Antitumor Effects of Interferon-ω: In Vivo Therapy of Human Tumor Xenografts in Nude Mice

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ABSTRACT

The antitumor effect of the type I IFN, IFN-ω, was evaluated in both in vitro and in vivo studies of human cancer. For these studies, the cDNA for human IFN-ω was cloned into a eukaryotic expression pDNA (pDNA) driven by the cytomegalovirus promoter. Supernatants from UM449 cells transfected in vitro with IFN-ω pDNA had antiproliferative effects on 11 of 13 human tumor cell lines. For in vivo studies, nude mice were implanted s.c. with one of the following human tumors: NIH:OVCAR-3 ovarian carcinoma, A375 melanoma, or A431 epidermoid carcinoma. Direct intratumoral injection of 100 μg of a IFN-ω pDNA DMRIE/DOPE complex (1:1 DNA:DMRIE mass ratio) for 6 consecutive days resulted in a significant reduction in the tumor volume of NIH:OVCAR-3 ovarian carcinoma or A375 melanoma (P = 0.02). IFN-ω pDNA delivered by i.m. injection also had an antitumor effect. Nude mice bearing s.c. A431 epidermoid carcinoma and injected i.m. with 100 μg of IFN-ω pDNA, twice per week for 3 weeks, had a significant reduction in tumor volume (P = 0.009). These results demonstrate for the first time that IFN-ω can have in vivo antitumor effects in several models of human cancer.

INTRODUCTION

IFNs are a large family of proteins having antiviral, antiproliferative, and immunomodulatory effects (1). Among the family of IFNs are two major classes, type I and type II, designated primarily by differences in receptor binding and nucleotide sequence. Type I IFNs consist of IFN α, B, τ, and ω and bind to the type I IFN receptor, whereas IFN-γ is the only type II IFN and is specific for the type II IFN receptor. The type I IFN, IFN-ω, was identified by three independent groups in 1985 and has 60% nucleotide sequence identity to the family of IFN-α genes (2–5). Although both human IFN-ω and IFN-α are produced by virally induced leukocytes and have similar antiviral activities on human cell lines, IFN-ω is antigenically distinct from IFN-α and differs in its interaction with the type I IFN-α receptor (6–9).

Compared with IFN-ω, IFN-α has been more comprehensively studied and has been found to have a wide range of activities including inhibition of tumor cell growth, activation of T and natural killer cell cytotoxicity, stimulation of macrophages, up-regulation of cell surface MHC class I molecules, promotion of T helper type 1 responses, and inhibition of angiogenesis (10). Human IFN-ω delivered by either recombinant protein or by viral or plasmid vectors has been shown to have antitumor effects in preclinical mouse tumor models (11–17). Furthermore, recombinant IFN-α protein has been approved for treatment of patients with metastatic melanoma, hairy cell leukemia, and chronic myelogenous leukemia and has been found to have therapeutic benefits in treatment of follicular non-Hodgkin’s lymphoma (18, 19). In contrast, the antitumor efficacy of IFN-ω has not been well described and, to date, has been limited to in vitro studies. In one study, human IFN-ω inhibited the in vitro growth of bone marrow progenitor cells from chronic myelogenous leukemia patients, and in a second study, human breast and colon carcinoma cell lines incubated with human IFN-ω had increased cell surface expression of MHC class I molecules (20, 21).

One reason that the in vivo effects of IFN-ω have not been adequately described may be that no murine IFN-ω gene has been identified (22). Because IFNs, including IFN-ω, tend to be species specific in their actions, preclinical evaluation of the antitumor efficacy of IFN-ω requires the establishment of xenogeneic models of human cancer in immunodeficient mice. Therefore, in the present research, the human tumor cell lines, NIH:OVCAR-3 ovarian carcinoma, A375 melanoma, and A431 epidermoid carcinoma, were s.c. implanted into nude mice to establish several xenogeneic models of human cancer. For tumor therapy, IFN-ω was delivered by injection of a eukaryotic expression pDNA encoding the human IFN-ω gene. In the xenogeneic models, intratumoral delivery of IFN-ω pDNA significantly reduced the growth of s.c. NIH:OVCAR ovarian carcinoma and s.c. A375 melanoma, whereas i.m. delivery of IFN-ω pDNA significantly reduced the growth of s.c. A431 epidermoid carcinoma. The results of this work demonstrate for the first time that IFN-ω can inhibit the growth of human tumors in vivo and suggest a promising new candidate for human cancer therapy.

MATERIALS AND METHODS

Plasmids. The backbone pDNA VR1055 is derived from pUC19, with the β-lactamase (ampicillin resistance) gene replaced by the aminoglycosidase acetyltransferase (kanamycin resistance) gene from pET9a (Novagen, Madison, WI, Ref. 23). VR1055 directs eukaryotic gene expression from a cassette containing the human cytomegalovirus immediate early 1 gene promoter/enhancer, 5′ untranslated sequence, and the cytomegalovirus intron A sequence. The regulatory region is followed by the cloning polylinker and a transcriptional terminator region derived from the rabbit β-globin gene. To construct the IFN-ω pDNA used in these studies, VR4151, the human IFN-ω gene, was amplified from human genomic DNA and inserted into the polylinker region of VR1055.

Plasmid Purification. Plasmid was produced by bacterial fermentation and isolated by a modified lysis procedure (24), followed by standard double CsCl-ethidium bromide gradient ultracentrifugation. pDNA was ethanol precipitated and resolubilized in saline at 4°C and dialyzed against saline. All pDNA preparations were free of detectable RNA. Endotoxin content was determined by the Limulus Amebocyte Lysate assay (Associates of Cape Cod, Inc., Falmouth, MA), and all sera were obtained from HyClone (Logan, Utah). The human cell lines were obtained from the American Type Culture Collection (Bethesda, MD) and grown in DMEM supplemented with 10% fetal bovine serum. UM449 cells were kindly provided by Mark Cameron and Gary Nabel at the University of Michigan (Ann Arbor, MI) and were grown in RPMI 1640 with 10% fetal bovine serum.

In Vitro Transfections. UM449 cells were plated at a concentration of 2 × 10^5 cells/well in a six-well plate and incubated for 24 h. Medium was

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[2] The abbreviations used are: pDNA, plasmid DNA; EMC, encephalomyocarditis virus; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt; DMRIE, (N,N-diethyl)imidazole-2,3-bis(tetradecyloxyl)-1-propanaminium bromide; DOPE, dioleoylphosphatidylethanolamine.

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removed from the cells, which were washed with PBS followed by addition of the 1 μg of pDNA complexed with 1 μg of the cationic lipid DMRIE/DOPE (25) in a total volume of 1 ml OptiMEM medium (Life Technologies, Inc., Gaithersburg, MD). After incubation of 4–5 h at 37°C, 1 ml of OptiMEM with 30% FCS was added to each well, followed by the addition of 1 ml of OptiMEM with 10% FCS the next day. Tissue culture supernatants were collected 48 h after the start of the in vitro transfection.

Antiviral assay. In vitro transfections were performed as described previously, and supernatants were collected from cells transfected with either the IFN-ω or control pDNA. An EMC virus plaque reduction assay was used to determine the antiviral activity of the supernatants (IIT Research Institute, Chicago, IL). Briefly, tissue culture supernatants were added to monolayers of mouse L929 or human A549 cells in 96-well plates and incubated for 24 h. The cells were washed, and murine EMC virus was added at a multiplicity of infection of 0.04 and incubated for 24 h. The cells were fixed with 5% formalin and stained with 1% crystal violet. As a positive control, cells were incubated with an IFN reference standard (NIH, Bethesda, MD) prior to addition of EMC virus.

Cell Proliferation Assay. In vitro transfection of UM449 cells with the IFN-ω and control pDNA was performed as described previously, and the supernatants were tested in a cell proliferation assay of human tumor cell lines using the Boehringer Mannheim (Indianapolis, IN) Cell Proliferation Kit II (XTT). Human tumor cells were plated as monolayers in 96-well plates, incubated for 24 h, followed by the addition of tissue culture supernatants from UM449 cells transfected in vitro with either IFN-ω or control pDNA. Human leukocyte IFN protein (Sigma Chemical Co., St. Louis, MO) was used as a positive control. The XTT labeling reagent was added 24–48 h later, and the absorbance (A) at 490 nm was determined 6–24 h later. The percentage of reduction in cell proliferation due to addition of the supernatants was determined by the formula:

\[
\% \text{ reduction in cell proliferation} = \frac{A_{\text{untreated}} - A_{\text{treated}}}{A_{\text{untreated}}} \times 100
\]

Xenogeneic Tumor Models. Nude (nu/nu) female mice between the ages of 6 and 8 weeks were obtained from Harlan Sprague Dawley (San Diego, CA). All animal experiments were conducted in accordance with Vical’s Institutional Animal Care and Use Committee as well as the standards set forth in the National Research Council guidelines concerning animal care and use.

The NIH:OVCAR-3 ovarian carcinoma model was established by s.c. injection of 5 × 10^6 cells in nude mice, whereas the A375 melanoma and A431 squamous cell carcinoma lines were established by s.c. injection of 5 × 10^3 cells. Tumor dimensions were measured by calipers (length × width × height) three times per week, and the values were inserted into the formula: Tumor volume (mm^3) = 0.52 (length × width × height) (Ref. 26).

Intratumoral Injections. For intratumoral therapy of s.c. tumors in nude mice, pDNA was complexed with the cationic lipid, DMRIE/DOPE. DMRIE/DOPE has been shown to be effective for in vivo transfection of established tumors (27, 28) and consists of the cationic lipid DMRIE and the neutral lipid DOPE at a pDNA:DMRIE mass ratio of 1:1, according to the following protocol. Briefly, 100 μg of pDNA was diluted in 50 μl of 0.9% saline (Radix Labs, Eau Claire, WI). In a second vial, DMRIE/DOPE lipid containing 100 μg of DMRIE (DMRIE/DOPE 1:1 mol:mol) was diluted in 50 μl of 0.9% saline. Plasmid and DMRIE/DOPE were combined, vortexed for 5 s, and injected into mice shortly thereafter. For intratumoral therapy, 100 μg pDNA complexed with 100 μg DMRIE/DOPE in a 100 μl total volume was injected directly into s.c. tumors (70–300 mm^3) using a 1-ml tuberculin syringe fitted with a 28-gauge ½ needle (Becton Dickinson) modified with a plastic collar cut from a 200-μl micropipette tip. The collar length was adjusted to limit the needle from penetrating beyond 2 mm into the rectus femoris muscle. For i.m. tumor therapy, nude mice bearing s.c. tumors of 40–200 mm^3 were injected i.m. with 100 μg (50 μg/kg) of either IFN-ω or control pDNA twice per week for 3 weeks.

Determination of Serum IFN-ω. To determine the serum levels of IFN-ω, naive nude mice were injected i.m. once with 100 μg of either IFN-ω pDNA or control pDNA. Serum was collected daily for 2 weeks after injection (five mice/day) and analyzed using a human IFN-ω ELISA kit (Alexis, San Diego, CA), which was sensitive to 2 pg/ml.

Statistical Analyses. Tumor volume was analyzed using the Mann-Whitney U nonparametric statistical test to identify groups having significantly different tumor sizes. Differences were considered statistically significant when p < 0.05.

RESULTS

IFN-ω pDNA Has Antiviral and Antiproliferative Activity. The human IFN-ω gene was cloned into the eukaryotic expression plasmid VR1055 (23) to create the IFN-ω pDNA, VR4151, used in these studies. The biological activity of the IFN-ω pDNA was evaluated in several in vitro systems. Because type I IFNs, including IFN-ω, have been shown to have antiviral effects (1, 7), supernatants from UM449 cells in vitro transfected with IFN-ω pDNA were tested for the ability to protect human or murine cells from infection with EMC virus. IFN-ω pDNA supernatants markedly reduced the cytopathic effects of EMC virus on the human A549 cell line (Table 1). Supernatants from the IFN-ω pDNA-transfected cells had 30,000 units/ml of antiviral activity on the human cells, whereas supernatants from control pDNA-transfected cells (backbone plasmid without IFN-ω coding sequence) had <1 unit/ml of antiviral activity on the human cells. IFN-ω pDNA supernatants had a less marked antiviral effect when tested on the murine L929 cell line, having only 300 units/ml of antiviral activity. The 100-fold reduction in IFN-ω antiviral activity on murine cells compared with human cells is consistent with the known species-specific activity of type I IFNs (1, 7).

Because IFN-ω has been shown to have antiproliferative effects in vitro (21), supernatants from UM449 cells transfected with IFN-ω pDNA were assayed for antiproliferative activity on human tumor cell lines. The majority of the human tumor cell lines evaluated (11 of 13) had reductions in cell proliferation of 14–60% after incubation with IFN-ω pDNA supernatants, as compared with proliferation after incubation with control pDNA supernatants (Table 2). The NIH:OVCAR-3 ovarian line was the most sensitive to the IFN-ω antiproliferative effects with a 60% reduction in cell proliferation. The ACHN renal carcinoma, SCC-4 squamous carcinoma, and U-87 MG glioblastoma cell lines were the next most sensitive cell lines having 41, 36, and 36% reduction in cell proliferation after incubation with the IFN-ω pDNA supernatants, respectively. The degree of sensitivity to IFN-ω was not necessarily tumor type specific, because the NIH:OVCAR-3 ovarian carcinoma cell line was the most sensitive (60% reduction in proliferation), whereas the SK-OV-3 ovarian carcinoma cell line was one of the least sensitive (<10% reduction in proliferation). Overall, the results from the antiviral and antiproliferative assays confirmed that the IFN-ω encoding pDNA used in these studies, VR4151, was able to produce biologically active IFN-ω.

Table 1 Antiviral activity of tissue culture supernatants from IFN-ω pDNA-transfected cells

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Human cell line</th>
<th>Murine cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-ω pDNA</td>
<td>30,000</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Control pDNA</td>
<td>300</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

Interferon activity (units/ml)
Table 2. Antiproliferative activity of tissue culture supernatants from IFN-α pDNA-transfected cells

<table>
<thead>
<tr>
<th>Human tumor cell line</th>
<th>% reduction in cell proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH:OVCAR-3 (ovarian)</td>
<td>60</td>
</tr>
<tr>
<td>ACHN (renal)</td>
<td>41</td>
</tr>
<tr>
<td>SCC-4 (squamous)</td>
<td>36</td>
</tr>
<tr>
<td>U-87 MG (glioblastoma)</td>
<td>36</td>
</tr>
<tr>
<td>SCC-15 (squamous)</td>
<td>29</td>
</tr>
<tr>
<td>A431 (epidermoid)</td>
<td>24</td>
</tr>
<tr>
<td>A375 (melanoma)</td>
<td>24</td>
</tr>
<tr>
<td>PC-3 (prostate)</td>
<td>20</td>
</tr>
<tr>
<td>MCF-7 (breast)</td>
<td>18</td>
</tr>
<tr>
<td>A549 (lung)</td>
<td>17</td>
</tr>
<tr>
<td>UM-UC-3 (bladder)</td>
<td>14</td>
</tr>
<tr>
<td>SK-OV-3 (ovarian)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>K-562 (leukemia)</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

*a The reduction in cell proliferation values after incubation with IFN-α supernatant is relative to the reduction in proliferation after incubation with control pDNA supernatant.

Intratumoral Therapy with IFN-α pDNA Reduces the Growth of Human Tumors. To begin to address the issue of whether IFN-α can have in vivo antitumor effects, nude mice were implanted s.c. with one of the following human tumor cell lines: NIH:OVCAR-3 ovarian carcinoma, A375 melanoma, or A431 epidermoid carcinoma. When the mice had developed palpable s.c. tumor nodules of 70–300 mm³ (by day 43 after tumor cell injection for NIH:OVCAR-3, day 27 for A375, and day 19 for A431), they were treated with six consecutive intratumoral injections of 100 μg of either IFN-α pDNA or control pDNA complexed with the cationic lipid, DMRIE-DOPE. Mice bearing either the NIH:OVCAR-3 or A375 tumors and treated intratumorally with IFN-α pDNA had a significant reduction in tumor volume compared with mice treated with the control pDNA (P = 0.02; Fig. 1, A and B). By day 98 after tumor cell injection, NIH:OVCAR-3-bearing mice treated with IFN-α pDNA had a 75% reduction in tumor volume compared with mice injected with control pDNA. In the A375 model, a 56% reduction in tumor volume after IFN-α treatment was evident by day 49 after tumor cell injection. In contrast, intratumoral therapy with IFN-α pDNA in the A431 model was not statistically significant (P = 0.058), yet did reduce tumor growth by 41% compared with control pDNA-treated tumors (Fig. 1C). The cumulative results from the three xenogeneic human tumor models suggest that some human tumors can be sensitive to the in vivo antitumor effects of IFN-α.

Delivery of IFN-α pDNA by i.m. Injection Has Antitumor Effects. Recently, we demonstrated that i.m. injection of a pDNA encoding murine IFN-α could dramatically inhibit the growth of distant s.c. and metastatic B16F10 melanoma tumors in C57BL/6 mice (29). We were therefore interested in applying this type of therapy to the xenogenic models of human cancer to determine whether i.m. injection of IFN-α pDNA could also affect distant tumor growth. For this study, nude mice were again implanted s.c. with one of the following human tumor cell lines: NIH:OVCAR-3 ovarian carcinoma, A375 melanoma, or A431 epidermoid carcinoma. Upon development of palpable tumor nodules (40–200 mm³ on day 49 for NIH:OVCAR-3, day 21 for A375, and day 19 for A431), mice were injected i.m. with either 100 μg of IFN-α pDNA or control pDNA, twice per week for 3 weeks. The i.m. IFN-α pDNA therapy was only effective for the A431 epidermoid model (Fig. 2) and had no antitumor effect in the NIH:OVCAR-3 or A375 models (data not shown). In the A431 model, mice treated with IFN-α pDNA by i.m. injection had a significant 66% reduction in tumor volume by day 42 compared with mice treated by i.m. injection of control pDNA (P = 0.009). This result suggests that, in some tumor types, systemic delivery of IFN-α pDNA may be therapeutic.

Serum Pharmacokinetics of IFN-α. Because i.m. injection of IFN-α pDNA was able to significantly reduce the growth of human A431 tumors in nude mice, we were interested in determining whether serum levels of IFN-α could be detected after i.m. injection. For this study, naive nude mice received a single i.m. injection of 100 μg of IFN-α pDNA, followed by daily collections of serum for 2 weeks. Mice treated in this manner had mean serum levels of 133 pg/ml IFN-α as early as 1 day after i.m. injection of IFN-α pDNA (Fig. 3). By day 7 after i.m. injection, mice had mean IFN-α serum levels of 648 pg/ml, and by day 14, IFN-α serum levels were still present, with mean levels of 134 pg/ml. Mice injected i.m. with control pDNA had no detectable serum levels of IFN-α (data not shown). Thus, i.m. administration of the IFN-α pDNA can lead to systemic circulation of IFN-α.

Fig. 1. Intratumoral IFN-α pDNA therapy of human NIH:OVCAR-3 ovarian carcinoma (A), A375 melanoma (B), and A431 epidermoid carcinoma (C) in nude mice. Nude mice were injected s.c. with 5 × 10⁶ NIH:OVCAR-3 cells, 5 × 10⁶ A375 cells, or 5 × 10⁶ A431 cells. When tumors were between 70 and 300 mm³ (day 43 after tumor cell injection for NIH:OVCAR-3, day 27 for A375, and day 19 for A431), they were injected intratumorally with 100 μg of either IFN-α or control pDNA complexed with the cationic lipid, DMRIE-DOPE, at a 1:1 pDNA/DMRIE mass ratio. Mice received the intratumoral pDNA therapy for 6 consecutive days. For the NIH:OVCAR-3 and A375 tumor models, a significant reduction in tumor volume was found after intratumoral IFN-α pDNA therapy compared with control pDNA therapy (P = 0.02). The mean tumor volume for 10 mice per group is shown.
IFN-ω. If mice are reinfected with 100 μg of IFN-ω pDNA on day 14, a similar pattern of expression is elicited, with peak serum levels 7 days later and a decline in serum levels 14 days after the second injection (data not shown).

**DISCUSSION**

This is the first demonstration that IFN-ω can significantly reduce the growth of human tumors *in vivo*. Although recombinant IFN-ω has been shown to be effective in preclinical as well as clinical tumor studies and is, in fact, an approved therapy for several cancers (18), the *in vivo* antitumor effect of human IFN-ω has not been described previously. In this report, we found that either intratumoral or i.m. administration of a plasmid encoding IFN-ω can have significant antitumor effects in several xenogeneic nude mouse models of human cancer.

Because there is no murine homologue of human IFN-ω and IFNs are very species specific in their actions (1, 7, 22), we were restricted to evaluation of the effect of IFN-ω on human tumors implanted into immunodeficient mice. For the intratumoral therapy studies, nude mice were implanted s.c. with one of the following human tumors: NIH:OVCAR-3 ovarian carcinoma, A375 melanoma, or A431 epidermoid carcinoma. Treatment of either the NIH:OVCAR-3 or A375 human tumor models with intratumoral IFN-ω pDNA resulted in significantly reduced tumor growth compared with treatment with the control pDNA. Thus, direct intratumoral therapy with IFN-ω delivered by a plasmid vector can be therapeutic for established human tumors.

An alternative method of IFN-ω pDNA delivery, that of i.m. injection, was also evaluated in the three xenogeneic tumor models. Although i.m. delivery of IFN-ω pDNA had no effect on the growth of NIH:OVCAR-3 and A375 tumors, there was a significant reduction in growth of A431 tumors. The fact that i.m. delivery of IFN-ω pDNA was not effective in two of three of the xenogeneic models is not surprising because in previous research we found that i.m. delivery of murine IFN-α pDNA for treatment of B16F10 melanoma required CD8+ T cells (29). Because nude mice lack functional T cells, the antitumor effect of IFN-ω in the xenogeneic models is most likely due to a direct antiproliferative effect, and this may be more efficient when the IFN-ω is delivered intratumorally, rather than systemically.

The finding that the A431 epidermoid model was somewhat more sensitive to systemic IFN-ω therapy compared with intratumoral IFN-ω therapy (66% *versus* 41% reduction in tumor growth for i.m. *versus* intratumoral therapy) deserves further investigation. This result may, in part, be due to the fact that the A431 tumors injected intratumorally with control pDNA complexed with lipid grew more slowly than the A431 tumors treated by i.m. injection of control pDNA, possibly reflecting a nonspecific effect of intratumoral injection of control pDNA and lipid.

In a pharmacokinetics study, serum levels of IFN-ω were detected after a single i.m. injection of the IFN-ω pDNA. This result, combined with the fact that i.m. IFN-ω pDNA therapy of A431 tumors led to a significant reduction in tumor growth, suggests that circulating levels of IFN-ω may have a therapeutic effect on some human tumors. The importance of this finding is that IFN-ω pDNA may be administered in a manner that does not require identification of tumor nodules. This type of treatment can be beneficial for patients having tumors in internal organs that are difficult to access or who may have developed distant tumor metastases.

In summary, we have demonstrated that IFN-ω can have antitumor effects on human cancers *in vivo*. Because this research was conducted in immunodeficient mice, the application of IFN-ω therapy to human cancer patients may possibly result in even greater therapeutic effects. As discussed previously, another type 1 IFN, IFN-α, has a broad range of immunomodulatory activities including enhancement of T-cell cytotoxicity, up-regulation of MHC class I, and promotion of T helper-1 cell-mediated immune responses, all of which can enhance the antitumor effect (10). Furthermore, IFN-ω may have additional antitumor or immunological effects that have yet to be elucidated. The results of this research suggest that further studies on IFN-ω and its mechanism of action, as well as its potential as an antitumor agent, are warranted.

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