Synergistic Antitumor Effects of a Combination of Interferons and Retinoic Acid on Human Tumor Cells in Vitro and in Vivo

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ABSTRACT

Solid tumors are relatively resistant to growth inhibition by IFNs. To enhance sensitivity, we assessed combinations of IFNs with all-trans-retinoic acid (RA). Antiproliferative studies in vitro suggested that the growth of three human breast carcinomas (MCF-7, MDA-MB-231, and MDA-MB-468), an ovarian carcinoma (NIH-OVCA-3), and a malignant melanoma (SK-MEL-1) was inhibited to a greater degree by combination treatment with human IFN-β and RA compared to single agents. Some of these cell lines were resistant to 10–100 IU/ml human IFN-α2b or IFN-β or to 0.1–1.0 μM RA. Growth was inhibited significantly by combinations of IFNs and RA in all cell lines tested, and in some cases, cytotoxicity was observed. Sequential treatment of MCF-7 cells with RA followed by IFN-β was more effective at inhibiting growth than treatment with IFN-β followed by RA, suggesting that RA modulated the antiproliferative response of IFN-β rather than the converse. In nude mice, the growth of MCF-7 and NIH-OVCA-3 tumors was suppressed completely when combination treatment was started 2 days after tumor inoculation. Established, 6-week-old NIH-OVCA-3 tumors underwent regression when treated with the combination of IFN-β and RA but not with single-agent therapy. Together with our recent studies that demonstrated enhancement of IFN-stimulated gene expression by RA pretreatment in IFN-resistant cells, these data suggest that combination treatment with RA and IFNs may increase IFN-stimulated gene expression in IFN-resistant tumors, leading to augmented antitumor effects.

INTRODUCTION

IFNs regulate cellular, antitumor, antiviral, and immunological responses. IFNs have been successfully used in the treatment of malignancies, viral infections, and multiple sclerosis. However, despite proven clinical effectiveness, not all patients respond therapeutically to IFNs, and in other patients, clinical resistance emerges over time. RA, a potent biological response-modifying metabolite of vitamin A, is like IFNs, capable of inducing gene expression. RA also inhibits growth of some human tumors in the xenograft model (1–6) and has clinical effectiveness in squamous carcinoma and acute promyelocytic leukemia (7, 8).

Our previous studies using F9 embryonic carcinoma cells indicated that these cells were unresponsive to IFNs and exhibited no detectable transcription of ISGs (9). Upon exposure to RA, F9 cells readily differentiate in vitro. Differentiated F9 cells respond to IFNs and express ISGs. Similarly, RA treatment of MCF-7 breast cancer cells, which are relatively IFN resistant, resulted in restoration of IFN responsiveness that was mediated by elevations of transcription factor STAT1 protein levels. Elevation of STAT1 levels allowed formation of the IFN-regulated transcription factor, ISGF-3, and effective transcription of ISGs (10).

The inference from these studies was that treatment with RA might enhance the antiproliferative and antitumor effects of IFNs via enhanced ISG expression. In this study, we extended our previous studies on the effects of IFNs and RA, alone and in combination, to the proliferation of human tumor cell lines and the growth of xenografts in a nude mouse model. Our observations verify predictions from biochemical data and suggest that RA can overcome resistance to cellular effects of IFNs. They provide a rationale for the clinical use of IFNs and RA in combination against several different tumor types.

MATERIALS AND METHODS

Mice. Female athymic nude (nu/nu) BALB/c mice, 3–4 weeks of age, were oophorectomized by the supplier (Harlan Sprague Dawley, Indianapolis, IN) at ages 2–3 weeks. Mice were housed at a density of no more than five mice in each cage with microisolator tops and autoclaved bedding. They were fed autoclaved Purina Lab Rodent Chow 5010 (Menomonee Falls, WI) and HCl-acidified (pH 3) tap water ad libitum and were placed in rooms with controlled temperature (22–24.5°C), humidity (40%), and 12-h light-dark cycles. Mice were housed in the animal facility for at least 1 week prior to use.

Animal Procedures. Procedures involving animals and their care were conducted in conformity with the institutional

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3 The abbreviations used are: RA, all-trans-retinoic acid; ISG, IFN-stimulated gene; STAT1, signal transducing activator of transcription 1; ER, estrogen receptor.

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**Cell Lines.** MCF-7, MDA-MB-231, MDA-MB-468 human breast carcinoma, NIH-OVCAR-3 human ovarian carcinoma (11), and SK-MEL-1 human malignant melanoma (American Type Culture Collection, Rockville, MD) were cultured in Eagle’s MEM containing phenol red, with 5% fetal bovine serum (Life Technologies, Inc., Grand Island, NY) in a humidified atmosphere of 95% air/5% CO2 at 37°C. NB4 cells were grown in RPMI 1640 with 10% fetal bovine serum. Of the breast carcinomas, MCF-7 is ER positive; MDA-MB-231 and MDA-MB-468 are ER negative (12). Medium was changed every 3 days. Cells were removed from culture flasks for passage by washing once with HBSS, followed by a 5-min incubation with 0.5 mM EDTA and 0.05% trypsin at pH 7.4 and 37°C. For implantation, cells were harvested with trypsin-EDTA and washed twice with PBS at pH 7.4. Their viability was determined by microscopic examination of cells stained by 0.1% trypan blue. Viable cells (2 x 106) were inoculated into mice in a volume of 0.1 ml of PBS.

**Reagents.** Human IFN-β (Berlex Laboratories, Richmond, CA), specific activity 3.2 x 107 IU/mg protein, was diluted in normal saline to a concentration of 104 IU/ml. Human IFN-α2b (Schering, Kenilworth, NJ) was adjusted to the same concentration. Mice received 104 IU IFN-β in a single dose once a day (0.1 ml/day s.c.). RA (Sigma Chemical Co., St. Louis, MO) was suspended in 2% (w/v) hydroxy propyl cellulose vehicle at 3 mg/ml. Mice received 0.1 ml (0.3 mg) by gavage daily. 17β-Estradiol (Sigma) was mixed in a 1:3 ratio (w/w) with 3112 RTV silicone elastomer (Dow Corning, Corning, NY) into a slurry. The slurry was injected into 2-cm long, 0.125-inch outside diameter silastic tubing and the ends were sealed with silicone adhesive. Estradiol containing capsules were sterilized with 200,000 Rads γ-irradiation. Because MCF-7 tumors do not grow without estradiol, all athymic oophorectomized nude (nu/nu) mice inoculated with MCF-7 tumors received s.c. estradiol capsule implants 7 days prior to tumor inoculation. Following anesthesia with 0.3 ml avertin i.p., capsules were inserted into the dorsal s.c. region through a 4-mm incision that was closed with a stainless steel clip. Depleted estradiol capsules were removed and replaced with fresh ones after 6 weeks. Mice inoculated with ER-negative tumor cells received no estradiol supplementation. Xenograft experiments using NIH-OVCAR-3 cells used two different dose schedules, early and delayed. Mice in the early schedule received IFN and/or RA beginning 2 days after tumor cell inoculation. In the delayed schedule, tumors were allowed to grow without IFN or RA treatment for 6 weeks, at which time treatment was started.

**Tumors.** Mice were inoculated with 2 x 106 cultured tumor cells into the dermis overlying the mammary glands nearest the axillae. Tumor volume was calculated using the formula, \( V = 4/3\pi r^3/3 \), where 2x, 2y, and 2z are the three perpendicular diameters of the tumor. Each experimental group contained 10 mice, and each experiment was performed twice. The first time an experiment was performed, each mouse was inoculated with a single tumor. In the repeat experiments, each mouse carried two tumors. The variability in tumor volume observed among individual mice bearing a single tumor was similar to the variability in tumor volume among tumors on the same mouse. Student’s two-tailed t test was used to assess the statistical significance of difference between pairs of means of tumor volume.

**Antiproliferative Assay.** MCF-7, MDA-MB-321, MDA-MB-468, and NIH-OVCAR-3 cell monolayers were harvested with trypsin-EDTA, washed with PBS, and resuspended in phenol red-free Eagle’s MEM containing 5% charcoal-stripped calf serum. Cells were plated in 200 μl of medium in 96-well plates at densities of 2000 cells/well. IFN-β and RA in various concentrations were added to the assay plate. Quadruplicates of each treatment were performed. Cells in the assay plate were exposed to drugs continuously for 4–6 days. In sequential drug treatment assays, MCF-7 cells were treated with one agent for 24 h, washed, and treated with the second agent for 4 days. Cells that received continuous treatment were exposed to IFN-β, RA, or the combination for 5 days. Cells in the assay plate were then fixed by the addition of trichloroacetic acid at 4°C (final concentration, 10%) and incubation at 4°C for 1 h. The assay plate was rinsed with H2O and allowed to air dry. A dye uptake method was used to quantitate cell number (13). Cells were stained with 0.4% sulforhodamine B w/v (Sigma) in 1% acetic acid for 1 h. Dye was removed by inversion, and wells were washed with 1% acetic acid. Dye was eluted from cells with 100 μl of Tris-Cl (pH 10.5), and absorbance was measured at 570 nm. This method of quantitation of cell number gave similar values to results obtained with a Coulter counter.

**RESULTS**

**Effect of IFN and RA on Human Tumor Cell Growth in Vitro.** MCF-7 cells were relatively resistant to the antiproliferative effects of IFN-β (Fig. 1A, columns 2 and 3). Concentrations of IFN-β up to 100 IU/ml resulted in minimal growth inhibition (Fig. 1A, column 3). Treatment with 1 μM RA inhibited MCF-7 cell growth by 30% (Fig. 1A, column 5). Combination of 0.1 μM RA with 10 IU/ml IFN-β caused 80% growth inhibition (Fig. 1A, column 6). The combination of 100 IU/ml IFN-β and 1 μM RA resulted in cytotoxicity (Fig. 1A, column 7, represented as a decrease in cell number relative to the initial number plated). IFN-α2b treatment yielded a similar sensitivity pattern in MCF-7 cells compared to IFN-β (Fig. 1B). Treatment with 100 IU/ml IFN-α2b and 1 μM RA also caused cytotoxicity (Fig. 1B, column 7). Experiments with NIH-OVCAR-3 ovarian carcinoma cells (Fig. 1C) gave a similar pattern of response compared to MCF-7 cells. NIH-OVCAR-3 cells were relatively resistant to 100 IU/ml IFN-β (Fig. 1C, column 3) or 1 μM RA (Fig. 1C, column 5) as single agents, but the combination resulted in cell death (Fig. 1C, column 7) after 5 days of exposure to these agents. Compared to ER-positive MCF-7 cells, the ER-negative breast carcinoma cell lines MDA-MB-231 (Fig. 1D) and MDA-MB-468 (data not shown) were more sensitive to IFN-β (Fig. 1D, columns 2 and 3) and more resistant to RA (Fig. 1D, columns 4 and 5). The combinations of IFN-β and RA (Fig. 1D, columns 6 and 7) resulted in an enhancement of antiproliferative activity compared to IFN-β alone in these two ER-negative cell lines. The human promyelocytic leukemia
Pretreatment with RA resulted in 45% growth inhibition treatment with 1FN43 (Fig. 3A, cell line NB4 was resistant to 100 lU/mi IFN-3 (Fig. 3A, column 2, 100 lU/ml IFN-3; column 3, 100 lU/ml IFN-3; column 4, 0.1 lM RA; column 5, 1 lM RA; column 6, 10 lU/ml IFN-3 + 0.1 lM RA; and column 7, 100 lU/ml IFN-3 + 1.0 lM RA. In B, MCF-7 cells were treated with IFN-α2b instead of IFN-β. At the end of 5 days, cell number was quantitated by sulforhodamine B dye uptake and expressed as a percentage of control (untreated) cell growth. Bars, SE.

Fig. 2 Effect of IFNs and RA on NB4 cell growth in vitro. NB4 promyelocytic leukemia cells were treated with: column 1, no drug; column 2, 100 lU/ml IFN-β; column 3, 1 lM RA; and column 4, 100 lU/ml IFN-β + 1.0 lM RA. In B, NB4 cells were treated with IFN-α2b instead of IFN-β. Bars, SE.

Fig. 3 Effect of sequential treatment on MCF-7 cells and NB4 cells. MCF-7 cells (A) were exposed to one agent for 24 h, washed, and then exposed to a second agent for 4 days: column 1, untreated; column 2, 100 lU/ml IFN-β; column 3, 1 lM RA; column 4, 100 lU/ml IFN-β; then 1.0 lM RA; column 5, 1 lM RA, then 100 lU/ml IFN-β; and column 6, 1.0 lM RA + 100 lU/ml IFN-β. NB4 cells (B) were treated similarly, except that IFN-α2b was used instead of IFN-β. In addition, untreated: column 2, 100 lU/ml IFN-α2b; column 3, 1.0 lM RA; column 4, 100 lU/ml IFN-α2b; column 5, 1.0 lM RA; and column 6, 1.0 lM RA + 100 lU/ml IFN-α2b. Cell number was quantitated as in Fig. 1. Bars, SE.

cell line NB4 was resistant to 100 lU/ml IFN-β (Fig. 2A, column 2), moderately sensitive to 1 lM RA (Fig. 2A, column 3), and most sensitive to the combination of IFN-β and RA (Fig. 2A, column 4). NB4 cells exhibited similar sensitivity to IFN-α2b when compared to equivalent concentrations of IFN-β, as a single agent (Fig. 2B, column 2), and in combination with RA (Fig. 2B, column 4).

To determine whether one biological response modifier was augmenting antiproliferative activity of the other, experiments using sequential treatment of MCF-7 cells with IFN-β and RA were performed. The greatest antiproliferative effect was obtained when both agents were present continuously (Fig. 3A, column 6). Pretreatment with RA, followed by washing and treatment with IFN-β (Fig. 3A, column 5), was more effective than pretreatment with IFN-β followed by RA (Fig. 3A, column 4). Pretreatment with RA resulted in 45% growth inhibition compared to IFN-β pretreatment, which yielded only 18% growth inhibition (P < 0.01). Similarly, pretreatment of NB4 cells with 1 lM RA followed by 100 lU/ml IFN-α2b (Fig. 3B, column 5) was more effective at growth inhibition than IFN-α2b followed by RA (Fig. 3B, column 4). These data suggest that pretreatment with RA sensitized MCF-7 cells and NB4 cells to the antiproliferative effects of IFNs, rather than the converse.

Effect of IFN and RA on ER-positive MCF-7 Tumors. To test the antitumor effects of IFN-β and RA in vivo, human MCF-7 breast carcinoma cells were inoculated into nude mice and were treated with single agents or the combination. When treatment was begun 2 days following tumor inoculation, IFN-β or RA as single agents were effective at growth inhibition (Fig. 4). Treatment was continued for 10 weeks in the groups that received RA or IFN-β alone. At the end of 10 weeks, mean IFN-β-treated tumor volume was 33% of control tumor volume (P < 0.001), and RA-treated mean tumor volume was 62% of...
control tumor volume ($P < 0.001$). However, no tumors were detectable in mice treated with the combination of IFN-β and RA. Thus, neither IFN-β nor RA as single agents were completely effective, but the combination eliminated all tumor growth. In the combination group, RA and IFN-β treatment was stopped after 6 weeks. Estradiol administration was continued for all mice in the IFN-β and RA group for 1 year, replacing depleted estradiol capsules every 6 weeks. Tumors failed to grow, even after the withdrawal of IFN-β and RA treatment, despite the persistent growth-stimulatory effects of estradiol.

**Effect of IFN-β and RA on Estrogen-independent Tumors.** Because the combination of IFN-β and RA synergistically inhibited growth of estrogen-dependent MCF-7 tumors, we next examined whether it could also inhibit the growth of estrogen-independent breast tumors in vivo. RA treatment alone inhibited growth of MDA-MB-231 human breast carcinoma by 52% at the end of 14 weeks ($P = 0.001$; Fig. 5). IFN-β as a single agent inhibited growth by 71% ($P = 0.0001$). The combination of IFN-β and RA inhibited growth by 90% at the end of 14 weeks ($P < 0.0001$). The combination was significantly more effective than IFN-β alone ($P = 0.024$), suggesting that the action of this combination is independent of the ER status of this tumor. Unlike MCF-7 tumors, there was not complete suppression of MDA-MB-231 tumors in mice that received the combination.

MDA-MB-468, another ER-negative breast carcinoma, showed a different pattern of response compared to MDA-MB-231. RA treatment alone had no measurable effect (Fig. 6) on the growth of MDA-MB-468 tumors. IFN-β as a single agent inhibited growth by 48% at the end of 10 weeks ($P = 0.02$). The combination of IFN-β and RA inhibited growth by 53% at the end of 10 weeks ($P = 0.018$). The degree of growth inhibition achieved with the combination was better than the effect of IFN-β alone ($P = 0.041$). Thus, the combination of IFN-β and RA was effective against a tumor that was not inhibited in growth by RA.

To determine whether the combination of IFN-β and RA was effective in nonmammary tumors, similar studies were performed with an ovarian carcinoma cell line. NIH-OVCAR-3 human ovarian carcinoma is estrogen independent in its growth (11). IFN-β and RA were both effective as single-agent therapy and resulted in growth inhibition of 87 and 79%, respectively, after 14 weeks (Fig. 7A). As with MCF-7 tumors, IFN-β plus RA begun 2 days after inoculation resulted in total suppression of tumor growth. Discontinuation of treatment did not result in emergence of occult tumor, even after 1 year of observation.

To examine whether the combination of IFN-β and RA would affect larger established tumors, NIH-OVCAR-3 tumors were allowed to grow without any treatment for 6 weeks. Mice were stratiﬁed such that mean initial tumor volume in the group receiving combination treatment was ﬁve times that of initial tumor volume in the other three groups. When treatment was started on these established tumors (delayed treatment), single-agent treatment resulted in growth rates similar to untreated tumors for approximately 6 weeks (Fig. 7B). After 6 weeks, tumor volumes in mice receiving the combination of IFN-β and
RA started to decrease, declining from a maximum of 0.17 to 0.081 cm³, a 52% reduction over 3.3 weeks \((P = 0.022)\). Control tumors continued to increase in size from 0.189 to 0.329 cm³ after 9 weeks, a 74% increase over the same period of time. Single-agent treatment caused a slowing of tumor growth, but regression was not observed with single-agent treatment (Fig. 7B).

Comparison of tumor growth rates in untreated and the combination group indicated that the mean tumor doubling time was 23 days in the untreated group versus 54 days in the combination group between weeks 1–5 \((P = 0.003)\).

The drug combination was also effective for SK-MEL-1 human malignant melanoma. Each mouse was inoculated with \(2 \times 10^6\) NIH-OVCAR-3 cells (one site/mouse) on day 0. No estradiol supplementation was given. Treatments and graph notations are identical to Fig. 4. RA inhibits growth of one estrogen-independent breast tumor (MDA-MB-231), MCF-7, and NIH-OVCAR-3 human ovarian carcinoma, and SK-MEL-1 malignant melanoma. However, IFN-β by itself did not eliminate tumor growth completely. RA inhibited growth of one estrogen-independent breast tumor (MDA-MB-231), MCF-7, and NIH-OVCAR-3 tumors but had minimal effect on SK-MEL-1 or MDA-MB-468 tumors. Yet, when combined with IFN-β, RA caused a potentiation of growth suppression in these six tumors compared to single-agent treatment.

Growth suppression by RA in \textit{in vivo} occurred in estradiol-independent as well as -dependent cells. Most ER-positive breast carcinoma cell lines are inhibited in their growth by RA in \textit{in vitro}, whereas most ER-negative breast carcinoma cell lines are not (14, 15), consistent with our findings (Fig. 1, D and E). However, other estradiol-independent, non-breast cell lines were inhibited by RA in \textit{in vitro}, including rectal adenocarcinoma (16), neuroblastoma (17), prostate adenocarcinoma (18), ovarian carcinoma (19), acute promyelocytic leukemia (20–22), and small cell lung cancer (23).

IFN-β probably acts directly on human tumor cells growing as xenografts. It is unlikely that human IFN-β, a highly species-specific agent, induces any host effects, because it had no effect on growth \textit{in vitro} or gene induction in murine cells at concentrations of 10,000 IU/ml (data not shown). IFN-α and...
IFN-β appeared to have comparable antiproliferative and anti-tumor activity (Figs. 1B, 2B, and 3B).

Growth arrest and cell death were induced by the combination of IFN-β and RA but not by single agents in vitro, suggesting that the antitumor effects were mediated by direct activity of both agents on the tumor cells. Although transforming growth factor β has been implicated as a mediator of growth suppression by RA, we did not observe enhanced antiproliferative activity as a result of treatment with transforming growth factor β and IFN-β in vitro (data not shown). In the absence of this correlation, an intracellular event, such as induction of a specific cellular gene(s), appears more likely to be the cause of growth arrest (10). We are investigating gene products that might mediate growth inhibition by the combination of IFN-β and RA.

Lastly, these data suggest that the combination of IFN-β and RA may possess greater antitumor efficacy in a wide variety of different tumors compared to single-agent therapy. The combination of IFN-β and RA was particularly effective in the treatment of MCF-7 breast and NIH-OVCAR-3 ovarian carcinomas (Figs. 3 and 6A). In both cell types, the IFN-β and RA combination totally prevented tumor growth when treatment was started 2 days after inoculation. The combination caused partial regression of large, established NIH-OVCAR-3 tumors (Fig. 7B).

Combination of IFN-α or IFN-β with RA has resulted in additive or synergistic growth inhibition of breast carcinoma (10, 24, 25) and cervical carcinoma (26) in vitro. Combinations of IFN-α or IFN-β and retinoids may enhance clinical responses in patients with squamous cell carcinoma of the skin and cervix (27, 28), renal cell carcinoma (29, 30), neuroblastoma (31), bronchogenic carcinoma (32), melanoma (33, 34), and breast carcinoma (35). Augmented antitumor effects of IFN-β may be mediated by enhancement of ISG expression by RA. RA increases expression of STAT1 levels in tumor cells, which facilitates IFN-β-induced gene expression (10). IFN-activated STAT1 protein directly regulates the cell cycle-dependent kinase inhibitor p21/WAF1/CIP-1 gene, resulting in growth suppression (36). Alternatively, the apparent curative effect of this drug combination may be due to enhanced antiangiogenic activity (37, 38) or perhaps due to augmented apoptosis (39). Although studies of intratumoral apoptosis were not performed, there were increased apoptotic bodies in cells exposed to the combination in vitro (data not shown). Despite our lack of knowledge of the identity of the gene product responsible for these antitumor effects, treatment with a combination of IFNs and RA provides a new approach for therapy of tumors that are resistant or partially sensitive to single agents.

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