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IL-8 Reduced Tumorigenicity of Human Ovarian Cancer In Vivo Due to Neutrophil Infiltration

Li-Fen Lee,*† Ronald P. Hellendall, † Ying Wang, † J. Stephen Haskill,*‡ Naofumi Mukaida, ‡§ and Jenny P.-Y. Ting*†

Paclitaxel is a frontline therapy for ovarian cancer. Our laboratory has shown that paclitaxel induces IL-8, a member of the C-X-C family of chemokines, in subsets of human ovarian cancer cells. However, the critical issue concerns the biological significance of this chemokine in human ovarian cancer. To study the influence of IL-8 on tumor growth, human ovarian cancer cell lines were transfected with an expression vector for human IL-8 and tested for their ability to form tumors in nude mice. IL-8 expression by the transfected cells did not alter their growth properties in vitro. In contrast, tumor growth in vivo was significantly attenuated in animals receiving IL-8-expressing cells when compared with mice injected with control cells. As additional evidence that IL-8 is a crucial factor in tumor growth, it was noted that ovarian cell lines in which constitutive IL-8 expression is elevated did not form tumors. Injection of neutralizing Ab to IL-8 reverted the phenotype and caused tumor growth in vivo. Examination of tissue from the inoculation site revealed a dramatically elevated cellularity, containing neutrophils and macrophages, in mice receiving IL-8-expressing tumor cells. These results suggest that IL-8 production by human ovarian tumor cells can play a role in reducing the rate of tumor growth; this effect may be mediated by the increased targeting of neutrophil and other mononuclear cells to the tumor injection site. These studies indicate a role for IL-8 in ovarian cancer control and suggest that chemotherapy-induced IL-8 may have a positive role in controlling tumor growth.

The current report examines the role of elevated IL-8 expression in subsets of human ovarian cancers (24). Paclitaxel, an inhibitor of microtubule dissociation, represents a new class of antineoplastic agents and has shown efficacy in several malignant tumors. The compound has achieved specific prominence in the treatment of ovarian cancer (25, 26) because the majority of ovarian cancer patients have advanced disease (stage III or IV) at the time of diagnosis and the prognosis of these patients is poor in spite of aggressive intervention. Therefore, chemotherapy has attained a fundamental role in the therapy of ovarian cancer (27). Paclitaxel and docetaxel (Taxotere, Aventis Pharmaceuticals, Collegeville, PA) have shown significant success as single agents in treating recurrent ovarian cancer and provide viable options for patients with advanced cancers, including those that are resistant to Vinca alkaloids or anthracycline. Currently, paclitaxel, plus a platinum analogue, is considered a first-line therapy for advanced ovarian cancer and is used in relapsed or cisplatin-refractory ovarian cancer. Although the primary molecular target of paclitaxel is the microtubule, other chemotherapies, which display similar but not identical activity, are less effective in the clinic. These data suggest that paclitaxel may have additional effects on tumor cells. The earlier finding that the drug can induce IL-8 expression (24) suggests that this cytokine is involved in one of the alternative pathways that mediate the action of paclitaxel on ovarian tumor cells.

The current report examines the role of elevated IL-8 expression in a mouse model of human ovarian cell tumorigenesis. Our results indicate a striking correlation between IL-8 production, neutrophil-monocyte infiltration, and the regression of human ovarian cancer. The relevance of this finding to the therapy of human ovarian cancer is discussed.

Materials and Methods

Cells and plasmids

The human ovarian cancer cell lines OVCA 420, 429, 194, and 494 have been described previously (24, 28). The cells were maintained as
monolayer cultures in DMEM/F-12 medium supplemented with 5% FBS (complete media). The expression plasmids pIL-8BCMGSNeo and pBCMGSNeo have been described previously (16). Briefly, IL-8 cDNA was digested with the restriction enzyme Smal and BamHI from pUC 19 and inserted into the Bluescript KS + vector. The Bluescript KS +/IL-8 plasmid was digested with XhoI and NotI, and the resultant fragment was cloned into the XhoI-NotI site of BCMGS neo vector.

DNA transfection

Human ovarian cancer cells OVCA 194 and 494 cells were seeded in 100-mm plates (Falcon Plymouth, U.K.) and grown in complete media until they reached 75–80% of confluence. Subconfluent cultures were transfected by electroporation with pIL-8BCMGSNeo or BCMGS neo plasmids. After 48 h, G418 (800 μg/ml) was added to the cells; medium was changed every 3 days. Ninety-nine percent of cells in the control plate died whereas transfected cells produced colonies, which survived 3 wk of G418 treatment. G418-resistant clones were randomly selected, isolated, and expanded individually.

Northern blot

To assess the level of IL-8 expression, total RNA was isolated from stably transfected cells using the guanidinium isothiocyanate/CsCl method (29). From 3 to 5 μg of purified RNA were loaded in each lane of a denaturing agarose gel (30). Northern blotting was performed using a 32P-labeled IL-8 probe on a blot of the separated RNA as described (24).

IL-8 ELISA

To determine the release of IL-8 by transfected cells, conditioned medium was collected and IL-8 was measured as previously described (24). Briefly, a goat anti-human IL-8 Ab (R&D Systems, Minneapolis, MN) was used as the coating Ab, rabbit polyclonal anti-human IL-8 (Endogen, Cambridge, MA) as the primary Ab, and alkaline phosphatase-conjugated goat anti-rabbit Ab (Organon Teknika, Durham, NC) as the secondary reagent (31). The ELISA can accurately detect nanograms per milliliter of cytokine in the complete medium.

Animal studies

Female BALB/c- nu/nu mice, 4–6 wk old, were purchased from Taconic (Germantown, NY). They were maintained under pathogen-free conditions in the facilities of the Division of Laboratory Animal Medicine at the University of North Carolina (Chapel Hill, NC). Tumor cells were suspended in PBS at a concentration of 5 × 10^6/ml. For most experiments presented in the text, the total number of tumor cells injected per animal was 10^6. Injections (200 μl) were given s.c. in the right lower back using a 1-ml syringe. Tumor volumes were measured in cubic millimeters with a vernier caliper and calculated by the formula tumor size = a × b^2/2. The symbol a is the larger and b is the smaller of the two dimensions (32). All animal experiments were in accordance with guidelines provided by the UNC Institutional Animal Care and Use Committee.

Ab injection

Neutralization of endogenous IL-8 in vivo was examined by i.p. administration of 50 μg mouse anti-human IL-8 (R&D Systems) or mouse IgG1 isotype control Ab. The Abs were injected i.p. at day 0 and on every fifth day thereafter.

Histological analysis

Tissue from the site of tumor injection was fixed by immersion in 4% paraformaldehyde/70 mM phosphate buffer and subsequently embedded with paraffin. Five micron sections were stained with hematoxylin and eosin.

Immunohistochemical staining

Inoculation site tissue destined for immunohistochemistry was embedded in OCT compound (Miles, Elkhart, IN) and snap frozen in isopentane chilled to −100°C by liquid nitrogen. Sections (5 μm) were prepared and stained for NK cells (biotin-conjugated mouse anti-mly-49C, PharMingen, San Diego, CA), neutrophils (biotin-conjugated rat anti-mly-6G (Gr-1), PharMingen), macrophage (biotin-conjugated rat anti-Mac-1, PharMingen), or B cells (biotin-conjugated rat anti-B220/CD45R, PharMingen). Biotin-conjugated isotype control Abs were applied for each corresponding Ab. Slides were fixed in 100% acetone at −20°C for 5 min and then air dried. Nonspecific binding sites were blocked by treatment with 1% serum for 30 min followed by exposure to the Ab or isotype control. Sections were subsequently treated for 30 min with fluorescein-conjugated avidin

![FIGURE 1.](http://www.jimmunol.org/DownloadedFrom/10.4049/jimmunol.88.1.2770-fig1) Human ovarian cancer cell lines OVCA 194, OVCA 494, OVCA 429, and OVCA 420 (1 × 10^7) were individually injected s.c. into nude mice. Groups of five mice were inoculated, and tumor growth was monitored as described in Materials and Methods. Data presented as mean ± SEM.

Results

Production of IL-8 in human ovarian cancer cells

Four human ovarian cancer cell lines 420, 429, 194, and 194 were tested for tumorigenicity in nude mice. The IL-8 nonproducers OVCA 194 and 494 (24) formed tumors in vivo when injected s.c. into nude mice. In contrast, tumor formation by OVCA 429 cells was very slow, whereas animals injected with the IL-8-producing OVCA 420 cells were devoid of tumors (Fig. 1). No tumors were observed in these latter animals even 3 mo after injection.

IL-8 Ab reverses tumor growth of an IL-8-producing human ovarian cell line

The dichotomy in the growth pattern of IL-8 producers and non-producers is of interest and suggests that IL-8 may suppress the growth of human ovarian cancers. OVCA 420 cells have a basal
The amount of hIL-8 in culture supernatants was measured by ELISA and determined as the quantity of IL-8 secreted by $5 \times 10^6$ cells/24 h.

Table I. IL-8 secretion after gene transfer into human ovarian carcinoma cell lines

<table>
<thead>
<tr>
<th>Tumor Cells</th>
<th>Doubling Time (h)</th>
<th>Secretion (ng/ml)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>194 hIL-8</td>
<td>ND</td>
<td>23.9 ± 0.7</td>
</tr>
<tr>
<td>194 neo</td>
<td>ND</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>194 hIL-8 clone 6</td>
<td>24.7 ± 4.3</td>
<td>100.3 ± 10.2</td>
</tr>
<tr>
<td>194 neo clone 6</td>
<td>23.2 ± 1.9</td>
<td>4.1 ± 2.5</td>
</tr>
<tr>
<td>494 hIL-8</td>
<td>ND</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>494 neo</td>
<td>ND</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>494 hIL-8 clone 2</td>
<td>18.1 ± 0.5</td>
<td>27.1 ± 0.6</td>
</tr>
<tr>
<td>494 neo clone 8</td>
<td>20.8 ± 0.2</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ Each clone was seeded at $10^5$ cells/well in 12-well plates, and after 2 days the cells were harvested by trypsin treatment and counted. Doubling time was calculated as follows: Growth rate = $\ln$ (number of final cells/initial cells)/2 and doubling time = 0.693/growth rate.

$^b$ The amount of hIL-8 in culture supernatants was measured by ELISA and determined as the quantity of IL-8 secreted by $5 \times 10^6$ cells/24 h.

FIGURE 3. IL-8 gene expression in OVCA 194 and OVCA 494 transfectants. Control OVCA 494 and OVCA 194 express no or little IL-8, respectively (lanes 1 and 3). Bulk culture of OVCA 494 and OVCA 194 cells transfected with hIL-8-containing vector express substantial levels of IL-8 transcripts (lanes 2 and 4). Isolated clones of these transfectants produces an even higher quantity of IL-8 (lanes 5 and 6). An ethidium bromide-stained gel shows rRNA, shown as a control for equal loading. Total RNA (5 µg/lane) was analyzed by Northern analysis for IL-8 expression.

FIGURE 4. Comparison of the growth of control and hIL-8-transfected OVCA 194 cells. Groups of five mice were injected with $1 \times 10^7$ tumor cells, and tumor size was measured as discussed in Materials and Methods.
of 194 hIL-8 and 494 hIL-8 displayed a marked elevated cellular-
ity (Fig. 6, C, D, G, and H). In contrast, few infiltrating cells were
observed at the 194 neo and 494 neo injection sites (Fig. 6, A, B,
E, and F). This suggests that IL-8 production in human ovarian
tumors mediate cellular infiltration to the tumor injection site.

Immunohistochemical staining shows a predominance of
neutrophils and some monocytes

To identify the cells found in the infiltrate, immunocytochemistry
was performed on frozen tissue from the s.c. tumor injection site.
Application of Abs to identify neutrophils (Gr-1) or macrophages
(Mac-1) demonstrated a marked increase of both phenotypes at the
injection site of both IL-8-expressing clonal lines (Figs. 7 and 8).
We also detected the presence of B cells in these tumors, although
the signal was weak (Fig. 8). NK cells were not found. These
results suggest that infiltrating granulocytes, particularly neutro-
phils, are attracted by IL-8 and are likely to play a role in initiating
immune responses to human ovarian tumor cells. This response
appears to be correlated with a significant reduction in the rate of
tumor growth.

Discussion

The role of IL-8 in human ovarian tumor growth has not been
previously studied. To better understand the involvement of IL-8
in tumorigenesis, we have directly tested the effect of this chemok-
line in human ovarian cancer cell growth in nude mice. Our data
strongly suggest that IL-8 can retard the growth of human ovarian
tumors (1). The constitutive expression of IL-8 in human ovarian
tumors was associated with poor growth in nude mice (2). Neu-
ralization of IL-8 increased tumor growth (3). IL-8 transfection
into human ovarian tumor cells retarded tumor growth in nude
mice. This growth attenuation was correlated with neutrophil and
monocyte infiltration. Combined with our previous work indicat-
ing IL-8 induction by paclitaxel in selected ovarian tumors, these
data strongly suggest a model in which chemokine induction is of
benefit to the ovarian tumor-bearing host.

IL-8 is a member of the C-X-C chemokine family and has mul-
tiple biological functions (22). In vitro, IL-8 can inhibit tumor cell
proliferation (33) but does not cause tumoricidal activity. Based on
other reports, IL-8 is a chemotactic factor for T cells, neutrophils,
and basophils (34–36). In our model, the local production of IL-8
and the subsequent neutrophilic infiltration result in markedly
decreased tumor growth. If neutrophils are responsible for the inhi-
bition of tumor growth in nude mice, there are direct and indirect
mechanisms whereby IL-8-activated neutrophils can exhibit their

Table II. Tumorigenicity after IL-8 gene transfer

<table>
<thead>
<tr>
<th>Tumor Cells</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>494 hIL-8 clone 2</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>494 neo clone 8</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>194 hIL-8 clone 10</td>
<td>3/3</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>194 neo clone 6</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

*Mice were injected s.c. with 1 $\times$ 10^6 tumor cells.

**Tumor-bearing mice/total of mice refer to day 35 postinjection.

FIGURE 6. Cellular infiltration at the tumor inoculation site of IL-8-
producing OVCA 194 and OVCA 494 cells. A–D, Injection site of 194
hIL-8 and its corresponding control. E–H, Injection site of OVCA 494
hIL-8 and its corresponding control. Tumor cells were injected s.c. into
nude mice, and the tumor injection sites were analyzed 3 days after chal-
lenge with tumor cells and stained with hematoxylin and eosin. A, C, E, and
G, $\times$50; B, D, F, and H, $\times$400. The area shown in H corresponds to
the area in G where apparent infiltrates are detected. Such areas of mononu-
clear infiltrates are not detected in the control panels, E and F.
effect. Direct mechanisms may involve IL-8-activated neutrophils inducing shape change, granular release, and generation of oxygen radicals and proteases, which may lead to tumor cell death (37). Alternatively, an indirect mechanism may involve the production of mediators by neutrophils in response to chemokines, e.g., TNF, IL-1, and IFNs (38).

Although IL-8 was originally shown to attract and activate neutrophils, this chemokine has multiple biological functions. Some of these functions appear to involve pro-oncogenic activity: IL-8 can induce the migration of some tumor cells (20); it can enhance angiogenesis (39, 40); and it enhances metastasis of melanoma (19). Several papers have also demonstrated that the inhibition of IL-8 attenuates angiogenesis in bronchogenic carcinoma (18) and the expression level of IL-8 correlates with the metastatic potential of human melanoma cells in nude mice (17). Moreover, inhibition of IL-8 reduces tumorigenicity of human non-small cell lung cancer in SCID mice (21).

In contrast, we have observed an antitumorigenic effect of IL-8 in human ovarian cancer cells. This is consistent with a recent report by Hirose et al. (32) showing that the introduction of IL-8 into Chines hamster ovary cells, a hamster ovarian tumor cell line, reduces tumorigenicity in nude mice. Our use of human ovarian tumor cells may provide the impetus to further investigate the clinical relevance of IL-8. We have performed an in vitro invasion assay to determine whether human ovarian cancer cells treated with IL-8 exhibit increased invasion. We have also examined whether IL-8-transfected ovarian cell lines exhibit increased metastasis. Both findings were negative (data not shown). A pattern in melanoma and lung cancer cell lines may be explained by differences in the metastatic potential of different tumor cell types. There may be a balance between neutrophils/macrophages chemotaxis and angiogenesis/metastasis. The overall effect of IL-8 appears to require additional factors necessary for metastasis on human ovarian cancer cells (17, 32).

**FIGURE 7.** Immunohistochemical staining of Gr-1 and Ly-49C in tumor tissue from the s.c. injection site. A, 194 hIL-8 tumor stained with the isotype control of anti-Gr-1 (Rat IgG2b); B, 194 neo tumor with anti-Gr-1 Ab; C, 194 hIL-8 tumor with anti-Gr-1 Ab; D, 194 hIL-8 tumor with isotype control of anti-Ly-49C (mouse IgG2b); E, 194 neo tumor with anti-Ly-49C Ab; F, 194 hIL-8 tumor with anti-Ly-49C Ab.

**FIGURE 8.** Immunohistochemical staining of Mac-1 and B220 in tumor tissue from the s.c. injection site. A, 194 hIL-8 tumor stained with an isotype control of Mac-1 (Rat IgG2b); B, 194 neo tumor stained with anti-Mac-1 Ab; C, 194 hIL-8 tumor stained with anti-Mac-1 Ab; D, 194 hIL-8 tumor stained with an isotype control of B220 (Rat IgG2a); E, 194 neo tumor stained with anti-B220 Ab; F, 194 hIL-8 tumor stained with anti-B220 Ab.
Paclitaxel is highly effective as a clinical therapy for ovarian cancer, whereas its use against melanoma has not shown promise. The differential effect of paclitaxel-induced chemokine on the growth of different tumors may be an underlying factor for these distinct clinical responses and is worthy of further investigations. Interestingly, we have found two squamous lung cancer cell lines which produce IL-8 in response to paclitaxel (T. Collins, L.-F. Lee, and J. Ting, personal observation), and we are currently analyzing their outcomes under conditions similar to those of this study. The comparison is relevant because pulmonary and ovarian tissues are epithelial tumors which represent primary tumors that are clinically responsive to paclitaxel to a degree. Previously, we noted that ~50% of ovarian carcinoma lines synthesized IL-8 after paclitaxel treatment (24). This observation has been extended to primary freshly explanted ovarian cancer cells (24). The analysis of ovarian cancer cell lines that represent each of the two phenotype shows that the nonresponsive phenotype exemplified by OVCA 194 and OVCA 494 can be artificially engineered to express IL-8 gene resulting in reduced tumor growth.

The histological examination clearly shows more Gr-1+ and Mac-1+ cells at the site of 194 and 494 injection when hIL-8 isexpressed. In most reports, the inhibition of tumor growth is mediated through an infiltration of T lymphocytes and or macrophages into the tumor site (41). Previously, Tepper et al. (6, 42) devised a very similar methodology to study IL-4 and found that IL-4 production effectively suppressed a wide range of tumor cells in vivo. The antitumor effect is mediated by an inflammatory infiltrate composed of predominantly eosinophils and macrophages. Consistent with their findings, in our model, we found that the inhibition of tumor growth in immunocompetent mice was associated with macrophages and neutrophils. The possible mechanisms by which neutrophils kill tumors were discussed above. In tumor immunity, macrophages are thought to be important in tumor lysis and killing. Several reports showed that activated monocytes stimulated by M-CSF kill tumor cells both in vivo and in vitro (43, 44). Activation of macrophages has been suggested to have antitumor effects. In addition to chemotaxis of macrophages and neutrophils, IL-8 has been demonstrated in controlling the trafficking of T cells (35, 36). Similar experiments remain to be tested in immunocompetent mice.

In conclusion, artificial expression of IL-8 in human ovarian cancer cells retarded tumor growth. This was associated with an immune cells infiltrate composed of neutrophils and to a lesser degree, macrophages. Our data provide evidence that IL-8 mediates the beneficial effects of paclitaxel toward ovarian cancer. It would be useful to study the cytokine production in response to paclitaxel treatment in the xenograft mouse model and to determine cellularity. Experiments are currently under way to evaluate this postulate.

References


