from ovarian cancer patients was centrifuged at 1000 rpm for 10 min and passed through a 0.22- μ m filter. ELISA kits were used to determine the concentrations of TNF- α (Smith-Kline-Beecham, King of Prussia, PA; R&D Systems), IL-1 α , and IL-1 β (R&D Systems). The assays were able to detect \geq 50 (Smith-Kline-Beecham) or \geq 4.8 pg/ml (R&D Systems) TNF- α , \geq 0.2 pg/ml IL-1 α , and \geq 0.3 pg/ml IL-1 β .

Cell Proliferation. Cell proliferation was measured by [³H]thymidine incorporation. OVCA 432 cells (10⁴/well) were placed in 96-well flat bottom plates and incubated for 3 days at 37°C, in 5% CO₂, and 95% humidified air until the monolayers were nearly confluent. The medium was removed and TNF- α (1 ng/ml) or IL-1 β (1 ng/ml) diluted in culture medium was added to each well. Following 24 h of incubation, 1 μ Ci of [³H]thymidine was added to each well 4–6 h before the cells were washed and solubilized with 2 N NaOH. Radioactivity was measured in a beta-counter. To neutralize TNF- α activity, monoclonal anti-TNF- α antibody or soluble TNF- α receptor was preincubated with TNF- α or IL-1 β (1 ng/ml) for 2 h at 37°C before addition to cell monolayers. Stimulation of [³H]thymidine incorporation was then measured as above. Percentage of inhibition of cytokine-induced [³H]thymidine incorporation with anti-TNF- α antibody and soluble TNF- α receptor was calculated as:

$$1 - \frac{\text{Cytokine induced increase (cpm) with antibody or receptor}}{\text{Cytokine induced increase (cpm)}} \times 100\%$$

All assays were performed with at least four replicates. In previous studies increases in [³H]thymidine incorporation by OVCA 432 cells correlated with increases in cell number (7).

Statistical Analysis. Student's *t* test was used to compare [³H]thymidine incorporation in control and experimental groups. Data were expressed as mean cpm \pm SD.

RESULTS

TNF- α Gene Expression in Ascites Tumor Cells, Ovarian Cancer Cell Lines, and Normal Ovarian Epithelial Cells. A [³²P]-labeled human TNF- α cDNA probe was hybridized with total cellular RNAs from 4 normal ovarian epithelial cell cultures, 5 ovarian cancer cell lines, and 4 preparations of ovarian cancer cells purified directly from ascites. TNF- α expression was seen in all four highly purified ascites tumor cell preparations, but not in any of the normal or malignant ovarian epithelial cell lines (Fig. 1).

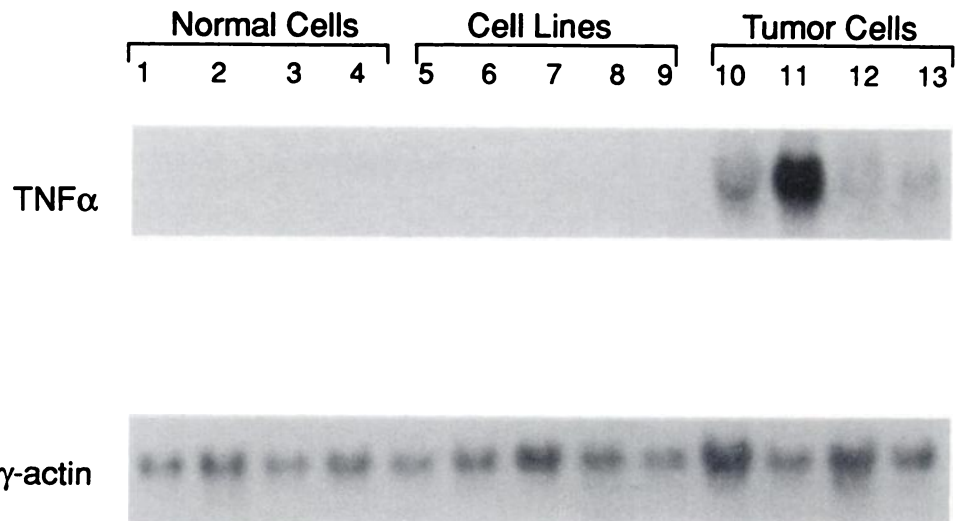


Fig. 1. TNF- α expression in normal ovarian epithelial cells, ovarian cancer cell lines, and tumor cells from ovarian cancer ascites fluid. Northern transfers of total cellular RNA (10 μ g/lane) from 4 normal ovarian epithelial cell lines (Lanes 1–4), 5 ovarian cancer cell lines (Lanes 5–9), and 4 preparations of ovarian cancer cells isolated directly from ascites fluid (Lanes 10–13) were hybridized with ³²P-labeled cDNA probes for human TNF- α and τ -actin.

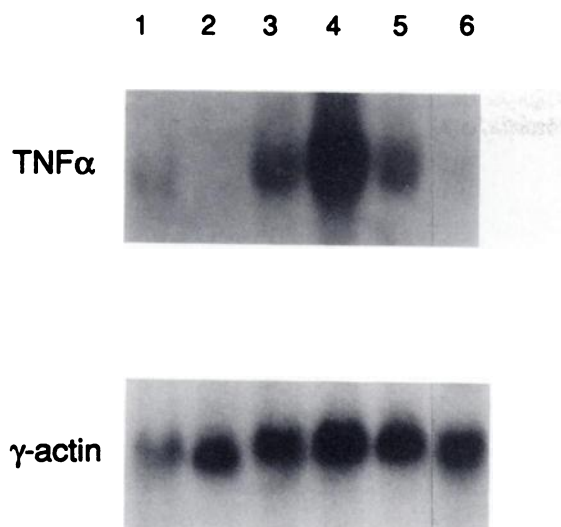


Fig. 2. Loss and reinduction of TNF- α expression in OVCA 189 ovarian cancer cells isolated directly from ascites fluid. Total cellular RNA (20 μ g/lane) from freshly isolated OVCA 189 cells (Lane 1), from 7 day cultures of OVCA 189 (Lane 2), from 7 day cultures of OVCA 189 treated with 1 ng/ml of IL-1 β for 1 (Lane 3) or 2 h (Lane 4), and 7 day cultures of OVCA 189 treated with TNF- α for 1 (Lane 5) or 2 h (Lane 6) were studied by Northern analysis using 32 P-labeled cDNA probes for human TNF- α and γ -actin.

Table 1 Concentration of TNF- α in the ascites fluid of ovarian cancer patients

Ascites fluid	TNF- α (pg/ml)
OVCA 34	1450.0 \pm 118.4 ^a
OVCA 87	329.1 \pm 123.7
OVCA 93	156.1 \pm 32.9
OVCA 119	116.0 \pm 13.0
OVCA 189	171.2 \pm 14.2

^a Mean \pm SD determined by ELISA.

Loss and Reinduction of TNF- α in Ascites Tumor Cells by TNF- α or IL-1 β . Since TNF- α expression was observed in ovarian cancer cells from ascites, but not in ovarian cancer cell lines, we tested the possibility that TNF- α expression might be lost in cell culture. After 7 days in culture, expression of TNF- α was no longer observed in OVCA 189 cells that had expressed TNF- α when first isolated (Fig. 2). Expression of TNF- α could, however, be reinduced by treatment either with TNF- α or IL-1 β (Fig. 2).

Concentrations of TNF- α , IL-1 α , and IL-1 β in Ascites Fluid. Ascites fluids were obtained from the same patients who had donated tumor cells. When TNF- α , IL-1 α , and IL-1 β were assayed, the concentration of TNF- α was from 116–1450 pg/ml, comparable to concentrations of TNF- α required to induce endogenous production of

TNF- α by ovarian cancer cells (Table 1). IL-1 β could not be detected in ascites fluid, but IL-1 α concentrations ranged from 2 to 14 pg/ml.

Induction of TNF- α in Normal and Malignant Ovarian Epithelial Cells. When ovarian cancer cell lines were treated with TNF- α or IL-1 β , induction of TNF- α mRNA occurred after 1 h treatment with either cytokine. The induction of TNF- α mRNA depended on the concentration of cytokines and the duration of treatment. TNF- α mRNA levels reached a maximum after 1–2 h of treatment with IL-1 β or TNF- α and subsequently declined (Fig. 3). Similar kinetics were observed after treatment with IL-1 α (data not shown). As little as 0.01 ng/ml IL-1 β induced TNF- α expression, whereas 0.1 ng/ml TNF- α was required to induce detectable endogenous expression of TNF- α (Fig. 4). Similar induction of TNF- α could be achieved with normal ovarian cell cultures and with several other ovarian cancer cell lines including OVCA 420, OVCA 429, OVCA 433, OVCAR3, and SK-Ov3. To determine whether expression of TNF- α transcripts was associated with production of TNF- α protein, culture supernatants from TNF- α or IL-1 β treated OVCA 432 cells were assayed for TNF- α by ELISA. TNF- α could not be detected in control supernatants, but TNF- α protein was detected after treatment with IL-1 β . In the presence of IL-1 β , TNF- α levels increased from 50–2000 pg/ml over 8 h (Table 2). In the presence of 1 ng/ml of exogenously added TNF- α , it was not possible to measure a time dependent increase in TNF- α levels (Table 2). Induction of TNF- α was also observed with freshly isolated ovarian cancer ascites cells. After 24 h in culture 3×10^5 OVCA 189 cells produced up to 135 pg/ml TNF- α in the culture supernatant. After 5 days in culture <4.8 pg/ml TNF- α could be detected. TNF production could, however, be reinduced (52.4–63.6 pg/ml) by incubation with 1 ng/ml IL-1 β .

Transcription of TNF- α mRNA in Ovarian Cancer Cells. To determine whether increased levels of TNF- α mRNA resulted from increased transcription, nuclear run-on analysis was performed with OVCA 432 cells following stimulation with TNF- α or IL-1 β (Fig. 5). Transcription of TNF- α message was increased by both TNF- α and IL-1 β .

Stimulation of [3 H]Thymidine Incorporation by TNF- α and IL-1 β . In an earlier study we had shown that treatment for 24 h with TNF- α or IL-1 could stimulate a dose dependent increase in [3 H]-thymidine incorporation and cell number by ovarian cancer cell lines. Around a 30–200% increase in proliferation by these parameters was observed in sensitive cell lines including OVCA 429 (7), OVCA 432 (7), OVCA 433 (7), OVCAR-3, and SKOV3 (Table 3). In the present study we have determined whether TNF- α or IL-1 β could stimulate proliferation of normal ovarian epithelial cells. After treatment for 24 h with IL-1 β , or TNF- α , increased [3 H]thymidine incorporation was observed in normal ovarian epithelial cells (Fig. 6).

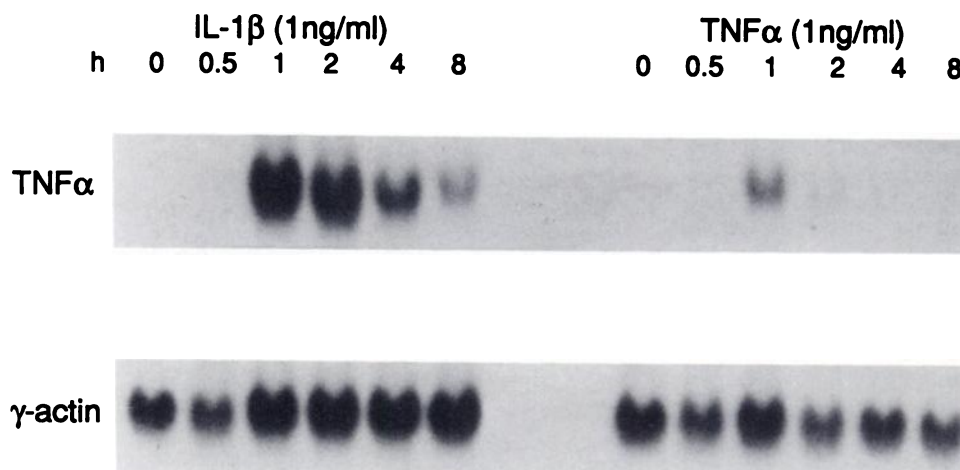


Fig. 3. Induction of TNF- α expression in the OVCA 432 ovarian cancer cell line with TNF- α or IL-1 β . OVCA 432 cells were treated with 1 ng/ml of IL-1 β or TNF- α for different intervals. Northern transfers were prepared from total cellular RNA (20 μ g/lane) and hybridized with 32 P-labeled cDNA probes for human TNF- α and γ -actin.

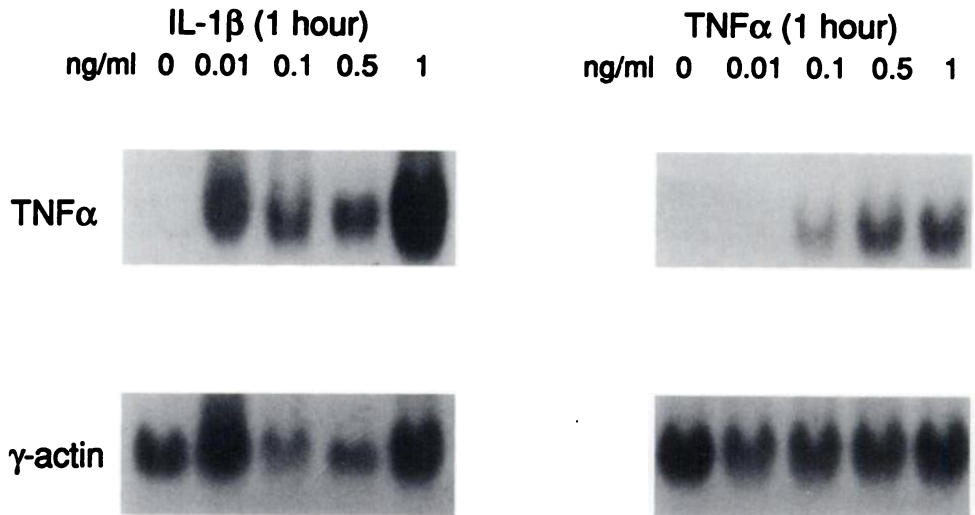


Fig. 4. Effect of IL-1 β and TNF- α on the expression of TNF- α in OVCA 432 ovarian cancer cells. OVCA 432 cells were treated with different concentrations of IL-1 β or TNF- α for 1 h. Total cellular RNA (20 μ g/lane) was transferred and hybridized with 32 P-labeled cDNA probes for human TNF- α and γ -actin.

Table 2 Concentration of TNF- α in the supernatants of TNF- α or IL-1 treated OVCA 432 ovarian cancer cells

Treatment ^a	TNF- α (pg/ml)
Control	<50
TNF- α , 1 h	1706.3 \pm 169.3 ^b
TNF- α , 2 h	1620.5 \pm 186.1
TNF- α , 4 h	2002.7 \pm 244.6
TNF- α , 8 h	1809.7 \pm 219.8
IL-1 β , 1 h	364.6 \pm 0.0
IL-1 β , 2 h	715.5 \pm 299.6
IL-1 β , 4 h	655.3 \pm 30.1
IL-1 β , 8 h	2104.9 \pm 206.5

^a OVCA 432 cells were treated with 1 ng/ml of TNF- α or 1 ng/ml of IL-1 β for the intervals indicated above. Supernatants were collected and the concentrations of TNF- α were assayed by ELISA.

^b Mean \pm SD.

Blocking of TNF- α or IL-1 β Induced [3 H]Thymidine Incorporation with Anti-TNF- α Antibody or Soluble TNF- α Receptor. Since IL-1 β could stimulate proliferation of ovarian cancer cells as well as induce TNF- α expression, we have evaluated the possibility that TNF- α could act as an autocrine growth factor. To test whether endogenous expression of TNF- α might be a critical factor triggering tumor cell proliferation, blocking studies were performed in two separate experiments with anti-TNF- α antibody (Fig. 7A) and TNF- α receptor (Fig. 7B). TNF- α mediated stimulation of [3 H]thymidine incorporation by ovarian cancer cell line OVCA 432 could be blocked with anti-TNF- α antibody (Fig. 7A) or TNF- α receptor (Fig. 7B). TNF- α induced stimulation was inhibited by 80% with anti-TNF- α antibody (1:50) and by 60% with soluble TNF- α receptor. In this experiment, blocking of proliferation could relate to neutralization either of TNF- α added to the cultures or of TNF- α produced endogenously and released into the culture medium. More definitive data could be obtained by blocking IL-1 β induced stimulation of [3 H]thymidine incorporation. Consistent with the possibility that endogenous production of TNF- α was linked to cell proliferation, IL-1 β induced stimulation was inhibited by 53% with anti-TNF- α antibody (1:50) and by 92% with soluble TNF- α receptor (Fig. 7, A and B).

DISCUSSION

Our studies support the possibility that TNF- α acts as both a paracrine and an autocrine growth factor for normal and malignant ovarian epithelium. Ovarian cancer cells produce macrophage colony stimulating factor, a potent chemoattractant for mononuclear phagocytes. Macrophages are associated with ovarian cancer cells both in ascites fluid (15) and in solid tumors (14, 15). Cytokines produced by macrophages, including IL-1, interleukin 6, and TNF- α can stimulate proliferation of ovarian cancer cells (7). Significant levels of TNF- α have been found in the plasma of certain cancer patients (24). Ascites fluid from the patients in our study all contained detectable TNF protein ranging from 116 to 1450 pg/ml. Although IL-1 β could not be detected, IL-1 α levels ranged from 2 to 14 pg/ml. Since we tested only 5 ovarian cancer patient ascites fluids for IL-1, it is possible that other patients may have detectable levels of IL-1. Continued exposure to these cytokines may be important not only for proliferation, but also for endogenous expression of TNF- α .

Our study confirms earlier observations that ovarian cancer cells can express TNF- α both *in vitro* and *in vivo* (8, 19). In one study, TNF- α was found in tumor cells from 4 of 5 ascites and in 16 of 20 tissue sections (19). TNF- α or TNF- α -like molecules were also produced by some tumor cell lines *in vitro* (8), but the expression of TNF- α was not constitutive and the endogenous production of TNF could be induced by exogenous TNF- α . In our present report, each of 5 tumor cell preparations from ascites expressed TNF- α , but TNF- α expression was lost during 7 days of incubation in culture. Endogenous expression of TNF- α could be reinduced by treatment with TNF- α or with IL-1 β . Although many of the experiments in our present report have been performed with IL-1 β , IL-1 α has stimulated proliferation of ovarian tumor cell lines (7) and can induce TNF- α with kinetics similar to those produced by IL-1 β . Expression of TNF- α by ovarian cancer cell lines was induced by TNF- α or IL-1 in a time and dose dependent manner. Higher levels of TNF- α mRNA resulted from increased transcription judged by nuclear run-on assays.

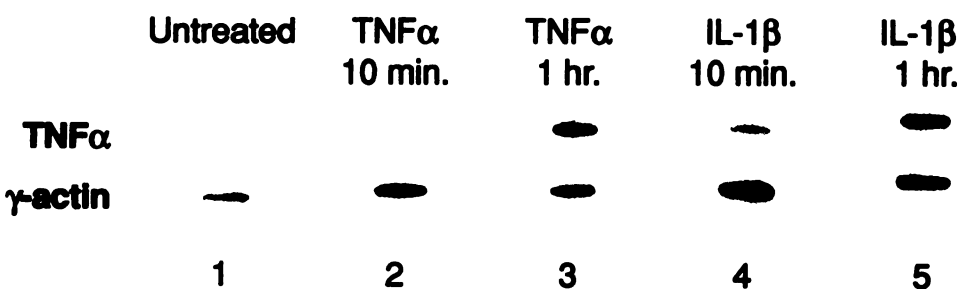
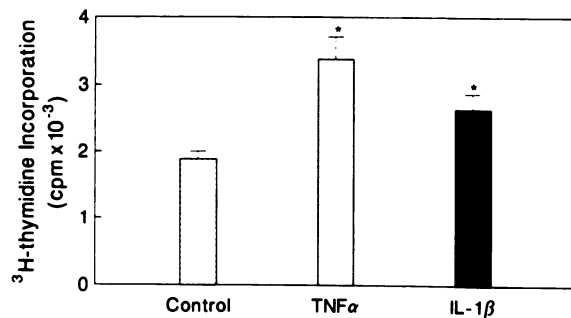
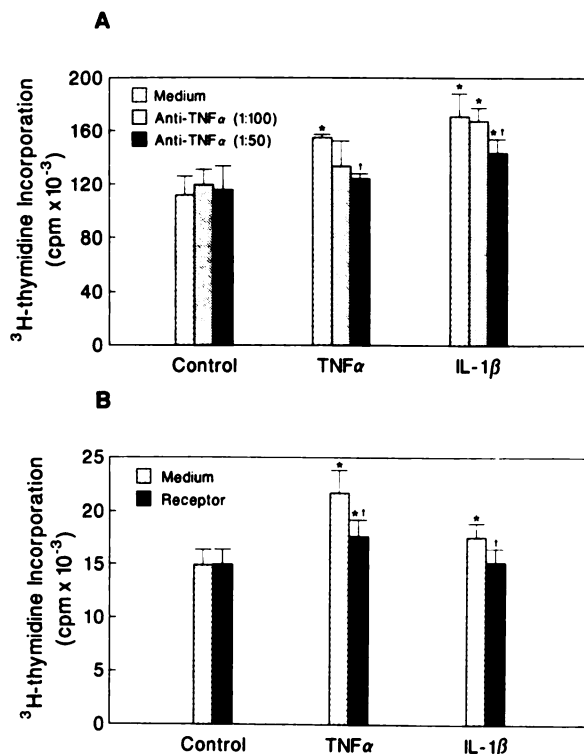


Fig. 5. Nuclear run-on assays of TNF- α transcription in OVCA 432 ovarian cancer cells treated with IL-1 β or TNF- α . Nuclei were harvested without treatment (Lane 1) or after treatment with 1 ng/ml of TNF- α for 10 min (Lane 2) or 1 h (Lane 3) or with 1 ng/ml of IL-1 β for 10 min (Lane 4) or 1 h (Lane 5). Nascent mRNA was radiolabeled and hybridized with nylon membranes prepared with equal amounts of cDNA for human TNF- α and γ -actin. Intensity ratios: 0.2 (Lane 1); 0.3 (Lane 2); 0.9 (Lane 3); 0.4 (Lane 4); 1.0 (Lane 5).

Table 3 Effect of IL-1 and TNF- α on [3 H]thymidine incorporation by ovarian cancer cell lines

Treatment	[3 H]Thymidine incorporation (cpm)	
	OVCAR-3	SKOv3
Control	24,077 \pm 6,364 ^a	69,060 \pm 8,241
IL-1 α , 1 ng/ml	62,812 \pm 3,619 ^b	78,490 \pm 2,780
IL-1 α , 10 ng/ml	67,631 \pm 5,376 ^b	87,686 \pm 5,719 ^b
IL-1 β , 1 ng/ml	62,286 \pm 10,709 ^b	80,849 \pm 3,274 ^b
IL-1 β , 10 ng/ml	67,676 \pm 7,552 ^b	89,502 \pm 7,023 ^b
TNF- α , 1 ng/ml	25,436 \pm 3,355	90,060 \pm 12,831
TNF- α , 10 ng/ml	43,573 \pm 9,395 ^b	85,589 \pm 3,678 ^b

^a Mean \pm SD.^b $P < 0.05$ by Student's t test compared to control.Fig. 6. Effect of TNF- α and IL-1 β on [3 H]thymidine incorporation by normal ovarian epithelial cells. Normal ovarian cells were plated at 5×10^4 /well. [3 H]Thymidine incorporation was measured after incubation with TNF- α or IL-1 β for 24 h. Data reflect the mean cpm \pm SD. *, statistically significant stimulation ($P < 0.05$).Fig. 7. Effect of anti-TNF- α antibody or soluble TNF- α receptor on the stimulation of proliferation induced by IL-1 β or TNF- α in ovarian cancer cells. IL-1 β (1 ng/ml) or TNF- α (1 ng/ml) was preincubated with medium, different concentrations of monoclonal anti-TNF- α antibody (A) or soluble TNF receptor (B) for 2 h at 37°C, before addition to cultures of OVCAR-3 cells. [3 H]Thymidine incorporation was measured after 24 h. Results are expressed as mean cpm \pm SD. *, significant stimulation ($P < 0.05$) relative to the medium control. †, significant reduction ($P < 0.05$) relative to the IL-1 β or TNF- α stimulated cells.

The expression of TNF- α mRNA was also accompanied by translation of TNF- α protein since the supernatants of IL-1 treated cultures contained TNF- α that could be detected by ELISA.

Endogenous expression of TNF- α may contribute to the proliferative activity of exogenous TNF- α or IL-1 β . When IL-1 β was used to stimulate proliferation and to induce TNF- α , the growth stimulating activity of IL-1 β was partially blocked by monoclonal anti-TNF- α antibodies or soluble TNF receptor. Failure to block IL-1 β -induced proliferation completely may relate to several factors. (a) The concentration of anti-TNF- α -antibody might be insufficient to neutralize the high levels of TNF induced by IL-1. The concentrations of antibodies and receptors used could neutralize approximately 1 ng/ml of TNF- α . (b) Intracellular TNF- α might provide a mitogenic signal. The anti-TNF- α antibodies and receptors can probably neutralize only extracellular TNF- α . TNF has exerted a mitogenic effect after microinjection into macrophages (25). (c) IL-1 could also act through pathway(s) that do not utilize TNF- α to mediate cell growth (26). Taken together, however, our data suggest that TNF- α might act through autocrine as well as paracrine pathways to stimulate rather than to inhibit ovarian cancer growth.

The ability to respond to TNF- α or IL-1 might be acquired by ovarian tumor cells during malignant transformation or might simply reflect the physiological response of the normal ovarian surface epithelial cells from which these tumors are derived. In our present study, TNF- α and IL-1 β significantly stimulated proliferation of normal ovarian epithelial cells in culture. Both IL-1 α and IL-1 β are expressed by human ovarian granulosa cells obtained from preovulatory follicular aspirates (27). IL-1 has also been detected in follicular fluid and might provide a critical signal to stimulate the focal proliferation of ovarian epithelium at the time of ovulation, facilitating repair of the defect produced when the follicle ruptures.

Several clinical trials have been conducted using TNF- α to treat ovarian cancer (28, 29). The clinical efficacy of TNF- α appears to be poor with an overall response rate of $<5\%$ (28). A poor response rate may relate to the innate resistance of ovarian tumor cells to TNF- α mediated cytotoxicity. Endogenous expression of TNF- α has correlated with resistance to the cytotoxic effects of TNF- α in some tumor cells (11). Given the technical difficulties in monitoring ovarian tumor growth within clinical trials, it is also possible that treatment with the cytokine might have actually accelerated tumor growth in some patients. Conversely, strategies to inhibit TNF- α expression and activity might provide a novel approach to treatment of at least some ovarian cancer patients.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent secretarial assistance of Linda Coyle and Lisa Hilliard and the bibliographical assistance of Peggy Haythorn.

REFERENCES

1. Matsushima, K., Akahoshi, T., Yamada, M., Furutani, Y., and Oppenheim, J. J. Properties of a specific interleukin 1 (IL-1) receptor on human Epstein Barr virus-transformed B lymphocytes: identity of the receptor for IL-1 α and IL-1 β . *J. Immunol.*, 136: 4496-4502, 1986.
2. Beutler, B. A., and Cerami, A. Recombinant interleukin 1 suppresses lipoprotein lipase activity in 3T3-L1 cells. *J. Immunol.*, 135: 3969-3971, 1985.
3. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA*, 72: 3666-3670, 1975.
4. Onozaki, K., Matsushima, K., Aggarwal, B. B., and Oppenheim, J. J. Human interleukin 1 is a cytotoxic factor for several tumor cell lines. *J. Immunol.*, 135: 3962-3968, 1985.
5. Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A., Jr., and Shepard, H. M. Recombinant human tumor necrosis factor- α : effects on proliferation of normal and transformed cells *in vitro*. *Science (Washington DC)*, 230: 943-945, 1985.
6. Guy, G. R., Chua, S. P., Wong, N. S., Ng, S. B., and Tan, Y. H. Interleukin 1 and tumor necrosis factor activate common multiple protein kinases in human fibroblasts. *J.*

- Biol. Chem., 266: 14343-14352, 1991.
7. Wu, S., Rodabaugh, K., Martinez-Maza, O., Watson, J. M., Silberstein, D. S., Boyer, C. M., Peters, W. P., Weinberg, J. B., Berek, J. S., and Bast, R. C., Jr. Stimulation of ovarian tumor cell proliferation with monocyte products including interleukin-1, interleukin-6, and tumor necrosis factor- α . *Am. J. Obstet. Gynecol.*, 166: 997-1007, 1992.
 8. Spriggs, D. R., Imamura, K., Rodriguez, C., Sariban, E., and Kufe, D. W. Tumor necrosis factor expression in human epithelial tumor cell lines. *J. Clin. Invest.*, 81: 455-460, 1988.
 9. Malik, S. T., Griffin, D. B., Fiers, W., and Balkwill, F. R. Paradoxical effects of tumor necrosis factor in experimental ovarian cancer. *Int. J. Cancer*, 44: 918-925, 1989.
 10. Oliff, A., Defeo-Jones, D., Boyer, M., Martinez, D., Kiefer, D., Vuocolo, G., Wolfe, A., and Socher, S. H. Tumors secreting human TNF/cachectin induce cachexia in mice. *Cell*, 50: 555-563, 1987.
 11. Spriggs, D., Imamura, K., Rodriguez, C., Horiguchi, J., and Kufe, D. W. Induction of tumor necrosis factor expression and resistance in a human breast tumor cell line. *Proc. Natl. Acad. Sci. USA*, 84: 6563-6566, 1987.
 12. Richardson, G. S., Scully, R. E., Nikrui, N., and Nelson, J. H., Jr. Common epithelial cancer of the ovary (2). *N. Engl. J. Med.*, 312: 415-424, 1985.
 13. Bast, R. C., Jr., Jacobs, I. J., and Berchuck, A. Editorial: malignant transformation of ovarian epithelium. *J. Natl. Cancer Inst.*, 84: 556-558, 1992.
 14. Kabawat, S. E., Bast, R. C., Jr., Welch, W. R., Knapp, R. C., and Bhan, A. K. Expression of major histocompatibility antigens and nature of inflammatory cellular infiltrate in ovarian neoplasms. *Int. J. Cancer*, 32: 547-554, 1983.
 15. Haskill, S., Becker, S., Fowler, W., and Walton, L. Mononuclear-cell infiltration in ovarian cancer. I. Inflammatory cell infiltrates from tumour and ascites material. *Br. J. Cancer*, 45: 728-736, 1982.
 16. Kacinski, B. M., Bloodgood, R. S., Schwartz, P. E., Carter, D. C., and Stanley, E. R. Macrophage colony stimulating factor is produced by human ovarian and endometrial adenocarcinoma-derived cell lines and is present at abnormally high levels in the plasma of ovarian carcinoma patients with active disease. *Cancer Cells (Cold Spring Harbor)*, 7: 333-337, 1989.
 17. Ramakrishnan, S., Xu, F. J., Brandt, S. J., Nidel, J. E., Bast, R. C., Jr., and Brown, E. L. Constitutive production of macrophage colony-stimulating factor by human ovarian and breast cancer cell lines. *J. Clin. Invest.*, 83: 921-926, 1989.
 18. Wang, J. M., Griffin, J. D., Rambaldi, A., Chen, Z. G., and Mantovani, A. Induction of monocyte migration by recombinant macrophage colony-stimulating factor. *J. Immunol.*, 141: 575-579, 1988.
 19. Takeyama, H., Wakamiya, N., O'Hara, C., Arthur, K., Kufe, D., Sakurai, K., and Spriggs, D. Tumor necrosis factor expression by human ovarian carcinoma *in vivo*. *Cancer Res.*, 51: 4476-4480, 1991.
 20. Naylor, M. S., Stamp, G. W. H., and Balkwill, F. R. Investigation of cytokine gene expression in human colorectal cancer. *Cancer Res.*, 50: 4436-4440, 1990.
 21. Bast, R. C., Jr., Feeney, M., Lazarus, H., Nadler, L. M., Colvin, R. B., and Knapp, R. C. Reactivity of a monoclonal antibody with human ovarian carcinoma. *J. Clin. Invest.*, 68: 1331-1337, 1981.
 22. Rodriguez, G. C., Berchuck, A., Whitaker, R. S., Schlossman, D., Clarke-Pearson, D. L., and Bast, R. C., Jr. Epidermal growth factor receptor expression in normal ovarian epithelium and ovarian cancer. II. Relationship between receptor expression and response to epidermal growth factor. *Am. J. Obstet. Gynecol.*, 164: 745-750, 1991.
 23. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*, 18: 5294-5299, 1979.
 24. Balkwill, F., Osborne, R., Burke, F., Naylor, S., Talbot, D., Durbin, H., Tavernier, J., and Fiers, W. Evidence for tumour necrosis factor/cachectin production in cancer. *Lancet*, 2: 1229-1232, 1987.
 25. Smith, M. R., Munger, W. E., Kung, H., Takacs, L., and Durum, S. K. Direct evidence for an intracellular role for tumor necrosis factor- α 1. Microinjection of tumor necrosis factor kills target cells. *J. Immunol.*, 144: 162-169, 1990.
 26. Lin, J. X., and Vilcek, J. Tumor necrosis factor and interleukin-1 cause a rapid and transient stimulation of *c-fos* and *c-myc* mRNA levels in human fibroblasts. *J. Biol. Chem.*, 262: 11908-11911, 1987.
 27. Hurwitz, A., Loukides, J., Ricciarelli, E., Botero, L., Katz, E., McAllister, J. M., Garcia, J. E., Rohan, R., Adashi, E. Y., and Hernandez, E. R. Human intraovarian interleukin-1 (IL-1) system: highly compartmentalized and hormonally dependent regulation of the genes encoding IL-1, its receptor, and its receptor antagonist. *J. Clin. Invest.*, 89: 1746-1754, 1992.
 28. Balkwill, F. R. Tumor necrosis factor. *Br. Med. Bull.*, 45: 389-400, 1989.
 29. Mizuno, D., and Soma, G. I. Endogenous and exogenous TNF therapy (EET therapy). Conceptual and experimental grounds. *Ann. Inst. Pasteur Immunol.*, 139: 282-285, 1988.