Interferon-α-induced Activation of Signal Transducer and Activator of Transcription Proteins in Malignant Melanoma

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INTRODUCTION

The worldwide incidence of malignant melanoma is rising faster than any other cancer. Almost 40,000 new cases of melanoma were diagnosed in the United States in 1995, and over 70% of patients with primary melanomas thicker than 4 mm or metastasis to the regional lymph nodes will die of disseminated disease within 5 years of diagnosis (1). Kirkwood et al. (2) recently reported increased overall and disease-free survival in patients with high-risk melanomas who received IFN-α2b as an adjuvant to surgical resection. High doses of IFN-α were used, and toxicities necessitating treatment delays or dose adjustments were encountered frequently. It has been difficult to determine the optimal dose of IFN-α in melanoma patients, in part, because its cellular target(s) and mechanism of action are unknown. In theory, an exogenously administered cytokine might exert an antitumor effect via stimulation of the host immune system or by a direct effect upon the tumor cell or its immediate environment. As for IFN-α, it is possible that both mechanisms of action are in effect, with administration of the cytokine leading to changes within the tumor cell, which make it more susceptible to detection and/or destruction by effector cells of the host immune system (3). IFN-α has distinct immunomodulatory effects, but it is also able to directly inhibit the proliferation of tumor cells, up-regulate their expression of adhesion molecules and MHC class I antigens, and inhibit tumor angiogenesis (4–7). It has been shown that IFN-α can mediate the regression of established melanomas in immunodeficient mice (8, 9), which implies that IFN-α has activity against melanoma cells even in the absence of a fully functional immune system. These studies suggest that the direct effects of IFN-α on the melanoma cell may be important to its antitumor activity. If this is the case, then the actions of IFN-α in the setting of malignant melanoma would be dependent upon its ability to bind to specific receptors on the surface of the melanoma cell and activate an intracellular signaling pathway.

Previous work has demonstrated that IFN-α can directly inhibit the proliferation of melanoma cells in vitro (3, 4), and that malignant melanoma cell lines express receptors for IFN-α (10, 11). However, IFN-α-induced signal transduction and gene regulation in melanoma cells has not been defined. In other cell types, the binding of IFN-α to its receptor stimulates receptor-associated Janus kinases (JAK1 and TYK2) to phosphorylate cytoplasmic proteins belonging to the STAT3 family. These activated STAT proteins (STAT1, STAT2, and to a lesser ex-
tent, STAT3) form high-affinity DNA binding complexes that rapidly translocate to the cell nucleus, where they interact with specific sequences within the promoters of IFN-α-responsive genes and initiate transcription. The prototypical IFN-α signaling reaction recruits a DNA binding complex known as ISGF3, which consists of STAT1α (or STAT1β), STAT2, and a non-phosphorylated protein, p48 (12). ISGF3 binds to a specific DNA sequence known as the ISRE (13). The EMSA has been used to study STAT protein activation and serves as a sensitive tool for the analysis of IFN-induced DNA binding complexes (14). In the present study, we have used the EMSA to analyze IFN-induced activation of STAT proteins in malignant melanoma. I provide data showing that IFN-α stimulates the JAK-STAT signaling pathway in malignant melanoma cell lines and in tumor samples obtained directly from patients. We also demonstrate that this signaling event can be enhanced by pretreatment of melanoma cells with IFN-γ.

MATERIALS AND METHODS

Cell Lines and Reagents. The HT144 cell line (American Type Culture Collection, Rockville, MD) is a melanoma cell line derived from a s.c. metastatic deposit in the lower extremity of a 29-year-old male (15). This cell line was maintained in McCoy’s 5A media supplemented with 10% FCS. The SKMe1S melanoma cell line (American Type Culture Collection) was derived from a metastatic deposit in the lymphatic system of a 24-year-old female (16). This cell line was maintained in Eagle’s MEM supplemented with 10% FCS. The HeLa cell line was used as a control and was maintained in RPMI 1640 supplemented with 10% FCS (17). Cell lines were harvested from culture via trypsinization, then washed, and counted prior to use. A polyclonal rabbit anti-STAT1 Ab was purchased from Transduction Laboratories (Lexington, KY) and used in supershift assays as described previously (18). Polyclonal rabbit anti-STAT2, STAT3, and STAT4 Abs were obtained from Sigma Chemical Corp. (St. Louis, MO) and resuspended in 0.1% DMSO. Recombinant human IFN-α2b (specific activity, 2 × 10⁸ IU/mg) was obtained from Schering-Plough, Inc. (Nutley, NJ). Recombinant human IFN-γ (specific activity, 3 × 10⁷ units/mg) was obtained from Genentech, Inc. (San Francisco, CA). All cytokines were resuspended in PBS supplemented with 0.1% human albumin (Armour Pharmaceutical Co., Kankakee, IL).

Preparation of Patient Tumor Samples and Cytokine Treatments. Patient tumors in excess of the pathologist’s requirements were procured at the time of surgery according to a Human Investigations Committee-approved protocol and placed immediately into RPMI 1640 supplemented with 20% FCS. Tumors were minced with scalpel blades, and the resultant cell suspension was filtered through sterile wire mesh to remove particulate matter. Tumor cells were counted and examined for viability via vital dye exclusion. Fresh tumor cell preparations were routinely >85% viable. Frozen tumors were thawed rapidly at 37°C and handled in a similar fashion. Two representative sections of each tumor were processed for routine histology and examined by a staff pathologist. All tumor specimens used in these experiments exhibited the characteristic features of malignant melanoma on light microscopy and were composed entirely of malignant cells. Sites of disease included the axillary or inguinal lymph node basins (patient nos. 1–11), abdominal viscera (patient nos. 12–15), and in transit metastases (patients nos. 16 and 17). None of the patients in this study had received IFN-α previously. Unless otherwise stated, melanoma cells were treated with IFN-α2b for 15 min prior to the preparation of cell lysates (see below). Tumor cells (5 × 10⁶) were used per condition. In some experiments, tumor cells were pretreated with IFN-γ prior to stimulation with IFN-α. After pretreatment with IFN-γ, cells were washed three times, cultured for an additional hour in cytokine-free medium, and washed once again prior to the addition of IFN-α. Overnight culture of melanoma cells in IFN-γ had no significant effect upon cell viability or proliferation.

Proliferation Assays and Flow Cytometric Analysis of MHC Class I Expression. HT144 or SKMe1S cells (2 × 10⁶) were plated in 96-well U-bottomed plates and incubated at 37°C for 24 h in the presence or absence of varying concentrations of IFN-α. Proliferation was measured by incorporation of [methyl-³H]thymidine during the last 12 h of culture as described previously (19). Results represent the mean of triplicate wells and are expressed as cpm of [³H]thymidine incorporation. In one set of proliferation experiments, HT144 cells were treated with IFN-γ (100 ng/ml) or control solution for 24 h prior to the addition of IFN-α. MHC class I expression of IFN-α-treated melanoma cell lines was measured via flow cytometric analysis using a fluorescence-conjugated mAb as described previously (19).

EMSA. Whole-cell extracts were prepared according to the method of Sadowski et al. (14). A double-stranded oligonucleotide representing a modified version of the SE of the c-fos promoter (5'-GGCTGACATTTCCTCCCTCCTTACGGAG-3') served as an indicator for the DNA binding of activated STAT proteins with broadest specificity and highest affinity (14, 18). A double-stranded ISRE oligonucleotide (5'-GGCTGACATTTCCTCCCTCCTTACGGAG-3') was used to assay for the formation of the ISGF3 DNA binding complex (20). Oligonucleotides were end-labeled using T4 kinase and [³²P]dATP. Whole-cell extracts (5 μg) were preincubated in 20 μl of reaction buffer [50 mM HEPES (pH 7.9), 250 mM KCl, 5 mM EDTA, 25 mM MgCl₂, 50% glycerol, 25 mM DTT, and 5 mg/ml BSA] containing 5 μg of poly(deoxyinosinose-deoxycytidylic acid) for 15 min on ice. Labeled probe (20,000 cpm) was then added, and the binding reaction was continued for 15 min at room temperature. Ten μl of the reaction mixture were loaded onto a 4% polyacrylamide gel in 0.5× Tris-borate EDTA buffer and electrophoresed at 120 V. The radioactive pattern was visualized by overnight autoradiography. In supershift assays, the binding reaction was carried out in the presence of anti-STAT Abs for 1 h on ice prior to the addition of radiolabeled oligonucleotide. In one set of experiments, the binding reaction was supplemented with 200 ng of excess unlabeled SIE probe. A 21-bp double-stranded oligonucleotide corresponding to the consensus sequence of the NFκB binding site (5'-AGTGT-GAGGGGACTTCTCCAGGC-3') was used as a control (21).
IFN-α Inhibits Proliferation and Up-Regulates MHC Class I Expression in a Melanoma Cell Line. In our initial experiments, we set out to identify a series of melanoma cell lines that were able to respond to IFN-α at the cellular level. It has been demonstrated previously that IFN-α can inhibit the proliferation of melanoma cell lines as well as up-regulate their MHC class I expression. In our experiments, we used the HT144 cell line for our studies.

Western Blot. The HT144 melanoma cell line was cultured for 18 h in medium alone or medium supplemented with IFN-γ (100 ng/ml). Cell lysates were prepared from these cultures, quantitated, equally loaded (30 μg per lane), separated by SDS-PAGE, and transferred to nitrocellulose as described previously (18). Preblocked nitrocellulose sheets were incubated with rabbit polyclonal antibodies to STAT1, STAT2, p48, or IFN-αR (Santa Cruz) for 1 h at room temperature. Immune complexes were detected via the enhanced chemiluminescence reaction (Amersham).

Statistical Analysis. Statistical analysis of IFN-α induced growth inhibition was performed using Student’s t test, with P < 0.05 being designated as significant. Quantitative measurements of SIE binding complexes were made on an Apple OneScanner. For each condition to be compared, background was subtracted from the SIE signal and analyzed using Scan Analysis software (Biosoft, Ferguson, MO).

RESULTS

IFN-α Inhibits Proliferation and Up-Regulates MHC Class I Expression in a Melanoma Cell Line. In our initial experiments, we set out to identify a series of melanoma cell lines that were able to respond to IFN-α at the cellular level. It has been demonstrated previously that IFN-α can inhibit the proliferation of melanoma cell lines as well as up-regulate their MHC class I expression.
expression of MHC class I antigens (4, 6). We examined the HT144 and SKMel5 human melanoma cell lines to determine their responsiveness to IFN-α. In our proliferation assay, melanoma cells were treated with varying concentrations of IFN-α for 12 h and then pulsed with tritiated thymidine. After an additional 12-h incubation, cells were harvested and analyzed for thymidine incorporation. IFN-α inhibited proliferation of the HT144 cell line in a dose-dependent fashion (Fig. 1A). Maximal inhibition occurred at 10^5 units/ml IFN-α, whereas significant inhibition was apparent at concentrations as low as 10^4 units/ml. Similar results were obtained with the SKMel5 cell line (data not shown). MHC class I expression was also up-regulated in the HT144 cell line after an 18-h incubation in 2 * 10^5 units/ml IFN-α, as determined by flow cytometric analysis (Fig. 1B).

Identification of Activated STAT1 and STAT2 in Melanoma Cell Lines after Treatment with IFN-α. The identity of the STAT proteins activated by treatment of melanoma cells with IFN-α was investigated using a supershift assay. As seen in Fig. 3, and as noted previously, the HT144 cell line exhibits strong SIE binding activity in response to treatment with 10^5 units/ml IFN-α. The addition of an anti-STAT1 Ab to the binding reaction resulted in a supershifted band (18), thus identifying the presence of activated STAT1 in the SIE binding complex. There was no evidence for activation of STAT3 or STAT4. Use of excess unlabeled SIE probe in the binding reaction resulted in the complete elimination of the IFN-α-induced gel shift. In contrast, the addition of excess unlabeled oligonucleotide corresponding to the consensus sequence for the NFκB binding site (21).

Inhibition of STAT Activation with a Protein Tyrosine Kinase Inhibitor. IFN-α signaling normally results in the phosphorylation of specific tyrosine residues on STAT1 and STAT2 (12). This step in the JAK-STAT signaling pathway can be inhibited by compounds such as genistein, which are specific inhibitors of tyrosine phosphorylation (22). HT144 cells were treated with either 10 μM genistein or carrier solution (0.1% DMSO) prior to treatment with IFN-α (10^5 U/ml) or control for 15 min. Results are shown in Fig. 5 and reveal that melanoma cell lines did not exhibit significant STAT activation in the absence of cytokine stimulation. However, dose-dependent DNA binding activity consistent with STAT activation was observed for both cell lines after treatment with IFN-α. Maximal STAT activation was observed at an IFN-α concentration of 10^5 units/ml, and treatment of cells with higher concentrations of IFN-α did not result in a greater degree of SIE binding, as measured by this assay (data not shown).
cells pretreated with genistein do not exhibit evidence of STAT activation in response to optimal concentrations of IFN-α, as determined by DNA binding to the SIE probe. Identical results were obtained with the HeLa cell line (Fig. 5). These data suggest that IFN-α-induced activation of STAT proteins in melanoma cell lines requires the activity of tyrosine kinases.

**Time Course of IFN-α-induced STAT Activation in a Melanoma Cell Line.** Fig. 6 depicts the results of a time course experiment that was undertaken using HT144 cells that had been treated with 10^5 units/ml IFN-α for varying periods of time. Detectable SIE binding activity was apparent within 30 s of cytokine treatment, and maximal binding activity was seen within 2–5 min. A significant decrease in SIE binding activity was noted within 60 min of cytokine treatment, possibly due to the activity of cellular phosphatases (23). Similar results were obtained with the SKMe15 cell line (data not shown). These results indicate that STAT activation in melanoma cell lines after treatment with IFN-α is rapid and similar to that seen for other cytokines that use the JAK-STAT pathway of signal transduction (24, 25).

**Identification of IFN-α-activated STAT Proteins in Melanoma Patient Samples.** Melanoma cells were prepared from fresh or frozen tumor samples obtained directly from patients at the time of surgery. Aliquots of these cells were then exposed to various cytokine treatments, followed by the preparation of whole-cell lysates for use in an EMSA. Fig. 7A depicts the SIE binding activity generated by the treatment of tumor cells with 10^5 units/ml IFN-α or control for 15 min. SIE binding activity was not observed in control-treated cells, but a strong gel shift consistent with STAT activation was seen in all 17 patient tumors in response to treatment with this high dose of IFN-α. Supershift analysis confirmed the presence of activated STAT1 within this DNA binding complex (Fig. 7B). Lysates from IFN-treated tumor cells also exhibited a gel shift in an

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**Fig. 4** Binding of IFN-α-activated STAT proteins to an ISRE probe. In A, HT144, SKMe15, and HeLa cells were harvested from culture and treated for 15 min with IFN-α (10^5 units/ml) or control. Whole-cell extracts were prepared and used in an EMSA with the ISRE radiolabeled probe. In B, HT144 cells were harvested from culture and treated for 15 min with IFN-α (10^5 units/ml) or control. The binding reactions were supplemented with an anti-STAT2 Ab or PBS and incubated on ice for 1 h prior to the addition of radiolabeled ISRE probe. Addition of an anti-STAT3 or anti-STAT4 Ab (Santa Cruz Biotechnology) to the binding reaction had no effect upon the ISRE binding complex (data not shown). The above results are representative of three independent determinations.

**Fig. 5** Inhibition of STAT activation with a protein tyrosine kinase inhibitor. HT144 cells and HeLa cells (5 × 10^6 cells/condition) were harvested from culture and incubated for 2 h in 10 μM genistein (Gen.) or carrier solution (0.1% DMSO). Cells were then washed twice and treated for 15 min with IFN-α (10^5 units/ml) or control (Ctl.). Whole-cell extracts were prepared and used in an EMSA with the ISRE radiolabeled SIE probe. These results are representative of three separate experiments.

**Fig. 6** Time course of IFN-α-induced STAT activation in a melanoma cell line. HT144 cells were harvested from culture and exposed to IFN-α (10^5 units/ml) for varying periods of time. Whole-cell extracts were prepared and used in an EMSA with the SIE probe. This experiment was performed twice with similar results.
Fig. 7 Identification of IFN-α-activated STAT proteins in melanoma patient samples. In A, tumor samples taken from 17 different patients at the time of surgery were placed immediately into RPMI 1640 supplemented with 20% FBS and minced using scalpel blades. The resulting cell suspension was filtered and counted, and aliquots of cells were treated with 10^5 units/ml IFN-α (+) or control (−) for 15 min (5 × 10^6 cells/condition). Whole-cell extracts were then prepared and used in an EMSA with the radiolabeled SIE probe. Panels 16 and 17, tumors that had been previously procured and frozen in DMSO solution. These tumors were thawed at 37°C and handled as above.

B, supershift analysis using the SIE probe. Whole-cell extracts were prepared from two patient tumors that had been treated with 10^4 units/ml IFN-α or control. These lysates were then used in an EMSA with the radiolabeled SIE probe. The binding reactions were supplemented with an anti-STAT1 mAb or PBS and incubated on ice for 1 h prior to the addition of radiolabeled probe. C, supershift analysis using the ISRE probe. Whole-cell extracts were prepared from patient tumors that had been treated with 10^5 units/ml IFN-α or control and used in an EMSA with the ISRE radiolabeled probe. The binding reactions were supplemented with an anti-STAT2 Ab or PBS and incubated on ice for 1 h prior to the addition of radiolabeled probe. D, IFN-α dose-response curve. Cells from a patient tumor were exposed to varying concentrations of IFN-α for 15 min. Whole-cell extracts were then prepared and used in an EMSA with the double-stranded SIE radiolabeled probe. Cells (5 × 10^6) were used for each condition. Each of these experiments is representative of at least three separate determinations.

EMSA using an ISRE probe, and addition of an anti-STAT2 Ab resulted in the disruption of the ISRE binding complex (Fig. 7C). The response of patient tumors to IFN-α was distinctly dose dependent, with STAT activation being noted at concentrations of IFN-α as low as 10^2 units/ml (Fig. 7D). Collectively, these data show that treatment of patient melanoma cells with IFN-α results in activation of the JAK-STAT signaling pathway.

Effects of IFN-γ Pretreatment on IFN-α-induced STAT Activation in Melanoma Cell Lines and Patient Tumors. Previous work by Schindler et al. (17) has shown that pretreatment of IFN-α-responsive cell lines with IFN-γ results in an enhanced response to IFN-α (17), possibly via the up-regulation of STAT1 and/or the p48 protein (26, 27). Expression of the IFN-αR can also be up-regulated by exposure of cells to IFN-γ (28). We therefore investigated whether pretreatment of melanoma cells with IFN-γ would increase their sensitivity to IFN-α. As expected, short-term treatment of melanoma cell lines and patient samples with IFN-γ (100 ng/ml) resulted in the rapid induction of DNA binding activity with affinity for the SIE probe. However, this activity returned to baseline after a 1-h incubation in cytokine-free medium (data not shown). Fig. 8A reveals that untreated tumor cells from a melanoma patient did not respond to IFN-α at concentrations below 10^2 units/ml. In contrast, tumor cells pretreated with IFN-γ, followed by a 1-h rest, were capable of responding to IFN-α at concentrations as low as 10^{-1} units/ml. Similar results were obtained for the HT144 and HeLa cell lines (Fig. 8A, lower two panels). Additional experiments with the HT144 cell line revealed that IFN-γ pretreatments of 4 h or more resulted in statistically significant enhancement of SIE binding in response to 10^6 units/ml IFN-α. Also, a 4-h pretreatment with IFN-γ was nearly as effective as an 18-h pretreatment (Fig. 8B). We subsequently held the pre-
Fig. 8  Effects of IFN-γ pretreatment on IFN-α-induced STAT activation in malignant melanoma. In A, patient tumor (Pt. Tumor) cells, HT144 cells, and HeLa cells were incubated for 18 h in 100 ng/ml IFN-γ or control solution. At the end of the incubation period, cells were washed three times in RPMI 1640 with 20% FBS, cultured for an additional hour at 37°C, and washed once more prior to stimulation with varying concentrations of IFN-α for 15 min. Whole-cell extracts were prepared from control-treated cells (left panels) and IFN-γ-treated cells (right panels), and these were used in an EMSA with the SIE probe. Pre-Tx, pretreatment. In B, HT144 cells were pretreated with 100 ng/ml IFN-γ for varying periods of time, washed, rested for 1 h, and then stimulated with control (Cntl.), 10², or 10⁵ units/ml IFN-α for 15 min. Whole-cell extracts were prepared from these cells and used in an EMSA with the SIE probe. As a control, HT144 cells were incubated in medium supplemented with PBS for 18 h prior to stimulation with IFN-α (far right panel). The response of these cells to 10⁵ units/ml IFN-α was similar to that of freshly isolated cells. Densitometric analysis of these results revealed that IFN-γ pretreatments of 4 h or more significantly enhanced the response to 10⁵ units/ml IFN-α. In C, HT144 cells were pretreated for 18 h with control or varying concentrations of IFN-γ (0.01–100 ng/ml), washed, rested for 1 h, and then stimulated with 10³, 10², or 10¹ units/ml of IFN-α for 15 min. Whole-cell extracts were prepared from these cells and used in an EMSA with the SIE probe. Densitometric analysis revealed that statistically significant enhancement of IFN-α-induced STAT signaling (10³ units/ml) was achievable with concentrations of IFN-γ as low as 0.01 ng/ml. Each of the above experiments is representative of at least three separate determinations.
IFN-α Signaling in Melanoma

Fig. 9 The HT144 cell line was cultured for 24 h in medium supplemented with IFN-γ (100 ng/ml) or control solution. Following this, the culture medium was removed, and cells were washed twice with fresh medium and allowed to rest for 1 h. Cells were then treated with varying concentrations of IFN-α for an additional 24 h. Proliferation was measured by determining the incorporation of [methyl-3H]thymidine incorporation during the final 24 h of culture. Results represent the mean of duplicate wells (cpm); bars, SE. IFN-γ pretreatments (Pre-Tx) significantly enhanced the antiproliferative effects of IFN-α at each dose level (P < 0.05).

IFN-γ Treatments Result in Enhanced Expression of JAK-STAT Signaling Intermediates in a Melanoma Cell Line. IFN-γ pretreatments enhance IFN-α-induced JAK-STAT signaling and also significantly potentiate the antiproliferative actions of IFN-α. In an attempt to detail the mechanism of this effect, we examined HT144 cells for levels of the IFN-αR and JAK-STAT signaling intermediates via Western blot analysis. HT144 cells were incubated for 18 h in medium alone or medium supplemented with IFN-γ (100 ng/ml). Levels of the IFN-αR subunit were only slightly up-regulated after overnight culture of cells in IFN-γ (Fig. 10). However, there was significant enhancement of STAT1, STAT2, and p48 protein levels. These findings suggest that IFN-γ may mediate its effects on IFN-α signaling via alterations in cellular levels of critical signaling intermediates.

DISCUSSION

In the present study, I have demonstrated that IFN-α activates STAT1 and STAT2 in melanoma cell lines and in melanoma tumor samples obtained directly from patients. A distinct dose-response curve was noted for IFN-α-induced STAT signaling. Maximal STAT activation occurred at IFN-α concentra-
tions of $10^4$--$10^5$ units/ml, whereas detectable signaling was routinely observed at concentrations as low as $10^2$ units/ml. Activation of STAT proteins by IFN-α was rapid and could be inhibited by genistein, a tyrosine kinase inhibitor. Although I did not directly demonstrate the presence of the p48 protein in the binding complex induced by IFN-α, the work of Kessler et al. (29) and others (17) suggests that STAT1 and STAT2 cannot bind efficiently to the ISRE sequence in the absence of this factor. Therefore, I would cautiously interpret these data as evidence that treatment of melanoma cells with IFN-α results in the formation of a classic ISGF3 binding complex. To my knowledge, this is the first demonstration of IFN-α-induced STAT signaling in malignant melanoma. The binding of activated STAT proteins to the SIE and ISRE DNA sequences implies that IFN-α is able to stimulate the transcription of IFN-responsive genes in melanoma cells (12). Thus, exogenously administered IFN-α likely exerts a direct effect upon melanoma cells in vivo in addition to any immunomodulatory actions. Whether this direct effect of IFN-α is important to its antitumor activity has yet to be determined. It is clear that IFN-α is able to improve disease-free and overall survival when administered adjunctively to patients who are at high risk for recurrence after surgical resection (2). However, metastatic disease will eventually develop in almost 60% of melanoma patients who have received adjuvant IFN-α therapy (2). In addition, the overall response rate to IFN-α in the setting of metastatic disease is just 15--20% (2). Thus, there appears to be a distinct subset of melanoma patients who will respond favorably to IFN-α. Prospective analysis of STAT signaling in patient tumors may provide important insights regarding the mechanism of action of IFN-α. If the direct antitumor effects of IFN-α are indeed an important component of its mechanism of action, then one might expect resistant tumors to exhibit diminished STAT signaling in response to clinically relevant concentrations of IFN-α (e.g., $10^4$--$10^5$ units/ml). Similarly, it will be important to determine the expression of IFN-αR components, JAK-STAT signaling intermediates, and STAT inhibitory factors in tumors that have recurrent during the course of adjuvant IFN-α therapy (30).

I have found that maximal STAT activation occurs at IFN-α concentrations of $10^4$--$10^5$ units/ml. However, these concentrations of IFN-α cannot be achieved in the serum of melanoma patients, even with current high-dose regimens. For example, Kurzrock et al. (31) found that a s.c. IFN-α dose of $4 \times 10^6$ units/m2/day resulted in peak serum levels of approximately $2 \times 10^2$ units/ml. Similar serum levels of IFN-α were achieved in the Eastern Cooperative Oncology Group Study conducted by Kirkwood et al. (2). In view of these findings, I investigated methods of enhancing signal transduction in malignant melanoma in response to low concentrations of IFN-α. It is known that pretreatment of IFN-responsive cell lines with IFN-γ leads to increased levels of critical signaling intermediates and enhanced signal transduction in response to subsequent challenges with IFN-α (17, 25, 26). IFN-γ can also up-regulate expression of the IFN-αR (28), yet we observed only a modest increase in cellular levels of the IFN-αR subunit after overnight culture of melanoma cells in 100 ng/ml IFN-γ. However, culture of a melanoma cell line in IFN-γ did result in strong up-regulation of STAT1, STAT2, and p48. In vitro studies have shown that IFN-α and IFN-γ exhibit synergistic antiproliferative effects on human melanoma cells and leukemia progenitor cells (32, 33), and there is data from several clinical trials to suggest that the concurrent or sequential administration of IFN-γ and IFN-α may lead to improved antitumor activity (31, 34--38). I found that pretreatment of melanoma cells with IFN-γ consistently resulted in a 4 log-fold decrease in the concentration of IFN-α required for STAT activation. Moreover, IFN-γ pretreatments significantly enhanced the antiproliferative effects of IFN-α. These data suggest that pretreatment of patients with IFN-γ might enhance the actions of low concentrations of IFN-α. I plan to investigate this regimen in a murine model of human malignant melanoma to learn more about its potential applicability to the clinical situation.

In summary, I have shown that IFN-α activates the JAK-STAT signaling pathway in malignant melanoma cell lines and patient tumors, and that pretreatment with IFN-γ drastically reduces the concentration of IFN-α required for STAT activation. These data provide new insights into how IFN-α may mediate its antineoplastic effect in malignant melanoma and how such an effect may be modulated. Analysis of IFN-α signaling pathways may also lead to the identification of specific genes that can serve as targets for antineoplastic strategies.

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