Inhibition of Growth and Metastasis of Orthotopic Human Prostate Cancer in Athymic Mice by Combination Therapy with Pegylated Interferon-α-2b and Docetaxel

Samuel F. Huang, Sun-Jin Kim, Anh T. Lee, Takashi Karashima, Cora Bucana, Daniel Kedar, Paul Sweeney, Badar Mian, Dominic Fan, David Shepherd, Isaiah J. Fidler, Colin P. Dinney, and Jerald J. Killion 1


INTRODUCTION

IFNs are multifunctional regulatory cytokines involved in control of cell function and replication, and IFN-α and IFN-β directly inhibit the proliferation of tumor cells of different histological origins (1–4). Recent studies indicate that IFN-α and IFN-β can also down-regulate the expression of proangiogenic molecules, such as bFGF (5–7), IL-8 (8, 9), and the metalloproteases, MMP-2 and MMP-9 (10–12), and activate host effector cells (4, 13).

For therapy of human bladder carcinoma grown in athymic nude mice, the optimal biological dose (i.e., the dose that exerts a maximal antiangiogenic effect) is a relatively low dose, e.g., 10,000 units administered on a daily schedule (7). Pharmacokinetic studies have demonstrated that the half-life of IFNs in the circulation of patients is on the order of minutes (14), thus making therapeutic levels difficult to sustain, which may in turn compromise the effectiveness of IFN in the therapy of solid tumors (14, 15). Pegylation of IFN is a biochemical method of delaying clearance and reducing immunogenicity (16), thus making therapeutic levels difficult to administer on a daily schedule (7). Pharmacokinetic studies have shown that PEG-IFN-α is significantly more effective than free-form IFN-α in the treatment of chronic hepatitis C (18, 19), and differences in clinical outcome have been related to the ability of pegylation to sustain absorption, restrict the volume of distribution, and reduce clearance of IFN-α (19).

We evaluated the therapeutic effect of PEG-IFN-α-2b in combination with docetaxel against the metastatic human prostate cancer PC3-MM2 implanted in the prostates of athymic nude mice. The growth and metastatic potential of PC3-MM2 is dependent upon its secretion of proangiogenic factors bFGF, VEGF, MMP-9, and IL-8 (20). Because IFN-α down-regulates the expression of these factors in different human tumors (5–10, 12) and because the microtubule destabilizer docetaxel is currently being evaluated in clinical trials against advanced prostate cancer (21–24), we hypothesized that a combination therapy for prostate cancer using the antiangiogenic properties of IFN-α together with the cytotoxic properties of docetaxel might result in a potent two-compartment attack (via the tumor vasculature and tumor cells) upon the growth and metastasis of prostate cancer.

MATERIALS AND METHODS

Cell Culture. The highly metastatic PC3-MM2 human prostate cancer variant line was originally selected from the PC-3 cell line (25). The cells were grown as monolayer cultures in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, and nonessential amino acids. The tumor cells were free of Mycoplasma, reovirus type 3, pneumonia virus of mice, mouse adenovirus, ectromelia virus, and lactate dehydrogenase virus (assayed by Microbiological Associates, Bethesda, MD).

Mice. Male NCr-nu/nu nude mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (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 according to institutional guidelines when they were 8–10 weeks of age.

Orthotopic Implantation of Tumor Cells. To produce prostate tumors, subconfluent cultures of PC3-MM2 cells were given fresh medium 24 h before being harvested by a brief treatment with 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. Only single-cell suspensions with a viability of >90% were used for injections. Mice anesthetized with an i.p. injection of nembutal were placed in a supine position. A lower midline abdominal incision was made; the prostate was exteriorized, and tumor cells (5 × 10^7/0.05 ml HBSS) were injected into the right lobe of the prostate. A well-localized bleb was a sign of a technically satisfactory injection. The abdominal incision was closed in one layer using metal clips.

Determination of Optimal Biological Dose of Human PEG-IFN-α-2b and Therapeutic Dose of Docetaxel. Three days after implantation of PC3-MM2 cells, groups of mice were randomized to receive different doses of s.c. PEG-IFN-α-2b (Schering-Plough Corp., Kenilworth, NJ; specific activity, 5720}
6 \times 10^3 \text{ IU/mg). Twenty-three days after orthotopic implantation of tumor cells, five mice from each group were killed, and their prostates were removed and processed for immunohistochemical analysis. The remaining mice were killed at 4 weeks. The primary prostate tumors were weighed, and the metastasis to regional lymph nodes was determined. Other groups of mice were randomized to receive different doses of docetaxel (Aventis, Paris, France). Docetaxel was administered weekly by i.p. injection of either 2.5, 5, or 10 mg/kg. Vehicle was used as a control. The mice were killed at 4 weeks, and the extent of prostatic tumor burden was determined.

**Systemic in Vivo Therapy with Combination Docetaxel and PEG-IFN-α-2b.** Mice were randomized into the following groups 3 days after implantation of tumor cells: saline (control); docetaxel administered i.p. once weekly (10 mg/kg); PEG-IFN-α-2b s.c., administered weekly (70,000 IU) or the combination of both therapies. Treated mice were killed when moribund (5–6 weeks), and the weight of the primary prostate tumors and the incidences of regional (pelvic and paraaortic) lymph node metastasis were recorded. Histopathology confirmed the nature of the disease. For immunohistochemical and histological 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.

**ELISA for Serum bFGF.** The level of bFGF protein in the serum was measured by the Quantikine ELISA kit (R&D Systems, Minneapolis, MN). The minimal level of bFGF detectable by this assay is 1 pg/ml.

**Immunohistochemical Determination of bFGF, MMP-9, VEGF, IL-8, E-Cadherin, PCNA, and CD31.** Paraffin-embedded tissues were used for identification of bFGF, MMP-9, VEGF, IL-8, E-cadherin, PCNA, and CD31. 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 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 5 min for “antigen retrieval” as described previously (26). All other paraffin-embedded tissues were treated with pepsin (Biomeda) for 15 min at 37°C. Tissues used for identification of CD31 were sectioned (8 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 (27–31). A positive reaction was 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 1 min, and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining.

**Immunofluorescence Double Staining for Apoptotic Endothelial 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 (v/v; 5 min), and acetone (5 min) and washed with PBS. Immunohistochemical procedures were performed as described previously (27–31). A positive reaction was 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 1 min, and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining.

**In Vitro Antiproliferative Effects Mediated by PEG-IFN-α-2b or Docetaxel against PC3-MM2 Cells.** PC3-MM2 cells were incubated for 6 days in medium containing increasing concentrations of either PEG-IFN-α-2b (0–1000 IU/ml) or docetaxel (0–100 ng/ml). Growth inhibition of the cultured tumor cells by PEG-IFN-α-2b was <30% at the highest concentration. Hence, this cell line is relatively resistant to the antiproliferative effects of PEG-IFN-α-2b. The IC50 of docetaxel was determined to be 2.3 ng/ml (data not shown).

**Inhibition of Prostate Carcinoma Growth and Metastasis by PEG-IFN-α-2b: Determination of Optimal Biological Dose.** The prostates of nude mice were injected with PC3-MM2 cells on day 0. Three days later, the mice were randomized into the following treatment groups. As shown in Table 1, therapy with PEG-IFN-α-2b or docetaxel on inhibition of growth and metastasis was determined to be 3.34 IU/mg, and 853–1091 mg, respectively. The incidence of lymph node metastasis was compared by using the χ2 test. In vitro protein expression by ELISA was compared using the unpaired Student’s t test.

<table>
<thead>
<tr>
<th>Table 1 Effect of PEG-IFN-α-2b on inhibition of growth and metastasis of human PC-3M-M2 prostate tumors in nude mice</th>
<th>Median tumor weight, mg</th>
<th>LN metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-IFN-α-2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (control)</td>
<td>1370</td>
<td>7/10</td>
</tr>
<tr>
<td>35,000 IU</td>
<td>1007</td>
<td>853–1091</td>
</tr>
<tr>
<td>70,000 IU</td>
<td>511</td>
<td>328–708</td>
</tr>
<tr>
<td>350,000 IU</td>
<td>759</td>
<td>653–846</td>
</tr>
<tr>
<td>Docetaxel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1268</td>
<td>7/10</td>
</tr>
<tr>
<td>2.5</td>
<td>776</td>
<td>75–1230</td>
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<tr>
<td>5.0</td>
<td>483</td>
<td>49/10</td>
</tr>
<tr>
<td>10.0</td>
<td>457</td>
<td>132–1607</td>
</tr>
</tbody>
</table>

* a P < 0.006, compared with control mice.
* b P < 0.001, compared with mice treated with either 35,000 or 350,000 IU.
* c Incidence of mice with tumor of 11 mice.
* d P < 0.002.
at all three doses significantly decreased median tumor when compared with the control (1007, 511, 759, and 1370 mg, respectively). Moreover, the use of PEG-IFN-α-2b at the optimal biological dose of 70,000 IU resulted in a greater decrease in primary tumor weight than that observed using 35,000 IU or 350,000 IU. Thus, the therapeutic effect of PEG-IFN-α-2b was biphasic. Histologically positive regional lymph nodes were found in 100% of control mice and in 100% of the mice treated with PEG-IFN-α-2b at the low dose of 35,000 IU weekly. However, treatment with either 70,000 IU or 350,000 IU or PEG-IFN-α-2b resulted in a significant reduction in the incidence of lymph node metastasis from 100% in control mice to 22 and 30%, respectively.

MVD and the Presence of Proangiogenic Factors in the Microenvironment of Prostate Carcinoma Treated with PEG-IFN-α-2b.

Groups of mice were given intraprostatic injections of cultured PC3-MM2 cells on day 0 and treated with PEG-IFN-α-2b as described above (35,000, 70,000 or 350,000 IU/week). All mice were killed on day 23. Tumors harvested from the different groups were processed for histology and stained for the presence of bFGF, MMP-9, MMP-2, and VEGF. Tumors from mice treated with PEG-IFN-α-2b demonstrated decreased expression of these proangiogenic molecules (Fig. 1). Treatment with 70,000 IU/week resulted in the lowest expression of bFGF, MMP-9, and MMP-2 as determined by quantitative imaging. There was no difference in expression of VEGF and IL-8. Moreover, treatment of mice with PEG-IFN-α-2b at 70,000 IU/week resulted in a significant decrease in the MVD of tumors as compared with the control (16 ± 8.6 and 47 ± 6.8, respectively). Tumors from mice treated with 35,000 IU/week and 350,000 IU/week also demonstrated a lower mean MVD (35.6 ± 9.9 and 36.2 ± 7.5) versus control; however, these differences were not significant.

Serum bFGF levels measured by ELISA were lower in mice treated with 70,000 IU/week as compared with controls (45 ± 3.5 ng/ml and 52 ± 3.3 ng/ml; P < 0.015). Levels were not significantly decreased in mice treated with 35,000 IU/week or 350,000 IU/week as compared with controls (57 ± 10.0 ng/ml and 51 ± 9.9 ng/ml).

**Inhibition of Prostate Carcinoma Growth and Metastasis by Docetaxel and Determination of Maximal Tolerated Dose.**

We determined the therapeutic dose of docetaxel to be used as a single agent or in combination therapy. Results are also shown in Table 1. Athymic nude mice received intraprostatic injections of PC3-MM2 cells. Three days later, the mice were randomized into four groups for treatment with either docetaxel at 2.5, 5, or 10 mg/kg/week or diluent as a control. All mice were killed on day 32 after tumor cell injection. Detailed necropsy revealed that each group had 2 mice that did not have primary prostate tumors. Weekly i.p. injections of docetaxel at all doses significantly decreased median tumor weight when compared with the control (776, 483, 457, and 1286 mg, respectively). There were no significant differences between the groups of treated mice, although the lowest median tumor weight was observed at the highest dose of 10 mg/kg. However, a significant reduction in the

![Fig. 1. Immunohistochemical analyses of PC3-MM2 tumors in the prostate of control and PEG-IFN-α-2b-treated nude mice. Tumor cells (5 × 10⁴/mouse) were implanted into the prostas of nude mice. Three days later, groups of mice (n = 5) began receiving s.c. injections of PEG-IFN-α-2b at doses of 35,000, 70,000, and 350,000 IU/week. Saline-treated mice with prostate tumor implants were used as controls. Treatment continued for 3 week. Bar, 50 μm.](downloaded_from_cancerres.aacnjournals.org_on_september_23_2017.2002アメリカンアセオニグナルカンセラリサーチ)
combination therapy resulted in a greater reduction of tumor burden than the use of either agent alone \( (P < 0.008) \). Histologically positive regional lymph node metastases were found in 10 of 12 control mice, 1 of 12 mice treated with docetaxel, 6 of 13 mice treated with PEG-IFN-\(\alpha\)-2b, and 1 of 12 mice given combination therapy. Hence, the metastatic spread of primary tumor appeared to be limited by therapy with docetaxel.

**Histological and Immunohistochemical Analyses.** Representative tumors harvested from each group were processed for histological and immunohistochemical analyses (Fig. 2). All tumors contained necrotic zones and a large number of infiltrating cells in their central portions. Protein expression was assessed only at the periphery of the tumors in areas not containing necrosis. Immunohistochemistry using specific anti-bFGF and anti-MMP-9 antibodies demonstrated that
when compared with controls, tumors treated with docetaxel alone expressed similar levels of these markers, whereas tumors treated with PEG-IFN-α-2b or PEG-IFN-α-2b plus docetaxel expressed decreased levels. Immunohistochemistry using specific anti-MMP-2 antibodies demonstrated that, when compared with controls, tumors treated with docetaxel alone or PEG-IFN-α-2b alone expressed similar levels of these markers. Lastly, immunohistochemistry using specific anti-E-cadherin antibodies demonstrated that tumors from mice treated with docetaxel alone expressed similar levels of these markers, whereas tumors from mice treated with PEG-IFN-α-2b or combination therapy had increased staining for this cellular protein.

Cell proliferation and apoptosis were evaluated using anti-PCNA antibodies and the TUNEL method, respectively. Results are shown in Fig. 3 and Table 3. The median number of PCNA-positive tumor cells in control tumors was 581. After therapy with docetaxel alone or docetaxel plus PEG-IFN-α-2b, this number decreased to 385 and 284, respectively (P < 0.01). Treatment with PEG-IFN-α-2b alone did not result in a significant reduction in cell proliferation when compared with the control (515 and 581, respectively).

The mean number of TUNEL-positive cells was inversely related to the number of PCNA-positive cells. The average value of TUNEL-positive cells in control tumors was 3.5; in docetaxel-treated animals, 13 (P < 0.005); in tumors from mice treated with PEG-IFN-α-2b-treated animals, 5; and in tumors from mice given combination therapy, 11.5 (P < 0.005).

MVD (measured by staining antibodies against CD31) was directly proportional to expression of bFGF. Tumors from control mice contained an average of 19.5 blood vessels/high-power field. MVD was not significantly reduced in tumors of mice treated with docetaxel (15.5) but was in tumor of mice treated with PEG-IFN-α-2b alone or combination therapy (8.4 and 6.5, respectively; P < 0.003).

Finally, apoptosis of endothelial cells within tumors was determined by using a method for simultaneously labeling markers for CD31 and apoptosis (TUNEL). Whereas tumors treated with PEG-IFN-α-2b or combination therapy contained apoptotic endothelial cells, tumors in control mice and in mice treated with docetaxel as a single agent did not (yellow reaction, Fig. 3).

**DISCUSSION**

Our results confirm the therapeutic approach of targeting both the tumor endothelium as well as the tumor cells within a primary prostate cancer. The use of pegylated IFN combined with docetaxel was...
superior to the use of either agent alone. The antiangiogenic effects of the type I IFN PEG-IFN-α-2b on tumor cells were established by molecular techniques that demonstrated a decrease in synthesis and secretion of bFGF and a decrease in expression of MMP-9, genes that correlate with growth and metastasis of prostate cancer (20). The PC3-MM2 cells used in our study are resistant to the anti proliferative effects of PEG-IFN-α-2b, and reduction in tumor burden appears to be solely attributable to reduction of proangiogenic gene expression. Treatment of a variety of human cancer cell lines have demonstrated the ability of IFN-α to down regulate proangiogenic factors such as bFGF, IL-8, MMP-9, and MMP-2 (5, 7–9, 11, 12). Our use of pegylated IFN-α paralleled the results observed in the treatment of orthotopic human bladder cancer or liver metastasis of human colon cancer in nude mice with free-form IFN-α (33, 34). The results presented here demonstrate that therapeutic effects can also be achieved by systemic low-dose administration of PEG-IFN-α-2b.

The rationale for the use of chronic, low-dose IFN-α has been exemplified in its use as therapy for infantile hemangiomas (35–38). It has been shown recently that the antiangiogenic properties of IFN-α may be the basis of regressions observed in Kaposi’s sarcoma and the effect of IFN-β on murine leukemia and melanoma (39–42). Similar to melanoma, renal cell carcinoma, and transitional cell carcinoma, metastatic prostate carcinoma has also been treated with IFN-α in clinical trials (21, 43). However, responses in these trials have been modest, and the optimal biological dose has yet to be determined. This inability to achieve an optimal therapeutic use of high-dose IFN-α may be attributable to the induction of a recently described family of proteins that negatively regulate cytokine signaling (46, 47). IFN signaling is mediated by JAK receptor-associated tyrosine kinases and latent cytoplasmic transcription factors called STATs (48). Suppressors of cytokine signaling (SOCS) negatively regulate IFN signaling through inhibition of the JAK/STAT pathway (46, 49, 50). Although the inhibition of STAT3 activation by SOCS3 is well described (51, 52), the relationship between maximal tolerated doses of IFN-α used in clinical trials and the induction of down-regulation of STAT1 signaling has yet to be fully elucidated.

Treatment of tumor cells with IFN has been shown recently to up-regulate the expression of E-cadherin, a cell-surface molecule involved in the cohesive properties of cells within tissue (53). Indeed, the relative expression of E-cadherin (a measure of the cohesive properties of tumor) and of metalloproteases, such as MMP-9, reflects the loss of E-cadherin as well as abundant expression of the enzyme(s) used by invasive tumor cells, the higher the risk of metastasis, our studies demonstrated that biological doses of PEG-IFN-α-2b produce significant antiangiogenic effects, and they expand the preclinical rationale for use of cytotoxic therapeutics in combination with inhibitors of angiogenesis.

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