Constitutive Production of Interleukin 6 by Ovarian Cancer Cell Lines and by Primary Ovarian Tumor Cultures

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ABSTRACT

We examined the production and utilization of interleukin 6 (IL-6), a multifunctional cytokine with diverse biological effects, by both ovarian cancer cell lines and primary ovarian tumor cultures. We have found that epithelial ovarian cancer cell lines (CAOV-3, OVCAR-3, and SKOV-3) constitutively produce varying amounts of IL-6. This molecule is biologically active as determined by the proliferation of an IL-6-dependent hybridoma cell line, MH60.BSF-2, and is detectable by an IL-6 enzyme-linked immunosorbent assay. By cytoplasmic immunoperoxidase staining, >98% of the cells produce at least some IL-6, with variation in the staining intensity between individual cells. The ovarian cancer cell-produced protein has a molecular weight of approximately 24,000, and exhibits some molecular weight heterogeneity, with M, 27,000 and 28,000 minor forms of IL-6. The levels of IL-6 produced by ovarian cancer cells can be modulated by other inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1β, and interferon-γ. Our results suggest that IL-6 is not an autocrine growth factor for these established ovarian tumor cell lines, because the addition of either exogenous IL-6 or antibodies to IL-6 did not affect the cellular proliferation of the cell lines. We also found significant levels (>3 ng/ml) of IL-6 in ascitic fluids of ovarian cancer patients and in the supernatants of primary cultures from freshly excised ovarian tumors. The production of IL-6 by epithelial ovarian cancer cells may prove to be a useful diagnostic tool and aid in investigation of the host immune response to ovarian cancer.

INTRODUCTION

Of all gynecological cancers, ovarian carcinoma has the highest rate of mortality. At the time of diagnosis most patients already present with advanced disease (1). Failure to detect the disease in the early stages results part from the lack of suitable secreted markers, of which the ovarian tumor-specific marker CA 125 has been the most widely pursued (2). Also, epithelial ovarian cancers are insidious and have a high frequency of subclinical metastasis that frequently remains localized within the peritoneal cavity.

Tumor development has often been associated with either oncogene expression and amplification or inappropriate growth factor regulation. With the exception of the recently identified HER-2/neu oncogene (3), which was found to be overexpressed in approximately 30% of ovarian cancers and was usually indicative of poor clinical prognosis, there has been little success in correlating significant oncogene expression and amplification with ovarian tumors (4). The HER-2/neu oncogene encodes an epidermal growth factor-like receptor (5). Therefore, these findings support the concept that ovarian tumor development and progression are more likely related to inappropriate growth factor regulation. One such factor that offers potential relevance to the progression of ovarian carcinomas is IL-6.6 Also known as BSF-2, IFN-β2, hybridoma/plasmacytoma growth factor, and M, 26,000 protein (6–9), IL-6 is a pleiotropic cytokine involved in numerous diverse biological functions (10–12). IL-6 has been shown to be produced by tumor cells such as cardiac myxoma cells (13) and myeloma cells and will in fact augment growth of myeloma cells in an autocrine loop as demonstrated by the growth inhibition of myeloma cells by anti-IL-6 antibodies (14). Recent evidence has shown IL-6 to be produced by both human epidermal cells and epidermoid carcinoma cell lines (15), as well as other epithelium such as bladder carcinoma cells (16) and renal cell carcinoma (17). As the majority of ovarian carcinomas often arise from the coelomic epithelium that covers the ovarian surface (1), we examined the production of IL-6 from several established ovarian carcinoma cell lines (3 of epithelial origin and 1 of germ cell origin) and from freshly excised epithelial ovarian tumors.

In this study we show that 3 of 4 ovarian carcinoma cell lines, as well as primary cultures taken from ovarian tumors, secrete IL-6. By all criteria examined, this IL-6 is indistinguishable from IL-6 produced by other epithelial, lymphoid, or monocytic cell lines. These epithelial ovarian tumor cell lines do not appear to produce IL-6 as an autocrine growth factor. The results of this study will allow further characterization of the role of cytokine production by ovarian tumor cells and provide information on the role of IL-6 as a growth factor for ovarian cancer cells, as well as provide a new potential marker useful in the diagnosis and treatment of ovarian carcinoma.

MATERIALS AND METHODS

Cells. CAOV-3, NIH-OVCAR-3, SKOV-3, and PA-1 are epithelial ovarian cancer cell lines obtained from and maintained according to directions from American Type Culture Collection. Small portions of fresh primary ovarian tumors were minced with sterile scissors in RPMI 1640, the resultant supernatant was seeded into 25-cm² tissue culture flasks (Corning Glass Works, Corning, NY); fed with complete media (RPMI 1640; Whittaker Bioproducts, Inc., Walkersville, MD). 10% FCS (Irvine Scientific, Irvine, CA), 1% l-glutamine, and 1% penicillin/streptomycin (Gibco, BRL, Bethesda, MD); and grown at 37°C with 5% CO₂. The cells were washed in PBS and fed prior to reaching confluence (2–3 weeks), and the supernatant was collected 14 days later. Ascitic fluid from ovarian cancer patients was clarified by low-speed centrifugation for 20 min. An aliquot of the supernatant was stored at 4°C until tested for IL-6 activity as described below.

IL-6 Assays. Supernatants were determined to have IL-6 biological activity by bioassay using the IL-6-dependent murine hybridoma cell line MH60.BSF-2 (kindly provided by Drs. T. Kishimoto and T. Hirano, Osaka, Japan) as described previously (18). Briefly, MH60.BSF-2 cells were maintained in RPMI 1640 supplemented with 10% FCS, 1% antibiotics, 1% glutamine, 2% 2-mercaptoethanol, and 5 units/ml rhIL-6 (provided by Dr. T. Kishimoto). Duplicate supernatants were serially diluted in 96-well flat-bottomed plates (Nunc, Inc., Roskilde, Denmark). A total of 1 x 10⁴ washed MH60.BSF-2 cells

6The abbreviations used are: IL-6, interleukin 6; IFN, interferon; FCS, fetal calf serum; PBS, phosphate-buffered saline; rhIL-6, recombinant human interleukin 6; ELISA, enzyme-linked immunosorbent assay; TNF, tumor necrosis factor; IL, interleukin; SDS, sodium dodecyl sulfate; BSF-2, B-cell stimulatory factor-2.
were added per well and the plates were incubated at 37°C for 2 h. At this
time, the cells were pulsed with 1 μCi of [3H]thymidine (ICN
Biochemicals, Irvine, CA) for 6 h and harvested onto glass membrane
filters, and the radioactivity was counted. The concentration of IL-6
that gave 50% maximal proliferation was assigned the value of 0.03
unit/ml. Relative concentration of IL-6 in the culture supernatants was
also determined using an IL-6-specific sandwich ELISA (18), which
differs from the bioassay by the failure to detect complexed forms of
IL-6 (19). This ELISA used a monoclonal anti-human IL-6 primary
antibody (kindly provided by Dr. F. Takatsuki, Ajinomoto Co. Inc.,
Kawasaki, Japan) coated onto plastic 96-well flat-bottomed microwells
overnight. Supernatants or rhIL-6 (provided by Dr. T. Kishimoto)
standards were added to coated wells, incubated overnight, and then
incubated further in the presence of a rabbit anti-IL-6 polyclonal
immunoglobulin fraction (Genzyme Corp.). The plates were then in-
cubated with an anti-rabbit horseradish peroxidase-conjugated goat serum
(Tago, Inc., Burlingame, CA) prior to development with the o-pheny-
lenediamine substrate (Sigma Chemical Co., St. Louis, MO).

Immunoperoxidase Staining for Cytoplasmic IL-6. Ovarian cancer
cells cultured on slides were washed in PBS, fixed in precooled acetone
for 10 min, and air dried. Prior to staining, cells were washed in PBS
for 10 min and incubated with blocking reagent (mouse serum) for 20
min at room temperature. Primary antibody (murine monoclonal anti-
IL-6; 80 μg/ml) or isotype control (IgG1) was then added, incubated
for 90 min, and washed with PBS for 10 min. After this, the biotinylated
antibody (horse anti-mouse IgG1) was added, incubated for 30 min, and
washed with PBS for 10 min. Following this, the ABC kit (Vector
Laboratories, Burlingame, CA) reagents were added according to the
manufacturer's instructions, incubated for 30 min, and washed with
PBS for 10 min, and 3-amino-9-ethyl-carbazole substrate solution (freshly prepared) was added and incubated for 10 min, with color
development checked by light microscopy. The enzyme reaction was
stopped with tap water wash to remove substrate.

Neutralization of IL-6 Activity. Fifty μl of culture supernatant col-
lected 5 days postpassage were incubated for 2 h at 37°C in the presence
of anti-IL-6 polyclonal antisera (Genzyme) at a final antibody concen-
tration of 0.2, 0.1, or 0.05 μg/μl. The supernatants were further incu-
bated at 4°C until tested in MH60.BSF-2 bioassy as described.

Modulation of IL-6 Secretion by Cytokines. Cells were plated in 96-
well flat-bottomed plates at 1×10⁵ cells/well to which either TNF-
α (Amgen, Thousand Oaks, CA), IL-1β (Amgen), or IFN-γ (Amgen)
was added in various concentrations (1–10,000 units/ml final concen-
tration). The final volume was 200 μl/well. The plates were incubated
for 48 h at which time the supernatants were removed and tested for
IL-6 concentration by ELISA.

IL-6 mRNA Analysis. Isolation and slot-blot analysis of mRNA from
the ovarian cancer cell lines were performed as previously described
(20) using the guanidinium isothiocyanate/cesium chloride ultracent-
trifugation method (21). The blot was probed with the 32P-labeled 440-
base pair Tagl-BanII fragment of BSF-2/IL-6 complementary DNA
(pBSF-2.38, kindly provided by Dr. T. Hirano) (22).

Immunoprecipitation and Gel Electrophoresis. To determine the mo-
lecular weight of the IL-6 produced by the ovarian cancer cell lines, 10 ×
10⁵ cells were plated in the presence of 100 units/ml IL-1β and
incubated for 48 h prior to pulsing the cells with 200 μCi of 35S-Trans
(ICN Biochemicals) in methionine-deficient RPMI 1640 (ICN Bio-
chemicals), supplemented with 5% FCS for 24 h. The supernatant was
incubated overnight at 4°C with anti-IL-6 polyclonal antisera (kindly
provided by Dr. F. Takatsuki, Ajinomoto Co. Inc., Kawasaki, Japan)
at an antibody dilution of 1:100, followed by a 3-h incubation at room
temperature after the addition of 150 μl of a 10% solution of protein
A-Sepharose (Pharmacia, Inc., Piscataway, NJ). The beads were washed
5 times in radioimmunoprecipitation assay buffer (0.05 M Tris-HCl,
pH 8, 0.1% SDS, 1.0% Triton X-100, 2.0 mM phenylmethylsulfonyl
fluoride, 0.15 M NaCl) once in 0.0625 M Tris, pH 6.8, and resuspended
in sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10%
2-mercaptoethanol). Antibody-antigen complexes were released from
protein A-Sepharose by boiling for 5 min, then incubated on ice for 10
min prior to separation on a 12.5% SDS-polyacrylamide gel. The
gel was fixed overnight in 50% ethanol and 7% glacial acetic acid, washed
twice in distilled water, incubated for 20 min in 1 M sodium salicylate,
and dried prior to autoradiography.

Statistics. Statistical analysis was done by using Student's t test.

RESULTS

Epithelial Ovarian Cancer Cell Lines Produce IL-6. IL-6
activity in culture supernatants from several well-characterized
established ovarian tumor cell lines and supernatants from primary
ovarian tumor cells grown in vitro was examined. Culture supernatants from human ovarian cancer cell lines
CAOV-3, NIH-OVCAR-3, and SK-OV-3 were found to sup-
port the growth of the IL-6-dependent murine hybridoma cell line
MH60.BSF-2, indicating the presence of biologically active
IL-6 in these supernatants (Fig. 1). This MH60.BSF-2 cell line,
with a lower sensitivity limit of 0.03 unit/ml, has been shown
to be solely responsive to IL-6; other cytokines, including IL-1
α, IL-1 β, IL-2-5, IFN-β, IFN-γ, and granulocyte-colony stim-
ulatory factor, do not induce cell growth (18). Only PA-1
supernatant failed to produce any detectable IL-6 activity.
Preincubation of these culture supernatants with a polyclonal
anti-IL-6 antibody significantly reduced the proliferation of
the MH60.BSF-2 cells (data not shown). As shown in Table 1 and
Figure 1, these supernatants not only produce a molecule that
has IL-6 biological activity, but also contain a molecule that
from cells plated at 10^4 cells/ml. The production of this activity is clearly not dependent on cell concentration and is linear with time (data not shown). It is interesting to note that the levels of IL-6 produced by each of the cell lines are dramatically different: CAOV-3 produces significantly greater quantities than does SKOV-3, whereas OVCAR-3 produces severalfold less than does SKOV-3 at all times and cell concentrations tested. Increases in the IL-6 levels were noted to coincide with the time the cultures reached confluency, after 72 h for SKOV-3, 5 days for OVCAR-3, and between days 5 and 7 for CAOV-3.

In light of the differences obtained in IL-6 production from plating varying cell concentrations, we sought to determine the relative IL-6 contribution on a per-cell basis using cytoplasmic immunoperoxidase staining. We found that 100% of CAOV-3, OVCAR-3 and SKOV-3 cells are immunoperoxidase-positive using a monoclonal anti-IL-6 antibody (Table 2). There was slight heterogeneity in the intensity of the staining for cytoplasmic IL-6. However, this staining heterogeneity was also seen when the cells were stained for major histocompatibility class I (data not shown). The PA-1 cell line, which failed to secrete IL-6 as determined by bioassay and ELISA of culture supernatants, appeared to be lightly positive for cytoplasmic IL-6. Both ovarian cancer cells isolated from a random selection of solid tumors and ascitic fluids also stained lightly positive for IL-6. Both ovarian cancer cell lines were producing IL-6 without exogenous stimulation, such as endotoxin contamination of the fetal calf serum used to culture the cell lines, we varied the culture conditions and again measured the IL-6 concentration in the supernatants. CAOV-3 produced biologically active IL-6 detectable in all supernatants, including both low serum conditions and Iscoves medium supplemented with albumin and transferrin (Fig. 2), although the level of IL-6 decreased by approximately 75% from 158 units/ml to 39 units/ml. Like CAOV-3, SKOV-3 appears to exhibit a relative or partial dependence upon serum for the production of biologically active IL-6; IL-6 decreased in culture supernatants grown in either 2% FCS by 30% (from 0.57 to 0.41 unit/ml) or by 60% when grown in Iscoves medium supplemented with albumin and transferrin (0.22 unit/ml) as compared with the control. OVCAR-3 produced IL-6 in all media tested, which was at least 90% that of the control (0.49 unit/ml at 5 days). When the cells were also grown in screened FCS-containing medium known not to induce IL-6 from unstimulated peripheral blood mononuclear cells (lipopolysaccharide concentration of this medium was 0.16 ng/ml as determined by the Limulus assay), comparable relative concentrations of IL-6 were detected from confluent cultures of each of the cell lines grown in such FCS-supplemented medium as compared with cells grown in medium containing untested FCS: CAOV-3, 6.31 and 7.54 units/ml; OVCAR-3 0.36, 0.48 unit/ml; and SKOV-3 1.38, 2.18 units/ml, respectively.

As the production of IL-6 can be induced in different cell types by various different agents, we examined the effects on production of IL-6 by the ovarian cancer cells grown in the presence of other inflammatory cytokines, TNF-α, IL-1β, and IFN-γ. IL-6 production by CAOV-3 was found to be enhanced (P < 0.01) in the presence of either TNF-α (all concentrations) or IFN-γ (>100 units/ml), while being unaffected by the presence of IL-1β (Table 3). OVCAR-3 modulated IL-6 production in a vigorous dose-dependent fashion to IL-1β (P < 0.005 at >10 units/ml), with a significant response to TNF-α (P < 0.01) and a negligible response to IFN-γ (Table 2). SKOV-3 showed dose-dependent modulation of IL-6 production by all 3 cytokines tested with significant increases in IL-6 production seen at the highest doses used (P < 0.01). The most dramatic enhancement of IL-6 secretion by SKOV-3 was induced by 100 units/ml of IL-1β where the IL-6 level increased 10-fold over the control level (Table 3). Neither TNF-α, IL-1β, nor IFN-γ was capable of stimulating production of IL-6 from PA-1 (data not shown).

Fibroblasts and monocytes have been shown to produce multiple forms of IL-6 (23). To determine what molecular weight form(s) of IL-6 were being synthesized and secreted by these ovarian cancer cell lines, we immunoprecipitated IL-6 from the culture supernatants of metabolically labeled cells. It appears that the IL-6-producing lines all secrete a major species of IL-6 that shares antigenic reactivity with human IL-6 as detected in an IL-6 ELISA (sensitive to 0.05 ng/ml). The media preparation alone failed to support the growth of the MH60.BSF-2 hybridoma cells (Table 1), indicating that the media itself did not contain any IL-6 activity.

Figure 1 shows that 3 of 4 of these ovarian cancer cell lines constitutively exhibit IL-6 activity. The production of this activity is dependent on cell concentration and is linear with time (data not shown). It appears that the levels of IL-6 are higher than those previously reported (3) and that the expression of IL-6 is not dependent on the concentration of FCS used in the culture medium. The levels of IL-6 are higher than those previously reported (3) and the expression of IL-6 is not dependent on the concentration of FCS used in the culture medium. The levels of IL-6 are higher than those previously reported (3) and the expression of IL-6 is not dependent on the concentration of FCS used in the culture medium.

Table 1: Ovarian cancer cell lines produce IL-6

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IL-6 activity (units/ml)</th>
<th>IL-6 concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAOV-3</td>
<td>8.4 ± 0.9a</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>0.2 ± 0.02</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>PA-1</td>
<td>≤ 0.03</td>
<td>≤ 0.05</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>2.5 ± 0.3</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>Culture medium</td>
<td>≤ 0.03</td>
<td>≤ 0.05</td>
</tr>
</tbody>
</table>

a Results determined by MIH60.BSF-2 bioassay.

Table 2: Ovarian cancer cell lines and primary tumor cells stain positive for cytoplasmic IL-6 by immunoperoxidase staining

<table>
<thead>
<tr>
<th>Established lines</th>
<th>% of positive cells</th>
<th>Staining intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAOV-3</td>
<td>&gt;98</td>
<td>++/+++</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>&gt;98</td>
<td>+++</td>
</tr>
<tr>
<td>PA-1</td>
<td>&gt;75</td>
<td>±</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>&gt;98</td>
<td>++</td>
</tr>
<tr>
<td>Primary tumor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>449s</td>
<td>&gt;75</td>
<td>±</td>
</tr>
<tr>
<td>451</td>
<td>&gt;80</td>
<td>±</td>
</tr>
<tr>
<td>453</td>
<td>&gt;80</td>
<td>±</td>
</tr>
<tr>
<td>455</td>
<td>&gt;80</td>
<td>±</td>
</tr>
</tbody>
</table>

±, faint, cytoplasmic staining; +, majority of cells lightly stained; ++, areas of dark staining; ++++, areas of intense staining.

Fig. 2. Constitutive production of IL-6 by ovarian cancer cell lines. Ovarian cancer cells were grown in the presence of 10% FCS, 5% FCS, 2% FCS-supplemented RPMI, or transferrin-albumin-supplemented Iscoves medium for 5 days. The IL-6 concentration was measured by bioassay. CAOV-3; II, OVCAR-3; OVCAR-3; OVCAR-3; OVCAR-3; OVCAR-3. Points, mean of triplicate samples.
Table 3  Modulation of IL-6 secretion from ovarian cancer cells by TNF-α, IL-1β, and IFN-γ

<table>
<thead>
<tr>
<th>IL-6 concentration (ng/ml)*</th>
<th>CAOV-3 cell line</th>
<th>OVCAR-3 cell line</th>
<th>SKOV-3 cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35.3 ± 0.7</td>
<td>0.1 ± 0.03</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 units/ml</td>
<td>51.4 ± 0.1</td>
<td>0.3 ± 0.09</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>100 units/ml</td>
<td>54.9 ± 3.0</td>
<td>0.9 ± 0.2</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>1,000 units/ml</td>
<td>52.1 ± 1.9</td>
<td>0.8 ± 0.3</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 unit/ml</td>
<td>35.0 ± 0.3</td>
<td>0.04 ± 0.0</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>10 units/ml</td>
<td>37.2 ± 9.3</td>
<td>1.1 ± 0.07</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>100 units/ml</td>
<td>33.4 ± 1.2</td>
<td>10.3 ± 1.3</td>
<td>6.2 ± 1.8</td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 unit/ml</td>
<td>45.6 ± 10.6</td>
<td>0.1 ± 0.02</td>
<td>0.9 ± 0.04</td>
</tr>
<tr>
<td>100 units/ml</td>
<td>50.6 ± 6.7</td>
<td>0.1 ± 0.05</td>
<td>1.4 ± 0.05</td>
</tr>
<tr>
<td>1,000 units/ml</td>
<td>52.8 ± 1.7</td>
<td>0.1 ± 0.03</td>
<td>1.5 ± 0.06</td>
</tr>
</tbody>
</table>

*Mean ± SD of triplicate samples from one representative experiment (n = 4).

6 that has a molecular weight of 24,000 (Fig. 3A). CAOV-3 also secretes isoforms of IL-6 at M, 27,000 and 28,000. This heterogeneity has been seen with other epidermal cells and epidermoid carcinoma cell lines (15). It is possible that OVCAR-3 and SKOV-3 also secrete other forms of IL-6 but in concentrations too low to be detected by immunoprecipitation. As a control, IL-6 was immunoprecipitated from the supernatants both from a human monocytic cell line (THP-1) and from lipopolysaccharide-activated peripheral blood mononuclear cells (Fig. 3B). The major species immunoprecipitated with this antibody is M, 24,000, with minor species of protein detectable at slightly higher molecular weights.

Slot-blot RNA analysis revealed the presence of IL-6-specific mRNA in SKOV-3 and CAOV-3 cell lines but failed to detect OVCAR-3 and PA-1 mRNA specific for IL-6 (Fig. 4). The levels of mRNA do not appear to directly correlate with the relative protein activity detectable in the culture supernatants because CAOV-3, which secretes more IL-6 protein than SKOV-3, has less specific mRNA than SKOV-3. The cause of this variation is unknown but could be the result of either greater mRNA stability or translation efficiency in the CAOV-3 cell line as opposed to the SKOV-3 line. Precedent for detection of a biologically active protein and subsequent failure to detect associated mRNA, as in the case of OVCAR-3-produced IL-6, has been demonstrated. Ramakrishnan et al. (24) have shown detectable macrophage-colony stimulating factor activity in culture supernatants of ovarian cancer cell lines, including OVCAR-3, while failing to detect corresponding specific cytokine mRNA.

IL-6 Production by Freshly Isolated Ovarian Cancer Cells. Since ovarian cancer cell lines represent fully transformed cells that have been selected for their ability to grow in vitro, the unstimulated constitutive production of IL-6 by these cell lines may be a consequence of such selection. To determine the relevance of ovarian cancer cell line-produced IL-6 to an in vivo condition, we examined freshly isolated ovarian tumors for the production of IL-6. In this study, all primary culture supernatants (n = 19) from freshly excised ovarian tumors and all ovarian cancer patient ascitic fluids (n = 24) were found to have detectable IL-6 levels (Table 4). The concentration ranged from 3 to 40 ng/ml and was detectable in both the IL-6 ELISA and MH60.BSF-2 bioassay (data not shown). The high (>3 ng/ml) IL-6 activity in all ascitic fluids (n = 24) tested cannot be directly linked to IL-6 produced by ovarian tumor cells. Ascitic fluid, obtained from one patient with a gynecological condition other than ovarian cancer, was found to have an IL-6 level of <3 ng/ml (1.8 ng/ml). Peritoneal fluid obtained from healthy women undergoing voluntary tubal ligations (n = 7) was found to contain IL-6 levels comparable with that of the non-cancer patient (2.1 ng/ml).

IL-6 is Not an Autocrine Growth Factor for Epithelial Ovarian Cancer Cells. To determine whether IL-6 is an autocrine growth factor for ovarian cancer cells, we examined the cellular proliferative response of the ovarian cancer cells in the presence of either exogenous rhIL-6 over a range of concentrations (0.01
constitutively produce a molecule with the biological and antigenic characteristics of IL-6. SKOV-3 and CAOV-3 are cell lines that constitutively produce moderate to high levels of IL-6 and detectable IL-6 mRNA. OVCAR-3 produces low amounts of IL-6 (<0.5 unit/ml) even at culture confluency and has no detectable IL-6 mRNA. The IL-6 produced by these ovarian cancer cell lines has a molecular weight of approximately 24,000, and exhibits some of the molecular weight heterogeneity often associated with IL-6 produced by epithelial cells (15).

Significant levels (>3 ng/ml) of IL-6 activity were also detected in the supernatants of primary ovarian tumor cultures and in ovarian cancer patient ascitic fluid. Although every effort was made to ensure that there were no contaminating peritoneal monocyte/macrophage cells in the cultures, the possibility of such cells contributing to IL-6 production cannot be excluded. However, by nonspecific esterase staining of a random selection of the primary cultures, we found that there was <10% macrophage contamination in these cultures (data not shown), whereas the cultures stained for cytoplasmic IL-6 by the immunoperoxidase technique expressed >75–80% IL-6 reactivity. The levels of IL-6 in the primary tumor cultures are significant in that tumor cells are exposed to no additional stimulus other than being placed in culture and go through changes in culture medium prior to testing the supernatant, removing any IL-6 secreted by contaminating monocytes upon adherence to the tissue culture flask.

In this study, we emphasize the production of IL-6 from the tumor cells themselves, unlike Erroi et al. (25), who have reported that IL-6 is constitutively produced by tumor-associated macrophages in ovarian carcinomas, without significant contribution from the neoplastic elements. Differences in isolation procedures and cell line selections probably account for the variation between their reported results and ours. However, the original source from which their cell lines were derived is not stated, and as shown here, cell lines from germ cell tissue (PA-1) did not secrete IL-6, whereas those of epithelial origin (CAOV-3, OVCAR-3, and SKOV-3) were seen to produce and secrete IL-6. Our results support those of Tabibzadeh et al. (26), who examined IL-6 immunoreactivity in human tumors by immunohistological staining and found that the majority of human tumors of epithelial or mesenchymal origin stain positive for IL-6. From such immunohistological procedures it was not apparent whether the tumors actually produced IL-6 or simply utilized and stored IL-6 produced by other cells. Our results suggest that the neoplastic elements themselves produce IL-6.

The source of the activity detected in the ascites fluids cannot be ascribed to IL-6 solely produced by the ovarian tumors. Despite the uncertain source of the ascitic fluid IL-6, it is apparent that there are extremely high levels of this cytokine within the peritoneal cavity of patients with ovarian cancer. Because the accumulation of ascitic fluid is an abnormal condition per se, the appropriate control for examining ascitic fluid IL-6 levels would be ascitic fluid from women with gynecological conditions other than ovarian carcinoma. Interestingly, the one such fluid tested contained only low levels (<3 ng/ml) of detectable IL-6, comparable with that found in peritoneal fluid from healthy women undergoing voluntary tubal ligations. The high concentrations of IL-6 within a confined and localized region reported here are not unique because high levels (>1000 units) of IL-6 have been reported to be present in amniotic fluid (27), in the synovial fluid of rheumatoid arthritis patients (28), and in localized regions of acute bacterial infections (29). These
high levels of IL-6 do not appear to directly contribute to the immunosuppressive effects of ascitic fluid (30) because neutralization of the IL-6 in the ascitic fluid was not capable of restoring proliferation to phytohemagglutinin-stimulated peripheral blood mononuclear cells (data not shown). It remains to be determined what immune function IL-6 plays in such localized regions, and whether in high, sustained concentrations IL-6 actively suppresses or enhances other immune functions or induces a state of nonresponsiveness through indirect means.

Although IL-6 has been implicated as an autocrine growth factor in other tumors (14), the results presented here showing that the levels of IL-6 continue to rise even after the cultures have reached confluence, the lack of proliferative inhibition in the presence of antibodies, and the lack of proliferative response to exogenous IL-6 suggest that these ovarian carcinoma cell lines are not producing this cytokine as an autocrine growth factor. Recent evidence from Klein et al. (31) suggested that IL-6 regulated myeloma cell growth by a paracrine (as opposed to an autocrine) mechanism (14). The possible role of ovarian cell-produced IL-6 as an exocrine or paracrine growth factor for other cells of the peritoneal cavity is intriguing, especially if considered in light of the high levels of the cytokine that are produced and retained within the peritoneal cavity and the lack of metastasis outside of the cavity. It has been proposed by Tamm et al. (32) that IL-6 may aid in tumor metastasis and invasiveness, since exogenous IL-6 was shown to increase motility and decrease adhering-type junctions in the breast carcinoma cell lines T47D and Zr-75-1. It is not known whether autonomously produced IL-6 would affect the motility and adherence of the ovarian cancer cell lines, but this could provide an explanation for the high degree of local seeding that occurs throughout the peritoneal cavity in patients with ovarian carcinoma.

All the primary ovarian tumors and ovarian cell lines that produced IL-6 activity were classified as adenocarcinoma tumors. The single ovarian carcinoma cell line that failed to produce any IL-6, PA-1, was originally derived from a teratocarcinoma (33). This germ cell tumor is derived from undifferentiated cells exhibiting characteristics of embryonal carcinoma cells, as opposed to the adenocarcinomas, which characterized contain more differentiated cells. The relevance of the state of differentiation to IL-6 production cannot be drawn from this study. However, it would be interesting to examine this further to determine if such a correlation exists, since this may provide a useful diagnostic tool. The ovarian carcinoma marker CA 125 has been demonstrated to be associated with approximately 85% of serous ovarian tumors and 70% of undifferentiated tumors, and only rarely with mucinous tumors (2). Likewise there has been some association between other markers such as CEA and CA 19-9 and other types of ovarian carcinoma (mucinous and endometroid). However, none of these particular tumor markers shows specificity for cancer or correlates with their respective secreted marker serum values. Studies to examine circulating serum IL-6 levels in ovarian cancer patients are currently underway. If a correlation is found between circulating IL-6 levels and ovarian tumor status, IL-6 may prove useful as another marker for ovarian cancer.

Evidence from Boltz et al. (4), regarding the lack of any significant amplification of the c-ras-Ki oncogene in ovarian tumors, and the results of this study suggest that ovarian tumor progression may be related to abnormal growth factor regulation as opposed to inappropriate oncogene expression and amplification. Evidence relating abnormal growth factor regulation to ovarian tumor progression has been demonstrated by the amplification and overexpression (3) of an epidermal growth factor-like receptor (5) encoded by the HER-2/neu oncogene. In further support of this idea, we have shown here that 2 of the IL-6-producing cell lines (CAOV-3 and SKOV-3) constitutively express IL-6 mRNA. Although the overexpression of IL-6 has been implicated in the pathogenesis of numerous diseases, including some lymphoid cancers (34), it remains to be determined what role such deregulated expression of IL-6 has in the development and progression of ovarian cancer.

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REFERENCES


Constitutive Production of Interleukin 6 by Ovarian Cancer Cell Lines and by Primary Ovarian Tumor Cultures
