Constitutive expression of TGF-bêta1, interleukin-6 and interleukin-8 by tumor cells as a major component of immune escape in human ovarian carcinoma

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ABSTRACT. Tumors could use several mechanisms to coexist with the host's immune system or to protect themselves from an immune response. Thus, insufficient expression of cell surface molecules on tumor cells, which are important for T cell recognition or activation, could lead to induction of a state of tolerance. Tumor cells could also produce cytokines that would inhibit the immune response and allow tumor progression. Here, we studied, *in vitro*, the cell surface expression of immunologically important molecules in seven ovarian carcinoma (OVCA) cell lines and the constitutive expression of cytokines. All OVCA cell lines expressed MHC class I molecules, ICAM-1 and LFA-3 adhesion molecules, necessary to induce a specific cytotoxic T-cell response, as well as the CD40 costimulatory molecules. Conversely, the lack of the dominant costimulatory molecules, CD80 (B7.1) and CD86 (B7.2) could be a possible explanation of poor immunogenicity of OVCA tumors. Immunosuppressive TGF-β1 was detected at the mRNA level in all cell lines but was weakly secreted in supernatants. By contrast, IL-10 was never found. Most of them constitutively produced IL-8 and IL-6, two cytokines known as tumor promoting factors whereas the proinflammatory cytokines TNF-α, IL-1β and GM-CSF were rarely produced. Data from this study could be useful for designing new strategies of immunotherapy to improve immunogenicity and/or limit protumor cytokine production.

Keywords: ovarian carcinoma, TGF-β1, IL-6, IL-8, differentiation markers

INTRODUCTION

Ovarian carcinoma (OVCA), which derives from the ovarian surface epithelium, is the main cause of death in patients with gynecological cancers. These tumors are often diagnosed late, spread within peritoneal cavity in advanced disease, and are associated with ascitic effusion. Current treatment, consisting of surgery in association with chemotherapy or radiotherapy, achieves good response but in the majority of patients the disease recurs.

Recent evidence based particularly on the discovery of tumor-associated antigens (TAA), specifically recognized by cytotoxic T lymphocytes (CTLs) with the ability to kill tumor cells, have provided a strong basis for the development of immunotherapeutic protocols against cancer [1, 2]. However, until now, success in human clinical trials has been limited, probably because of the capacity of tumors to induce a tolerance state and/or to escape from immune-surveillance [3].

Optimal T-cell activation requires a first signal mediated by the interaction of the T-cell receptor (TCR) with peptide-MHC complex. The second signal is provided by the interaction of surface receptors on the T cells, with costimulatory/adhesion molecules expressed on antigen-presenting cells (APC). Low or absent expression of MHC or costimulatory/adhesion molecules by tumor cells might lead to an insufficient immune response or induce tumor antigen-specific tolerance or anergy [4].

Innate and adaptive antitumor responses are controlled by several immunological mediators including cytokines [5]. Cytokines such as interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α) are known to deliver a "danger signal", and promote maturation and differentiation of dendritic cells (DC) which could generate an efficient antitumoral T-cell response [6]. Conversely, the production of inhibitory cytokines in the immediate microenvironment of tumors has been suggested as one of the major mechanisms by which tumor cells can avoid immune recognition [7]. The detection of the main immunosuppressive cytokines, IL-10 and transforming growth factor-β (TGF-β), in peritoneal fluid from patients with ovarian cancer supports this hypothesis [8, 9]. In fact, these cytokines share many immunosuppressive activities that inhibit the function of APC, and the generation and activity of immune effectors cells [4, 10]. Likewise, it is well established that other cytokines such as IL-6 or IL-8, constitutively produced by a variety of tumor types, can also promote tumor progression, acting on tumor growth and survival [11, 12]. Here, we studied the phenotypic charac-

Table 1
Clinical characteristics of the ovarian carcinoma patients and methods used to obtain tumor cell suspensions

Patients	Histological type	Histological grade	FIGO* stage	Type	Enzymatic digestion	Discontinuous gradient
O65	Papillary serous cystadenocarcinoma	3	IV	ascite	–	–
O114	Papillary serous cystadenocarcinoma	2	IIIc	ascite	–	–
O135	Papillary serous cystadenocarcinoma	3	IIIc	solid	+	–
O151	Papillary serous adenocarcinoma	2	ND	solid	+	+
O170	Serous cystadenocarcinoma	1	III	ascite	–	+

*FIGO: International Federation of Obstetrics and Gynecology. ND, Not determined.

teristics of cell lines generated in our laboratory from OVCA biopsies, and of two established OVCA cell lines.

We focused on the molecules necessary for mounting an effective antitumor response: MHC class I and class II, costimulatory molecules B7.1 (CD80), B7.2 (CD86) and CD40 as well as adhesion molecules ICAM1 (CD54) and LFA-3 (CD58). On the other hand, we evaluated the level of "key" cytokines produced by OVCA cell lines: cytokines which contribute either to the elaboration of an effective anti-cancer immune response – IL-1β, granulocyte-macrophage-colony stimulating factor (GM-CSF), and TNF-α, or to the suppression of the immune response – IL-10 and TGF-β1, as well as cytokines known to favor tumor growth and survival: IL-6 and IL-8. In OVCA, the majority of the published studies examining cytokine production have been carried out at the mRNA level from tissue biopsies, which does not allow precise determination of the original source of the cytokine production [13-15]. Moreover, analysis of cytokine transcripts alone is insufficient, since control of cytokine secretion can occur at a post-transcriptional level. So, we have investigated the constitutive expression of cytokines at the mRNA level, and simultaneously measured the amounts of soluble protein in OVCA cell lines generated in our laboratory from biopsies and in two established OVCA cell lines.

Data from this study may help to provide a better understanding of the interactions between host and tumor cell, and enable the design of novel therapeutic strategies to increase tumor immunogenicity or limit the negative effects of cytokines.

PATIENTS AND METHODS

Patients

Clinical characteristics of five patients with ovarian carcinoma (OVCA) included in this study are shown in Table 1, as well as the nature of the biopsies (solid or ascite), and the methods used to obtain tumor cell suspensions. Ascitic fluid samples from 3 patients with advanced disease were obtained under sterile conditions by paracentesis using an ascitic drain. Solid tumor tissue specimens were collected from two patients at the time of surgery.

Tumor cell collection

Ascitic samples collected in sterile bottles were washed twice in Ca^{2+}-, Mg^{2+}-free HBSS (Hank's Balanced Salt Solution) (Sigma, Saint Quentin Fallavier, France), and centrifuged at 400 g for 20 min to pellet the cells. Solid tumor specimens were processed rapidly under sterile conditions after surgery. The tumor was minced to 1-2 mm^3 fragments using a scalpel, and placed in a sterile Erlenmeyer with RPMI 1640 (Eurobio, Les Ulis, France) containing 1 µg/ml collagenase (Sigma), 2.5 U/ml hyaluronidase (Roche Biochemicals, Meylan, France) and 65 Kunits/ml DNase II-S (Sigma) supplemented with 10 µg/ml gentamycin, 100 µg/ml penicillin, 100 IU/ml streptomycin, 1.25 µg/ml amphotericin B, 2% L-glutamine and 5mM HEPES (ICN Biomedicals, Aurora, USA). Enzymatic digestion was performed under agitation for 4 hours at 37 °C or overnight at room temperature. The cell suspension obtained was filtered through a sterile gauze to remove clumps and washed twice in Ca^{2+} –, Mg^{2+}-free HBSS. Single-cell suspensions from ascitic effusion or solid tumor were cultured directly in flasks as described below, or separated over a 75%/100% discontinuous ficoll (Histopaque®, Sigma) gradient by centrifugation at 900 g for 30 min. The lower lymphocyte-enriched and upper tumor cell-enriched interfaces were collected separately, washed twice and distinct populations were counted for viability using trypan blue exclusion.

Tumor cell lines

Tumor cells isolated from ascitic or solid samples were cultured in 25 cm^2 flasks (Cellstar®, Greiner Labortechnik, Germany), in a humidified 5% CO_2 incubator, and in RPMI-1640 medium plus 10% fetal calf serum (FCS) (Gibco, Life technology, Germany) supplemented by 100 µg/ml penicillin, 100 IU/ml streptomycin and 5mM HEPES, and defined as complete medium. In primary cultures from solid tumors or ascitic fluid, we observed fibroblast-like cells that could grow faster than tumor cells. So, to prevent this overgrowth, we used a differential trypsinization method based on selective detachment and attachment properties of fibroblastoid and tumor cells to plastic flasks, as described by Polinger [16].

In one case (O135 cell line), it was not possible to separate tumor cells from fibroblasts by the procedure described above so tumor cells were isolated by magnetic cell sorting. Briefly cells were incubated with Anti-Fibroblast MicroBeads (Miltenyi Biotec, Paris, France) before separation on a depletion column. "Magnetically" labeled fibroblasts were retained in the column, while unlabeled

cells ran through. The negative tumor cell-enriched cell fraction was then cultured as detailed above.

Once confluent, the tumor cell cultures were split by trypsinization. Each sub-culture was maintained in culture flasks and used at different passages (P).

Established cell lines

The established human ovarian carcinoma cell line, NIH:OVCAR-3, was purchased from the American Type Cell Collection (ATCC). The IGROV1 cell line generated from a solid ovarian carcinoma, was kindly provided by J Bénard (Institut Gustave Roussy, France). These cell lines used as control, were maintained in culture flasks in complete medium as described above.

Phenotype of ovarian carcinoma cell lines by flow cytometric analysis

In order to determine the tissue origin of the cells maintained in culture, cells were stained by indirect immunofluorescence using unconjugated mAbs (monoclonal antibodies) directed against the tumor cell-associated marker CA125 (clone OC125) and directed against fibroblast marker vimentin (clone V9), or directly stained by fluorescein-isothiocyanate (FITC) mAb directed against the epithelial marker cytokeratin (clone MNF116). All these antibodies were purchased from DAKO (Glostrup, Denmark).

The OVCA cell lines were characterized for the expression of cell surface molecules involved in immune interaction, using FITC or phycoerythrin (PE)-conjugated mAbs from Immunotech (Marseilles, France): costimulatory molecules were detected by mAbs specific for B7.1 (anti-CD80, clone MAB104), B7.2 (anti-CD86, clone B.T7) and CD40 (clone mAB89); adhesion molecules by mAbs directed against lymphocyte-function associated antigen-3 (LFA-3) (anti-CD58, clone AICD58) and intercellular adhesion molecule-1 (ICAM-1) (anti-CD54, clone 84H10); MHC molecules by mAbs specific for MHC class I (anti-HLA ABC, clone B9.12.1) or MHC class II (anti-HLA DR, clone Immu-357).

Expression of surface markers by OVCA was assayed by single or dual-color immunofluorescence analysis after trypsin-EDTA treatment of cultures. Cells were washed with phosphate - buffered saline (PBS), and direct cell surface staining was performed by incubating cells in tubes at 2.5 to 5×10^5 cells in 50 µl of PBS supplemented with 0.5% bovine serum albumin (BSA) (Eurobio) with conjugated mAbs for 30 min at 4 °C. After washing with PBS-BSA, cells were fixed in 0.37% formaldehyde until analysis. For indirect immunostaining, cells were incubated with unconjugated primary mAbs for 30 min at 4 °C, washed and stained with FITC-labeled sheep $(Fab')_2$ anti-mouse IgG (Silenus, Amrad Biotech, Australia). Direct or indirect intracellular immunostaining with mAbs against cytokeratin or vimentin was carried out in a permeabilization buffer containing PBS with 0.5% BSA and 0.2% saponin according to the conditions described above. In parallel, isotype-matched murine fluorochrome-conjugated or uncoupled immunoglobulins were used as negative controls. Data were acquired using a FACSCalibur cytometer (Becton Dickinson, Moutain View, USA), and analysis was performed using Cell Quest software.

RT-Multiplex PCR (RT-MPCR) assay for expression of cytokine mRNA

Cells were grown to confluence and total RNA from 7 OVCA cell lines was isolated using a SV Total RNA Isolation System kit (Promega, FRANCE) according to the manufacturer's instructions. cDNA was prepared by a standard method using reverse transcriptase and an oligo-dT primer from SuperScript kit (Gibco).

We used a multiplex PCR-based technique (CytoXpress Inflammation Cytokine Set 1 kit, BioSource, Belgium) to screen for gene expression of IL-1β, IL-6, IL-8, TNF-α GM-CSF, and TGF-β1. This method allows amplification of different cDNA in the same reaction tube using multiple primer pairs. Semiquantitative amplification of each desired amplicon was made possible by inclusion in each tube of primer pairs for the constitutively expressed housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GADPH).

cDNA (5%) or control cDNA from the supplier were added in a 50 µl reaction mixture containing 0.5 U of AmpliTaq (Applied Biosytems, Roche Molecular Systems, USA), 5 µl 10X buffer, 5 µl 10X primers, and 4 µl dNTP (3.12 mM). The reaction mixture was amplified with a DNA thermal cycler (GenAmp type 9700, Applied Biosystems). After an initial denaturing step at 96 °C for 1 min, the temperature profile consisted of a denaturation step (96 °C for 1 min) and an annealing step (58 °C for 4 min) for 2 cycles followed by 33 cycles at 94 °C for 1 min and 58 °C for 2.5 min. Thermocycling was completed by an incubation step at 70 °C for 10 min. Ten µl of each PCR product were analyzed on ethidium bromide-stained 2% agarose gel.

Densitometry of the resulting bands was performed using the Syngene gel analyzer (Syngene, Ozyme, France) with Genetools analysis software.

Quantitative measurements of cytokine production

Cells were grown to confluence before trypsin-EDTA treatment in order to obtain cell suspensions, which were washed, counted and incubated at the indicated density in RPMI 1640 medium supplemented with 10% FCS for 72h, in 12-well culture plates (Falcon®, Becton Dickinson, USA). Cell viability was controlled by staining with trypan blue, and culture supernatants were collected, centrifuged and frozen at – 80 °C until analysis. Assays with serum-free medium had been performed in preliminary experiments. For all cytokines, levels of detected proteins were very weak, in agreement with low cell viability. So subsequent investigations were performed with cells grown in medium with 10% FCS.

TGF-β1 is secreted by cells in an inactive complex called the latent form, which can be cleaved by proteases to release active or mature TGF-β1 [17]. Only the mature form is bioactive, and can be detected by commercially available kits for Enzyme-linked Immunosorbent Assay (ELISA). Latent TGF-β1 can be activated by low pH, enabling assay of total (active + latent) TGF-β1. The concentration of the latent form is obtained by subtracting the amount of the active form from total TGF-β1. Culture supernatants were assayed in duplicate in 96-well, flat-bottomed microtiter plates (Corning-Costar Corp), using a specific ELISA according to the manufacturer's instructions (Promega). To measure total TGF-β1, 100 µl aliquots

Table 2
Phenotypic characteristics of ovarian carcinoma cell lines

OVCA	Tissue markers			MHC molecules		Costimulatory molecules			Adhesion molecules	
	CA-125	Cytokeratin	Vimentin	Class I	Class II	CD80	CD86	CD40	CD54	CD58
IGROV1	–	–	++	+++	–	–	–	++	–	++
OVCAR3	++	++	–	++	–	–	–	++	++	++
O65	++	+++	–	+++	–	–	–	++	+++	++
O114	+++	++	–	++	–	–	–	+	++	++
O135	+	++	–	+++	–	–	–	++	++	+++
O151	–	++	–	+++	–	–	–	++	+++	++
O170	++	++	–	+++	–	–	–	++	+++	++

Levels of expression were determined by the measurement of the mean fluorescence intensities (MFI) and ranked as: +++ = MFI > 100; ++ = MFI from 10-100; + = MFI < 10; – = identical to control staining.

were first acid treated with 5 µl 1 M HCl for 15 min and then neutralized with 5 µl 1 M NaOH. As significant levels of active and latent TGF-β1 present in the bovine sera are necessary to maintain cells in culture, the background in control medium was determined and subtracted from samples of conditioned medium. GM-CSF was assayed using a specific ELISA (Pharmingen).

We used a bead-based immunoassay kit, combined with flow cytometry (Cytometric Bead Array, CBA, Becton Dickinson) for the quantification of IL-1β, IL-6, IL-8, IL-10 and TNF-α in the supernatants of OVCA cell lines. This recent technique is analogous to ELISA, and provides the advantage of the simultaneous assay of several cytokines in a single and small sample of biological fluids (50 µl). The CBA assay used fluorescent beads coated with antibodies as the solid support for classical immunosorbent assays.

50 µl of mixed bead populations coated with capture antibodies specific for each cytokine were mixed with 50 µl of test samples or recombinant standards, and 50 µl of (PE)-conjugated detection antibodies, and maintained for 3 hours at room temperature. Beads were washed with 1ml of buffer by centrifugation for 5 minutes at 200 g. Supernatants were carefully discarded and beads were resuspended in 300 µl of buffer before FACS analysis. Human recombinant cytokines used as standards allowed calibration curves (between 0 and 5000 pg/ml) to be obtained. The concentration of each cytokine was calculated using the calibration curves. Data from 1800 events were acquired using a FACSCalibur cytometer and analyzed automatically with the BD CBA Analysis software.

RESULTS

Characterization of ovarian carcinoma cell lines

Although 85% of all malignant ovarian tumors are known to originate from the epithelial surface, short-term cultures from tumor biopsies could contain mixed cell populations consisting of epithelial cells and fibroblast-like cells derived from fibroblasts in solid tumors or mesothelial cells in ascitic effusions. Initially, cultures grew slowly with few epithelial islets arranged in a cobblestone pattern and surrounded by fibroblast-like cells with fusiform morphology. Fibroblastoid cells were removed by differential trypsinization or magnetic cell sorting as described in Materials and methods, and after 3 to 5 passages, no such cells were visible under inversion microscopy.

Using flow cytometric analysis of tissue markers, we characterized cell lines to ensure their epithelial origin and to guarantee absence of contaminating cells (Table 2). Most of the cell lines (5/7) expressed CA125, a mucin-like protein and tumor-associated antigen used as a serum marker in OVCA. The level of expression, as represented by mean fluorescence intensities was high in 4/5 samples, OVCAR-3, O65, O114 and O170 cell lines, and weak for the O135 cell line (Figure 1A).

The epithelial origin of the cells was confirmed by positive staining for cytokeratin (6/7) in the absence of vimentin staining detected at the intracellular level. In some samples, we noted a weak percentage (< 6%) of double-positive cells for cytokeratin and vimentin. Only IGROV1 was negative for cytokeratin and positive for vimentin.

OVCA cell lines were then characterized for cell surface molecules of potential immunological interest. All of the seven cell lines have been shown to express MHC class I

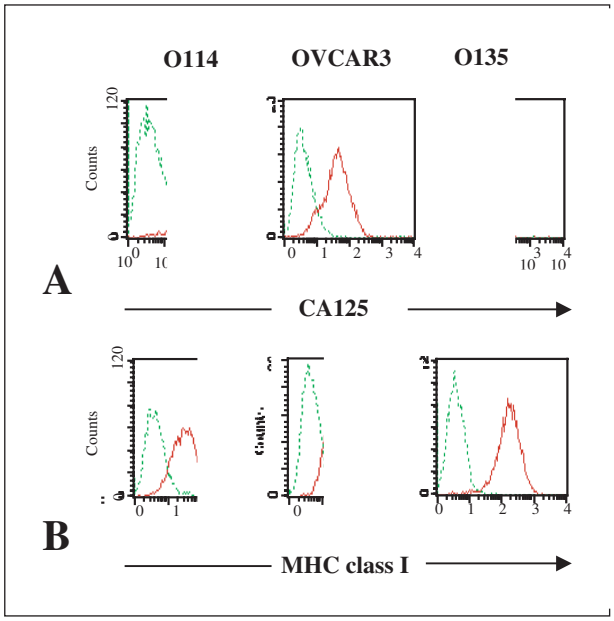

Figure 1
Expression of ovarian carcinoma marker CA125 and MHC class I molecules in OVCA cell lines as detected by flow cytometry. Representative data were obtained by cytofluorometric analysis of OVCA cells, after trypsin-EDTA treatment and immunofluorescence staining. Cells were marked by mAbs specific for CA125 (A) or MHC class I molecules (solid line)(B); or by an isotype mAb (dotted line). Results are expressed in histogram form, as the number of cells by channel of fluorescence intensity.

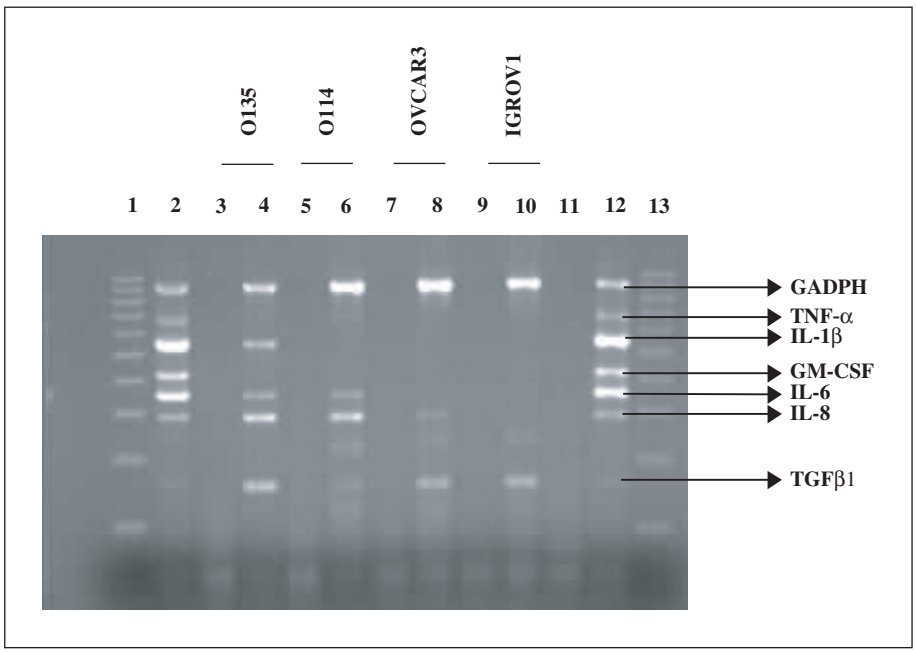

Figure 2

Specific expression of cytokine mRNA by ovarian carcinoma cell lines. OVCA cell lines were grown to confluence, total mRNA was isolated and RT-Multiplex PCR with multiple pair primers was performed. A representative electrophoresis gel image is shown. Lanes 1 and 13 correspond to molecular weight markers, lane 2 and 12 to cDNA control from supplier. Lanes 4, 6, 8, 10 represent results from cell lines O135, O114, OVCAR3 and IGROV1 respectively. Lanes 3, 5, 7, 9 are results from the same cell lines but obtained without adding reverse transcriptase (RT-). Expected products size are: GADPH, 921 pb; TNF-α, 680 pb; IL-1β, 555 pb; GM-CSF, 424 pb; IL-6, 360 pb; IL-8, 300 pb; TGF-β1, 161 pb.

molecules. The expression level was high for IGROV1, O65, O135, O151 and O170 cell lines. Only modest expression was noted for OVCAR-3 and O114 (Figure 1B). By contrast, none of the OVCA cell lines expressed MHC class II molecules. All cell lines tested were negative for B7.1 (CD80) or B7.2 (CD86) costimulatory molecules but, on the other hand, they expressed CD40. Likewise, adhesion molecules ICAM-1 (CD54) and LFA3 (CD58) were expressed on all OVCA cell lines except for IGROV1, which expressed only CD58.

Analysis of cytokine expression at mRNA and protein levels

Representative cytokine expression profiles, defined by the Multiplex PCR method on cDNA obtained after reverse transcription of mRNA isolated from cell lines at various passages, are presented in Figure 2. Semiquantitative determination of cytokine gene expression was obtained by normalizing its expression against GADPH expression, using densitometry. The absence of signal when reverse transcriptase was omitted (RT-) confirmed the specificity of cDNA amplification and excluded possible contamination by genomic DNA. TGFβ1 was demonstrated in all OVCA cell lines, with some quantitative variations between cell lines and passages (Table 3). The highest levels, although inconsistent, were observed in O65, O135, O151 and O170 cell lines. In contrast to the data obtained for RT-MPCR, the level of TGF-β1 in the supernatants of OVCA cell lines was usually low. When bioactive TGF-β1 was found, the majority of the protein was secreted in latent form, which was detectable by ELISA after an acid treatment (Figure 3). Nevertheless, the concentration did not exceed 100 pg/ml/10^5 cells, except for O135 on one occasion (260 pg/ml/10^5 cells at 19th passage). IL-10 was not detectable in any supernatants by CBA assay with a threshold of 10 pg/ml (data not shown).

Most of the carcinoma cell lines (5/7) expressed IL-8 mRNA constitutively. As detected by CBA, IL-8 was produced by all the OVCA cell lines except for IGROV1 (Figure 4A). Results obtained from detection of IL-8 mRNA and soluble protein quantification, were concordant. Only O65 was weakly detected by CBA but not by RT-MPCR. Cell lines producing IL-8 could be sorted into two groups.

The first group including O135, O151 and O170 cell lines, was characterized by a high level of IL-8 production, above 1ng/ml/10^5 cells and up to 14 ng/ml/10^5 cells for cell line O135 at the 19th passage. The second group, with OVCAR3, O65 and O114 cell lines, produced smaller amounts, below 1 ng/10^5 cells. Interestingly, in the case of the O114 cell line, expression of IL-8 mRNA turned negative with increasing passage number, as observed at the protein level: the amount of IL-8 decreased from 1000 after 14 passages to less than 100 pg/ml/10^5 cells after 102 passages.

Proinflammatory IL-1β and TNF-α cytokine gene expression was infrequent in OVCA cell lines whereas, IL-6 was found in the supernatants of most cell lines at variable levels. The high level of IL-6 production observed in the two cell lines O135 and O170, which released considerable amounts of IL-6 (superior to 5 ng/ml/10^5 cells), was associated with detection of IL-6 mRNA (Figure 4B). Conversely, in the 3 cases where the amounts of IL-6 were weak or modest – about 100 pg/ml/10^5 cells for IGROV1 and OVCAR3 cell lines, and 400 pgml/10^5 cells for the

Table 3
Cytokine expression in ovarian carcinoma cell lines

Cell lines	Number of passages (P)	TGF-β1	IL-8	IL-6	GM-CSF	IL-1β	TNF-α
O65	20	+ + +	–	–	–	–	–
	23	+	–	–	–	–	–
O114	10	+	+	+	–	–	+
	12	+	+	–	–	–	+
	76	+	+	–	–	–	–
	88	+	–	–	–	–	–
	102	+	–	–	–	–	–
O135	8	+	+	+	–	+	–
	14	+ + +	+ + +	–	–	+	–
	19	+	+	+	–	+	+
O151	5	+ +	+	–	–	–	–
	9	+ + +	+	–	–	–	–
O170	13	+ +	+	+	–	–	–
IGROV1	/	+	–	–	–	–	–
		+	–	–	–	–	–
OVCAR3	/	+	+	–	–	–	–
		+	+	–	–	–	–

Cytokines expressed by OVCA cell lines were analyzed at distinct passages (P). Band density of each amplicon was indexed to GADPH (index 100) and expressed in relative densitometric units (RDU) which were ranked as follows: - = no band; + = RDU < 100; + + = RDU from 100 to 200; + + + = RDU > 200.

O151 cell line – IL-6 transcripts were not demonstrated. IL-6 secretion by the O114 cell line was weak, about 100 pg/ml/10^5 cells, and disappeared with passages as noted at the mRNA level, and in a manner identical to that for IL-8 expression. This cell line also transiently expressed TNF-α transcripts before the 12^{th} passage, an observation which was not confirmed at the protein level for TNF-α.

Only O135 co-expressed IL-1β, IL6 and TNF-α, but at fluctuating levels as seen at the mRNA and protein levels; amounts of TNF-α and IL-1β not exceeding 200 pg/ml/10^5 cells at 2 tested passages (data not shown). A modest production of GM-CSF (less than 450 pg/ml/10^5 cells) was observed in the supernatants of 3/7 cell lines, but its transcript was not detected at the mRNA level (Figure 4C). In addition, we detected one unexpected signal with a low intensity at 250 pb in some experiments, which did not correspond to any tested cytokine. Taken together, our results show that cytokine transcript expression correlates with cytokine production in tumor cell supernatants, but occasionally some discordances have been noted as mentioned above (Table 4).

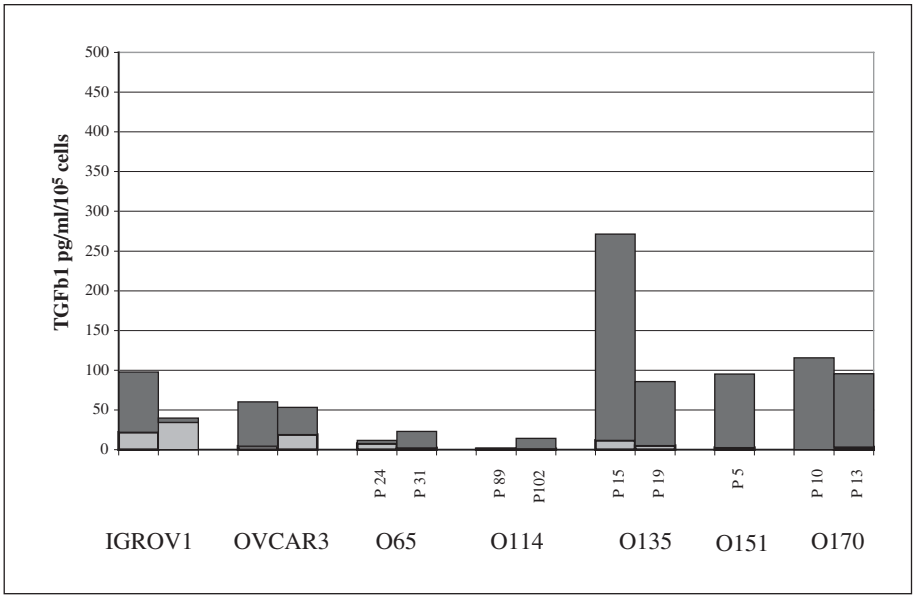

Figure 3
TGF-β1 production by ovarian carcinoma cell lines. At various passages (P), OVCA cells (2 to 5 × 10^5) were incubated in 12-well plates in complete medium, and supernatants were collected after 72 h. Active (▒) or latent (■) TGF-β1 were assayed by ELISA. Concentrations are expressed in pg/ml for 10^5 cells initially seeded. Results of two independent experiments are shown except for O151 (one experiment).

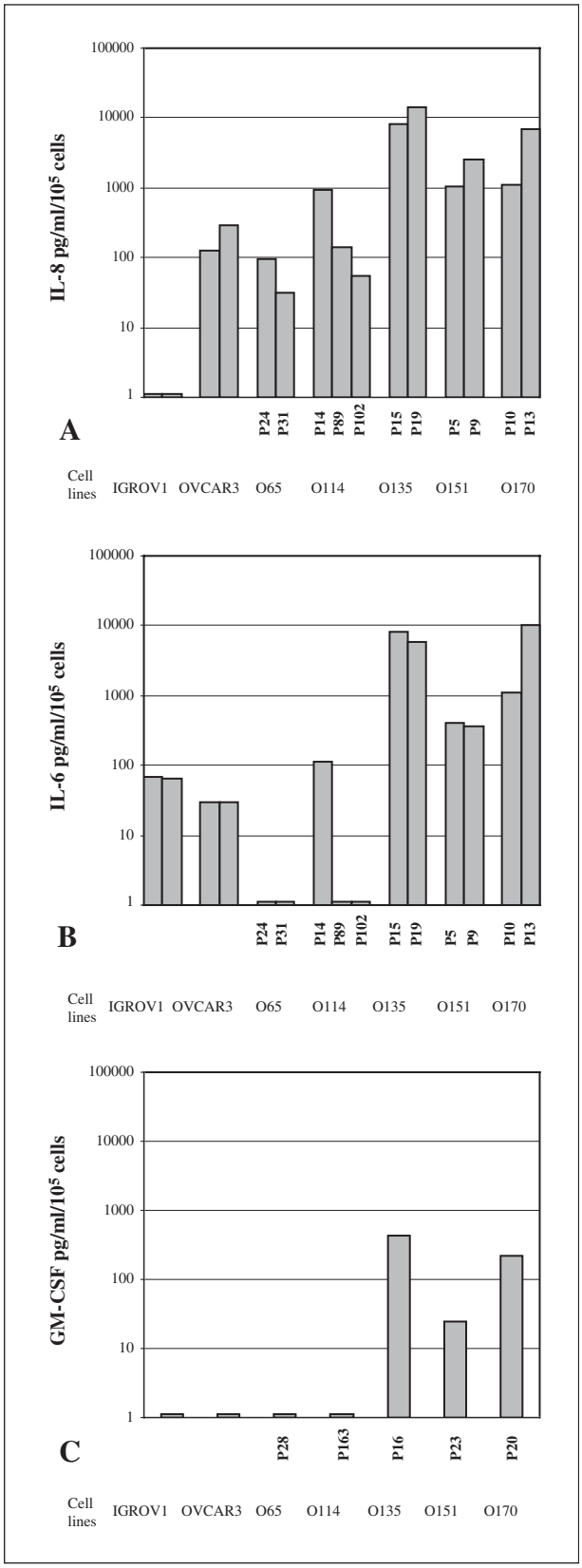

Figure 4
IL-6, IL-8 and GM-CSF production by ovarian carcinoma cell lines. At various passages (P), OVCA cells (2 to 5×10^5) were incubated in 12-well plates in complete medium, and supernatants were collected after 72 h. Cytokines IL-8 (A) and IL-6 (B) were assayed by Cytometric Beads Array (CBA) and by a specific ELISA for GM-CSF (C). Concentrations are expressed in pg/ml for 10^5 cells initially seeded. Results of independent experiments are shown.

DISCUSSION

In the present study, we evaluated ovarian carcinoma cell lines for cell surface expression of immunologically important molecules and secreted cytokines. These cell lines were confirmed as epithelial in origin on the basis of cytokeratin expression in the absence of vimentin detection. Only IGROV1 was negative for cytokeratin but, others studies have certified its epithelial origin, as attested by certain criteria such as the presence of cellular junctions, microvilli and abundant secretions [18]. Moreover, IGROV1 cells express folate receptor, a tumor-associated antigen described in most ovarian carcinomas and recognized by mAb Mov18 [19]. On the other hand, 5/7 cell lines were positive for the tumor-associated-antigen CA125 described in 80% of epithelial ovarian cancers [20]. The lack of CA125 staining in two cases (IGROV1 and O151) might be due to more rapid or intense shedding at the membrane level [21, 22].

All OVCA cell lines expressed the MHC class I molecules necessary for the induction of the specific cytotoxic T cell response and/or for conferring sensitivity to lysis by cytotoxic T lymphocytes, as well as the ICAM-1 and LFA-3 adhesion molecules required to stabilize immunological synapses and to induce an efficient antitumor response [23]. Further investigations are in progress to study immunogenicity of ovarian carcinoma cell lines, *in vitro*. However, despite adequate expression of MHC class I molecules, the lack of the dominant costimulatory molecules, CD80 and CD86, on OVCA appears to be a major limitation for carcinoma cells to drive a T cell response, since TCR signaling in the absence of costimulation results in T cell inactivation or anergy. An attractive strategy to induce or reactivate pre-existing immune responses would be to increase costimulatory capacity in tumor cells themselves [24]. For example, introduction of the B7.1 gene in human ovarian, breast, pancreatic or renal carcinoma cell lines has been shown to improve their immunogenicity *in vitro* and in some cases, can lead to specific CTL activation [25, 26].

Interestingly, CD40, a member of the TNF receptor superfamily was found on the surface of all tested OVCA cell lines. This costimulatory molecule has been described in several types of cancer including bladder, breast and colon carcinomas [27]. Previous studies have shown that CD40 engagement on carcinoma cells by its ligand CD154 (CD40 ligand) results in growth inhibition, modulation of cytokine release and increased antigen presentation function, suggesting that this molecule is a potential therapeutic target [28-30].

It has recently been proposed that the cytokine-rich inflammatory microenvironment of tumors may contribute to cancer growth and spread, rather than the immune response to cancer [31]. For these reasons, we have chosen to identify "key" cytokines produced by ovarian carcinoma cells.

In our experiment, a signal obtained by RT-MPCR was usually associated with cytokine detection in tumor cell supernatants, but with some discordance. TGF-β1 transcripts were clearly demonstrated in all OVCA cell lines; by contrast, TGF-β1 protein appeared to be synthesized at weak levels, and mainly in latent form, in tumor cell supernatants. It was previously shown that TGF-β1 production as assessed by bioassay with the CCL-64 mink

Table 4
Comparative data of cytokine mRNA and protein expression by ovarian carcinoma cell lines.

Cell lines	TNF-α		IL-1-β		GM-CSF		IL-6		IL-8		TGF-β1		IL-10	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
IGROV1	–	–	–	–	–	–	–	+	–	–	+	++	ND	–
OVCAR3	–	–	–	–	–	–	–	+	+	++	+	++	ND	–
O65	–	–	–	–	–	–	–	–	–	+	+	+	ND	–
O114	±	–	–	–	–	–	±	±	±	++	+	+	ND	–
O151	–	–	–	–	–	++	–	++	+	+++	+	++	ND	–
O170	–	–	–	–	–	+	+	+++	+	+++	+	++	ND	–
O135	±	+	+	++	–	++	±	+++	+	+++	+	++	ND	–

RT-Multiplex was used to detect cytokine expression in 7 ovarian carcinoma cell lines. A " + " sign indicates expression, a "-" sign indicates no expression and a " ± " designates a variable expression with passages. The results from quantitative measurements of cytokines were expressed in pg/ml for 10^5 cells and ranked as following: – = undetectable level; + = < 0.1 ng/ml; ++ = from 0.1 to 1ng/ml, +++ = from 1 to 10 ng/ml. ND, Not determined.

lung epithelial cell line, varies widely among ovarian carcinoma cell lines [32].

Our results are consistent with the data obtained by Abenstein and coworkers, who used an ELISA assay and found a modest production in most ovarian carcinoma cell lines [33]. The discrepancies between the strong expression of TGF-β1 mRNA and the weak level of protein have already been reported by some authors and may be explained by two reasons [34, 35].

Firstly, it is known that TGF-β1 synthesis is tightly regulated, especially at the post-transcriptional level [36]. TGF-β1 mRNA contains an unusually long, 5'-untranslated region (UTR), involved in the regulation of the translation and deletion sequence experiments involving this region, indicating that it includes both stimulatory and inhibitory sequences [37, 38]. Morrisey *et al.* have shown that increased TGF-β1 secretion in insulin-stimulated renal cells does not require *de novo* gene transcription, but does involve the stimulation of translation [39]. Poor translatability of TGF-β1 mRNA in ovarian carcinoma cells, could therefore explain our results. Secondly, TGF-β1 could be localized at the cell surface through binding to its specific receptors expressed by ovarian tumor cells or several other molecules such as extracellular matrix proteoglycans [40-42]. Consequently, because of TGF-β1 bioavailability, an important fraction of this cytokine may be not accessible to ELISA, which may have led to an underestimation of the real amount of TGF-β1 produced by our cell lines.

TGF-β1 production by OVCA cell lines could play an important role in suppression of the anti-tumor immune response by inhibiting antigen presentation, proliferation of specific T cells and generation of the cytotoxic response [10, 43]. In this regard, it should be noted that the small amount of TGF-β1 produced by human carcinoma could prevent the activation and cytotoxic activity of autologous blood lymphocytes [44]. In addition, it was suggested that surface-bound TGF-β1 on CD4+ CD25+ regulatory T cells could contribute to immunosuppression by cell contact [45].

IL-10 has already been demonstrated in ovarian cancer biopsies, but not in OVCA cell lines [13, 46]. In agreement with these observations, we did not detect IL-10 in any OVCA cell line supernatants.

In our study, the fact that IL-6 was secreted constitutively, at a high level, by the majority of the tumor cell lines is in agreement with observations from other groups [47, 48]. Some studies have described the increase of serum IL-6 as an indicator of poor prognosis in ovarian and other carcinomas [49-51]. The protumor effects of IL-6 might first result from its ability to induce a decrease in cell-cell adhesion and increased cellular motility as shown in breast carcinoma cells, suggesting a role for IL-6 in tumor metastasis [52].

Failure to detect mRNA, despite IL-6 production, in supernatants of the cell line has been reported for OVCAR-3 [47]. Such discordant data were also observed in GM-CSF evaluations: no PCR signal was detected for this cytokine in any OVCA cell line, whereas GM-CSF protein was constitutively secreted at modest levels by 3 OVCA cell lines. These data are in favor of the hypothesis that small amounts of cytokine mRNA, or unstable mRNA, are not detected by RT-MPCR, and that a CBA assay can demonstrate low levels of cytokine, probably because of protein accumulation in supernatants.

In our experiment, most OVCA cell lines proceeding from advanced stage of disease, expressed IL-8 mRNA and protein, often in association with IL-6. Several authors have also demonstrated IL-8 transcripts in ovarian cancer biopsies or cell lines, and noted that its expression correlated with disease progression [14, 53]. IL-8/CXCL8, is a member of the chemokine superfamily and a potent chemotactic factor for leukocytes. Host cells, e.g. neutrophils attracted to the tumor site have been shown to have a tendency to limit tumor growth, but also are capable of producing factors involved in tumor promotion [54]. The role of IL-8 as a growth factor has been established in many types of tumors, but the controversy remains open in ovarian cancer [12]. By contrast, the involvement of IL-8 in tumor angiogenesis has been well demonstrated in OVCA [55].

The mRNAs of the proinflammatory cytokines IL-1β, TNF-α and GM-CSF have been detected in ovarian carcinoma biopsies [14, 56]. However, other cell types such as tumor-associated macrophages (TAM), which could also produce various cytokines are present in adjacent stroma. It is thus difficult to compare our data with other studies analyzing gene expression directly from tumor biopsies [57, 58]. In our experiments, IL-1 β and TNF-α were observed only in one case; and GM-CSF in 3 cases.

We cannot exclude the possibility that the properties of *ex vivo* expanded cell lines differ from those expressed by tumors cells in their *in vivo* physiological environment.

During testing at different passages, we observed that tumor cell lines maintain their characteristic properties in culture. Nevertheless, some modifications of the cytokine profile were noted: for example, expression of IL-6, IL-8 and TNF-α mRNA may disappear with increased passages, as observed in cell line O114.

In conclusion, our results document the presence of tumor promoting cytokines TGF-β1, IL-6 and IL-8 in OVCA cell lines. Additionally, we noted that proinflammatory cytokines IL-1β, TNF-α and GM-CSF, involved in the development of immune responses, are rarely produced. Such a cytokine profile in OVCA would be more likely to be responsible for tumor growth, spread and immunosuppression than for elaboration of a successful immune response against potentially immunogenic tumor cells. In other words, tumor cells could subvert inflammation mechanisms and escape the immune system, using a repertoire of cytokines produced by themselves or infiltrating leukocytes.

Taken together, data from this study may helpful for future development of novel strategies to prevent protumor cytokine production.

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