Administration of IFN-α Enhances the Efficacy of a Granulocyte Macrophage Colony Stimulating Factor–Secreting Tumor Cell Vaccine

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Abstract
IFN-α is approved for the treatment of multiple cancers. Its pleiotropic properties include inhibition of proliferation and angiogenesis and induction of apoptosis. Type I IFNs also exert immunomodulatory effects, which make it an appropriate candidate to combine with cancer vaccines. The studies reported herein show that 50% of mice reject established B16 tumors following treatment with the combination of a granulocyte macrophage colony-stimulating factor–secreting tumor cell vaccine (B16.GM) and subclinical doses of recombinant murine IFN-α delivered at the vaccine site. Similarly, 80% of mice treated with the combination reject established B16 tumors when recombinant murine IFN-α is given at the challenge site, suggesting that in the latter case its antiproliferative, proapoptotic, and antiangiogenic properties may be involved in controlling tumor growth. In contrast, fewer than 10% of mice reject the tumors when either one is used as a monotherapy. Furthermore, a 30-fold increase in the frequency of melanoma-associated antigen (Trp-2 and gp100) specific T cells was observed in mice treated with the combination when compared with unvaccinated controls. These data show that IFN-α combined with a granulocyte macrophage colony-stimulating factor–secreting tumor cell vaccine significantly enhances vaccine potency and may represent a potential new approach for tumor immunotherapy. (Cancer Res 2005; 65(6); 2449-56)

Introduction
IFN-α, which is a type I IFN, was originally described as an antiviral agent (1). Under physiologic conditions, IFN-α is constitutively expressed at low levels from a wide variety of cell types. High levels of IFN-α are induced by viral or bacterial infections leading to the activation of natural killer cells and macrophages and to the induction of genes that block virus replication (2–5). IFN-α exhibits antiproliferative (6), proapoptotic, and antiangiogenic properties (7–10) as well as immunomodulatory properties such as up-regulation of MHC class I (11), enhancement of maturation and activation of dendritic cells (12–14), and potentiating humoral immunity by promoting isotype switching (15). Although IFN-α was first developed as an antiviral agent, it is also widely used in the clinic as a cancer therapy for lymphoma, leukemia (haairy cell), chronic myeloid leukemia, Kaposi’s sarcoma, and melanoma (16–18). However, systemic infusion of clinically relevant doses of IFN-α (6–50 × 10⁶ units/m² thrice/wk) has been associated with significant toxicities but only modest therapeutic benefits. Therefore, its local effects have been evaluated in preclinical tumor models in the context of cellular vaccines modified to secrete IFN-α (19–21). These studies showed that local expression of IFN-α from cellular vaccines enhanced the generation and promoted the survival of tumor-specific CTL responses (22, 23), suggesting that combining IFN-α with vaccines might increase overall vaccine potency.

Cytokine-secreting tumor cell vaccines elicit systemic tumor-specific immune responses protecting against tumor challenge in various preclinical models. Originally, the ability of different cytokines to enhance the immunogenicity of tumor cells was evaluated in the B16 melanoma model, by transducing B16 cells with retroviral vectors expressing various cytokines, irradiating the cells, and using them as cellular vaccines (24). These studies showed that B16 cells modified to secrete granulocyte macrophage colony-stimulating factor (GM-CSF) stimulated a potent, long-lasting and specific antitumor immune response that required both CD4⁺ and CD8⁺ T cells. These findings have been confirmed in numerous preclinical models and pursued in the clinic in melanoma (25, 26), non–small cell lung carcinoma (27–29), pancreatic cancer (30), and prostate cancer (31).

The studies reported herein were done to evaluate the combination of IFN-α with GM-CSF-secreting tumor cell vaccines in preclinical models. We show in the B16 melanoma model that local administration of subclinical doses (2,500 units; 4 × 10⁵ units/m²) of IFN-α either at the tumor challenge site or at the vaccine site enhances the potency of a GM-CSF-secreting tumor-cell vaccine resulting in prolonged survival. Increased Trp-2- and gp100-specific T-cell responses could be detected in animals treated with the combination of a GM-CSF-secreting tumor cell vaccine and recombinant murine IFN-α (rmIFN-α), when compared with animals treated with either monotherapy. Furthermore, using ovalbumin-specific OT-1 T-cell receptor (TCR) transgenic T cells, an increase in the overall number of OT-1 T cells was detected in the tumor draining lymph nodes and vaccine draining lymph nodes when IFN-α was given near the site of tumor inoculation or vaccination respectively.

Materials and Methods

Mice. Female C57Bl/6 mice and OT-1 TCR transgenic (OT-1 TCR Tg) mice were purchased from Taconic (Oxnard, CA) and The Jackson Laboratory (Bar Harbor, ME), respectively, and maintained according to institutional and NIH guidelines. All mice were used between 8 and 12 weeks of age. Water and food were provided ad libitum.

Cell Lines, Culture Media, and Reagents. The CT26 (colon carcinoma), 4T1 (breast cancer), LLC (Lewis lung carcinoma), and 3T3 cell lines (fibrosarcoma) were purchased from American Type Culture Collection (Manassas, VA). The B16F10 melanoma and the retrovirally transduced GM-CSF-secreting B16.GM cell lines were previously described (25).
The latter generates 150 ng per 10^6 per 24 hours of mouse GM-CSF. The Renca cell line (renal cell carcinoma) was kindly provided by Dr. Hong-Ming Hu (Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR). Cells were maintained in DMEM (Hyclone, Logan, UT) supplemented with 10% heat inactivated fetal bovine serum (Hyclone), 2 mmol/L L-glutamine, and 1× penicillin/streptomycin (JRH, Lenexa, KS) in a humidified incubator with 5% CO2 at 37°C. The Kβ binding peptide derived from ovalbumin (SIINFEKL), Trp2 (SVYDFFVWL), or gp100 (TGWKYQWY) were purchased from Genmed Synthesis, Inc (South San Francisco, CA). Cytokine anti-CD8 was obtained from BD Pharmingen (San Diego, CA) and PE-conjugated Kβ-ova tetramer was purchased from Immunomics (San Diego, CA). rmIFN-α was purchased from R&D Systems (Minneapolis, MN).

In vitro Proliferation Assay. Tumor cells (1×10^5 cells per well) were plated in 96-well flat-bottomed plates (Costar, Milpitas, CA) in the presence of the indicated concentrations of rmIFN-α. Plates were incubated for 48 hours at 37°C and 5% CO2. Cells were pulsed with 1 Ci of [3H]-thymidine for the final 8 hours and proliferation was measured by radioactivity uptake in a Wallac beta counter (Perkin-Elmer, Norwalk, CT).

Apoptosis Assay. Murine tumor cells were plated as described in the section above. After 48 hours, cells were trypsinized and stained with the Annexin V-PE Apoptotic Kit (BD Pharmingen) according to the manufacturer’s instructions. Samples were analyzed using a FACScan instrument and its associated software (Becton Dickinson, San Jose, CA).

Chorioallantoic Membrane Assay. Chorioallantoic membrane (CAM) assays were done as previously described (32). Briefly, hydrocortisone-containing filter discs were soaked with 25 ng basic fibroblast growth factor (bFGF; R&D Systems) in the presence or absence of the indicated doses of rmIFN-α. Discs were aseptically placed on top of the CAM of 11-day-old chick embryos. Seventy-two hours later, the discs were surgically removed. The CAMs were harvested and analyzed for bFGF-induced angiogenesis under stereomicroscopy. The mean number (±SE) of branch points of blood vessel sprouts beneath the applied filter disc was enumerated from multiple (n = 8-10) samples.

Tumor Growth CAM Assay. B16F10 tumor cells (5×10^5) were premixed with the indicated concentration of rmIFN-α aseptically placed on top of the CAM of 11-day-old chick embryos and incubated at 37°C. Seven days later, the tumors were surgically removed and their weight determined. Alternatively, B16F10 tumor cells (5×10^5), were placed on the CAM of 11-day-old chick embryos, and 24 hours later, the indicated dose of rmIFN-α was injected i.v. (100 µL) into the circulation of the chick embryo. Seven days later the tumors were surgically removed and their weight determined. Mean weight (±SE) of tumors was determined from multiple (n = 8-10) vivo samples.

Tumor Treatment Experiment. Female C57Bl/6 mice were challenged (dorsal s.c.) with B16F10 tumor cells (1×10^5) and vaccinated (ventral s.c.) 3 days later with 1×10^6 irradiated B16F10 or GM-CSF-secreting B16F10 (B16.GM) tumor cells. Four days after vaccination, rmIFN-α treatment was initiated. IFN-α [2,500 units/d (4×10^6 units/mg)] was injected (i.p.) to 100 µL at the site of tumor challenge (dorsal) or vaccination (ventral). Animals were monitored for the formation of palpable tumors twice weekly and sacrificed if tumors became necrotic or exceeded 150 mm^3 in size.

OT-1 Adoptive Transfer Tumor Model. Spleen and LN cells (inguinal, axillary, lateral axillary, mesenteric, mandibular, and iliac) from OT-1 mice were homogenized, treated with ACK lysis buffer to remove RBC, and washed thrice in phenol red free HBSS. Lymphocytes (1-2×10^6 cells) were transferred into sex-matched naive C57Bl/6 mice by tail vein injection. Two days later recipients were challenged s.c. with 1×10^5 B16-OVA cells (day 0). On days 3 and 17, mice were vaccinated s.c. with 1×10^5 irradiated B16.GM-OVA cells. IFN-α was injected i.p. as described above. Seven days after the second vaccination (24 days post-tumor challenge) spleen cells were isolated and assayed for ovalbumin, Trp2, or gp100-specific responses by ELISPOT analysis.

ELISPOT Assay. Antigen-specific responses were enumerated by an IFN-γ ELISPOT assay (R&D Biosystems) according to the manufacturer’s instructions. Briefly, 96-well filtration ELISPOT plates (Millipore, Bedford, MA) were coated with the specified amount of capture antibody in 100 µL of reagent diluent for 2 hours at 37°C. Plates were washed twice with wash buffer (PBS with 0.5% Tween 20) and blocked for 2 hours at room temperature in blocking buffer containing PBS with 1% bovine serum albumin and 5% sucrose. Erythrocyte-depleted splenocytes (1×10^6 or 5×10^5) were plated and incubated for 24 hours at 37°C, 5% CO2 with 1 µmol/L of the Kβ binding peptide derived from ovalbumin (SIINFEKL), Trp2 (SVYDFFVWL), or gp100 (TGWKYQWY) in a final volume of 200 µL per well. ELISPOT plates were developed according to the manufacturer’s specifications. Wells containing media and spleen cells only served as negative control. Spots were enumerated by an automated plate scanning service from Cellular Technology Ltd. (Cleveland, OH).

Results

Direct Effects of IFN-α on Proliferation and Apoptosis of Murine Tumor Cells. IFN-α has been shown to exert antiproliferative and proapoptotic activity on a limited number of human and murine cancer cell lines in vitro (8, 33–35). To get a better understanding of the breadth of these responses, the effect of rmIFN-α was tested in vitro on a panel of murine tumor cell lines, including melanoma (B16F10), renal cell carcinoma (Renca), colon carcinoma (CT26), breast cancer (4T1), lung cancer (LLC), and fibrosarcoma (3T3). The antiproliferative activity of rmIFN-α was most pronounced against the B16F10 melanoma cell line (Fig. 1A). rmIFN-α inhibited B16F10 proliferation in a dose-dependent manner, with >80% inhibition at the highest concentration of rmIFN-α tested (5,000 units/mL), which is consistent with that previously reported (35). The other tumor cell lines tested were also sensitive to the antiproliferative activity of rmIFN-α, however to a lesser extent than B16F10. In these cell lines, proliferation was maximally inhibited by only 30% to 40% at a dose of 5,000 units/mL of rmIFN-α (Fig. 1A).

The ability of rmIFN-α to induce apoptosis was also tested. Cells were cultured in the presence of various doses of rmIFN-α and assayed for apoptosis 72 hours later. Of the cell lines tested, Renca, 4T1, B16F10, and 3T3 were relatively resistant to rmIFN-α-induced apoptosis, with only a 2-fold increase detected at the highest dose of rmIFN-α tested (5,000 units/mL, Fig. 1B). In contrast, apoptosis was increased ≥2-fold in LLC and CT26 tumor cells with only 300 units/mL of rmIFN-α, with a nearly 5-fold increase in apoptosis in CT26 cells at 5,000 units/mL of rmIFN-α (Fig. 1C).

Direct Effects of IFN-α on Angiogenesis. Another mechanism by which IFN-α may affect tumor growth is by down-regulating expression of angiogenic factors such as bFGF, vascular endothelial growth factor, metalloproteinase 9, and interleukin 8 (35–38). As a measure of direct antiangiogenic activity of recombinant human IFN-α, the ability of rmIFN-α to inhibit bFGF-induced angiogenesis was tested in the CAM assay. As shown in Fig. 2, bFGF stimulated a high degree of vessel formation, with 135±14 vessel sprouts per CAM. Coincubation with rmIFN-α significantly reduced the number of vessel sprouts to 34±5 per CAM, which is consistent with previous reports demonstrating antiangiogenic activity of recombinant human IFN-α (38, 39).

The CAM assay can also be used as an in vivo screen for compounds with direct antitumor activity, which could result from a combination of antiproliferative, antiangiogenic, and proapoptotic activity. To evaluate the overall immune-independent antitumor effects of rmIFN-α, a series of experiments were done to determine whether rmIFN-α could inhibit B16 tumor formation on the CAM.
Subcutaneous injection of rmIFN-α directly at the tumor challenge site (dorsal) or at a distal (ventral) site did not alter tumor growth, demonstrating that this dose is subtherapeutic as a monotherapy (Fig. 4A). To verify bioavailability and systemic distribution of the s.c. injected IFN-α, serum levels were measured at 6 and 24 hours post-injection. IFN-α could be detected at 6 hours (500 pg/mL) but was below detection limits by 24 hours and was never detected in untreated controls.

To test whether delivery of rmIFN-α at the tumor challenge site could enhance survival in combination with B16.GM vaccine treatment, mice were vaccinated with irradiated B16 or B16.GM cells 3 days after live tumor cell challenge. All mice subsequently were injected with 2,500 units of rmIFN-α at the challenge site twice per week for 4 weeks starting 4 days after vaccination. All of the control mice challenged with B16 tumor cells were sacrificed by day 40 due to progressive tumor growth with a median survival of 29.5 days (Fig. 4B). Similarly, 90% of the mice vaccinated with B16 or B16.GM alone succumbed to tumor burden by day 50, despite a slight delay in tumor growth rate in mice, which was reflected by an increase in median survival (40 and 42 days, respectively). The combination of a B16.GM vaccine and rmIFN-α given at the tumor challenge site lead to a significant delay in tumor growth, resulting in 80% survival of animals at day 80, when all surviving mice were tumor-free.

Because IFN-α has been shown to have immunomodulatory properties, we also evaluated whether administering rmIFN-α at the vaccine site would increase the potency of the cellular vaccine. Survival of mice injected with rmIFN-α as a monotherapy was not different from mice that received HBSS, whereas vaccination with B16.GM led to a slightly prolonged survival (Fig. 4C). Ultimately, only 10% of the mice survived after treatment with rmIFN-α or the B16.GM vaccine as a monotherapy. In contrast, 50% of mice vaccinated with B16.GM and injected with 2,500 units of rmIFN-α at the site of vaccination survived and remained tumor-free for >80 days. Taken together these data show that administration of rmIFN-α enhances the efficacy of a GM-CSF-secreting tumor cell vaccine independent of the site of administration.

Administration of IFN-α Enhances the Efficacy of a GM-CSF-Secreting Tumor Cell Vaccine. It is well documented in preclinical models that GM-CSF-secreting tumor cell vaccines stimulate potent, long-lasting, and specific antitumor immunity (24, 40, 41). However, the vaccines are typically less effective in mice with large tumor burdens. The ability of rmIFN-α to enhance the antitumor efficacy of a GM-CSF-secreting tumor cell vaccine was evaluated in the B16/F10 tumor model due to its direct antitumor efficacy as well as potent immunomodulatory properties (4, 12–15, 22, 42–45).

Initially, B16F10 tumor cells were premixed with either 1,000 or 5,000 units of rmIFN-α or left untreated and placed on the CAM of 11-day-old chick embryos (Fig. 3A). Premixing of B16F10 tumor cells with rmIFN-α significantly inhibited tumor growth compared with untreated controls. Tumor weight was inhibited 56% and 71% by 1,000 and 5,000 units of rmIFN-α, respectively. To evaluate whether systemic (i.v.) administration of rmIFN-α to the chick embryo could inhibit tumor growth on the CAM, B16F10 tumor cells were placed on the CAM, and 24 hours later, chick embryos were injected i.v. with 1,000 or 5,000 units of rmIFN-α (Fig. 3B). Intravenous administration of 1,000 and 5,000 units of rmIFN-α reduced tumor weight by 21% and 42%, respectively. These data show that IFN-α can inhibit tumor growth through both direct (antiproliferative and proapoptotic) and indirect (antiangiogenic) mechanisms independently of an antitumor immune response.

Figure 1. Dose-dependent effects of rmIFN-α on cell proliferation and induction of apoptosis in various murine tumor cell lines. Tumor cells (1 × 10⁴ cells per well) were cultured in a 96-well plate for 48 hours in the presence of the indicated concentrations of rmIFN-α. A, proliferation was measured by addition of [3H]-thymidine for the last 8 hours of the cultures. Cells were harvested and counted on a beta counter. B, induction of apoptosis was detected by flow cytometry following incubation with PE-conjugated Annexin-V and 7-AAD. Fold-increase in apoptosis as compared with controls for each tumor cell line. One example of two experiments.
Recent studies show that IFN-α increases the generation and survival of CD8+ effector T cells \textit{in vitro} and \textit{in vivo} (22, 44, 45), which could be one explanation for the increased survival observed in animals treated with the combination therapy. To test this hypothesis, an adoptive T-cell transfer system in which ovalbumin is expressed on the cell surface of the tumor cells (B16.OVA) and vaccine cells (B16.GM-OVA), serving as a surrogate tumor-associated antigen, was used. This model system permits one to track the expansion and survival of OT-1 T cells over time via tetramer staining. Studies were set up to determine whether IFN-α affected T cells in local draining lymph nodes only or also at distant sites. Mice were adoptively transferred with CD8+ T cells bearing a TCR-specific for the OVA-derived peptide SIINFEKL bound to H-2Kb. Mice were challenged with B16.OVA cells on day 0 and were left unvaccinated or vaccinated with irradiated B16.GM-OVA cells on day 3. IFN-α (2,500 units) was given at the site of tumor inoculation (dorsal) or vaccination (ventral) 4 and 7 days later. Tumor draining lymph nodes or vaccine draining lymph nodes were removed 24 hours after the last injection of IFN-α and analyzed for the total number of ovalbumin-specific T cells via tetramer analysis (Fig. 5A and B, respectively). Administration of IFN-α alone did not affect the overall number of ovalbumin-specific T cells in local lymph nodes or distant lymph nodes. Following vaccination with B16.GM-OVA cells, the number of OT-1* T cells detected in the tumor draining lymph nodes was similar to that in the vaccine draining lymph nodes (compare B16.GM in Fig. 5A and B). Administration of IFN-α after vaccination with B16.GM-OVA cells resulted in a moderate increase in the number of OT-1 T cells in both the tumor and vaccine draining lymph nodes when compared with vaccine alone (Fig. 5A and B). However, the effects were more apparent in the lymph nodes near the site of IFN-α injection (i.e., tumor draining lymph nodes following peritumoral injection and vaccine draining lymph nodes following injection at the site of vaccination). The results suggest that IFN-α may preferentially affect T cells in local lymph nodes but is also able to affect T cells systemically.

Figure 3. IFN-α inhibits B16F10 tumor growth in the CAM assay. A, B16F10 cells (5 x 10⁵) were admixed with IFN-α and placed on the CAM of 11-day-old chick embryos (n = 8-10 per group). Seven days later, the tumors were removed and weighed. B, B16F10 cells (5 x 10⁵) were placed on the CAM of 11-day-old chick embryos (n = 8-10 per group). Twenty-four hours later, rmIFN-α was injected i.v. into the CAM. Tumors were removed and weighed 7 days after rmIFN-α treatment. Columns, mean weight; bars, ±SE.

**IFN-α Enhances the Generation of Tumor-Specific T Cells.** Recent studies show that IFN-α increases the generation and survival of CD8* effector T cells \textit{in vitro} and \textit{in vivo} (22, 44, 45), which could be one explanation for the increased survival observed in animals treated with the combination therapy. To test this hypothesis, an adoptive T-cell transfer system in which ovalbumin is expressed on the cell surface of the tumor cells (B16.OVA) and vaccine cells (B16.GM-OVA), serving as a surrogate tumor-associated antigen, was used. This model system permits one to track the expansion and survival of OT-1 T cells in local draining lymph nodes only or also at distant sites. Mice were adoptively transferred with CD8* T cells bearing a TCR-specific for the OVA-derived peptide SIINFEKL bound to H-2Kb. Mice were challenged with B16.OVA cells on day 0 and were left unvaccinated or vaccinated with irradiated B16.GM-OVA cells on day 3. IFN-α (2,500 units) was given at the site of tumor inoculation (dorsal) or vaccination (ventral) 4 and 7 days later. Tumor draining lymph nodes or vaccine draining lymph nodes were removed 24 hours after the last injection of IFN-α and analyzed for the total number of ovalbumin-specific T cells via tetramer analysis (Fig. 5A and B, respectively). Administration of IFN-α alone did not affect the overall number of ovalbumin-specific T cells in local lymph nodes or distant lymph nodes. Following vaccination with B16.GM-OVA cells, the number of OT-1* T cells detected in the tumor draining lymph nodes was similar to that in the vaccine draining lymph nodes (compare B16.GM in Fig. 5A and B). Administration of IFN-α after vaccination with B16.GM-OVA cells resulted in a moderate increase in the number of OT-1 T cells in both the tumor and vaccine draining lymph nodes when compared with vaccine alone (Fig. 5A and B). However, the effects were more apparent in the lymph nodes near the site of IFN-α injection (i.e., tumor draining lymph nodes following peritumoral injection and vaccine draining lymph nodes following injection at the site of vaccination). The results suggest that IFN-α may preferentially affect T cells in local lymph nodes but is also able to affect T cells systemically.

Figure 4. IFN-α enhances the therapeutic efficacy of a GM-CSF-secreting tumor cell vaccine. C57Bl/6 mice (n = 10 per group) were challenged with 1 x 10⁵ B16F10 tumor cells and (A) injected with IFN-α (2,500 units/d; twice/wk) at the tumor site (dorsal) or at a distant site (ventral). Alternatively, mice were vaccinated 3 days after tumor challenge with 1 x 10⁵ irradiated B16F10 or GM-CSF-secreting B16F10 (B16.GM) cells. Four days after the vaccine, rmIFN-α administration was initiated as described above at the (B) site of tumor challenge or (C) site of vaccine administration. Mice were monitored twice weekly for the development of subcutaneous tumors. Mice were sacrificed if tumors became necrotic or once tumor size exceeded 150 mm². Kaplan Meier survival curves of % surviving mice as a function of time after tumor challenge. Comparable with three independent experiments.
specific IFN-γ were analyzed for their activation status by measuring SIINFEKL-removed 7 days after the second vaccination and OT-1 T cells vaccinated on days 3 and 17. IFN-γ-a adoptively transferred with OT-1 T cells, challenged on day 0 and OT-1 responses as well as responses to trp-2 and gp100. Mice were done in the B16.OVA model as this would permit one to measure directed against weakly immunogenic endogenous melanoma—could also increase the overall population of antigen-specific T cells when combined with a GM-CSF-secreting tumor cell vaccine, seems to be more potent than administration at the vaccine site when rmIFN-α is given in close proximity to the vaccine. The site of tumor challenge, in particular, appears to result in increased IFN-γ concentrations in the tumor microenvironment that could lead to direct antitumor activity as well as increased T-cell responses to tumor-associated antigens (46, 47). Due to the potential limitations of this model for measuring T-cell activation, we evaluated whether the observed improvement in tumor protection in animals treated with the combination of vaccine and rmIFN-α correlated with an increase in the frequency of T cells specific to the melanoma—specific tumor antigens Trp-2 and gp100. T-cell responses against Trp-2 and gp100 were tested by IFN-γ ELISPOT assay. The frequency of Trp-2 and gp100 specific cells (Fig. 6C and D) in naïve (control) mice and mice challenged with B16-OVA (HBSS) cells only was low, with fewer than 15 spots/10^5 spleen cells in either group. The frequency of Trp-2-specific cells did not increase with vaccine treatment whereas a 10-fold increase in gp100-specific events was detected in mice vaccinated with B16.GM-OVA alone compared with the control. In contrast, a 30-fold increase in the frequency of Trp-2- and gp100-specific cells was observed in mice treated with B16.GM-OVA plus rmIFN-α, which correlated with the survival benefit observed in mice treated with this combination (Fig. 4B). These findings show that use of a GM-CSF-secreting cellular vaccine in combination with rmIFN-α results in increased tumor-specific T-cell responses as well as survival of such T cells, leading to a significant survival advantage over animals treated with vaccine only.

Discussion

The findings reported herein show that local administration of subclinical doses of rmIFN-α at the site of tumor challenge, in combination with an irradiated GM-CSF-secreting tumor cell vaccine, improves tumor free survival compared with animals receiving either vaccine or rmIFN-α alone. These effects are observed following different rmIFN-α dosing regimens (20,000 units total dose; 3 × 10^5 units/mouse total dose) including four daily injections of 5,000 units, 2,500 units twice/wk for 4 weeks, or a metronomic dosing regimen that consists of 300 units/d, 5 days/wk for 8 weeks (data not shown). Furthermore, enhanced protection is also observed in animals treated with the combination therapy when rmIFN-α is given in close proximity to the vaccine. The observation that administration of rmIFN-α at the challenge site seems to be more potent than administration at the vaccine site (80% versus 50% survival; median survival of >80 versus 60 days), when combined with a GM-CSF-secreting tumor cell vaccine, suggests that multiple mechanisms could be involved with this combination. For example, local delivery at the tumor challenge site can result in high IFN-α concentrations in the tumor microenvironment that could lead to direct antitumor activity as well.
as antiangiogenic and immunomodulatory effects. In contrast, delivery at the site of vaccination increases the vaccine efficacy likely through the immunomodulatory properties of IFN-α because the concentration of IFN-α at the tumor site are too low to have an impact through direct antitumor mechanisms. This idea is supported by the observation that the concentration of IFN-α detected in the serum 6 hours after injection of 2500 units is ∼10-fold lower than the concentration required in vitro to significantly impact proliferation, apoptosis, and angiogenesis (500 pg/mL in serum versus 4.6 ng/mL in vitro).

Although IFN-α is approved for the treatment of various cancers (16–18), the mechanism(s) underlying its antitumor activity are not completely understood. Initially, the direct cytostatic effect of IFN-α was thought to be the major mechanism by which it inhibits tumor growth (33, 34, 48); however, subsequent studies revealed that inhibition of angiogenesis and induction of tumor cell apoptosis are other IFN-α mediated antitumor effects (7, 10, 49–52). Our in vitro studies revealed that B16F10 melanoma cells are more sensitive to the cytostatic effects of IFN-α, whereas the CT26 colon carcinoma cells are more sensitive to the proapoptotic properties of rmIFN-α in vitro. Furthermore, both premixing rmIFN-α with B16F10 tumors and i.v. administration of rmIFN-α in the embryos inhibited B16F10 tumor growth in the CAM assay demonstrating that local and systemic administration of IFN-α can directly inhibit tumor growth. Whereas it is not possible to delineate the individual involvement of the antiproliferative, antiangiogenic, and proapoptotic activities of rmIFN-α in the CAM assay, these data support and extend the in vitro data on the direct antitumor properties of IFN-α.

Another mechanism by which IFN-α may enhance the antitumor efficacy of a GM-CSF-secreting tumor cell vaccine is by augmenting the vaccine-induced immune response. The increased numbers of IFN-γ-secreting, melanoma-specific T cells (gp100 and Trp2) detected in mice treated with the combination but not in animals treated with either monotherapy emphasize the immunomodulatory properties of the IFN when it is combined with a GM-CSF-secreting vaccine. IFN-α has been shown to enhance the activation and differentiation of human and mouse dendritic cells in vitro and in vivo (12, 13, 15, 43), which may be one mechanism by which it enhances tumor-specific T cell responses when combined with a vaccine. The GM-CSF-secreting tumor cell vaccine has been shown to attract naive dendritic cells to the vaccine site, where these antigen presenting cells take up antigen released from dying tumor vaccine cells. We observed that administration of IFN-α at the vaccine site increases the number of CD11c+ cells at the site of vaccination (data not shown), which may increase CD4+ and CD8+ T cell activation. IFN-α has also been shown to promote the differentiation of Th1 lymphocytes and the generation and activity of tumor antigen–specific CTL by promoting T-cell survival (22, 44, 45, 53–55). Interestingly, a significant increase in the number of CD3+ cells was detected at the vaccine site following local injection of IFN-α plus vaccine.
when compared with vaccine alone (data not shown). Together, these immunostimulatory properties of IFN-α may enhance the initial priming phase of the antitumor response as well as increase the population of tumor-specific memory T cells as shown by the increased response to trp-2 and gp100 in mice treated with the combination.

Surprisingly, increased numbers of IFN-γ secreting OT-1 TCR Tg T cells were not detected in the spleen in mice treated with a B16.GM-OVA vaccine plus rmIFN-α compared with animals treated with vaccine alone. OT-1 T cells increased in animals challenged with OVA-expressing B16F10 cells but not vaccinated with B16.GM-OVA, demonstrating the weak stimulus apparently necessary for these cells to become activated. Vaccination with B16.GM-OVA increased the overall OT-1 response strongly, most likely reaching a plateau, because addition of rmIFN-α did not potentiate this response. However, we were able to detect a moderate increase in the number of OT-1 T cells residing in the local lymph nodes 24 hours after the second IFN-α injection as well as prolonged survival of these T cells only in animals that received GM-secreting tumor cell vaccine in combination with IFN-α. In contrast, IFN-α in combination with the GM-CSF-secreting tumor cell vaccine led to an increase in gp100- and Trp-2-specific responses over that observed with vaccine only. This discrepancy may reflect differences in the signals required to activate T-cell receptor transgenic T cells versus endogenous, naive, melanoma-specific T cells.

Whereas clinically meaningful antitumor activity has been shown for IFN-α systemic therapy in certain malignancies including, in particular, chronic leukemia, toxic side effects due to repeated systemic administration at high doses of IFN-α limits its clinical application (56, 57). The therapeutic benefit of IFN-α as a monotherapy may depend more upon its direct antitumor properties, which require high systemic concentrations. However, when used as an adjuvant with a GM-CSF-secreting tumor cell vaccine it may be possible to administer subclinical doses of IFN-α at the vaccine site, improving therapeutic benefits whereas limiting systemic toxicity. In the adjuvant setting its therapeutic potential may depend more upon its immunomodulatory properties (e.g., promoting APC maturation and increasing tumor-specific T-cell responses and survival rather than the direct antitumor mechanisms), although the direct mechanisms can not be dismissed as a possibility.

In summary, this study suggests that the various properties of IFN-α can be exploited in combination with immunotherapies for the treatment of cancer. More specifically, IFN-α in combination with a GM-CSF-secreting whole cell vaccine may represent a promising clinical combination for tumor immunotherapy, especially for cancers such as renal cell carcinoma, for which IFN-α is currently used as first-line therapy.

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