Administration of Optimal Biological Dose and Schedule of Interferon-α Combined with Gemcitabine Induces Apoptosis in Tumor-associated Endothelial Cells and Reduces Growth of Human Pancreatic Carcinoma Implanted Orthotopically in Nude Mice

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ABSTRACT

Purpose: We determined whether chronic administration of IFN-α at optimal biological dose inhibits angiogenesis of human pancreatic carcinoma growing in the pancreas of nude mice.

Experimental Design: Cells of the human pancreatic cancer cell line L3.6pl were implanted into the pancreas of nude mice. Seven days later, groups of mice received s.c. injection with IFN-α alone (50,000 units biweekly or 10,000 units daily), i.p. injection with gemcitabine alone (125 mg/kg biweekly), or injection with both daily IFN-α and biweekly gemcitabine for 35 days. In a survival study, the mice were treated until they became moribund.

Results: Biweekly treatments with 50,000 units of IFN-α alone were ineffective. In contrast, daily injections of IFN-α (10,000 units/day) alone, biweekly injections of gemcitabine alone, or the combination of IFN-α and gemcitabine reduced tumor volume by 53%, 70%, and 87%, respectively. Immunohistochemical analysis revealed that treatment with gemcitabine alone. These treatments also decreased the staining of proliferating cell nuclear antigen within the tumor and induced apoptosis in tumor-associated mouse endothelial cells (staining with CD31/terminal deoxynucleotidyl transferase-mediated nick end labeling), leading to a decrease in microvessel density.

Conclusions: These data show that administration of IFN-α at optimal biological dose and schedule in combination with gemcitabine induced apoptosis in tumor-associated endothelial cells and decreased growth of human pancreatic cancer cells in the pancreas, leading to a significant increase in survival.

INTRODUCTION

Extensive local invasion and early lymphatic and hematogenous metastasis characterize cancer of the exocrine pancreas. At the time of diagnosis, >80% of patients present with either locally advanced or metastatic disease (1, 2). The inability to detect pancreatic cancer at an early stage, its aggressiveness, and the lack of effective systemic therapy are all responsible for the high mortality of this disease. Only 3–4% of all patients with adenocarcinoma of the pancreas survive 5 years after diagnosis (3). Not even the recent introduction of the deoxycytidine analogue, gemcitabine, has extended median survival beyond 6 months for patients with advanced pancreatic cancer (4, 5).

IFNs are a family of natural glycoproteins first discovered in the 1950s because of their antiviral activity (6). IFN-α, for example, has been shown to directly inhibit the proliferation of tumor cells of different histological origins (7–9). More recently, IFN-α has been shown to down-regulate the expression of the proangiogenic molecules bFGF, MMP-2 and MMP-9 (10–12), and IL-8 (13, 14) and to activate host effector cells (9).

Unfortunately, several clinical trials of recombinant IFN-α combined with standard chemotherapy (15–17) in advanced pancreatic cancer were associated with significant toxicity, fail-

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3 The abbreviations used are: bFGF, basic fibroblast growth factor; IHC, immunohistochemical; IL, interleukin; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MVD, microvessel density; PCNA, proliferating cell nuclear antigen; VEGF, vascular endothelial growth factor; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PECAM-1, platelet/endothelial cell adhesion molecule 1.
ing to prove any benefit to the patients. More encouraging, a recent Phase II trial using IFN-α/cisplatin/5-fluorouracil-based adjuvant chemoradiation after pancreaticoduodenectomy reported the best-published 2-year survival for patients with resected pancreatic cancer (18). It should be noted, however, that in these trials, IFN-α was typically administered once weekly or 3×/week, and none of these trials used IFN-α in combination with gemcitabine (the most active cytotoxic agent against advanced pancreatic cancer; Ref. 5). These schedules may well be suboptimal, given that the reported half-life of IFN-α ranges from 4–8 h, with peak serum concentrations occurring at 3–8 h after i.v. or s.c. administration. Twenty-four h after i.v. or s.c. administration, little or no IFN-α is present in the serum (19).

Previous reports from our laboratory demonstrated that down-regulation of angiogenesis-related genes (20) and therapy of solid tumors are dependent on optimization of biological dose and schedule of IFN-α (21).

Using a murine orthotopic model of pancreatic cancer (22), we set out to determine the most effective of two IFN-α schedules plus a combined regimen of IFN-α and gemcitabine. We show that daily s.c. injections of IFN-α at the dose of 10,000 units in combination with i.p. injections of gemcitabine significantly inhibited the growth of human pancreatic carcinoma by directly down-regulating the expression of bFGF, MMP-9, IL-8, and VEGF/vascular permeability factor; by inducing apoptosis in tumor-associated endothelial cells; and by inhibiting tumor cell proliferation.

MATERIALS AND METHODS

Pancreatic Cancer Cell Lines and Culture Conditions. The metastatic human pancreatic cancer cell line L3.6pl (22) was maintained in DMEM supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD). Adherent monolayer cultures were maintained at 37°C in a mixture of 5% CO₂ and 95% air. The cultures were free of endotoxins as determined by the Limulus amebocyte lyase assay (sensitivity limit of 0.125 ng/ml) purchased from Associates of Cape Cod (Falmouth, MA).

Reagents. Recombinant human IFN-α-2a (IFN-α; Ro-feron; Hoffman-LaRoche, Nutley, NJ) was kept at 4°C and diluted in HBSS as necessary at the time of use. Gemcitabine (GENZAR; Eli Lilly Co., Indianapolis, IN) was kept at room temperature and made fresh in 0.9% NaCl on the day of use. Antibodies purchased were as follows: polyclonal rabbit anti-VEGF/vascular permeability factor (Santa Cruz Biotechnology, Santa Cruz, CA); peroxidase-conjugated goat antirabbit IgG (H&L; Jackson Research Laboratories); monoclonal mouse anti-PCNA clone PC-10 (DAKO A/S); peroxidase-conjugated rat antimouse IgG2a heavy chain (Srotec; Harlan Bioproducts for Science, Inc., Indianapolis, IN); polyclonal rabbit antihuman IL-8 (Bio-source International, Camarillo, CA); rabbit antihuman bFGF (Sigma); peroxidase-conjugated rat antimouse IgG1 (PharMingen); mouse antihuman MMP-9 (Oncogene, Boston, MA); and Texas Red-conjugated goat antirat IgG (Jackson Research Laboratories).

Hoechst Dye 3342 Mr 615.9 was purchased from Hoechst (Warrington, PA), stable 3,3’-diaminobenzidine was purchased from Research Genetics (Huntsville, AL), 3-amino-9-ethylcarbazole was purchased from Biogases Laboratories (San Ramon, CA), and Gill’s hematoxylin was purchased from Sigma. Promega, Madison, WI) with modifications.

In Vitro Cytotoxicity Assay. Tumor cells (1×10⁶) were seeded into triplicate wells of flat-bottomed 96-well plates in triplicate and allowed to adhere overnight. The spent medium was then removed, and the cultures were refed with new medium (negative control) or medium containing different concentrations of human IFN-α. After 4 days (control cultures did not reach confluence), the number of metabolically active cells was determined by MTT assay (23).

Animals and Orthotopic Implantation of Tumor Cells. Male athymic nude mice (NCI-nu) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were 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 in accordance with institutional guidelines when they were 8–10 weeks old.

To produce pancreatic tumors, L3.6pl cells 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 HBBS. Only suspensions consisting of single cells with >90% viability were used for the injections. Cells were injected into the pancreas as described previously (22). The mice were killed when moribund. The size and weight of the primary pancreatic tumors, the incidence of regional (celiac and para-aortic) lymph node metastasis, and the incidence of liver metastasis were recorded. Histopathology confirmed the nature of the disease. For histology and immunohistochemistry, 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 Pancreatic Carcinoma Tumors Growing in the Pancreas of Athymic Nude Mice.** Seven days after the implantation of tumor cells into the pancreas, five mice were killed, and the presence of tumor lesions was determined. At this time, the median tumor volume was 18 mm³. Histological examination confirmed the lesions to be actively growing pancreatic cancer. For the first experiment, the mice were randomized into the following five treatment groups: (a) daily s.c. administration of human IFN-α (10,000 units); (b) biweekly s.c. administrations of IFN-α (50,000 units); (c) twice a week i.p. injections of gemcitabine (125 mg/kg); (d) daily s.c. administrations of IFN-α (10,000 units) and twice a week i.p. injections of gemcitabine (125 mg/kg); and (e) biweekly s.c. administrations of IFN-α (50,000 units) and twice a week i.p. injections of gemcitabine (125 mg/kg). In the sixth group, control mice received saline and i.p. HBSS (vehicle). The mice were necropsied on day 35, and the volume of pancreatic tumors and incidence of lymph node and liver metastasis were recorded.

In a second experiment, the most effective dosage of IFN-α as determined in the first experiment was used. Three treatment groups were used as follows: (a) daily s.c. injections of human IFN-α (10,000 units) alone; (b) twice per week i.p. injections of gemcitabine (125 mg/kg) alone; and (c) daily s.c. injections of IFN-α (10,000 units) and twice per week i.p. injections of gemcitabine. The fourth group of control mice were treated s.c. and i.p. with HBSS. Mice were killed and autopsied on day 35, and the results were analyzed as described above. In a third therapy experiment, mice were randomized to the above-described four groups (n = 15), and five mice in each treatment group were killed after 14, 21, and 28 days of treatment. A final experiment determined overall survival of treated mice. Mice received implant of L3.6pl cells in the pancreas and were randomized on day 7 to the four groups (n = 10). The mice were killed and necropsied only when they became moribund. The volume of pancreatic tumors and the incidence of lymph node and liver metastases were recorded.

**Necropsy Procedures.** Mice were killed, and body weight was determined. Primary tumors in the pancreas were excised, measured, and weighed. Visible liver metastases were counted with the aid of a dissecting microscope and processed for H&E staining. All macroscopically enlarged regional (celiac and para-aortal) lymph nodes were harvested, and the presence of metastatic disease was confirmed by histology.

**IHC Determination of PCNA, CD31/PECAM-1, bFGF, MMP-9, IL-8, and VEGF.** Paraffin-embedded tissues were used for IHC identification of PCNA, CD31/PCAM-1, bFGF, MMP-9, IL-8, and VEGF. Sections (4–6-μm thick) were mounted on positively charged Superfrost slides (Fisher Scientific, 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.” All other paraffin-embedded tissues were treated with pepsin (Biomedica) 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. IHC procedures were performed as described previously (24). Positive reactions were visualized by incubating the slides with stable 3,3′-diaminobenzidine for 10–20 min. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 30 s, and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining.

**Double Immunofluorescence 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, acetone/chloroform (1:1) 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

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**Table 1  Therapy of human pancreatic carcinoma growing in the pancreas of nude mice**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Pancreatic tumors</th>
<th>Metastasis</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumorigenicity</td>
<td>Tumor volume (mm³)</td>
<td>Liver</td>
</tr>
<tr>
<td>Saline control</td>
<td>10/10</td>
<td>435</td>
<td>3/10</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>10/10</td>
<td>134^c</td>
<td>3/10</td>
</tr>
<tr>
<td>IFN-α (10,000 units)</td>
<td>10/10</td>
<td>111^f</td>
<td>2/10</td>
</tr>
<tr>
<td>IFN-α (50,000 units)</td>
<td>10/10</td>
<td>550</td>
<td>2/10</td>
</tr>
<tr>
<td>IFN-α (10,000 units) + gemcitabine</td>
<td>10/10</td>
<td>33^d</td>
<td>2/10</td>
</tr>
<tr>
<td>IFNα (50,000 units) + gemcitabine</td>
<td>10/10</td>
<td>100^g</td>
<td>2/10</td>
</tr>
</tbody>
</table>

^a^ L3.6pl human pancreatic cancer cells (1 × 10⁶) were injected into the pancreas of nude mice. Seven days later, groups of mice were treated with biweekly i.p. injections of gemcitabine (125 mg/kg) alone, daily s.c. injections of human IFN-α (10,000 units) alone, or with gemcitabine, biweekly s.c. injections of human IFN-α (50,000 units) alone or with gemcitabine, or saline (control). All mice were killed on day 35.

^b^ Number of positive mice/number of mice injected.

^c^ P < 0.001 versus control.

^d^ P < 0.001 versus control and P < 0.05 versus single-agent therapy.

^e^ P < 0.05 versus control.
Combination of gemcitabine and IFN-α/H9251/H18554, P (control).

Survival was analyzed by the Kaplan-Meier method and compared with the appropriate dilution (1:200) of secondary goat antirat conjugated to Texas Red for 1 h at room temperature. The equilibration buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme was added to the tissue sections and incubated in a humidified chamber at 37°C for 1 h in the dark. Immersing the samples in 2× SSC for 15 min terminated the reaction, and samples were washed 3 times for 5 min to remove unincorporated fluorescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 μg/ml Hoechst stain for 10 min at room temperature. Fluorescent bleaching was minimized by treating slides with an enhancing reagent (Prolong solution). Immunofluorescence microscopy was performed using a ×40 objective (Zeiss Plan-Neofluar) on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured using a chilled cooled charge-coupled device camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, United Kingdom) on a Macintosh computer. Images were further processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus (visualized by Hoechst stain) of apoptotic cells. Quantification of apoptotic endothelial cells was expressed as a percentage of TUNEL− endothelial cells (that made up the total endothelial cell population/field) in 5–10 random 0.011-mm² fields at ×400 magnification. For the quantification of total TUNEL expression, the number of apoptotic events was counted in 10 random 0.159-mm² fields at ×100 magnification (25).

Quantification of MVD and PCNA. For the quantification of MVD, 10 random 0.159-mm² fields at ×100 magnification were captured for each tumor, and microvessels were quantified according to the method described previously (26, 27). 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. Pancreatic tumor volume was compared by the Mann-Whitney U test, and the incidence of metastasis was evaluated by using Fisher’s exact test. Quantification of PCNA, TUNEL, CD31, and the number of apoptotic endothelial cells were calculated using the Mann-Whitney U test and the incidence of metastasis was evaluated by using Fisher’s exact test. Quantification of PCNA, TUNEL, CD31, and the number of apoptotic endothelial cells were calculated using the Mann-Whitney U test and the incidence of metastasis was evaluated by using Fisher’s exact test.

Table 2 Therapy of human pancreatic carcinoma growing in the pancreas of nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Pancreatic tumors</th>
<th>Metastasis</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor volume (mm³)</td>
<td>Liver</td>
<td>Lymph node</td>
</tr>
<tr>
<td>Saline control</td>
<td>10/10²¹</td>
<td>749</td>
<td>4/10²¹</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>10/10²¹</td>
<td>226²²</td>
<td>3/10²¹</td>
</tr>
<tr>
<td>IFN-α (10,000 units)</td>
<td>10/10²¹</td>
<td>352²³</td>
<td>2/10²¹</td>
</tr>
<tr>
<td>IFN-α (10,000 units) + gemcitabine</td>
<td>10/10²¹</td>
<td>100²⁴</td>
<td>0/10²¹</td>
</tr>
</tbody>
</table>

²¹ Number of positive mice/number of mice injected.
²² P < 0.05 versus control.
²³ P < 0.01 versus control.
²⁴ P < 0.001 versus control and P < 0.01 versus single-agent therapy.

Fig. 1 Therapeutic effects of IFN-α and gemcitabine. L3.6pl cells were injected into the pancreas of nude mice. Seven days later, the mice were randomized into four treatment groups (n = 10). Mice were killed when moribund. Survival was analyzed by the Kaplan-Meier method and compared by the log-rank test. Control group, ●; gemcitabine alone, ▲ (P < 0.05 compared with control); IFN-α alone, ○ (P = 0.06); combination of gemcitabine and IFN-α, □ (P < 0.001 compared with control).
thelial cells was compared by unpaired Student’s t test. Survival analysis was computed by the Kaplan-Meier method and compared by the log-rank test.

RESULTS

In Vitro IC₅₀ of IFN-α. L3.6pl cells were incubated for 4 days in medium containing increasing concentrations of IFN-α (0–10,000 units). The cells were relatively resistant to the antiproliferative effects of IFN-α, whose IC₅₀ was 5,200 units/ml.

Inhibition of Pancreatic Cancer Growth and Metastasis. L3.6pl cells were injected into the pancreas of athymic nude mice. Seven days later, the mice were randomized into six groups of 10 mice each. The first group received twice weekly i.p. injections of gemcitabine at 125 mg/kg, the second group received daily human IFN-α at 10,000 units s.c., the third group received twice weekly IFN-α at 50,000 units s.c., the fourth group received twice weekly i.p. gemcitabine and daily s.c. IFN-α (10,000 units), and the fifth group received twice weekly i.p. gemcitabine and twice weekly s.c. IFN-α (50,000 units); the sixth group (control) received HBSS i.p. and s.c. All mice were killed on day 35. All mice had tumors in the pancreas. The data summarized in Table 1 show that daily s.c. injections of IFN-α (10,000 units) or twice weekly i.p. injections of gemcitabine significantly decreased median tumor volume as compared with control mice (P < 0.001). Treatment with gemcitabine and daily IFN-α (10,000 units, s.c.) produced the greatest decrease in median volume of pancreatic tumor (P < 0.001). Administration of biweekly IFN-α (50,000 units, s.c.) was ineffective. The experiment was repeated using daily IFN-α (10,000 units, s.c.), gemcitabine, and daily IFN-α plus gemcitabine. Visible liver metastases (enumerated with the aid of a dissecting microscope)
were present in 40% of the control mice and 0–30% of the treatment groups (Tables 1 and 2). Histologically positive regional lymph node metastases were found in 70–100% of control- and gemcitabine-treated mice, whereas in mice receiving both gemcitabine and IFN-α (10,000 units), the incidence of regional lymph node metastasis was 40% (P < 0.05; Table 1). Treatments with IFN-α alone or in combination with gemcitabine were well tolerated as determined by maintenance of body weight (Tables 1 and 2).

A final experiment was carried out to determine survival. Mice were killed and necropsied only when they became moribund (Fig. 1). The median survival time for the control group was 38 days. After treatment with gemcitabine alone, daily IFN-α alone, and a combination of daily IFN-α and gemcitabine, the median survival time was 44, 38, and 78 days, respectively (control versus gemcitabine, P < 0.05; control versus IFN-α, P = 0.06; control versus IFN-α and gemcitabine, P < 0.001).

Histology and IHC Analyses. We next determined the in vivo effects of IFN-α (alone or in combination with gemcitabine). L3.6pl cells were injected into the pancreas of mice, and the mice were randomized into the above-mentioned four treatment groups. After 14, 21, and 28 days of treatment, five mice from each treatment group were killed and necropsied, and tumors were harvested and processed for routine histology and IHC analyses of cell proliferation and apoptosis using anti-PCNA antibodies and the TUNEL method, respectively (Fig. 2). At 28 days of treatment, the mean number of PCNA+ tumor cells in control tumors was 586 ± 125. After therapy with gemcitabine or IFN-α, the mean number was 400 ± 71 or 407 ± 65, respectively (Table 3). The lowest mean number of PCNA+ cells (361 ± 72) was found in tumors of mice treated with both IFN-α and gemcitabine (P < 0.05 versus other treatment groups).

Table 3  Immunohistochemical analysis of human pancreatic carcinoma in the pancreas of control and treated nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Human tumor cells</th>
<th>Mouse endothelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCNA+</td>
<td>TUNEL+</td>
</tr>
<tr>
<td>Saline control</td>
<td>586 ± 125</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>400 ± 71</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>IFN-α (10,000 units)</td>
<td>407 ± 65</td>
<td>58 ± 20</td>
</tr>
<tr>
<td>IFN-α (10,000 units) + gemcitabine</td>
<td>361 ± 72</td>
<td>84 ± 19</td>
</tr>
</tbody>
</table>

* L3.6pl human pancreatic cancer cells (1 × 10⁶) were injected into the pancreas of nude mice. Seven days later, groups of mice were treated with biweekly i.p. injections of gemcitabine (125 mg/kg) alone, daily s.c. injections of human IFN-α (10,000 units) alone, or with gemcitabine or saline (control). Data after 28 days of treatment are shown.

* Number (mean ± SD) of CD31/TUNEL-positive cells in 10 random 0.011-mm² fields at ×100 magnification. Fluorescent double-labeling was performed on frozen tissue sections.

* P < 0.05 as compared with controls.

* P < 0.05 as compared with single-agent therapy.

tumor cells in tumors treated with gemcitabine or IFN-α alone as compared with tumors in the combination therapy group (P < 0.05). In control mice and in mice treated with gemcitabine alone, IFN-α alone, and the combination of the two, the ratio of PCNA+/TUNEL+ cells was 65, 7, 7, and 4, respectively.

There was a significant reduction in tumor MVD (measured by staining with antibodies against CD31) per field after treatment with IFN-α or combination therapy as compared with control tumors or gemcitabine-treated tumors (Fig. 2; Table 3). There was no significant difference between MVD of tumors treated with IFN-α and MVD of tumors from mice given combination therapy (P = 0.3). IHC staining of tumors after 28 days of treatment with IFN-α and gemcitabine revealed a significant decrease in expression of bFGF, MMP-9, IL-8, and VEGF (P < 0.05 versus control, Fig. 3).

The CD31/TUNEL fluorescent double-labeling technique (25) revealed that many endothelial cells in tumors from mice treated with IFN-α or with IFN-α and gemcitabine underwent apoptosis (Fig. 4, yellow reaction). The number of apoptotic endothelial cells significantly increased in pancreatic tumors harvested 28 days after the initiation of treatment with IFN-α or IFN-α + gemcitabine as compared with tumors from control mice or from mice treated with gemcitabine (P < 0.001; Table 3; Figs. 4 and 5A).

Apoptosis of tumor-associated endothelial cells (CD31/TUNEL+ cells, Fig. 5A) was found as early as day 14 of treatment (Fig. 5). Endothelial cell apoptosis was higher in tumors from mice treated with IFN-α or IFN-α and gemcitabine than in tumors from control mice or those treated with gemcitabine alone (control versus IFN-α, P < 0.05; gemcitabine versus IFN-α and gemcitabine, P < 0.01; gemcitabine versus IFN-α, P < 0.05). There was no significant difference in the number of apoptotic endothelial cells between tumors from mice treated with IFN-α alone and tumors from mice treated with combination therapy (P = 0.07). In all treatment groups, overall cell death (TUNEL+ cells) increased with the duration of therapy (Fig. 5B).

DISCUSSION

We report that daily s.c. injections of 10,000 units of human IFN-α combined with twice a week i.p. injections of...
gemcitabine significantly inhibited the growth and metastasis of human pancreatic carcinoma cells implanted into the pancreas of nude mice. Twice weekly s.c. injections of 50,000 units of IFN-α were ineffective. As reported previously by our laboratory (20, 21), to produce maximal therapeutic effects, IFN-α must be administered daily at an optimal biological dose. Treatment with daily IFN-α alone or gemcitabine alone reduced the growth of primary neoplasms by 53% and 70%, respectively. Combination therapy with IFN-α and gemcitabine further decreased tumor growth by 87% and decreased the incidence of lymph node metastasis, significantly prolonging survival. Daily s.c. doses of IFN-α were well tolerated by nude mice. IHC analyses of the pancreatic cancers demonstrated a significant decrease in PCNA+ cells, an associated increase in TUNEL+ cells (apoptotic cells), and a significant decrease in MVD in the IFN-α-treated or IFN-α + gemcitabine-treated tumors. The reduction in MVD was due to a significant increase in endothelial cell apoptosis.

The reduction in MVD is noteworthy because the progressive growth and metastasis of malignant neoplasms depend on adequate neovascularization. The balance between the proangiogenic and antiangiogenic molecules released by tumor cells
and surrounding host cells (28, 29) determines the extent of angiogenesis. Among the major proangiogenic molecules are bFGF, MMP-9, IL-8, and VEGF (20, 21, 30–33). IFN-α has been shown to inhibit transcription and protein production of many of these proangiogenic molecules (11, 13, 20, 21, 28). This regulation, however, is associated with induction of a feedback mechanism involving a family of proteins called SOCS that negatively regulate cytokine signaling. The induction of SOCS directly correlates with the concentration of a given cytokine (34–36). At daily doses exceeding 10,000 units of IFN-α, the induction of SOCS may have been responsible for the failure of IFN-α to down-regulate the target genes (37).

In this study, the daily administration of human IFN-α at 10,000 units/dose decreased the expression of bFGF, MMP-9, IL-8, and VEGF in human pancreatic cancer cells. These are

Fig. 4 Immunofluorescent CD31 (endothelial cells), TUNEL (apoptosis) double labeling of human pancreatic cancer growing in the pancreas of nude mice. Pancreatic tumors (L3.6pl cells) from mice treated with HBSS (control), gemcitabine, IFN-α, or gemcitabine + IFN-α were harvested on day 28 of the treatment. The tissues were processed for immunohistochemistry using anti-CD31 antibodies (Texas Red) and TUNEL (FITC-green). A representative sample (×400) of this CD31/ TUNEL fluorescent double staining is shown. Fluorescent red, CD31 + endothelial cells; fluorescent green, TUNEL + cells; yellow, TUNEL + endothelial cells.

Fig. 5 A, apoptosis of tumor-associated endothelial cells. Tumors were harvested from control mice and mice treated with gemcitabine, IFN-α, or both IFN-α and gemcitabine after 14, 21, and 28 days of treatment. The sections were double-stained with CD31/TUNEL fluorescent antibodies as described in “Materials and Methods.”

Quantification of apoptotic endothelial cells was expressed as the percentage of total endothelial cells per field that were TUNEL + in 5–10 random 0.011-mm² fields at ×400 magnification. Numbers represent mean ± SD. *, *P < 0.05 versus gemcitabine and control. **, *P < 0.01 versus gemcitabine and control. B, induction of apoptosis. Tumors were harvested from control mice and mice treated with gemcitabine, IFN-α, or both IFN-α and gemcitabine after 14, 21, and 28 days of treatment. The sections were stained using the TUNEL technique as described in “Materials and Methods.” The number of TUNEL + cells/field (mean ± SD) was determined from measuring 10 random 0.159-mm² fields at ×100 magnification. *, *P < 0.05 versus control. **, *P < 0.05 versus others.
important survival factors for human and mouse endothelial cells (38–40). This decreased expression correlated with an increased apoptosis of endothelial cells. The administration of gemcitabine and the decrease in survival factor receptor signaling can clearly enhance apoptosis in tumor-associated endothelial cells. Decreased MVD, i.e., vascularization, leads to apoptosis of tumor cells and the surrounding stroma (41).

In summary, we show that a 10,000-unit dose of human IFN-α, which we consider the optimal biological dose, given daily in combination with biweekly gemcitabine significantly reduces the growth of human pancreatic carcinoma in nude mice. The inhibition of primary tumor growth and lymph node metastasis is mediated by both direct antitumor effects (gemcitabine) and antiangiogenic effects (IFN-α). This combination therapy may provide a new approach to the treatment of a devastating disease.

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REFERENCES

Administration of Optimal Biological Dose and Schedule of Interferon α Combined with Gemcitabine Induces Apoptosis in Tumor-associated Endothelial Cells and Reduces Growth of Human Pancreatic Carcinoma Implanted Orthotopically in Nude Mice
