Original Articles

Interferon Inducibility of STAT 1 Activation and Its Prognostic Significance in Melanoma Patients

(STAT 1 / malignant melanoma / signal transduction / interferons)

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Abstract. STAT 1, a member of latent cytoplasmic proteins, plays a pivotal role in mediating biological effects of interferons. Its transducing, DNA binding and transcriptional activity require phosphorylation at both Tyr 701 (Y 701) and Ser 727 (S 727) residues. Deficient phosphorylation or constitutive activation of the STAT 1 protein were observed in some human malignancies. Using immunoprecipitation and Western blots performed with lysates made of melanoma cells derived from patients with clinical stage II/III and employing specific anti-STAT 1 PS 727/PY 701 immunoprobes, we show that STAT 1 activation response induced by IFN-α/-γ is significantly impaired. On average, three quarters of patients were lacking phosphorylation at S 727. STAT 1 PY 701 was not inducible by IFN-α in 63% and by IFN-γ in 34% of samples. However, these STAT 1 activation defects showed no correlation with the disease outcome and immunotherapy response as indicated by progression-free survival profiles in patients treated with IFN-α2b.

STAT 1 belongs to a multigene family of signal transducers and activators of transcription (STATs), latent cytoplasmic proteins, comprising seven members so far identified. They mediate various biological effects of cytokines and peptide growth factors, thus participating in the maintenance of cellular homeostasis (for review see Stark et al., 1998). Dominant molecular events that follow ligand-receptor interactions and provide propagation of signals from the membrane to the nucleus initiate activation of Janus family receptor-associated protein kinases (JAKs) through their own phosphorylation at tyrosine residues. The activated JAKs phosphorylate the conserved tyrosine on the receptor, thereby creating a docking site for the STATs. Unphosphorylated receptor-bound STATs are phosphorylated by JAKs at tyrosine residues. Activated STATs subsequently dimerize through the interaction of the SH2 domain of one STAT with phosphotyrosine of the other one. These dimers translocate to the nucleus where they bind to specific DNA sequences in the promoters of responsive genes and modulate their expression (for review see O'Shea et al., 2002).

Although STAT phosphorylation at tyrosine seems to be crucial for signal transduction, investigation of the mechanisms regulating STAT-mediated transcriptional power showed that phosphorylation of STAT 1 and STAT 3 at serine residues by a variety of external stimuli also actively operates in signalling pathways elevating the transcriptional activity and enhancing the expression of target genes (Wen et al., 1995; Decker and Kovarik, 2000; Kovarik et al., 2001). Individual STATs differ in the physiological consequences of their activation. The final outcome mostly depends on the type of external signal and downstream target gene clusters that are transcribed.

In general, STATs exclusively function as mediators of all cytokine signals. Thus, for example, studies on STAT 1 mutant mice and STAT 1 knockout models showed that this protein constitutes a major transcription factor in the interferon type I and type II signal transduction pathways, and that the expression of impaired STAT 1 protein and/or deletion of the STAT 1 gene might associate with the abrogation of IFN biological responses (Durbin et al., 1996; Meraz et al., 1996; for review see Levy, 1999).

Given the importance of STATs in mediating signals for cell growth, differentiation and survival it is not surprising that STAT dysregulation has been described in several human pathological conditions such as immunodeficiencies or cancer. A number of reports described constitutive STAT 1 and STAT 3 activation in a variety of human tumour cell lines and primary human cancers, claiming that persistent phosphorylation of these proteins

Received June 4, 2003. Accepted June 12, 2003.

The study was supported in part by Grant NC/7139-3 from the Internal Grant Agency of the Czech Ministry of Health, Grant 301/00/0564 from the Grant Agency of the Czech Republic and by Institutional Project No. MZ 00020980501.

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Abbreviations: IFN – interferon, JAKs – Janus family receptor-associated protein kinases, STAT 1 – signal transducer and activator of transcription.

associates directly or indirectly with oncogenesis (Watson and Miller, 1995; for review see Frank, 1999; Bowman et al., 2000; Garcia et al., 2001). Inappropriate STAT 1 functioning due to impaired signal-induced activation has been observed in some malignant melanomas (Wong et al., 1997; Pansky et al., 2000). It is argued that defective STAT phosphorylation may constitute one of the mechanisms that attenuate or abolish the cellular response to interferons. Extended studies are needed to clarify to what extent STAT perturbances operate in real clinical situations. This study attempted to analyse potentially faulty activation of the STAT 1 protein in response to IFN-α/-γ signalling in high-risk human malignant melanoma and to correlate the STAT 1 phosphorylation status with the disease outcome in patients treated with IFN-α.

Material and Methods

Patients

Basal levels of STAT 1 phospho-forms (PY 701 and PS 727, respectively) as well as IFN-induced STAT 1 phosphorylation were examined in 35 melanoma patients (median age 57 years) of clinical stage II-III (UICC TNM classification, the fifth edition) whose metastatic lymph nodes were surgically dissected. The same indices were assessed in 15 melanoma patients (median age 52 years) of clinical stage II/III who, after regional lymph node dissection for metastatic disease, underwent postsurgical adjuvant immunotherapy with interferon (IFN)-α2b (Schering-Plough, Kenilworth, NJ) according to the following regimen: initial induction phase of 10 MU subcutaneously daily for 5 days during 4 weeks followed by a maintenance dosage of 10 MU subcutaneously, three times a week for 48 weeks. The inducibility of STAT 1 activation response to *in vitro* exposure of melanoma cells to either IFN-α or IFN-β at the time of immunotherapy commencement was correlated with the occurrence of progression and disease-free survival, respectively.

Melanoma cell cultures

Thirty-five primary cell cultures derived from lymph node metastasis were set up. Immunostaining of grown cells by means of two specific antibodies proved that the cellular suspension mostly contained over 95% of melanoma cells. All cells were grown in Dulbecco's modified Eagle's medium (DMEM, PAN Biotech GmbH, Aidenbach, Germany) supplemented with 10% foetal bovine serum (FBS, PAN Biotech GmbH). Melanoma primary cultures were maintained *in vitro* for a short period of time (up to one week) to be as representative as possible of *in vivo* conditions.

Reagents and antibodies

Recombinant human IFN-α and IFN-γ were purchased from Sigma (St. Louis, MO). For the detection of the STAT 1 protein and its phosphorylated forms, polyclonal antiserum against the C-terminal domain of STAT 1 (S1C) as well as monoclonal antibodies recognizing the STAT 1 protein (SM 1) and its S 727 phosphorylated form (pSM 1) developed in the authors' laboratory were used. Commercial STAT 1 anti-PY 701 polyclonal antibody (Sigma) was employed.

STAT 1 phosphorylation

Subconfluent cells were serum-starved overnight in DMEM before exposure to IFNs. Activation dosages of either IFN were selected from the dose-response curves. IFN-γ was used at a concentration of 10 ng/ml and IFN-α at concentrations of 1000 IU/ml and 5000 IU/ml, respectively. Cells were incubated with IFNs for 30 min at 37°C. Control samples were in parallel incubated without IFN treatment. Induction of STAT 1 phosphorylation at Y 701 and S 727 by IFNs was assessed in cellular lysates by means of Western blots. As a negative control (STAT 1 -/-), mouse embryonal fibroblasts (MEF) were used (the lysates were kindly provided by Dr. P. Kovarik, Institute of Microbiology and Genetics, Vienna Biocenter, Vienna, Austria).

Cellular lysates

Harvested cells were washed with PBS containing 1 mM Na_3VO_4 and then lysed for 5 min on ice in Frackleton buffer consisting of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM Na PPi, 50 mM NaF, 1% Triton X-100, supplemented with 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM Na_3VO_4, 3 μg/ml aprotonin, 1 μg/ml leupeptin and 1 μg/ml pepstatin. Extracts were cleared by centrifugation at 20 500 *g* (4°C) for 15 min and resulting supernatants were kept frozen until used for further analysis.

Immunoprecipitation and Western blot analysis

The protein content in whole-cell extracts (WCEs) was determined by means of Bradford assay (Bio-Rad, Munich, Germany). Supernatants were incubated overnight at 4°C with polyclonal antibody S1C (dilution 1 : 200) and protein A-Sepharose 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 to nitrocellulose (Bio-Rad, Hercules, CA). Membranes were blocked with skimmed milk or bovine serum albumin (BSA) in TBS buffer containing 0.1% Tween-20 (TBST) and incubated with the predetermined concentrations of specific antibodies. For the dilution of polyclonal antisera, TBST supplemented with 2% BSA (Sigma, St. Louis, MO) and 0.05% NaN_3 was employed. After washing, the blots were incubated with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated

secondary antibody (Amersham, UK) and developed using the enhanced chemiluminescence (ECL) detection system (Amersham, UK) according to manufacturer's instructions.

Statistical methods

Direct comparison of relative frequencies was based on standard Fisher's exact test, principal analyses were focused on time-related profile of progression (progression-free survival). Standard Kaplan-Meier analysis was applied to quantify progression-free survival profiles of patients stratified according to the STAT 1 inducibility on Y 701 and S 721, respectively. Stratified survival data were mutually compared by the log-rank test (Klein and Moeschberger, 1997).

Table 1. STAT 1 phosphorylation in IFN-α/-γ-treated and untreated primary cell cultures derived from melanoma patients.

Malignant melanoma patient # (n = 35)	**Inducibility PS 727 IFN-α**	**PS 727 IFN-γ**	**PS 727 untreated**	**PY 701 IFN-α**	**PY 701 IFN-γ**	**PY 701 untreated**
1	I	I	+	I	I	+
2	I	I	+	I	I	+
3	I	N	+	I	I	+
4	I	N	+	I	I	+
5	I	N	–	I	I	–
6	N	N	–	N	N	+
7	N	N	+	N	N	+
8	N	N	+	N	N	+
9	N	N	+	N	N	+
10	N	N	+	N	N	+
11	N	N	+	N	N	+
12	N	N	+	N	N	+
13	N	N	+	N	N	+
14	N	N	+	I	I	+
15	N	N	+	I	I	–
16	N	N	+	I	I	–
17	N	N	+	I	I	–
18	N	N	+	I	I	–
19	ND	ND	ND	I	I	–
20	I	I	+	N	N	+
21	N	N	+	N	I	–
22	N	N	+	N	I	+
23	N	N	+	N	I	+
24	N	N	+	N	I	+
25	N	N	+	N	I	–
26	N	N	+	N	I	+
27	N	N	+	N	I	+
28	ND	ND	ND	N	I	–
29	I	N	+	N	I	+
30	N	I	–	N	I	+
31	N	I	+	I	I	+
32	N	N	+	I	N	+
33	N	I	+	N	N	+
34	I	I	–	N	I	+
35	I	N	+	N	N	+
Percentage of non-responders	**74.3**	**80.0**		**62.9**	**34.3**	

I – inducible, N – not inducible, ND – not detectable, untreated: (+) – positive signal, (-) – negative signal

Results and Discussion

Assessment of STAT 1 phosphorylation induced by IFN signals in melanoma cell cultures

Western blot assays were used to investigate STAT 1 S 727 and Y 701 activation in melanoma cell cultures of 35 patients exposed *in vitro* to IFN-α and IFN-γ, respectively. IFN-induced STAT 1 phosphorylation at S 727 was detectable in a significant minority of patient samples, i.e. in 9 patients (25.7%) after IFN-α and in only 7 samples (20%) treated with IFN-γ (Table 1). In contrast, slightly more favourable IFN-induced STAT 1 activation at tyrosine (Y 701) could be recorded. There were 13 responders (37.1%) to IFN-α and 23 responders (65.7%) to IFN-γ (Table 1). Our findings demonstrate that human malignant melanoma associates with altered IFN-stimulated STAT 1 phosphorylation. Interestingly, samples of only 2 patients manifested STAT 1 activation response at both S 727 and Y 701 inducible by either IFN. In melanoma cells of 8 patients, STAT 1 phosphorylation could not be induced at either S 727 or Y 701, regardless of the type of IFN used (Table 1).

IFN-induced STAT 1 activation does not significantly correlate with the disease outcome in IFN-α treated melanoma patients

In a small group of patients we have analysed whether normal or defective STAT 1 phosphorylation by IFN signals as determined prior to the commencement of immunotherapy might have a clinical impact as to prediction of the disease outcome. Median follow-up was 13 months. IFN-induced STAT 1 activation responses in individual patients expressed by its phos-

Table 2. Inducibility of STAT 1 activation by IFNs in relation to the progression-free survival in melanoma patients

Patient	PY 701	PS 727	progression (months)	exitus (months)
1	I	I	10	13
2	N	N	12	
3	I	N	2	20
4	N	N		
5	N	N		
6	I	N	10	
7	I	I		
8	N	I	7	16
9	I	I		
10	I	I	5	6
11	N	N	4	
12	I	N		
13	I	N		
14	N	N		
15	I	N	4	

Summarized data:

		Y 701 activation	S 727 activation
Progression:	8/15	I 5/8 (62.5 %)	I 3/8 (37.5 %)
		N 3/8 (37.5 %)	N 5/8 (62.5 %)
No progression:	7/15	I 4/7 (57.1 %)	I 2/7 (28.6 %)
		N 3/7 (42.8 %)	N 5/7 (71.4 %)

I – inducible, N – not inducible

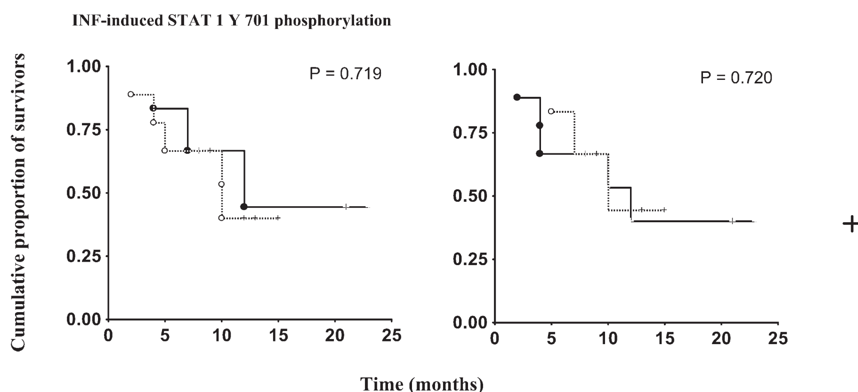

Fig. 1. Progression-free survival of melanoma patients treated with interferon in relation to the pretreatment inducibility of STAT 1 phosphorylation at Y 701 and S 727, respectively. No statistically significant difference was found between the inducible (full line) and non-inducible (stippled line) cohort of patients in relation to the disease outcome as analysed by standard Kaplan-Meier methodology and log-rank test.

phorylation at Tyr 701 and Ser 727, together with the proportion of inducible and non-inducible cases within groups with and without progression, is illustrated by Table 2. Positive response to at least one IFN tested has been considered as inducible case. Out of 15 clinically disease-free patients treated with IFN-α2b, eight patients manifested progression during IFN treatment and four of them died. When comparing STAT 1 activation at either Y 701 or S 727 in patients who manifested progression (62.5% and 37.5%, respectively) with those who remained progression-free (57.1% and 28.6%, respectively), no statistical difference between both groups of patients could be proved (see Table 2 and Fig. 1). Unexpectedly, in contrast to the STAT 1 Y 701 activation profiles, the IFN-induced STAT 1 phosphorylation at S 727 occurred in a smaller number of patients regardless of the disease outcome (see summarized data in Table 2). Melanoma-associated deficient Ser 727 phosphorylation by IFNs might reflect a diminished transcriptional power of the STAT 1 protein. However, the negative IFN inducibility of STAT 1 S 727 may be falsified by constitutive activation that causes a high level of PS 727 further not inducible.

Our results suggest that the *in vitro* examination of STAT 1 phosphorylation by IFNs prior to immunotherapy can hardly serve as a meaningful parameter of prognosis and beneficial response to IFN-based immunotherapy in patients with stage II/III melanoma. Nevertheless, the presented data should be considered as preliminary due to a small size of samples. Moreover, the possibility that a link between STAT 1 activation response and disease outcome might exist in patients with localized process should also be considered. To our knowledge, no similarly oriented study has been performed so far. The lack of correlation between STAT 1 or STAT 2 expression or phosphorylation and resistance to IFNs recently described in melanoma cells are in support of our findings (Chawla-Sarkar et al., 2002). On the other hand, the prognostic significance of STAT activation and correlation with IFN responsiveness have also been reported in malignant melanoma (Wong et al., 1997; Pansky et al., 2000), carcinoid tumors (Zhou et al., 2001) and breast carcinoma (Widschwendter et al., 2002). However, these studies including ours differ not only in the material explored, but mainly in the methods used to screen for defective STAT functioning. In view of the molecular complexity of the IFN signalling path-

ways involving additional STAT proteins, physiological suppressors of cytokine signalling (SOCS proteins) and recently described STAT 1-independent IFN-regulated gene expression (Ramana et al., 2001; for review see Ramana et al., 2002), further studies are needed to validate the role of STAT abnormalities in cancer growth and cytokine-based therapy response.

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