Defective IFNα/γ-induced STAT3 protein activation in human malignant melanoma cells

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Abstract. Signal transducer and activator of transcription 3 (STAT3) protein has been documented as a significant mediator of interferon (IFN) signaling. Physiological STAT3 phosphorylation involves tyrosine (Y705) and serine (S727) activation. Impairment of STAT3 protein levels and/or of STAT3 phosphorylation after IFN treatment has been found in many pathological conditions such as cancer, immunopathy and inflammatory disease. To analyze tumor-associated defective STAT3 response to IFNs, the induction of S727 and Y705 STAT3 activation after IFN exposure was evaluated in 18 human malignant melanoma cell lines and 68 primary cell cultures established from the lymph node metastases of melanoma patients. STAT3 expression and STAT3 phosphorylated forms were assayed by Western blot analysis employing specific STAT3 antibodies. All melanoma cell lines as well as samples derived from metastatic melanoma patients expressed STAT3 with variable signal intensities depending on the appropriate cell type. Significantly altered IFNγ-induced S727 STAT3 activation was found in both experimental models, with on average 94.1% of patients detected to be non-responders in lymph node cell cultures and 83.3% in melanoma cell lines. Moreover, a deficiency in IFNα-induced S727 induction was detected in 88.9% of melanoma cell lines. Defects in Y705 STAT3 phosphorylation were determined in clinical material (61.8% after IFNγ exposure) as well as in melanoma cell lines (absence of response to IFNα/γ in 83.3 and 55.5%, respectively). Our data clearly confirm STAT3 pathophysiological perturbances in human malignant melanoma cells. Depending on the induction of STAT3-activated phosphoforms by IFNs, three categories of melanoma cells were identified: a) phosphorylation on both the S727 and Y705 amino acid residues; b) STAT3 activation on Y705 only; c) phosphorylation at neither S727 nor Y705. The significance of *in vitro* STAT3 activation for predicting patient response to immunotherapy will be examined in a prospective clinical study by our group.

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Introduction

Cellular homeostasis, including growth and differentiation patterns, is regulated by a continuous flow of signals from the cellular environment. An important set of signals utilized by many, if not all, cell types is provided by various cytokines and growth factors. Molecular analyses of cytokine-induced signaling pathways disclosed key proteins that ensure the transmission of signals downstream from receptors to target genes. These mainly include receptor-associated Janus tyrosine kinases (JAKs), signal transducers and activators of transcription (STATs), suppressors of cytokine signaling (SOCS) and protein inhibitors of activated STAT (PIAS) proteins (1,2). Impairment of JAK/STAT/SOCS/PIAS cascades has been described in several human pathological conditions, including inflammatory diseases, immunopathies and cancers (3). Perturbances in some of the abovementioned proteins may be involved in the pathogenesis of human malignancies, and may serve as new tumor markers and promising targets for cancer therapy. Moreover, it is hypothesized that cancer-related defective JAK/STAT/SOCS/PIAS pathways might negatively affect responses to interferons (IFNs) and other cytokine-based immunotherapeutic interventions.

STATs are a family of seven proteins (1, 2, 3, 4, 5A, 5B and 6) that are unique in their ability to transduce extracellular signals and to directly regulate transcription. Molecular events that under physiological conditions follow their ligand-induced activation and proceed downstream from receptors to STAT-specific binding sites in the nucleus are well recognized and reviewed (4-6). Some individual STATs possess specific functional properties that sometimes exert even antagonistic effects on cellular functions. For instance, STAT 1 and 3 exhibit opposite effects on cellular proliferation and apoptosis. Likewise, cell types of different histogenetic origins often manifest diverse functional responses to the same STAT molecule (7-9). The magnitude of STAT transcriptional activity and its duration are tightly controlled by several negative regulators, including the SOCS and PIAS proteins. Eight SOCS and four PIAS family members have been identified to

date. They attenuate or inhibit cytokine/growth factor signals mostly at the JAK/STAT transduction cascades (2,10). Although the exact molecular mechanisms by which SOCS/PIAS molecules inhibit or modulate IFN signaling are not precisely understood, it is evident that their essential biological role is to control the duration and magnitude of signals so that the cell can meaningfully respond to a continuous flow of stimuli (2,11).

Although STAT phosphorylation at tyrosine residues seems to be crucial for signal transduction, the investigation of mechanisms regulating STAT-mediated transcriptional power has shown that serine activation by a variety of external stimuli also actively operates in signaling pathways, elevating transcriptional activity and enhancing the expression of target genes. Individual STATs differ in the physiological consequences of their phosphorylation; the final outcome mostly depends on the type of external signal and downstream target gene clusters that are transcribed. For example, the outcome of STAT3 activation can be positive or negative depending on the stimulus and cell type involved.

STAT3 has been identified as an important mediator of IFN signaling. Its transducing, DNA binding and transcriptional activities require phosphorylation at both the tyrosine 705 (Y705) and serine 727 (S727) amino acid residues. Constitutive activation or deficient phosphorylation of STAT3 in response to IFNs has been observed in various human malignancies. It has been demonstrated that an increasing number of relevant STAT3 target genes are involved in the formation of tumors. STAT3 regulates basic biological processes crucial to tumorigenesis, including cell-cycle progression, apoptosis, tumor angiogenesis, invasion, metastases and tumor-cell evasion of the immune system. A major role of STAT3 is the regulation of programmed cell death (apoptosis).

In this study, we investigated STAT3 gene expression/activation in response to IFNs in human malignant melanoma cells - both established cell lines and primary cell cultures derived from patient lymph node metastases.

Materials and methods

Cellular models. As a model, a unique collection of 18 well-characterized human malignant melanoma cell lines kindly provided by Professor M. Herlyn (the Wistar Institute, Philadelphia, PA, USA) was used, along with 68 primary cell cultures derived from lymph node metastases of human malignant melanoma. To verify their character, cells grown on slides were fixed and immunostained by means of four specific antibodies (melan-A and tyrosinase from Novocastra, S-100 and HMB-45 from BioGenex) considered to be melanoma phenotypic markers. Only cell cultures positive for at least one tested antibody in >90% of cells were considered malignant. All melanoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (Pan Biotech GmbH, Aidenbach, Germany), sodium bicarbonate (2 g/l), insulin (5 ng/ml) and antibiotics (penicillin and streptomycin). Cells were cultured in an incubator with 5% CO_2 in a humidified atmosphere. Melanoma primary cell cultures were maintained *in vitro* for a short period of time (no more than a month) to be as representative as possible of *in vivo* conditions.

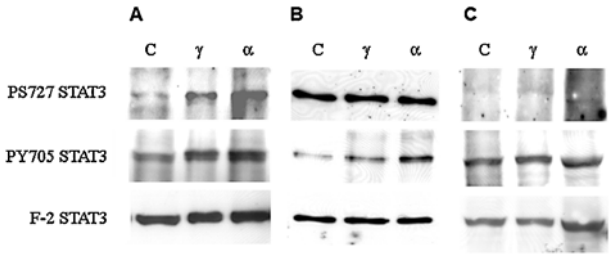

Figure 1. STAT3 protein activation in human malignant melanoma cell lines. Extracts from IFN-treated and untreated cells were subjected to immunoprecipitation with rabbit polyclonal antibody against STAT3 C-terminus (C-20). The immunoprecipitates were stained with anti-PS727 rabbit polyclonal antibody, anti-PY705 rabbit polyclonal antibody or normal anti-STAT3 mouse monoclonal antibody (F-2), as indicated. Three principle categories of human malignant melanoma cells, applicable to established cell lines as well as to primary cell cultures derived from metastatic patients, are shown. Western blotting revealed that, in general, human melanoma cells showed differential responses to IFN-induced STAT3 phosphorylation on S727 and Y705 amino acid residues. (A) Positive STAT3 activation on both S727 and Y705 after induction by both IFNs (cell line 1205 Lu). (B) Positive STAT3 phosphorylation induced by both IFNs on Y705 only (cell line WM 902B). (C) IFN-induced STAT3 activation on neither S727 nor Y705 residue (cell line WM 115).

Human epidermal melanocytes (manufacture code C-002-5C) isolated from lightly pigmented neonatal foreskin were purchased from Cascade Biologics, USA. The cells were grown in medium 254 supplemented with human melanocyte growth supplement (both from Cascade Biologics) in an incubator with 5% CO_2. The cells were obtained at the end of the secondary culture stage and passed 3-6 passages before being used in the experiments.

Patients. Sixty-eight melanoma patients of clinical stage II-III (UICC TNM classification) whose metastatic lymph nodes were surgically dissected were recruited for the study.

Reagents and antibodies. Recombinant human IFNα and IFNγ were purchased from Sigma (St. Louis, MO, USA). For the detection of STAT3 protein, rabbit polyclonal antibody against the C-terminal domain of STAT3 (C-20) as well as mouse monoclonal antibody recognizing STAT3 protein (F-2) were employed (Santa Cruz Biotechnology, CA, USA). For the detection of STAT3 phosphorylated forms, anti-PY705 rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA) and anti-PS727 rabbit polyclonal antibody (AbCam, Cambridge, UK) were used.

STAT3 activation. Activation doses of both IFNs were selected from dose-response curves. IFNα and IFNγ were used at concentrations of 5000 IU/ml and 10 ng/ml, respectively. Cells were incubated with the IFNs for 30 min at 37˚C. Control samples were incubated in parallel without IFN treatment. Induction of STAT3 phosphorylation on Y705 and S727 by the IFNs was assessed in cellular lysates by means of Western blotting.

STAT3 immunoprecipitation and immunoblot analysis. Protein content in whole-cell extracts was determined by means of the Bradford assay (Bio-Rad, Munich, Germany). Supernatants were incubated overnight at 4˚C with polyclonal

Table I. STAT3 phosphorylation in IFNα/γ-treated and untreated human malignant melanoma cell lines (n=18).

	STAT3 protein induction					
	PS727			PY705		
Cell line	IFNα	IFNγ	Untreated	IFNα	IFNγ	Untreated
1205 Lu	I	I	(+)	I	I	+
WM 278	I	I	(+)	N	I	+
WM 793B	N	N	++	I	I	+
WM 902B	N	N	+	I	I	+
WM 1158	N	N	+++	N	I	+++
WM 1341D	N	N	(+)	N	I	+++
WM 373	N	N	+	N	I	+
WM 983A	N	N	-	N	I	+
WM 1617	N	I	-	N	N	-
WM 852	N	N	++	N	N	++
WM 115	N	N	(+)	N	N	(+)
WM 35	N	N	+	N	N	++
WM 39	N	N	+	N	N	-
WM 9	N	N	(+)	N	N	+++
451 Lu	N	N	++	N	N	+
WM 239A	N	N	+	N	N	++
WM 164	N	N	++	N	N	++
WM 1552C	N	N	++	N	N	+++
Lack of activation (%)	(16/18) 88.9	(15/18) 83.3		(15/18) 83.3	(10/18) 55.5	

Inducibility: I, inducible; N, not inducible. Untreated levels: -, negative levels (negative signal); (+), very low levels (very slightly positive signal); +, low levels (slightly positive signal); ++, medium levels (positive signal); +++, high levels (strongly positive signal).

antibody C-20 (dilution 1:100) and protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Immunoprecipitates were washed four times with ice-cold Frackleton buffer. The beads were eluted by boiling in Laemmli sample buffer for 5 min. Equal amounts of proteins were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) in TBS buffer containing 0.1% Tween-20 (TBST) and incubated with the pre-determined concentrations of specific antibodies. For the dilution of anti-STAT3 antibodies, TBST supplemented with 5% BSA (Sigma) and 0.05% NaN_3 was employed. After washing, the blots were incubated with either anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham, UK) and developed using the enhanced chemiluminescence detection system (Amersham) according to the manufacturer's instructions.

Results

This study aimed to explore STAT3 expression and the induction of STAT3 activation (phosphorylation) by IFNα/γ in human malignant melanoma cells. STAT3 protein levels and IFN-induced STAT3 phosphorylation (PS727, PY705) were analyzed by Western blotting using immunoprecipitation and specific polyclonal or monoclonal anti-STAT3 antibodies.

Categories of Western blotting results. Immunostaining with monoclonal F-2 antibody recognizing STAT3 protein (Fig. 1, F-2 STAT3) showed that total STAT3 protein levels differed slightly between each cell line/patient in signal intensity. Fig. 1 shows three principle categories of human malignant melanoma cells, applicable to established cell lines as well as to primary cell cultures derived from metastatic patients. Western blotting revealed that, in general, human melanoma cells show differential responses to IFN-induced STAT3 phosphorylation on S727 and Y705 amino acid residues. Fig. 1 illustrates the results from the melanoma cell lines. Fig. 1A shows cases exhibiting positive STAT3 activation induced by both IFNs on both S727 and Y705 (cell line 1205 Lu), whereas B demonstrates the induction of STAT3 by both IFNs on Y705 only (cell line WM 902B). Fig. 1C shows cases that were completely non-inducible by IFN treatment; neither S727 nor Y705 activation was detected (cell line WM 115).

Defective IFN-induced STAT3 phosphorylation in human malignant melanoma cell lines. Table I summarizes the results of STAT3 activation in 18 IFN-treated and untreated melanoma cell lines. More than four-fifths of the cell lines were resistant to IFNα- or IFNγ-induced STAT3 phosphorylation on S727. A lack of serine activation was observed with IFNα in 16/18 (88.9%) cell lines and with IFNγ in 15/18 cell lines (83.3%). Similar results were observed with IFNα-induced

Table II. STAT3 phosphorylation in IFNγ-treated and untreated primary cell cultures derived from patients with metastatic melanoma (n=68).

	STAT3 protein induction			
	PS727		PY705	
Patient no.	IFNγ	Untreated	IFNγ	Untreated
1	I	-	I	++
2	I	+	I	+
3	I	+	I	(+)
4	I	+	I	+
5	N	++	I	++
6	N	-	I	+
7	N	-	I	+
8	N	-	I	+
9	N	-	I	+
10	N	+	I	+
11	N	-	I	+
12	N	++	I	+
13	N	+	I	+
14	N	++	I	+
15	N	+	I	++
16	N	-	I	+
17	N	+	I	+
18	N	++	I	++
19	N	++	I	+
20	N	+	I	+
21	N	+	I	+
22	N	++	I	+
23	N	+	I	+
24	N	-	I	-
25	N	+	I	+
26	N	+	I	+
27	N	+	N	+
28	N	-	N	+
29	N	-	N	++
30	N	-	N	-
31	N	-	N	+
32	N	-	N	-
33	N	+	N	(+)
34	N	-	N	+
35	N	++	N	+
36	N	+	N	+
37	N	+	N	(+)
38	N	++	N	+
39	N	+++	N	+++
40	N	++	N	+
41	N	+++	N	+
42	N	++	N	+
43	N	+++	N	+
44	N	+++	N	+++
45	N	+	N	++
46	N	++	N	++
47	N	++	N	++
48	N	(+)	N	(+)
49	N	++	N	+++
50	N	-	N	+
51	N	-	N	(+)
52	N	++	N	++
53	N	++	N	+
54	N	++	N	+
55	N	++	N	+
56	N	+	N	+
57	N	-	N	++
58	N	++	N	+
59	N	++	N	++
60	N	+	N	++
61	N	++	N	+++
62	N	++	N	++
63	N	+	N	+
64	N	+	N	+
65	N	-	N	+
66	N	+	N	+
67	N	+	N	+
68	N	+	N	+
Non-responders	(64/68)		(42/68)	
(%)	94.1		61.8	

Inducibility: I, inducible; N, not inducible. Untreated levels: -, negative levels (negative signal); (+), very low levels (very slightly positive signal); +, low levels (slightly positive signal); ++, medium levels (positive signal); +++, high levels (strongly positive signal).

phosphorylation on Y705 (83.3%). Defects in STAT3 activation on tyrosine after IFNγ treatment were less pronounced, with the absence of induction in 10/18 cell lines (55.5%).

Of note, only one (5.6%) melanoma cell line (1205 Lu) exhibited normal physiological STAT3 phosphorylation at both amino acid residues induced by either IFN. In addition, one cell line (WM 278) was lacking IFNα-induced Y705 activation only. Moreover, in the group of two (11.1%) cell lines (WM 793B and WM 902B), both IFNs induced STAT3 phosphorylation on Y705 only, without detectable induction on S727. Furthermore, four (22.2%) different melanoma lines (WM 1158 to WM 983A; Table I), including normal human melanocytes, exhibited IFNγ-induced Y705 STAT3 phosphorylation only. Notably, one line (WM 1617) responded on S727 only after IFNγ treatment. In contrast, nine (50%) cell lines (WM 852 to WM 1552C; Table I) were completely unresponsive to IFNα/γ-induced STAT3 activation.

In the majority of melanoma cell lines, basal levels of PS727 (16/18) or PY705 (16/18) were detectable in control (IFN-untreated) cells. In spite of the basal levels of both STAT3 phosphoforms, it remained possible to induce STAT3 activation with IFN. In contrast to total STAT3, there were differences in the protein levels of both phosphoforms between different cell lines.

IFN-induced STAT3 activation in malignant melanoma primary cell cultures. As short-term melanoma cell cultures better reflect the *in vivo* situation than malignant melanoma cell lines, the induction of STAT3 phosphorylation by IFNγ treatment was analyzed in melanoma cells derived from 68 patients. Primary cell cultures were established from regional lymph node melanoma metastases after surgery. The results from individual patients are shown in Table II. Samples from >94% patients (64/68) treated *in vitro* with IFNγ were lacking activation on S727. The absence of Y705 phosphorylation after IFNγ treatment occurred in 42/68 patients (61.8%). Table II also shows that PS727 and PY705 STAT3 levels were increased in a high percentage (73.5 and 95.6%; 50/68 and 65/68, respectively) of IFN-untreated control cells.

Based on the response seen in both amino acid residues as well as on STAT3 phosphorylation type, our patients can be classified into three different principal groups (Table II). The first group includes four (5.9%) patients (nos. 1-4) who exhibited normal physiological responses to IFNγ on both amino acid residues. These patients may speculatively be considered as normal responders to IFNγ as far as STAT3-mediated signaling is concerned. The second category involves 22 (32.3%) patients (nos. 5-26) and comprises cases that responded to IFNγ by STAT3 activation on Y705 only, without S727 phosphorylation necessarily being detectable. The biological consequences of this with respect to the clinical effectiveness of IFNγ remain to be clarified. In contrast, the third group of 42 (61.8%) patients (nos. 27-68) showed no STAT3 activation on either S727 or Y705. It is likely that the failure observed in these patients of STAT3 protein to undergo IFN-induced phosphorylation may negatively affect the biological effects of immunotherapy.

As in the melanoma cell lines, the basal levels of both STAT3 phosphoforms could also be determined in various primary melanoma cell cultures. There is clear evidence that the majority of melanoma cells at a metastatic stage expressed both phosphorylated STAT3 forms (Table II; untreated). In contrast to total STAT3, there were differences in the protein levels of both phosphoforms between individual patients.

Discussion

The most striking abnormalities observed in various human tumors comprise the downregulation or constitutive expression of STATs, their faulty activation, and the epigenetic silencing of genes (4,12). Important evidence directly linking STATs to cancer was revealed by studies showing that activated STAT3 and/or 5 function in a manner that promotes the growth and survival of malignant cells, blocks apoptosis and can mediate cellular transformation; i.e., features attributable to true oncogenes (4,6,13). In contrast, activated STAT 1 inhibits the growth and survival of tumor cells and promotes apoptosis, thus behaving as a tumor-suppressor protein (14). It seems probable that STATs 1, 3 and 5 maintain a critical biological balance in normal growth-regulated genes, and that any perturbations of this balance may result in oncogenesis and/or s transformed cellular phenotype. However, reliable data on STAT alterations in cancer patients and their clinical significance are to date lacking.

We recently demonstrated that human malignant melanoma is associated with significantly impaired STAT 1 phosphorylation induced by IFN (15), and that the absence of STAT 1 activation on Y701 in response to IFNγ in *ex vivo* melanoma cells positively correlates with disease outcome in melanoma patients (9). Constitutively active variants of STAT3 induce oncogenic transformation and tumorigenesis; i.e., they function as an oncogene (13,16).

In this study, we analyzed IFN-dependent STAT3 expression and activation, both in human melanoma primary cell cultures derived from lymph node metastases and in established melanoma cell lines. Phosphorylated STAT3 is considered to be an oncogene with anti-apoptotic and growth-promoting properties (17). Our study clearly demonstrates STAT3 functional abnormalities in human malignant melanoma. STAT3 alterations occurred in a prevailing number of melanoma cell lines as well as in primary metastatic melanoma cell cultures.

Immunoblot analysis showed constitutive STAT3 expression in all the samples analyzed, which is in good agreement with the function of STAT3 in tumor cell survival. Western blotting was employed to investigate S727 and Y705 STAT3 activation in melanoma cells treated *in vitro* with IFNs. Incubation with IFNα/γ caused varying increases in phosphorylated STAT3 forms. IFNγ-induced STAT3 phosphorylation on S727 was detectable in a minority - only 4 (5.9%) - patient samples. Conversely, more frequent IFNγ-induced STAT3 activation on Y705 was recorded. There were 26 responders (38.2%) to IFNγ. These findings demonstrated that human malignant melanoma is associated with altered IFN-stimulated STAT3 phosphorylation. Notably, samples from only four melanoma patients (5.9%) manifested a STAT3 activation response on both S727 and Y705 inducible by IFNγ, while in the melanoma cells of 42 patients (61.8%) STAT3 phosphorylation could not be induced on either S727 or Y705 (Table I).

On average, more than four-fifths of malignant melanoma cells were lacking IFNγ-induced STAT3 activation on S727. Our study showed that, in a prevalent number of established melanoma cell lines and melanoma cells derived from metastatic patients, STAT3 phosphorylation on S727 was not inducible by either of the IFNs. In general, activation on S727 was inducible by IFNγ in only approximately 5-15% of melanoma cells. The percentage of human non-responders more or less coincided with that obtained in melanoma cell lines. However, we found significant differences between the experimental models in terms of STAT3 activation on Y705. Whereas the induction of STAT3 on Y705 after IFNγ treatment in melanoma cell lines was detectable in almost half the cases (44.4%), STAT3 phosphorylation defects were more frequent in melanoma patients. The percentage of patient samples showing pathological STAT3 phosphorylation on Y705 was surprisingly high, with 61.8% being non-responders to IFNγ (Tables I and II).

Constitutively activated STAT3 is involved in the formation of multiple types of tumors, including breast cancer. Activated STAT3 protein plays a critical role in the induction of breast tumors induced by 4T1 cells by enhancing the expression of several important genes, including c-Myc and the metastatic regulator Twist (18). There is abundant evidence demonstrating that constitutively activated STAT3 is fre-

quently found in breast cancer cells (19). Studies of molecular mechanisms suggest that c-Myc is upregulated by activated STAT3, which plays a role in the transformation of mammary gland cells (20). Although STAT3 is constitutively activated in breast cancer, it is not clear what type of biological effects could result from the inhibition of STAT3 protein expression.

Transcription factors play a crucial role in oncogenic signaling in cases of inappropriate and sustained activation of STATs, especially STAT3, which is a trait of many different cancers and their derived cell lines. Constitutively active STAT3 has been reported to prevent programmed cell death (apoptosis) and to enhance cell proliferation, whereas the disruption of STAT3 signaling can inhibit tumor growth. The physiological activation of STAT3 by cytokines has been well established; however, little is known about altered, stimulation-independent STAT3 activation (21).

Human type I IFNs play an important role in the regulation of antiviral defense mechanisms, immunomodulatory activities and growth control. Recent efforts have demonstrated the importance of IFNs in the activation of STATs. The role of STAT1 and 2 in IFN-dependent JAK/STAT signaling is well established; however, the role of STAT3 and the mechanisms of its activation by the IFNs remains unclear. Understanding the IFN-dependent regulation of STAT3 is of increasing interest because recent studies have demonstrated that STAT3 may play a role in cancer. Studies have revealed that STAT3 is constitutively active in a number of cancer cell lines and that overexpression of an active form of STAT3 transforms normal fibroblasts. Therefore, STAT3 exhibits properties indicative of known oncogenes (22). STAT3 is also activated in response to IFN stimulation in a number of different cell types (23-25). This is of particular interest in that the expression of a constitutively active form of STAT3 has been shown to transform normal fibroblasts (13). In addition, constitutive expression of STAT3 has been demonstrated to occur in a number of cancer cell lines (26). Therefore, it has been proposed that STAT3 is an oncogene capable of transforming normal cells. The direct activation of STAT3 by type I IFNs has been demonstrated, and the specific cytoplasmic tyrosine residues present in IFNαR2c that are required for STAT3 activation have been identified (22). The activation of STATs is tightly controlled by tyrosine phosphorylation, and this control appears to be of universal importance, in that similar STAT activation mechanisms are present in organisms ranging from slime molds to humans (27). STAT 1, 3 and 5 have been implicated in a number of cancer types (28-30). It has been suggested that STAT3 itself has oncogenic properties, and is constitutively activated in a number of tumor cell lines (13). Although STAT3 and 1 are highly homologous, they appear to have divergent functions. IFN-dependent STAT3 activation has been documented in a number of studies, but the mechanism of activation by IFNs remains unclear.

Alterations in JAK/STAT components of IFN-mediated signaling can contribute to the mechanism of cell resistance to IFNs, leading to a lack of MHC class I inducibility. Studies have been conducted on the two distinct mechanisms of loss of IFNγ inducibility of HLA class I antigens due to epigenetic blocking of IFN-regulatory factor 1 (IRF-1) transactivation in two melanoma cell lines (31).

To conclude, our data indicate that human malignant melanoma is associated with defective STAT3-dependent IFN-induced signaling pathways, and that results concerning STAT3 inactivation obtained from established melanoma cell lines may not necessarily correlate with *in vivo* results. The significance of *in vitro* STAT3 phosphorylation for predicting patient response to IFN therapy will be examined in a prospective clinical study by our group.

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