Synergistic Therapy of Human Ovarian Carcinoma Implanted Orthotopically in Nude Mice by Optimal Biological Dose of Pegylated Interferon α Combined with Paclitaxel

Sean Tedjarati, Cheryl H. Baker, Sachin Apte, Suyun Huang, Judith K. Wolf, Jerald J. Killion, and Isaiah J. Fidler


ABSTRACT

The purpose of this study was to optimize the antitumor and antiangiogenic activities of pegylated IFN-α (PEG-IFN-α) alone or in combination with paclitaxel against SKOV3ip1 human ovarian cancer cells growing orthotopically in female nude mice. Seven days after the i.p. implantation of tumor cells, groups of mice (n = 10) were injected s.c. once per week (for 4 weeks) with different doses of PEG-IFN-α (3,500, 7,000, 35,000, and 350,000 units). PEG-IFN-α at 7,000 units significantly decreased tumor incidence and volume. At doses exceeding 7,000 units, PEG-IFN-α was less efficacious. In another set of studies conducted 7 days after the i.p. implantation of SKOV3ip1 cells, groups of mice (n = 10) received (once per week for 4 weeks) either s.c. administrations of PEG-IFN-α (7,000 units), i.p. injections of paclitaxel (100 μg wk), or a combination of PEG-IFN-α and paclitaxel. The mice were killed 7 days after the last treatment, and tumor burden was assessed. Administration of PEG-IFN-α at the optimal biological dose (7,000 units) in combination with paclitaxel significantly decreased angiogenesis and progressive growth of human ovarian carcinoma cells in a synergistic fashion. The combination therapy produced the most significant inhibition in expression of the proangiogenic molecules basic fibroblast growth factor and matrix metalloproteinase-9. Decreased microvessel density, decreased proliferating cell nuclear antigen staining, and increased endothelial cell apoptosis also correlated with therapeutic success. Collectively, the data suggest that combining the optimal biological dose of PEG-IFN-α with paclitaxel may provide a novel and effective approach to the treatment of human ovarian carcinoma.

INTRODUCTION

Epithelial ovarian carcinoma is the leading cause of death from gynecologic cancer in the United States and the fourth most frequent cause of cancer death in women (1). Despite extensive efforts aimed at improving methods of early detection and diagnosis, the majority of patients with ovarian cancer are diagnosed with disease that has spread beyond the ovary. Modern surgical cytoreduction combined with platinum-based chemotherapy has improved the 5-year survival rate from 36% in the early 1970s to 50% by 1994 (1). Overall survival, however, has been relatively unchanged from 1979 to 1995 (2). Despite initial response rates of 80% (3), the majority of women with advanced ovarian cancer will ultimately relapse and develop drug-resistant disease (4, 5). Therefore, new agents and innovative approaches to therapy are an important subject for research.

Among the candidates are the IFNs, a family of natural glycoproteins first discovered in the 1950s due to their antiviral activity (6). Subsequent studies concluded that the IFNs are multifunctional and can modulate the activities of regulatory cytokines involved in the control of cell function and replication (7–9). IFN-α has been shown to directly inhibit the proliferation of tumor cells of various histological origins (7–11). More recently, IFN-α has been shown to down-regulate the expression of the proangiogenic molecules bFGF (12–15), IL-8 (16–18), and MMP-2 and -9 (19–22) and to activate host effector cells (11, 23).

Several clinical trials have investigated the use of natural and recombinant IFN-α as a single agent or combined with standard chemotherapy. Early studies of systemic natural IFN-α therapy in advanced disease showed poor patient tolerance and an 18% partial response rate (24). Therefore, additional studies focused on regional, or i.p., administration. Two trials investigated the use of recombinant IFN-α alone in the setting of residual disease (25, 26). Response rates as high as 45% have been observed, but only in patients with documented residual disease less than 5 mm. Four recent Phase II and III trials studied the use of IFN-α in combination with platinum-based...
chemothry (27–30). These studies failed to prove any benefit of combining cytotoxic chemotherapy with IFN-α. Again, remissions were observed only in patients with small-volume residual disease.

Despite these modest results, it should be noted that in these trials, IFN-α was typically administered at weekly or longer intervals. Based on the pharmacokinetic properties of IFN-α, the efficacy may be compromised when administered at weekly intervals. The reported half-life of IFN-α ranges from 4 to 8 h, with peak serum concentrations occurring at 3–8 h after i.v. or s.c. administration. Twenty-four h after i.v. or i.p. administration, little or no IFN-α is present in the serum (31, 32). Previous reports from our laboratory demonstrated that down-regulation of angiogenesis-related genes (33) and therapy of solid tumors are dependent on optimization of biological dose and schedule of IFN-α (34). Therefore, we reasoned that injection of IFN-α in a sustained release composition at an optimal biological dose may overcome these kinetic constraints and demonstrate an optimal antiangiogenic effect.

Biomolecules conjugated to polyethylene glycol have been shown to be superior to their corresponding unmodified parent molecules (35–38). PEG-IFN-α is a potent, long-lasting form of IFN-α monopegylated with a $M_1$ 40,000 branched polyethylene glycol designed to enhance pharmacokinetic characteristics and reduce immunogenicity. It is administered on a weekly basis in doses equivalent to those used for unconjugated IFN-α. Clinical trials on patients with hepatitis C confirm the therapeutic superiority of PEG-IFN-α compared with unmodified IFN-α (39–42). A Phase I study has demonstrated the potential efficacy of PEG-IFN-α in renal cell carcinoma (43). Whether administration of PEG-IFN-α can produce sustained antiangiogenic effects has remained unknown.

The purpose of the present study was to determine the efficacy of human PEG-IFN-α-2b in a reliable, preclinical, in vivo model of human ovarian carcinoma growing in the peritoneal cavity of female nude mice. We show that weekly s.c. injections of PEG-IFN-α-2b at the optimal biological dose of 7000 units, alone or in combination with paclitaxel, produce significant therapeutic effects by directly down-regulating the expression of bFGF, MMP-9, VEGF, and IL-8; increasing apoptosis of tumor-associated endothelial cells; and inhibiting tumor cell proliferation.

**MATERIALS AND METHODS**

**Ovarian Cancer Cell Lines and Culture Conditions.** The highly tumorigenic SKOV3ip1 human ovarian cancer cells (44, 45) were grown as monolayer cultures in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, nonessential amino acids (Life Technologies, Inc., Grand Island, NY), and penicillin-streptomycin (Flow Laboratories, Rockville, MD). Adherent monolayers were maintained on plastic and incubated at 37°C in a mixture of 5% CO$_2$ and 95% air. The tumor cells were free of *Mycoplasma*, reovirus type 3, pneumonia virus of mice, mouse adenovirus, murine hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

**Reagents.** PEG-IFN-α, a monopegylated IFN-α-2b provided by Schering-Plough (Kenilworth, NJ), was dissolved in PBS (37). For IHC, antibodies were purchased from the following manufacturers: (a) rabbit anti-bFGF, Sigma Chemical Co. (St. Louis, MO); (b) rabbit anti-VEGF, Santa Cruz Biotechnology (Santa Cruz, CA); (c) polyclonal rabbit antihuman IL-8, Biosource International (Camarillo, CA); (d) rabbit polyclonal anti-MMP-9 antibody, Calbiochem (La Jolla, CA); (e) rat antirat mouse CD31/PECAM-1 and peroxidase-conjugated rat antimouse IgGl, PharMingen (San Diego, CA); (f) mouse anti-PCNA clone PC 10, DAKO A/S (Copenhagen, Denmark); (g) peroxidase-conjugated goat antirabbit IgG and peroxidase-conjugated goat antirat IgG, Serotec, Harlan Bioproducts for Science, Inc. (Indianapolis, IN); and (h) Texas Red-conjugated goat antirat IgG (Jackson Research Laboratories, West Grove, CA). Other reagents were Hoechst dye 3342 (M, 615,9; Hoechst, Warrington, PA), stable 3,3’-diaminobenzidine (Research Genetics, Huntsville, AL), and Gills hematoxylin (Sigma Chemical Co.) Prolong solution was purchased from Molecular Probes (Eugene, OR), and pepsin was purchased from Biomedia (Foster City, CA). TUNEL was performed using a commercial apoptosis detection kit (Promega, Madison, WI) with modifications.

**Northern Blot Analysis: SOCS1.** Tumor tissues from control and PEG-IFN-α-treated mice were homogenized in 15 ml of lysis buffer (2% SDS, 0.2 M NaCl, 0.02 M Tris, and 1 mM EDTA) using a power homogenizer (Polytron) to extract mRNA for analysis (33, 46). The cDNA probes used in this analysis were a 1.3-kb PstI cDNA fragment corresponding to rat GAPDH (47) and a 0.6-kb XbaI and NotI cDNA fragment corresponding to murine SOCS1 (48). Each cDNA probe was radiolabeled with the random primer technique using α-32P-labeled deoxyribonucleotide triphosphate (49). The level of expression of SOCS1 was quantitated by densitometry readings of autoradiograms using the IMAGE QUANT software program (Molecular Dynamics). Each measurement was expressed as the ratio of the average area under the curve of SOCS1-specific mRNA transcripts to 1.3-kb GAPDH mRNA transcripts.

**Animals and Orthotopic Implantation of Tumor Cells.** Female athymic nude mice (NCr-nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used according to institutional guidelines when they were 8–10 weeks of age.

To produce tumors, SKOV3ip1 cells (45) were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in HBSS. Single-cell suspensions with >95% viability were used for the in vivo injections. Tumor cells (1 × 106 cells/0.2 ml HBSS) were injected i.p. into female nude mice. The mice were killed at 5
or saline control. All mice were killed on day 35. The tumors were harvested and prepared for IHC.

with weekly s.c. injections of PEG-IFN-α/H9251 with once per week s.c. injections of PEG-IFN-α/H9251. Seven days after i.p. implantation of tumor cells, five mice were randomized into five groups (n = 10) to receive one s.c. injection of saline (control group), or 3,500, 7,000, 35,000, or 350,000 units of PEG-IFN-α.

In the next set of studies, the mice were randomized into four treatment groups (n = 10) to receive once per week an s.c. injection of saline and an i.p. injection of HBSS (control); an i.p. injection of 100 μg of paclitaxel; an s.c. injection of 7000 units of PEG-IFN-α; or an s.c. injection of 7000 units of PEG-IFN-α and an i.p. injection of 100 μg of paclitaxel. The mice were treated for 4 weeks.

Necropsy Procedures and Histological Studies. Mice were euthanized on day 35 of the study and weighed. Primary tumors in the peritoneum were excised and weighed. Malignant ascites was aspirated and measured. For IHC and H&E staining procedures, some lesions were fixed in formalin and embedded in paraffin. Other tumor lesions were embedded in OCT compound (Miles, Inc.), frozen rapidly in liquid nitrogen, and stored at −70°C.

IHC for bFGF, VEGF, IL-8, MMP-9, PCNA, and CD31/PECAM-1. Expression of bFGF, VEGF, IL-8, MMP-9, and PCNA was determined in paraffin-embedded tissues. Sections (8-μm thick) were mounted on positively charged Plus slides (Fisher Scientific Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylene, followed by treatment with a graded series of alcohol [100%, 95%, and 80% ethanol/double-distilled H2O (v/v)], and rehydrated in PBS (pH 7.5). Sections analyzed for PCNA were microwaved for 5 min for antigen retrieval (50). All other paraffin-embedded tissues were treated with pepsin (Biomedex) for 15 min at 37°C and washed with PBS. Frozen tissues used for identification of CD31/PECAM-1 were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific), and air-dried for 30 min. Frozen sections were fixed in cold acetone (5 min), acetone/chloroform [(v/v) 5 min], and acetone (5 min) and washed with PBS. Immunohistochemical procedures were performed as described previously (51). A positive reaction was visualized by incubating the slides with stable 3,3'-diaminoben-

 weeks. The size and weight of the solid peritoneal lesions and the volume of malignant ascites were recorded. Histopathology confirmed the nature of the disease. For IHC and histology staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin. Another part of the tumor was embedded in OCT compound (Miles, Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C.

Therapy of Established Human Ovarian Carcinomas in the Peritoneal Cavity of Athymic Female Nude Mice. Seven days after i.p. implantation of tumor cells, five mice were killed to ascertain the presence and size of tumor lesions. Mice were randomized into five groups (n = 10) to receive one s.c. injection of saline (control group), or 3,500, 7,000, 35,000, or 350,000 units of PEG-IFN-α.

In the next set of studies, the mice were randomized into four treatment groups (n = 10) to receive once per week an s.c. injection of saline and an i.p. injection of HBSS (control); an i.p. injection of 100 μg of paclitaxel; an s.c. injection of 7000 units of PEG-IFN-α; or an s.c. injection of 7000 units of PEG-IFN-α and an i.p. injection of 100 μg of paclitaxel. The mice were treated for 4 weeks.

Necropsy Procedures and Histological Studies. Mice were euthanized on day 35 of the study and weighed. Primary tumors in the peritoneum were excised and weighed. Malignant ascites was aspirated and measured. For IHC and H&E staining procedures, some lesions were fixed in formalin and embedded in paraffin. Other tumor lesions were embedded in OCT compound (Miles, Inc.), frozen rapidly in liquid nitrogen, and stored at −70°C.

### Table 1 Therapy of human ovarian carcinoma growing in the peritoneum of nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>N</th>
<th>Incidence</th>
<th>Tumor weight (g)</th>
<th>Ascites (ml)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>10</td>
<td>10/10</td>
<td>2.0 ± 0.6e</td>
<td>1.5 ± 0.05</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>PEG-IFN-α (350,000 units)</td>
<td>10</td>
<td>10/10</td>
<td>1.0 ± 0.3d</td>
<td>0.0e</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>PEG-IFN-α (350,000 units)</td>
<td>10</td>
<td>10/10</td>
<td>0.8 ± 0.3d</td>
<td>0.0e</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>PEG-IFN-α (7,000 units)</td>
<td>10</td>
<td>10/10</td>
<td>0.4 ± 0.2d</td>
<td>0.0e</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>PEG-IFN-α (3,500 units)</td>
<td>10</td>
<td>10/10</td>
<td>0.9 ± 0.3d</td>
<td>0.0e</td>
<td>22 ± 1</td>
</tr>
</tbody>
</table>

* SKOV3ip1 human ovarian cancer cells (1 × 10⁶) were injected into the peritoneum of nude mice. Seven days later, groups of mice were treated with once per week s.c. injections of PEG-IFN-α or saline (control). All mice were killed on day 35.

* Number of positive mice/number of mice injected.

a P < 0.001 as compared with control.

b P < 0.001 as compared with control.

c P < 0.05 as compared with the other treated mice.

d P < 0.01.

e P < 0.001.

### Table 2 Immunohistochemical analysis of human ovarian carcinoma in the peritoneum of control and treated nude mice

<table>
<thead>
<tr>
<th>Treatment groupa</th>
<th>Endothelial cells</th>
<th>Tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD31%</td>
<td>% TUNEL%</td>
</tr>
<tr>
<td>Control</td>
<td>95 ± 13b</td>
<td>0e</td>
</tr>
<tr>
<td>PEG-IFN-α (350,000 units)</td>
<td>70 ± 14d</td>
<td>0</td>
</tr>
<tr>
<td>PEG-IFN-α (350,000 units)</td>
<td>35 ± 7f</td>
<td>2d</td>
</tr>
<tr>
<td>PEG-IFN-α (7,000 units)</td>
<td>16 ± 5e</td>
<td>18f</td>
</tr>
<tr>
<td>PEG-IFN-α (3,500 units)</td>
<td>47 ± 13d</td>
<td>6f</td>
</tr>
</tbody>
</table>

* SKOV3ip1 human ovarian cancer cells (1 × 10⁶) were injected into the peritoneum of nude mice. Seven days later, groups of mice were treated with weekly s.c. injections of PEG-IFN-α or saline control. All mice were killed on day 35. The tumors were harvested and prepared for IHC.

a Mean ± SD.

b P < 0.001 as compared with the other treated mice.

c P < 0.001 as compared with control.

d P < 0.05 as compared with the other treated mice.

e P < 0.001.

f P < 0.01.

g P < 0.001.
Fig. 1 Immunohistochemical analysis of SKOV3ip1 tumors growing in the peritoneal cavity of control and PEG-IFN-α-treated female nude mice. Seven days after i.p. injection of SKOV3ip1 cells (1 × 10⁶ cells/mouse), groups of mice (n = 10) received a weekly s.c. injection of PEG-IFN-α (3,500, 7,000, 35,000, or 350,000 units). Treatment continued for 4 weeks. The mice were killed on day 35 and necropsied. Peritoneal tumors were resected, weighed, and prepared for IHC. Note a significant reduction in staining intensity of bFGF and MMP-9 in tumors from mice treated with 7,000 units of PEG-IFN-α (also see Table 2).
PEG-IFN-α

C 7K 35K 350K

PEG-IFN-α

SOCS-1

1.0 1.0 1.3 2.8

GAPDH

Fig. 2 Northern blot analysis of mRNA for SOCS1 in SKOV3ip1 tumors growing in the peritoneal cavity of control and PEG-IFN-α-treated female nude mice. Samples consisted of polyadenylated mRNA (5 μg). The probes used were a 0.6-kb cDNA fragment corresponding to murine SOCS1 (48) and a 1.3-kb cDNA fragment corresponding to rat GAPDH (47). The level of expression of SOCS1 was quantitated by densitometry readings of autoradiograms and is expressed as the ratio of the average area under the curve of SOCS1-specific mRNA transcripts to the 1.3-kb GAPDH mRNA transcripts. Note the increase in SOCS1 expression in tumors of mice treated with 35,000 or 350,000 units as compared with control mice or mice treated with 7,000 units of PEG-IFN-α.

Results of a 10–20 min. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 min, and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining.

To minimize bias and subjectivity, the data of this assay were recorded by two separate individuals who were both blinded to the slides. Specifically, a constant and uniform pattern of cytoplasmic staining recognition was developed by first calibrating the apparatus to the varying staining intensities. Areas representing true positive staining were then selected. Absorbance (A) was used to calculate the average intensity of staining. Ten different sections of each slide were scored using 10 different areas in one field. The mean A was then recorded.

Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and TUNEL (Apoptotic Cells). Frozen tissues were sectioned (8–10 μm), mounted on positively charged slides, air-dried for 30 min, and fixed in cold acetone for 5 min, 1:1 acetone:chloroform (v/v) for 5 min, and acetone for 5 min. Samples were washed three times with PBS, incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature, and then incubated with the appropriate dilution (1:400) of rat monoclonal antimouse CD31 antibody (human cross-reactive) for 18 h at 4°C. After the samples were rinsed four times for 3 min each with PBS, they were incubated with the appropriate dilution (1:200) of secondary goat antirat antibody conjugated to Texas Red for 1 h at room temperature in the dark. Samples were washed twice with PBS containing 0.1% Brij and washed with PBS for 5 min. TUNEL was performed using a commercially available apoptosis detection kit with the following modifications: samples were fixed with 4% paraformaldehyde (methanol-free) for 10 min at room temperature, washed twice with PBS for 5 min, and then incubated with 0.2% Triton X-100 for 15 min at room temperature. After they were washed twice for 5 min each with PBS, the samples were incubated with equilibration buffer (from the kit) for 10 min at room temperature. The equilibration buffer was drained, and the reaction buffer containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyltransferase enzyme was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h in the dark. The reaction was terminated by immersing the samples in 2× SSC for 15 min. Samples were washed three times for 5 min to remove unincorporated fluorescent-dUTP. To label nuclei of endothelial cells, the samples were incubated with 300 μg/ml Hoechst stain for 10 min at room temperature. Fluorescence bleaching was minimized by treating slides with an enhancing reagent (Prolong solution). Immunofluorescence microscopy was performed using a ×40 objective on a Zeiss Axioplan epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with an HBO 100 mercury lamp and narrow bandpass filters to individually select for green, red, and blue fluorescence (Chroma Technology Corp., Brattleboro, VT). Images were captured using a cooled charge-coupled device Hamamatsu C5810 camera (Hamamatsu Corp., Bridgewater, NJ) and Optimas Image Analysis software (Media Cybernetics, Silver Spring, MD) installed on a Compaq computer with a Pentium chip, a frame grabber, an optical disk storage system, and a Sony Mavigraph UP-D 7000 Digital color printer (Tokyo, Japan). Images were processed using Adobe PhotoShop software (Adobe Systems, Inc., San Jose, CA).

Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus of apoptotic cells. Quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to total number of endothelial cells in 5–10 random 0.011-mm² fields at ×400 magnification.

Quantification of MVD, PCNA, and Absorbance. For the quantification of MVD, 10 random 0.159-mm² fields at ×100 magnification were captured for each tumor, and microvessels were quantified as described previously (52, 53). For the quantification of the immunohistochemical reaction intensity, the absorbance of 100 bFGF-, VEGF-, IL-8-, and MMP-9-positive cells in 10 random 0.039-mm² fields at ×200 magnification of tumor tissues was measured using Optimas image analysis software (54–56). The samples were not counterstained, so the absorbance was due solely to the product of the immunohistochemical reaction. bFGF, VEGF, IL-8, and MMP-9 cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and expressed as the ratio of tumor cell expression to normal ovarian expression multiplied by 100 (52, 53). For the quantification of PCNA expression, the number of positive cells was quantified in 10 random 0.159-mm² fields at ×100 magnification.

Statistical Analysis. The volume of ovarian tumors; expression of bFGF, VEGF, IL-8, and MMP-9; quantification of CD31 and PCNA; and the percentage of apoptotic endothelial cells were compared by using the unpaired Student’s t test.

RESULTS

In Vivo Therapy of SKOV3ip1 Tumors by Weekly Administration of PEG-IFN-α: Effect of Dose. In the first set of experiments, we evaluated the therapeutic efficacy of weekly administration of different doses of PEG-IFN-α against human ovarian cancer cells growing in the peritoneum of nude mice.
We also determined whether the PEG-IFN-α treatment was associated with down-regulation of angiogenesis-regulating genes. Therapy began on day 7 after tumor cell implantation. Groups of mice (n = 10) received weekly s.c. injections of saline (control) or PEG-IFN-α at 3,500 (equivalent to 500 units/day), 7,000 (equivalent to 1,000 units/day), 35,000 (equivalent to 5,000 units/day), or 350,000 units (equivalent to 50,000 units/day) for 4 weeks. The data of one of two representative experiments are shown in Table 1. All doses of PEG-IFN-α completely inhibited formation of ascites. Whereas the volume of solid tumors was significantly decreased in all treatment groups, the greatest inhibition was found in mice receiving once per week s.c. injections of 7000 units of PEG-IFN-α (P < 0.0001). The inhibition in tumor size directly correlated with inhibition of tumor cell proliferation and tumor vascularization revealed by immunohistochemical analysis using antibodies against PCNA or CD31, respectively (Table 2). The number of PCNA+ cells was reduced from 97 ± 12 in tumors of control mice to 23 ± 6 in tumors growing in mice receiving weekly s.c. injections of 7000 units of PEG-IFN-α (P < 0.0001). The MVD was reduced from 95 ± 13 in control tumors to 16 ± 5 in tumors of mice receiving once per week s.c. injections of 7000 units of PEG-IFN-α (P < 0.0001). The intensity of bFGF and MMP-9 immunostaining in tumor tissues was also significantly reduced in mice treated with weekly s.c. injections of 7000 units of PEG-IFN-α (P < 0.0001) (Fig. 1 and Table 2). The intensity of IL-8 and VEGF immunostaining in the ovarian cancer cells was also reduced (P < 0.001 and P < 0.01, respectively, Table 2). As was the case in our previous studies of IFN-α (33, 34), exceeding the optimal biological dose of IFN did not increase inhibition.

IFN-α and -β inhibit the transcription and protein production of bFGF, MMP-2, and MMP-9 (12–15, 19–22). The expression of SOCS1 inversely correlates with the activity of IFN-α and -β (48, 57). Tumor tissue from control and PEG-IFN-α-treated mice was analyzed for SOCS1-specific mRNA transcripts by Northern blot analysis (Fig. 2). The SOCS1 expression level in tumors from control mice was similar to that of mice treated with 7,000 units of PEG-IFN-α. In contrast, the level of SOCS1 was higher in tumors of mice treated with 35,000 or 350,000 units of PEG-IFN-α (Fig. 2). Because expression of SOCS1 inversely correlates with the biological activity of cytokines, such as IFNs, the data suggest an explanation for the failure of high-dose IFN-α to inhibit transcription of bFGF and MMP-9.

To determine whether tumor-associated endothelial cells underwent apoptosis, we used the CD31/TUNEL fluorescent double-labeling technique (58). The data shown in Fig. 1 and Table 2 demonstrate a significant increase in the percentage of

### Table 3 Therapy of human ovarian carcinoma growing in the peritoneum of nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>N</th>
<th>Incidence</th>
<th>Tumor weight (g)</th>
<th>Ascites (ml)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline control</td>
<td>10</td>
<td>10/10b</td>
<td>2.2 ± 0.6c</td>
<td>1.8 ± 0.6</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>10</td>
<td>10/10d</td>
<td>0.2 ± 0.1d</td>
<td>0.0e</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>PEG-IFN-α (7000 units)</td>
<td>10</td>
<td>10/10</td>
<td>0.5 ± 0.2d</td>
<td>0.0e</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>PEG-IFN-α + paclitaxel</td>
<td>10</td>
<td>3/10</td>
<td>0.03 ± 0.04f</td>
<td>0.0e</td>
<td>24 ± 2</td>
</tr>
</tbody>
</table>

* SKOV3ip1 human ovarian cancer cells (1 × 10⁶) were injected into the peritoneum of nude mice. Seven days later, groups of mice were treated with once per week i.p. injections of paclitaxel (100 µg), once per week s.c. injections of PEG-IFN-α (7000 units), a combination of paclitaxel and PEG-IFN-α, or saline (control). All mice were killed on day 35.

* Number of positive mice/number of mice injected.

* Mean ± SD.

* P < 0.001.

* P < 0.0001.

### Table 4 Immunohistochemical analysis of human ovarian carcinoma in the peritoneum of control and treated nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>CD31+</th>
<th>% TUNEL</th>
<th>PCNA</th>
<th>bFGF</th>
<th>VEGF</th>
<th>IL-8</th>
<th>MMP-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36 ± 9b</td>
<td>0f</td>
<td>13 ± 12h</td>
<td>144 ± 8d</td>
<td>138 ± 11f</td>
<td>164 ± 14f</td>
<td></td>
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<tr>
<td>Paclitaxel</td>
<td>26 ± 10</td>
<td>0e</td>
<td>63 ± 19c</td>
<td>156 ± 8e</td>
<td>143 ± 12d</td>
<td>123 ± 7e</td>
<td>167 ± 12d</td>
</tr>
<tr>
<td>PEG-IFN-α (7000 units)</td>
<td>17 ± 6e</td>
<td>12c</td>
<td>45 ± 12e</td>
<td>128 ± 15c</td>
<td>116 ± 11e</td>
<td>118 ± 16c</td>
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<td>PEG-IFN-α + paclitaxel</td>
<td>13 ± 5e</td>
<td>29c</td>
<td>33 ± 6e</td>
<td>125 ± 14e</td>
<td>118 ± 16c</td>
<td>116 ± 13c</td>
<td>127 ± 19c</td>
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* SKOV3ip1 human ovarian cancer cells (1 × 10⁶) were injected into the peritoneum of nude mice. Seven days later, groups of mice were treated with once per week i.p. injections of paclitaxel (100 µg), once per week s.c. injections of PEG-IFN-α (7000 units), a combination of paclitaxel and PEG-IFN-α, or saline (control). All mice were killed on day 35.

* Mean ± SD, number of positive cells/field determined from measurement of 10 random 0.159-mm² fields at ×100 magnification.

* Percentage of CD31/TUNEL+ cells in 10 random 0.011-mm² fields at ×400 magnification. Fluorescence double labeling was performed on frozen tissue sections.

* Mean ± SD absorbance, determined as described in “Materials and Methods.”

* P < 0.0001.

* P < 0.01.

* P < 0.001.
Fig. 3  Vascular density and endothelial cell apoptosis. Peritoneal SKOV3ip1 tumors from mice treated once per week with vehicle (control), paclitaxel, PEG-IFN-α (7,000 units/dose), or PEG-IFN-α plus paclitaxel were harvested on day 35. Sections were immunostained for expression of CD31/PECAM-1 (MVD) or anti-CD31 antibodies (Texas Red) and TUNEL (FITC green). Multiple sections were examined, and a representative sample (×400) is shown. Treatment with PEG-IFN-α or paclitaxel decreased mean vessel density. Treatment with both PEG-IFN-α and paclitaxel produced a significant decrease of CD31⁺ endothelial cells, which directly correlated with the induction of apoptosis. Red, CD31⁺ endothelial cells; green, TUNEL⁺ cells; yellow, TUNEL⁺/CD31⁺ cells.
apoptotic endothelial cells in peritoneal ovarian cancer lesions of mice treated once per week with 7,000 units of PEG-IFN-α as compared with tumors from control mice or mice treated with 3,500, 35,000, or 350,000 units of PEG-IFN-α (P < 0.0001).

**PEG-IFN-α and Chemotherapy.** In the next set of experiments, we determined whether administration of the optimal biological dose of PEG-IFN-α combined with paclitaxel would produce additive or synergistic therapeutic effects. Groups of mice (n = 10) were implanted i.p. with SKOV3ip1 cells. One week later, treatment with saline (control), paclitaxel (100 μg, once per week), PEG-IFN-α (7000 units once per week), or paclitaxel and PEG-IFN-α commenced. The mice were treated once per week for 4 weeks, at which time all mice were necropsied (day 35 of the study). Peritoneal tumors were resected, weighed, and prepared for IHC. The data of one representative experiment (of two) are summarized in Table 3. All mice (10 of 10) in the control group, in the group treated with paclitaxel alone, or in the group treated with PEG-IFN-α alone had peritoneal disease (albeit to different degrees). In sharp contrast, the incidence of disease in mice receiving both PEG-IFN-α and paclitaxel was reduced to 3 of 10 (P < 0.0001), suggesting synergistic effects. The weight of peritoneal tumors was also significantly reduced from a mean weight of 2.2 ± 0.6 g in saline-treated mice to 0.5 ± 0.2 g in mice treated with PEG-IFN-α alone to 0.03 ± 0.04 g in mice treated with 7000 units of PEG-IFN-α plus 100 μg of paclitaxel. As observed in the previous experiments, all treatment groups had a significant reduction in malignant ascites as compared with control mice (P < 0.0001).

The inhibition of tumor growth correlated inversely with the intensity of bFGF and MMP-9 immunostaining (Table 4). Specifically, bFGF staining intensity was reduced from a mean of 153 ± 8 in control tumors to 125 ± 14 in tumors of mice treated with PEG-IFN-α plus paclitaxel (P < 0.0001). MMP-9 staining intensity was reduced from a mean of 164 ± 14 in control tumors to 127 ± 19 in tumors of mice treated with PEG-IFN-α plus paclitaxel (P < 0.0001). Interestingly, the staining intensity of VEGF and IL-8 were also reduced in tumors of mice receiving combination therapy.

The immunohistochemical data summarized in Table 4 demonstrate that the inhibition in tumor growth observed in tumors of mice treated with once per week injections of 7000 units of PEG-IFN-α plus paclitaxel was accompanied by a significant reduction in tumor cell proliferation and tumor vascularization. The number of proliferating tumor cells (measured by staining with antibodies against PCNA) was reduced from 95 ± 10 in tumors of control mice to 33 ± 6 in tumors of mice treated with the combination therapy (P < 0.0001). MVD (CD31 staining) directly correlated with expression of the proangiogenic molecule. MVD was reduced from 36 ± 9 in tumors of control mice to 13 ± 5 in tumors of mice treated with PEG-IFN-α and paclitaxel (P < 0.0001). In tumors from mice treated with PEG-IFN-α plus paclitaxel, we observed a significant increase in the percentage of apoptotic endothelial cells as compared with tumors of control mice (29 ± 8 and 0 ± 0; P < 0.0001; Fig. 3). Specifically, the percentage of apoptotic endothelial cells (yellow reactions) in tumors of mice treated with PEG-IFN-α plus paclitaxel was more than double that observed in tumors from mice treated with PEG-IFN-α alone.

**DISCUSSION**

We report that once per week injections of 7000 units of PEG-IFN-α (equivalent to 1000 units/day) into female nude mice bearing i.p. growing human ovarian carcinoma cells inhibited angiogenesis and tumor growth. Altering the dose of PEG-IFN-α significantly influenced therapeutic outcome, i.e., PEG-IFN-α administered at higher or lower doses was less effective. As previously reported from our laboratory (33, 34), to produce maximal therapeutic effects, IFN must be administered at an optimal biological dose. Furthermore, combination therapy of once per week s.c. injections of PEG-IFN-α at 7000 units plus once per week i.p. injections of 100 μg of paclitaxel produced a synergistic therapeutic response resulting in a highly significant reduction in MVD, tumor incidence, tumor growth, and production of ascites.

The progressive growth and metastasis of malignant neoplasms depend on adequate neovascularization. The extent of angiogenesis is determined by the balance between the proangiogenic and antiangiogenic molecules released by tumor cells and surrounding host cells (59–62). Among the major proangiogenic molecules are bFGF, VEGF, IL-8, and MMP-2/9 (33, 61–63). IFN has been shown to inhibit transcription and protein production of many of these proangiogenic molecules (14, 17, 20, 33, 34, 60). This regulation is associated with induction of a feedback mechanism involving a family of proteins called SOCS that negatively regulate cytokine signaling (57, 64, 65).

The induction of SOCS expression is directly correlated with the concentration of a given cytokine (57, 64, 65). Indeed, we found that the mRNA expression of SOCS1 in human ovarian cancer cells directly correlated with the dose of PEG-IFN-α. At doses exceeding 7000 units PEG-IFN-α/week, the induction of higher levels of SOCS1 may have been responsible for the failure of IFN-α to down-regulate its target genes, such as bFGF, MMP-9, and IL-8 (14, 17, 20, 33, 34). When the level of SOCS1 did not exceed that found in control tumors (not exposed to PEG-IFN-α), i.e., administration of 7000 units of PEG-IFN-α alone or in combination with paclitaxel, we found a significant reduction in the proangiogenic molecules bFGF and MMP-9, a reduction associated with a significant decrease in tumor cell proliferation and MVD.

In summary, we show that once per week injections of PEG-IFN-α at an optimal biological dose combined with once per week paclitaxel (4) produce significant inhibition of angiogenesis and growth of human ovarian carcinoma growing orthotopically in nude mice. The results recommend the examination of this combined therapy in autochthonous human ovarian carcinoma.

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**REFERENCES**


Synergistic Therapy of Human Ovarian Carcinoma Implanted Orthotopically in Nude Mice by Optimal Biological Dose of Pegylated Interferon α Combined with Paclitaxel

Sean Tedjarati, Cheryl H. Baker, Sachin Apte, et al.


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