Gene Expression Changes and Signaling Events Associated with the Direct Antimelanoma Effect of IFN-γ

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Abstract

IFN-γ plays a role in the response to melanoma indirectly through its effect on the immune system and directly through its antiproliferative and proapoptotic effects on melanoma cells. To understand the molecular basis for the direct antimelanoma effect of IFN-γ, we studied IFN-induced changes in gene expression and signaling among three human melanoma cell lines (DM6, DM93, and 501mel). These were resistant to the antimelanoma effect of IFN-α, and only DM6 cells exhibited growth inhibition and apoptosis with IFN-γ. Through DNA microarray analysis, we found that the antimelanoma effect of IFN-γ in DM6 was associated with the down-regulation of multiple genes involved in G-protein signaling and phospholipase C activation (including Rap2B and calpain 3) as well as the down-regulation of genes involved in melanocyte/melanoma survival (MITF and SLUG), apoptosis inhibition (Bcl2A1 and galectin-3), and cell cycling (CDK2). The antimelanoma effect of IFN-γ was also associated with the up-regulation of the proapoptotic dependence receptor UNC5H2 and the Wnt inhibitor Dkk-1. Whereas both IFNs were able to activate Stat1 in all cell lines, the delayed activation of the extracellular signal-regulated kinase, p38, and c-Jun NH₂-terminal kinase mitogen-activated protein kinases occurred only in DM6 with IFN-γ, and the effect of IFN-γ on cell growth and survival as well as gene expression in DM6 was dependent on the coordinate activation of MEK1 and p38. These findings provide new insights into the signaling events and gene expression changes associated with growth inhibition and apoptosis in melanoma and may thereby assist in identifying new targets for the treatment of melanoma.

Introduction

Immunotherapy, including the use of cytokines, vaccines, and adoptive transfer of lymphocytes, has dominated the treatment of melanoma for the past two decades (1). The development of agents and techniques to generate melanoma-specific T cells and maximally stimulate lymphocytes has been driven by the concept of immune escape, which underscores the importance of overcoming immune tolerance to treat melanoma (2, 3). Although novel vaccination strategies can generate larger numbers of antigen-specific cytotoxic T cells, and cytokines, such as interleukin (IL)-2, IL-12, IFN-α, and IFN-γ, can activate multiple different immune effector cells, few patients have benefited from these treatment modalities (4, 5).

One reason for this lack of success is the resistance of melanoma cells to growth inhibition and apoptosis following adequate recognition by activated effector cells. Resistance to chemotherapy- and death receptor–induced apoptosis is well described for melanoma and includes such mechanisms as Apaf-1 down-regulation and the up-regulation of IAP, survivin, FLIP, and Bcl-2 (6, 7). These are believed to be in part responsible for resistance to granzyme- and/or death receptor–mediated killing by T cells and natural killer (NK) cells. However, T and NK cells also produce IFN-γ and IFN-α following activation and in the context of tumor recognition, and it is well known that these IFNs can have direct antiproliferative and proapoptotic effects on melanoma cells (8, 9). However, the mechanism underlying these effects remains undefined, as does the basis for resistance to the direct antimelanoma effects of the IFNs.

IFN-γ production is central to the antitumor effect of IL-12 (10), and tumor cell sensitivity to IFN-γ is central to the antiangiogenic/antitumor effect of combination therapy with IL-12 and IL-18 (11). As one hallmark of overcoming immunologic tolerance through vaccination is the generation of IFN-γ-producing T cells (3), one has to consider the possibility that the direct effects of IFN-γ on melanoma may underlie the antitumor effect of immunostimulatory cytokines and vaccines. In that context, tumor cell resistance to IFN-γ may be as central to immune escape as effector cell tolerance.

To understand the molecular basis of the direct antimelanoma effects of IFN-γ, we undertook an analysis of the gene expression changes and signaling events associated with IFN stimulation in melanoma cell lines that were either sensitive or resistant to the antiproliferative and proapoptotic effects of IFN-γ and IFN-α. In this report, we show how the application of DNA microarray analysis to this system has yielded relatively small sets of genes whose change in expression is associated with the antimelanoma effect of IFN-γ and provide evidence linking MEK1 and p38 mitogen-activated protein kinase (MAPK) activation to these effects of IFN-γ on gene expression.

Materials and Methods

Cell culture. Human melanoma cell lines DM6 and DM93 were kindly provided by Drs. James Grichnik and Hilliard Seigler (Duke University Medical Center, Durham, NC), and 501mel was kindly provided by Dr. David Fisher (Dana-Farber Cancer Institute, Boston, MA). Cells were grown in MEM supplied with 5% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were treated with IFN-γ (1,000 units/ml, R&D Systems, Inc., Minneapolis, MN) or IFN-α2a (1,000 units/ml, PBL Biomedical Laboratories, New Brunswick, NJ) in the presence or absence of 10 µmol/L concentrations of either PD98059, SB203580, or SP600125 (Biomol, Plymouth Meeting, PA). Cultures were maintained at 37°C with 5% CO₂.
Immunofluorescence microscopy. Cell lines were cultured on coverslips and grown in MEM containing 5% FBS. The cells were treated with or without IFN-γ for 48 hours. This was followed by permeabilization and fixation using 0.5% Triton X-100 (3 minutes) and 100% methanol (8 minutes at 20°C). After a 30-minute incubation in blocking buffer (1.5% goat serum in PBS) and 60-minute incubation with anti-ki-67 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the cells were incubated with a FITC-conjugated goat anti-rabbit IgG antibody (diluted 1:750, Molecular Probes, Eugene, OR) for 1 hour at room temperature. Counterstaining was done with 4′,6-diamidino-2-phenylindole, and cells were analyzed under a fluorescence microscope (Nikon Optiphot, Melville, NY).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. Apoptotic cells were visualized by in situ terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay described by the manufacturer (Promega, Madison, WI). Briefly, the cell lines were grown on coverslips and treated with or without IFN-γ for 48 hours. The cells were fixed in 4% methanol-free formaldehyde solution (pH 7.4) for 25 minutes at 4°C. After permeabilizing cells in 0.2% Triton X-100 for 60 minutes at room temperature, the cells were incubated with rTdT buffer for 30 minutes at 37°C. The reaction was terminated using 2× SSC for 15 minutes at room temperature. The cells were analyzed under a fluorescence microscope.

Cell cycle analysis. Cell lines were split evenly into 100 mm dishes the day before transfection and grown in antibiotic-free MEM containing 5% FBS to 80% to 90% confluence. The cells were treated with or without IFN-γ for 48 hours. The suspension cells and adherent cells were harvested and combined. Cells were fixed with 3 mL absolute ethanol for at least 1 hour at 4°C. The cells were stained with a 50 μg/mL propidium iodide (BD Biosciences, San Jose, CA) solution containing 10 μg/mL RNase A (Roche Applied Science, Indianapolis, IN) for 3 hours at 4°C. The cells were analyzed by flow cytometry using the CelQuest program.

Arrays. Arrays were printed at the Duke Microarray Facility using the Genomics Solutions OmniGrid 100 Arrayer. The arrays contain the Operon Human Genome Oligo Set version 2.1 (Operon, Huntsville, AL) that possess 21,329 optimized 70-mer, representing 21,329 genes.

Probe preparation and microarray hybridization. Total RNA (10 μg) from each cell line and the reference (Universal Human Reference RNA, Stratagene, La Jolla, CA) was hybridized to oligo(dt) primers at 65°C and then incubated at 42°C for 2 hours in the presence of reverse transcriptase, Cy5- or Cy3-dUTP and Cy5- or Cy3-dCTP, and a deoxynucleotidyl mix. NaOH was used to destroy residual RNA. Cell line and reference cDNA were pooled, mixed with 1× hybridization buffer (50% formamide, 5× SSC, and 0.1% SDS), Cot-1 DNA, and poly-deoxyadenylate acid to limit nonspecific binding, and heated to 95°C for 2 minutes. This mixture was pipetted onto a microarray slide, coverslipped, and hybridized overnight at 42°C. The array was then washed at increasing stringencies and scanned on a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). All protocols are available in greater detail on the Duke Microarray Facility Web site (http://mgen.duke.edu/genome/dna_micro/core/spotted.htm).

Data processing and statistical analysis. Genespring 6.1 program (Agilent Technologies, Silicon Genetics, Redwood City, CA) was used to perform data analysis. Intensity-dependent (Lowess) normalization was done on the entire data set. Based on triplicates of each condition, a threshold of 2-fold increase or decrease in expression relative to the control and a two-way ANOVA with a P cutoff of 0.05 was done. The expression of each gene was reported as the ratio of the value obtained for each condition relative to the control conditions after normalization of the data. All raw data files and gene lists are found at http://data.cgt.duke.edu/inf.php.

Western blotting. Following indicated treatment, whole-cell extracts were prepared in lysis buffer [20 mmol/L Tris-HCl (pH 8.0), 1% Igepal/NP40, 10 mmol/L EDTA, 137 mmol/L NaCl, 100 mmol/L NaF; complete protease inhibitor mixture (Roche Molecular Biochemicals, Mannheim, Germany)] and complete phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Protein in the lysates was quantitated and equal amounts were subjected to SDS-PAGE and immunoblotting. Blots were incubated with the indicated primary antibody and then incubated with horseradish peroxidase–conjugated goat anti-mouse, goat anti-rabbit, or rabbit anti-goat IgG antibody for 1 hour at room temperature. Blots were then developed using the enhanced chemiluminescence system (Pierce, Rockford, IL). Primary antibodies used included antibodies to Bcl2A1 (Abgent, San Diego, CA), CDK2 (BD PharMingen, San Diego, CA), CDH3 (Zymed, South San Francisco, CA), tubulin (NeoMarkers, Fremont, CA), phospho-Stat1 (Santa Cruz Biotechnology, Stat1 (Santa Cruz Biotechnology), MEKK1 (NeoMarkers), MEKK2 (Abgent), and MEKK3 (amino acids 27-135; BD PharMingen). Antibodies to phospho–extracellular signal-regulated kinase (ERK) 1/2, ERK1/2, phospho-p38, p38, phospho–c-Jun NH2-terminal kinase (JNK), and JNK were all purchased from Promega.

Reverse transcription-PCR analysis. Total RNA was extracted from cells using TRIzol reagents. Reverse transcription-PCR (RT-PCR) was done according to the protocol provided by the manufacturer (Invitrogen Corp., Carlsbad, CA). Total RNA (50 ng) was used per RT-PCR reaction. The specific primers were as follows: MITF forward 5′-CAAGCGCGCAGGTAAGAAG-3′ and reverse 5′-GGCCAGTGTCTTGGCTTCA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5′-ACGTGTAGGTCCTCAGTGGC-3′ and reverse 5′-GCGGGCATCACCCGAGT-3′; calpain 3 forward 5′-GGCCGATGCTCTGCAATGCA-3′ and reverse 5′-TAGGGTGAGGGGACAGTAGC-3′; UNC5H2 forward 5′-AGACCCCTGAACACCCCTG-3′ and reverse 5′-TGAAGCTCTGGAGGAGGAAAG-3′; netrin-1 forward 5′-CAACACTGTCGCTGCAGGC-3′ and reverse 5′-TAGAAGTACGAGTCGGC-3′; Bcl2A1 forward 5′-GCTGGTCGACACATCTGTC-3′ and reverse 5′-GGGCCAATT- TGTCCGTGATAG-3′, CDK2 forward 5′-GCGCCATTGCAGGTGTGATG-3′ and reverse 5′-CCAGATGCACAGGTCGTTG-3′; Rap2B forward 5′-GCTCACTGCAGCTCTGCA-3′ and reverse 5′-GGCGATGCTCTGCAATGCA-3′, and JNK forward 5′-GCGCCACGTCTGTGAGTAC-3′ and reverse 5′-ACCTG- TGAAGTACGAGTCGGC-3′.

Results

Differential response of human melanoma cell lines to the antimalanoma effect of IFN-γ. To develop a system that could aid in identifying genes and signaling pathways involved in the antimalanoma effect of IFN-γ, we obtained cell lines that differed in their ability to exhibit growth inhibition or undergo apoptosis in response to IFN-γ. Among three human cell lines derived from melanoma metastases (DM6, DM93, and 501mel), IFN-α did not affect the growth or viability of any. Whereas DM93 and 501mel grew normally in the presence of IFN-γ, DM6 cells incubated with IFN-γ were fewer and the surviving cells were larger and flatter compared with cells cultured in medium alone (Fig. 1A). This effect of IFN-γ on DM6 took 48 hours to appear. IFN-γ inhibited proliferation and induced apoptosis in DM6 as shown by Ki-67 staining and TUNEL assay (Fig. 1B) as well as by propidium iodide staining and flow cytometry (Fig. 1C).

Genes involved in G-protein signaling, apoptosis, cell cycling, adhesion, and Wnt signaling are uniquely regulated by IFN-γ in DM6 cells. To find changes in gene expression involved in the antimalanoma effect of IFN-γ, we used our system of three melanoma cell lines in which growth inhibition and apoptosis occurred only in DM6 and only in response to IFN-γ. This permitted two levels of analysis regarding changes in gene expression: (a) the effects of IFN-γ versus IFN-α within each cell line and (b) the effects of IFN-γ and IFN-α across the three cell lines.

Changes in gene expression in each cell line in response to 1,000 units/mL IFN-γ or IFN-α following a 48-hour incubation, relative to cells incubated for the same time period in medium alone (control), were examined using the Operon Human Genome Oligo spotted array, permitting the analysis of 21,000 genes. Setting the threshold at 2-fold increase or decrease in expression relative to the control (two-way ANOVA, P cutoff of 0.05 for analyses done in triplicate), the expression of many genes was affected by either or both IFNs in all cell lines (DM6: 1,560 genes modulated by IFN-γ and 492 by IFN-α; DM93: 45 by IFN-γ and 462 by IFN-α; and 501mel: 889 by IFN-γ and IFN-α; of IFN-γ and 462 by IFN-α; and 501mel: 889 by IFN-α).
molecule implicated in the survival of pigmented epithelial cells in the macula and regenerative cells in hair follicles (Fig. 2C).

Genes up-regulated by IFN-γ included Dkk-1 (a secreted inhibitor of canonical Wnt signaling), the dependence receptor UNC5H2, DSCR1 (an endogenous calcineurin inhibitor), the tumor suppressor gene deleted in liver cancer-1, and the antiangiogenic thrombospondin-1 (THBS1; Fig. 2C). Both UNC5H2 and THBS1 were the only genes up-regulated by IFN-γ known to have direct proapoptotic effects.

The DNA microarray data were validated for select genes of interest by Western blot and/or semiquantitative RT-PCR (Fig. 3), with the effect of IFN-γ on gene expression matched by a similar effect on protein expression (Fig. 3A). Although the microarray data for MITF, a transcription factor involved in melanocyte and melanoma survival, were not consistent across multiple experiments, RT-PCR (Fig. 3D) and Western blotting (data not shown) reproducibly confirmed that its expression was also selectively down-regulated by IFN-γ in DM6 but not in DM93 or 501mel (Fig. 2C). The dependence receptor UNC5H2 induces apoptosis in the absence of its ligand, netrin-1, and it was therefore of interest that UNC5H2 up-regulation by IFN-γ in DM6 occurred in the absence of netrin-1 expression (Fig. 3C). The antiproliferative and proapoptotic effects of IFN-γ on DM6 took ~ 48 hours to appear. This corresponded to the observed period for gene expression down-regulation, as maximal suppression of those genes uniquely affected by IFN-γ in DM6 was not observed until 48 hours (Fig. 3D). The same pattern was seen for select genes up-regulated by IFN-γ, including UNC5H2, THBS1, and Dkk-1 (data not shown).

IFN-γ induces the delayed activation of extracellular signal-regulated kinase, p38, and c-Jun NH2-terminal kinase mitogen-activated protein kinases in DM6 cells but not in DM93 or 501mel cells. Signaling by IFN-γ and IFN-α is mediated largely through the Jak/Stat1 and MAPK pathways (12, 13). To determine which signal transduction events were mediating the effect of IFN-γ on the expression of genes modulated only in DM6, we analyzed Stat1 and MAPK activation by IFN-γ and IFN-α in the three melanoma cell lines. In DM6, both IFN-α and IFN-γ induced Stat1 tyrosine phosphorylation to a similar extent at 30 minutes (Fig. 4A). This activation had dissipated almost completely after 24 hours of continuous IFN exposure and was gone at 48 hours, although Stat1 protein expression was increased at 24 to 48 hours. A nearly identical pattern of Stat1 activation by both IFNs was observed in DM93 and 501mel cells. Although the early phase of Stat1 activation was more pronounced in the absence of its ligand, netrin-1, and it was therefore of interest that UNC5H2 up-regulation by IFN-γ in DM6 occurred in the absence of netrin-1 expression (Fig. 3C). The antiproliferative and proapoptotic effects of IFN-γ on DM6 took ~ 48 hours to appear. This corresponded to the observed period for gene expression down-regulation, as maximal suppression of those genes uniquely affected by IFN-γ in DM6 was not observed until 48 hours (Fig. 3D). The same pattern was seen for select genes up-regulated by IFN-γ, including UNC5H2, THBS1, and Dkk-1 (data not shown).

Antiproliferative and proapoptotic effects of IFN-γ in DM6 melanoma. DM6 cells were incubated for 48 hours in medium alone or IFN-γ. Cell number and morphology were evaluated using light microscopy (A). Proliferation and apoptosis were analyzed by fluorescence microscopy following Ki-67 staining or TUNEL assay (B) as well as by propidium iodide staining and flow cytometry (C).

IFN-γ and 767 by IFN-α. This showed that IFN-γ and IFN-α were modulating the expression of many genes in all cell lines, although the antimalanoma effect of IFN-γ occurred only in DM6.

To narrow this further to genes implicated in the antiproliferative and proapoptotic effects of IFN-γ, we generated two lists consisting of genes whose ≥2-fold decrease or increase relative to control was occurring only or, for a few genes, predominantly with IFN-γ in DM6 compared with DM93 or 501mel. This approach yielded 32 genes uniquely down-regulated by IFN-γ in DM6 and 40 genes that were up-regulated by IFN-γ (Fig. 2A and B). Genes included in the down-regulated list had an expression level of at least 2.0 in DM6 cells treated with medium alone, whereas genes included in the up-regulated list had an expression level of at least 2.0 in DM6 cells treated with IFN-γ. Among the genes down-regulated by IFN-γ were seven genes involved in G-protein signaling (including Ras and Rab GTPases, G-protein-coupled receptors, and calpain 3), two inhibitors of apoptosis (Bcl2A1 and LGALS3, also known as galectin-3), a transcription factor implicated in melanocyte survival (Slug), CDK2 (the cyclin-dependent kinase, which along with cyclin E is overexpressed in melanoma compared with normal melanocytes), the drug resistance gene glutathione S-transferase, and P-cadherin, an adhesion molecule implicated in the survival of pigmented epithelial cells in the macula and regenerative cells in hair follicles (Fig. 2C).

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incubation (Fig. 4B). Both IFN-α and IFN-γ weakly activated ERK1/2 at 30 minutes in DM6, but at the later 24 and 48 hour time points activation was seen only in response to IFN-γ. IFN-α failed to augment p38 or JNK phosphorylation at either early or late time points in DM6 (Fig. 4B). The stimulation of ERK1/2, p38, and JNK phosphorylation by IFN-γ in DM6 at 24 to 48 hours was not associated with an increase in protein levels for these MAPKs. The effect of IFN-γ on cell proliferation, apoptosis, and gene expression in DM6 is dependent on MEK1 and p38 activation. The delayed activation of the ERK1/2, p38, and JNK MAPKs by IFN-γ in DM6 corresponded to the delayed effect of IFN-γ on cell proliferation, apoptosis, and gene expression. The inability of IFN-α to exert this same effect on MAPK activation in DM6, along with the failure of IFN-α or IFN-γ to augment MAPK phosphorylation in DM93 and 501mel, suggested that one or more of these MAPKs were mediating the antimelanoma effect of IFN-γ. To determine whether this was the case, DM6 cells were cultured for 48 hours with IFN-γ either alone or in the presence of specific inhibitors of MEK1 (which usually results in ERK inhibition), p38, or JNK. The MEK1 and p38 inhibitors blocked the proapoptotic and antiproliferative effects of IFN-γ, whereas the JNK inhibitor had no effect (data not shown). However, MEK1 inhibition did not block ERK1/2 activation by IFN-γ; in fact, ERK1/2 phosphorylation by IFN-γ was augmented by the MEK1, p38, and JNK inhibitors (Fig. 5A). These inhibitors by themselves, in the absence of IFN-γ, had no effect on basal ERK1/2 phosphorylation (data not shown). These results suggested that both MEK1 and p38 activation were necessary for the antiproliferative and proapoptotic effects of IFN-γ in DM6, whereas ERK1/2 and JNK were not.

If the gene expression changes uniquely induced in DM6 cells by IFN-γ were central to the antimelanoma effect of IFN-γ, then inhibition of MEK1 or p38 should prevent these changes. To test this hypothesis, gene expression was examined through DNA microarray analysis in DM6 cells cultured with IFN-γ alone, MEK1 inhibitor alone, or IFN-γ plus MEK1 inhibitor. The MEK1 inhibitor partially or completely reversed the effect of IFN-γ on many of the genes uniquely down-regulated (Fig. 5B) or up-regulated (Fig. 5C) by IFN-γ in DM6 and affected only a few genes not included in the lists in Fig. 2A and B. The MEK1 inhibitor by itself was also able to up-regulate the expression of some genes down-regulated by IFN-γ.
(Fig. 5B) and likewise could by itself down-regulate some genes up-regulated by IFN-γ (Fig. 5C). Using semiquantitative PCR for select genes, such as Bcl2A1, CDK2, MITF, and UNC5H2, we observed that p38 inhibition had the same effect on IFN-γ-induced changes in expression, whereas JNK inhibition did not (data not shown).

IFN-γ does not modulate MEKK expression in DM6 cells. The ability of IFN-γ to stimulate the phosphorylation of ERK1/2, p38, and JNK in DM6 cells suggested that it was using MEKK1, MEKK2, and/or MEKK3 to coordinately activate all three MAPKs, because each of these MEKks is capable of activating ERK, p38, and JNK (14). To determine whether MEKK expression differed among the three cell lines or could be modulated by the IFNs, we examined MEKK1, MEKK2, and MEKK3 expression by Western blotting in untreated cells and in cells treated with IFN-γ or IFN-α. MEKK1, MEKK2, and MEKK3 were expressed in all of the cell lines, with higher expression of MEKK1 and MEKK3 in 501mel compared with the other two cell lines (Fig. 6A). Expression of these MEKks was unaffected following a 48-hour incubation with either IFN (Fig. 6A and B).

Discussion

The data shown here represent the first in-depth examination of the gene expression changes and signaling events associated with the direct antiproliferative and proapoptotic effects of IFN-γ in human melanoma cells. Despite the prominent role played by IFN-γ in the immune response to melanoma (15) and the established activity of IFN-α in stage III and IV melanoma (16), the mechanisms underlying the antimelanoma effect of these IFNs have remained undefined. Although the results presented in this report perhaps raise as many questions as they answer, we believe that they provide a basis for beginning to understand the determinants of melanoma cell sensitivity and resistance to IFN-γ.

The changes in gene expression associated with the direct antimelanoma effect of IFN-γ were striking, as these involved genes or groups of genes previously implicated in the malignant phenotype of melanoma as well as genes not previously thought to be involved in melanoma growth and survival. Of particular interest was the IFN-γ-induced down-regulation of seven different genes involved in G-protein signaling. Two prior reports have implicated G-protein signaling in the malignant phenotype of melanoma. One of these showed that Wnt5a was central to melanoma cell invasiveness and motility and suggested that G-protein-based noncanonical Wnt signaling, leading to protein kinase C (PKC) activation, was mediating this effect of Wnt5a (17). The other report found that genes involved in G-protein signaling and intracellular calcium regulation best discriminated melanoma from other cancer types (18). Genes highly expressed in melanoma compared with other malignancies included regulator of G-protein signaling 12 and 14, phospholipase C (PLC)-γ, inositol-1,4,5-triphosphate-3 kinase B and C, PKC-μ, and calpain 3. We found that IFN-γ down-regulated four proteins belonging to the Ras superfamily of small GTPases (RAB27A, RAB38, RAGD, and Rap2B).

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**Figure 3.** Validation of gene expression changes derived from DNA microarray data. Semiquantitative RT-PCR (A–C) and Western blotting (A) were used to analyze RNA and protein expression for the indicated genes in DM6, DM93, and 501mel cells following a 48-hour incubation with either medium alone, IFN-γ, or IFN-α2a. GAPDH and tubulin were used as controls for semiquantitative RT-PCR and Western blotting, respectively. The time course of gene down-regulation by IFN-γ in DM6 cells determined via semiquantitative RT-PCR is shown for the indicated genes in (D).
as well as two G-protein-coupled receptors. The down-regulation of Rap2B may be of particular significance, for studies have shown that Rap2B, following activation by heterotrimeric G-protein signaling, interacts with and activates PLC-ε, which in turn can affect cell growth by activating the Ras-Raf-ERK kinase-ERK pathway, increasing intracellular calcium, and activating PKC (19, 20). It is possible, therefore, that IFN-γ is affecting melanoma cell growth by inhibiting Rap2B/PLC-ε signaling. We also found that INF-γ down-regulated calpain 3, the calcium-regulated protease whose expression in melanoma is unique compared with other tumor types (18). Although the manner by which calpain 3 expression or cellular localization might contribute to the malignant phenotype of melanoma is unknown, it is of interest that PLC-β binding and activation by Gq/γ (a component of heterotrimeric G-proteins) is dependent on calpain-mediated cleavage of the PLC-β COOH terminus (21). Therefore, IFN-γ could conceivably be blocking signaling by both PLC-β and PLC-ε by down-regulating calpain 3 and Rap2B, respectively. Lastly, IFN-γ up-regulated the expression of DSCR1, which is an endogenous inhibitor of calcineurin that can block NFATc-mediated gene expression (22, 23). This represents another mechanism through which IFN-γ may have interfered with calcium-regulated signaling events in DM6.

The down-regulation of MITF and SLUG was also associated with the antimelanoma effect of IFN-γ. Both of these transcription factors have been implicated in melanocyte and/or melanoma cell viability (24, 25), and it is therefore significant that IFN-γ can modulate the expression of both in melanoma. However, although the effect of MITF on melanocyte and melanoma survival has been linked to its control of Bcl-2 expression (24), the down-regulation of MITF in the DM6 cell line was not associated with any change in Bcl-2 expression. Rather, the two antiapoptotic proteins down-regulated by IFN-γ in DM6 were Bcl2A1 (26, 27) and galectin-3 (28). The role of Bcl2A1 or galectin-3 in the resistance of melanoma to apoptosis is currently undefined, but the failure of Bcl-2 antisense

Figure 4. Differential effects of IFN-γ and IFN-α on Stat1 and MAPK signaling in DM6, DM93, and 501mel cells. Each cell line was cultured for the indicated times with either medium alone, IFN-γ, or IFN-α, and whole-cell lysates were then analyzed by Western blotting using the indicated antibodies to phosphorylated (p-) or total Stat1 (A) or antibodies to the various phosphorylated or total MAPKs (B-D).
to induce tumor regression in melanoma patients may be evidence that other antiapoptotic proteins like Bcl2A1 and galectin-3 are more central to melanoma cell viability. The only proapoptotic gene up-regulated by IFN-γ in DM6 was the dependence receptor UNC5H2. This is the only dependence receptor containing a type II death domain. Caspase-mediated cleavage occurring in the absence of netrin-1, the ligand for UNC5H2, somehow renders the death domain active, thereby stimulating apoptosis (29, 30). In DM6, it is therefore possible that the up-regulation of UNC5H2 in the absence on netrin-1 expression, combined with the down-regulation of Bcl2A1 and/or galectin-3, contributed to the proapoptotic effect of IFN-γ. THBS1 was also up-regulated by IFN-γ, and although this is not known to have direct proapoptotic effects on cancer cells, it does inhibit angiogenesis by promoting endothelial cell apoptosis (31).

The CDK2-cyclin E complex promotes the transition from G1 to S phase in melanoma, whereas the CDK2-cyclin A complex promotes the S-G2 transition (32). CDK2 expression has also been shown to increase during tumor progression in melanoma (33), and its depletion can suppress growth and cell cycle progression in melanoma (34). It is therefore significant that CDK2 was the one cell cycling protein strongly down-regulated by IFN-γ, suggesting that the modulation of CDK2 expression was operative in the antiproliferative effect of IFN-γ on DM6. IFN-γ also up-regulated Dkk-1, a soluble antagonist of canonical Wnt signaling (35). To our knowledge, this is the first time that IFN-γ has been shown to alter the expression of a protein that can affect canonical Wnt signaling. Although the inhibition of Wnt/β-catenin signaling could have both antiproliferative and proapoptotic effects (36), this was unlikely to be responsible for the antimelanoma effect of IFN-γ on DM6, as we were unable to detect basal canonical Wnt signaling in DM6 using the TOPFLASH reporter assay.4 Nonetheless, through its ability to down-regulate components of G-protein signaling as well as Dkk-1, IFN-γ seems to be capable of inhibiting both noncanonical (37) and canonical Wnt signaling in melanoma cells.

Our analysis of the signaling pathways involved in the antiproliferative and proapoptotic effects of IFN-γ on melanoma cells implicated the coordinate activation of multiple MAPKs in a...
pattern mimicking a stress response. Stat1 was activated in all cell lines by both IFN-α and IFN-γ, and activation occurred early, with dissipation by 24 to 48 hours. This makes it unlikely that Stat1 was involved in the antimelanoma effect observed only with IFN-γ and only in the DM6 cell line. In contrast, MAPK activation occurred late (24-48 hours) and was seen only in DM6 and only with IFN-γ.

The time course of this late activation of ERK1/2, p38, and JNK paralleled the time course of IFN-γ-induced changes in gene expression as well as the induction of growth inhibition and apoptosis in DM6, providing a compelling link among these three events. Among the three cell lines, 501mel had the strongest basal levels of ERK1/2 and p38 phosphorylation, whereas DM93 had the weakest, and these basal levels diminished over time. However, what seemed to be important was not the basal level of MAPK phosphorylation but rather the ability of IFN-γ to augment phosphorylation in a delayed manner following 24 to 48 hours of continuous exposure.

The experiments using the specific MAPK inhibitors showed that MEK1 and p38 were the critical regulators of the effects of IFN-γ on gene expression and cell growth/viability. Concurrent activation of both MEK1 and p38 was necessary for IFN-γ to elicit the changes in gene expression and affect cell growth and viability, whereas JNK seemed to be dispensable for those effects. ERK1/2 activation was clearly not important to the antimelanoma effect of IFN-γ, as it was unaffected by MEK1 inhibition. This result implies that MEK2 was responsible for ERK1/2 phosphorylation by IFN-γ. The MEK1 inhibitor also had no effect on basal ERK1/2 phosphorylation in all three cell lines, suggesting that this too was mediated largely via MEK2.

ERK activation has been implicated in melanoma cell proliferation and resistance to apoptosis, and activating mutations in B-Raf may contribute to the malignant phenotype of melanoma by promoting ERK activation (38). In the cell lines that we examined, the B-Raf mutational status was unknown. However, although B-Raf may promote ERK activation via either MEK1 or MEK2, our results suggest that MEK1 activation, in conjunction with p38 activation, will send an antiproliferative/proapoptotic signal to melanoma cells independent of the status of ERK activation. In that context, strategies that focus on blocking ERK activation (39) may be less successful in treating melanoma than strategies focusing on the coordinate activation of MEK1 and p38.

In order for IFN-γ to activate all three MAPKs in DM6 melanoma, it is likely to have activated one or more MEKKs (14). Proinflammatory cytokines, including IFN-γ, IL-1, and tumor necrosis factor-α, have been shown to activate MEK1 or MEK3 (40–42). In addition, MEKK3 activation has been linked to paclitaxel-induced, BRCAl-dependent apoptosis in breast cancer (43) and to TRAF7-mediated apoptosis (44) and MEKK3 has been shown to physically interact with BRCAl and TRAF7. Although we have not been able to successfully measure MEKK kinase activity in our system, we did find that MEK1, MEK2, and MEK3 were expressed in all three cell lines, and the degree of expression was not altered by either IFN-γ or IFN-α. It remains to be determined whether IFN-γ can activate one or more of these MEKKs in our system and in other melanoma cell lines and whether a defect in MEKK activation can lead to melanoma cell resistance to IFN-γ by virtue of the resultant inability to coordinateately activate MEK1 and p38.

Building on the findings presented in this report, future studies examining the role that signaling proteins proximal to the MAPKs, such as the MEKKs, may play in mediating the direct antimelanoma effect of IFN-γ could provide further insight into the molecular basis of melanoma cell sensitivity and resistance to IFN-γ. In addition, experiments aimed at determining which gene changes identified in this report are truly central to the antiproliferative and proapoptotic effects of IFN-γ may provide new targets for the treatment of melanoma.

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family, is highly expressed in multiple cancer cell lines and in normal leukocytes. Oncogene 1997;14:997–1001.


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