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Interleukin-6 dependent induction of the cyclin dependent kinase inhibitor $p21^{WAF1/CIP1}$ is lost during progression of human malignant melanoma

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Human melanoma cell lines derived from early stage primary tumors are particularly sensitive to growth arrest induced by interleukin-6 (IL-6). This response is lost in cell lines derived from advanced lesions, a phenomenon which may contribute to tumor aggressiveness. We sought to determine whether resistance to growth inhibition by IL-6 can be explained by oncogenic alterations in cell cycle regulators or relevant components of intracellular signaling. Our results show that IL-6 treatment of early stage melanoma cell lines caused G_1 arrest, which could not be explained by changes in levels of G_1 cyclins (D1, E), cdks (cdk4, cdk2) or by loss of cyclin/cdk complex formation. Instead, IL-6 caused a marked induction of the cdk inhibitor $p21^{WAF1/CIP1}$ in three different IL-6 sensitive cell lines, two of which also showed a marked accumulation of the cdk inhibitor $p27^{Kip1}$. In contrast, IL-6 failed to induce $p21^{WAF1/CIP1}$ transcript and did not increase $p21^{WAF1/CIP1}$ or $p27^{Kip1}$ proteins in any of the resistant lines. In fact, of five IL-6 resistant cell lines, only two expressed detectable levels of $p21^{WAF1/CIP1}$ mRNA and protein, while in three other lines, $p21^{WAF1/CIP1}$ was undetectable. IL-6 dependent upregulation of $p21^{WAF1/CIP1}$ was associated with binding of both STAT3 and STAT1 to the $p21^{WAF1/CIP1}$ promoter. Surprisingly, however, IL-6 stimulated STAT binding to this promoter in both sensitive and resistant cell lines (with one exception), suggesting that gross deregulation of this event is not the unifying cause of the defect in $p21^{WAF1/CIP1}$ induction in IL-6 resistant cells. In somatic cell hybrids of IL-6 sensitive and resistant cell lines, the resistant phenotype was dominant and IL-6 failed to induce $p21^{WAF1/CIP1}$. Thus, our results suggest that in early stage human melanoma cells, IL-6 induced growth inhibition involves induction of $p21^{WAF1/CIP1}$ which is lost in the course of tumor progression presumably as a result of a dominant oncogenic event.

Keywords: melanoma, IL-6; sensitivity; $p21^{WAF1/CIP1}$; $p27^{Kip1}$; STAT

Introduction

Perturbations in cell cycle control and loss of responsiveness to inhibitory growth factors are among the hallmarks of tumor development and progression. This is especially evident in human malignant melanoma where rapidly dividing tumor cells originate from melanocytes which are considered to be terminally differentiated and hence, mitotically inactive.

Mitogenic and growth inhibitory factors influence cell cycle progression during the G_1 phase. Passage through the G_1 phase of the cell cycle is mediated by a family of cyclin-dependent kinases (cdks). The activity of the cdks is regulated at several levels, e.g. by: (i) changes in cyclin levels, (ii) activating and inactivating phosphorylations of the cdk subunit and (iii) association with a number of small cdk inhibitory molecules. The latter can be classified as members of either the KIP family, of which $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ are the best known examples, and the INK4 family, which includes inhibitors such as $p15^{INK4b}$ and $p16^{INK4a}$ (Martin-Castellanos and Moreno, 1997; Sherr, 1996; Hirama and Koeffler, 1995; Morgan 1995; Sherr and Roberts, 1995). Members of the INK4 family (Sherr, 1996; Sherr and Roberts, 1995) bind specifically and inhibit the activity of cdk4 and cdk6 by displacement of the associated cyclin subunit (Parry *et al*., 1995; Sandhu *et al*., 1997). The gene encoding $p16^{INK4a}$ has generated considerable interest owing to the observation that it is frequently mutated, deleted, or transcriptionally repressed in human tumors and tumor derived cell lines (Kamb *et al*., 1994a; Nobori *et al*., 1994; Caldas *et al*., 1994; Spruck *et al*., 1994), in familial melanoma (Kamb *et al*., 1994b) and, to a lesser extent, also in sporadic melanoma (Maelandsmo *et al*., 1996; Flores *et al*., 1996; Reed *et al*., 1995). The gene encoding $p15^{INK4b}$ is located adjacent to the $p16^{INK4a}$ gene, and is frequently lost together with $p16^{INK4a}$ (Otsuki *et al*., 1995). Its functional role is linked to regulation of epithelial cell proliferation by growth inhibitory cytokines such as TGF-β (Hannon and Beach, 1994; Sandhu *et al*., 1997).

Unlike $p16^{INK4a}$ and $p15^{INK4b}$, members of the KIP family, p21 $^{WAF1/CIP1}$ and $p27^{Kip1}$ are thought to act as universal cdk inhibitors. They mediate effects on cell cycle progression by their ability to bind with cyclin/cdk complexes and inhibit cdk activity. $p27^{Kip1}$ was identified originally as an inhibitory activity upregulated in cells arrested in the G_1 phase by intercellular contact or treatment with TGF-β (Slingerland *et al*., 1994; Polyak *et al*., 1994a,b; Koff *et al*., 1993). An elevated $p27^{Kip1}$ protein level has also been associated with a quiescent, G_0 state (Flørenes *et al*., 1996; Reynisdottir *et al*., 1995; Hengst and Reed, 1996; Pagano *et al*., 1995) and diminished levels of $p27^{Kip1}$ protein have been recently associated with poor prognosis in breast and colon cancers (Catzavelos *et al*., 1997; Loda *et al*., 1997; Porter *et al*., 1997).

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The involvement of p21$^{WAF1/CIP1}$ in tumorigenesis has been widely studied since the gene was identified as a major transcriptional target of wild-type p53 (El-Deiry *et al*., 1993). p21$^{WAF1/CIP1}$ expression is induced by DNA-damage and p21$^{WAF1/CIP1}$ appears to play a role in p53 mediated growth arrest, DNA repair and possibly in apoptosis (Di Leonardo *et al*., 1994; El-Deiry *et al*., 1993). However, p53 independent effects of p21$^{WAF1/CIP1}$ have also been reported in relation to cell cycle arrest of senescent fibroblasts, terminal differentiation, and apoptosis (Noda *et al*., 1994). p21$^{WAF1/CIP1}$ also plays a role in TGF-β mediated growth inhibition (Flørenes *et al*., 1996; Zeng and El-Deiry, 1996; Shao *et al*., 1995; Jiang *et al*., 1994; Steinman *et al*., 1994). Of particular significance with respect to human melanoma, the gene encoding p21$^{WAF1/CIP1}$ was recently cloned as a melanoma differentiating antigen (mda6), the expression of which was shown to be upregulated in more differentiated melanoma cells and in melanocytes grown *in vitro* (Jiang *et al*., 1995). Conversely, decreased p21$^{WAF1/CIP1}$ mRNA and protein levels were detected in cell lines derived from advanced melanomas as well as in tumor specimens obtained from melanoma metastases, suggesting that loss of p21$^{WAF1/CIP1}$ expression may contribute to altered growth regulation during malignant melanoma progression (Maelandsmo *et al*., 1996; Jiang *et al*., 1995; Welch and Rieber, 1996).

Abnormal mitogenesis in tumors can be attributed to either intrinsic aberrations in the generation of mitogenic signals or to abnormal responses to various paracrine and autocrine growth stimulators or inhibitors. The best known growth inhibiting cytokine is TGF-β. Tumors, including melanoma, frequently develop resistance to growth inhibition by TGF-β, in part, through loss or deregulation of cdk inhibitors (Sandhu *et al*., 1997; Fynan and Reiss, 1993). Another cytokine which can mediate G_1 arrest is interleukin-6 (IL-6) which is a potent growth inhibitor of normal melanocytes and of early stage melanoma cells, but not of cell lines isolated from advanced or metastatic melanoma (Jennings *et al*., 1991; Swope *et al*., 1991; Lu and Kerbel, 1993). IL-6 is a pleiotropc cytokine produced by a variety of cells, including endothelial cells, fibroblasts, keratinocytes, monocytes, macrophages, B and T cells, as well as by some cancer cells. Other biological roles IL-6 is known to play include immunological and inflammatory reactions, cell differentiation, angiogenesis and acute phase responses (Kishimoto *et al*., 1995; Kishimoto, 1989). Many of these activities involve autocrine or paracrine regulation of cell growth (Okamoto *et al*., 1997; Chiu *et al*., 1996; Eustace *et al*., 1993; Miki *et al*., 1989; Zhang *et al*., 1989; Kawano *et al*., 1988), motility (Swope *et al*., 1991) or apoptosis (Mizutani *et al*., 1995; Borsellino *et al*., 1995).

The receptor for IL-6 (IL-6R) is a tetramer composed of two transmembrane proteins, the high affinity ligand binding alpha subunit (gp 80) and a signal transducing beta subunit (gp130), which is shared by receptors for oncostatin M (OSM), IL-11, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and cardiotrophin 1 (Kishimoto *et al*., 1995). Binding of IL-6 to the gp 80 protein, results in dimerization and activation of the gp130 subunits followed by activation of members of the tyrosine kinase family known as the Janus kinases (JAKs). Activated JAKs in turn phosphorylate and activate various members of the STAT (Signal Transducers and Activators of Transcription) family of transcription factors, especially STAT3 and STAT1 (Kishimoto *et al*., 1995).

In the context of the growth inhibitory activity of exogenous IL-6 on melanoma cells, it is interesting to note that the growth arrest mediated by certain other cytokines, such as INF-γ and high concentrations of EGF, has been recently associated with activation of STAT1 and STAT3 and their subsequent binding to the p21$^{WAF1/CIP1}$ promoter (Chin *et al*., 1996). Consistent with this finding, an increased level of p21$^{WAF1/CIP1}$ along with accumulation of hypophosphorylated pRb protein, have been detected in M1 leukemia cells induced to differentiate by IL-6 treatment (Resnitzky *et al*., 1992). Furthermore, treatment of B16–F10.9 mouse metastatic melanoma cells with IL-6 in the presence of exogenously added soluble IL-6R alpha subunit (gp 80) was shown recently to induce growth arrest, differentiation and expression of p21$^{WAF1/CIP1}$ (Oh *et al*., 1997).

Despite their IL-6 resistant phenotype, advanced stage-derived human melanoma cell lines express functional IL-6 receptors. Our previous Scatchard analysis revealed significant numbers of high affinity IL-6 binding sites on both IL-6 resistant and sensitive human melanoma cell lines, all of which express both gp 80 and gp130 receptor subunits (Lu and Kerbel, 1993). Furthermore, stimulation of human melanoma cells with IL-6, regardless of their subsequent growth response, leads to activation of JAK kinases and to binding of STAT3/APRF transcription factor to a standard alpha2 microglobulin promoter sequence, as well as to detectable upregulation of IL-6 responsive genes, such as VEGF (Lu and Kerbel, 1993; Rak *et al*., 1996; C Sheehan, unpublished observations). Thus, it appears that loss of responsiveness to the growth inhibitory activity of IL-6 in advanced stage human melanoma is unlikely to be related to a general defect in expression or function of IL-6 receptors. Instead, errors in post-receptor processing of IL-6 signaling and/or alteration in the responses by cell cycle effectors are more likely possibilities.

The purpose of the present study was to identify molecular events leading to cell cycle arrest in IL-6 sensitive melanoma cell lines and to explore the nature of the defects leading to IL-6 resistance in advanced stage cell lines. This aspect of melanoma pathobiology may bear considerable significance since the acquisition of IL-6 resistance is likely to confer a selective growth advantage on tumor cells *in vivo* and such a phenotype may also be an indicator of genetic lesions underlying progression of this disease.

Our results show that IL-6 dependent growth arrest of melanoma cells is associated with upregulation of both p21$^{WAF1/CIP1}$ and p27^{Kip1} in early stage cell lines. Furthermore, p21$^{WAF1/CIP1}$ induction by IL-6 is lost in cell lines derived from advanced primary and metastatic lesions. This change appears to be unrelated to alterations in immediate post-receptor signaling events such as binding of STAT3 and STAT1 to the p21$^{WAF1/CIP1}$ promoter, which is retained in almost all IL-6 resistant cell lines examined. Finally, the loss of p21$^{WAF1/CIP1}$ induction by IL-6 in advanced melanoma cells appears to be a dominant event as it cannot be

rescued in somatic cell hybrids made between IL-6 sensitive and resistant melanoma cell lines.

Results

IL-6 dependent G_1 arrest in early stage melanoma cell lines

We have previously shown that cell lines derived from early stage human melanoma are growth arrested by IL-6, and that this response is lost in cell lines derived from advanced stage lesions (Lu and Kerbel, 1993; Lu *et al*., 1992). Using flow cytometry, we confirmed that this growth inhibition is due to cell cycle arrest in the G_1 phase. Treatment of asynchronously growing early stage cell lines WM35, WM902B and WM1341B with 10 ng/ml IL-6 for 24 h resulted in an increase in the proportion of cells in G_1 and a fall in the S and G_2/M phase fractions. No such growth inhibition was observed following IL-6 treatment of the advanced stage cell lines WM983A, WM239, WM45.1, WM9 and MeWo. Representative data from several FACScan analyses are shown in Table 1.

During progression through G_1 phase of the cell cycle, several events can potentially be affected by IL-6 treatment. In order to determine the interval during the G_1 phase in which IL-6 exerts its inhibitory effect, WM35 cells were synchronized in G_0 by growth to confluence and serum deprivation, and subsequently released from quiescence by replating at low density in serum containing medium, in the presence or absence of 10 ng/ml IL-6. Whereas untreated cells moved through G_1 and into S phase by 16–18 h, entry into S phase was inhibited by addition of the cytokine (Figure 1a). However, IL-6 could only inhibit entrance into S phase if added to the culture no later than 8–10 h after release from G_0. Thus, sensitivity to IL-6-dependent growth arrest is limited to the first part of G_1 phase as has been shown for other growth inhibitory cytokines (Figure 1b) (Tam *et al*., 1994; Laiho *et al*., 1990).

IL-6 induced G_1 arrest is accompanied by inhibition of pRb phosphorylation and loss of cyclin dependent kinase activities

An important requirement for progression through the G_1 phase is phosphorylation of the pRb protein. We have previously shown that treatment of WM35 cells with TGF-β lead to G_1 arrest and accumulation of hypophosphorylated pRb protein (Flørenes *et al*., 1996). Similarly, addition of 10 ng/ml IL-6 to asynchronous cultures of WM35, caused loss of pRb phosphorylation within 12 h. By 24 h, the pRb protein was mainly present in the hypophosphorylated form (Figure 2a). Loss of pRb phosphorylation was paralleled by loss of cyclin D1-associated kinase activity and cyclin E- and cyclin A-associated cdk2 activities (Figure 2b).

$p21^{WAF1/CIP1}$ and $p27^{Kip1}$ accumulate in melanoma cell lines growth inhibited by IL-6

To clarify the mechanism of IL-6 mediated inhibition of cyclin–cdk activities in IL-6 sensitive cell lines, we examined the protein levels of cdks, their associated G_1 cyclins, and the cdk inhibitors, $p21^{WAF1/CIP1}$ and $p27^{Kip1}$, during IL-6-mediated arrest of asynchronously growing WM35 cells. Loss of cyclin D1 and cyclin E associated kinase activitites could not be explained by reduction in the levels of the cyclins or cdks, nor by loss of cyclin/cdk association (Figure 2c and data not shown). No change in cyclin D1 or cyclin E levels were observed up to 48 h after addition of IL-6 to asynchronously growing cells (see Figure 2c). A dramatic reduction in cyclin A protein was observed in IL-6 treated cells (Figure 2c), and was paralleled by the loss of cyclin A/cdk2 activity. Since the latter activity is involved late in the G_1 to S phase transition, its loss is likely secondary to the IL-6 dependent G_1 arrest.

IL-6 treatment induced a significant increase in the levels of KIP family members $p21^{WAF1/CIP1}$ and $p27^{Kip1}$. In this regard, it should be noted that WM35 cells do not express the INK4 inhibitors $p15^{INK4b}$ and $p16^{INK4a}$ (Flørenes *et al*., 1996). Asynchronously growing WM35 cells express moderate levels of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ proteins, both of which were dramatically increased following treatment with IL-6 (Figure 2c). $p21^{WAF1/CIP1}$ protein increased by fourfold during the first 6 h of treatment and remained elevated. In comparison, a rise in $p27^{Kip1}$ was noted by 12 h and continued to increase for up to 36 h reaching a maximum of sixfold above the control level (Figure 2c). Significantly, the increase in $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ levels was paralleled by their increased association with both cyclin D1/cdk4 and cyclin E/cdk2 complexes (data not shown).

Table 1 Cell cycle distribution of human melanoma cell lines treated with 10 ng/ml IL-6

	G_1		S		G_2/M	
IL-6 treatment (24 h)	*Control*	*IL-6*	*Control*	*IL-6*	*Control*	*IL-6*
Cell Lines						
Sensitive						
WM35	52	82	34	8	14	9
WM902B	58	73	26	18	16	9
WM1341B	69	80	20	12	11	8
Resistant						
WM983A	63	61	31	30	7	9
WM239	62	67	26	21	12	12
WM451	43	48	32	27	25	25
WM9	65	63	29	29	6	8
MeWo	63	63	27	26	10	11

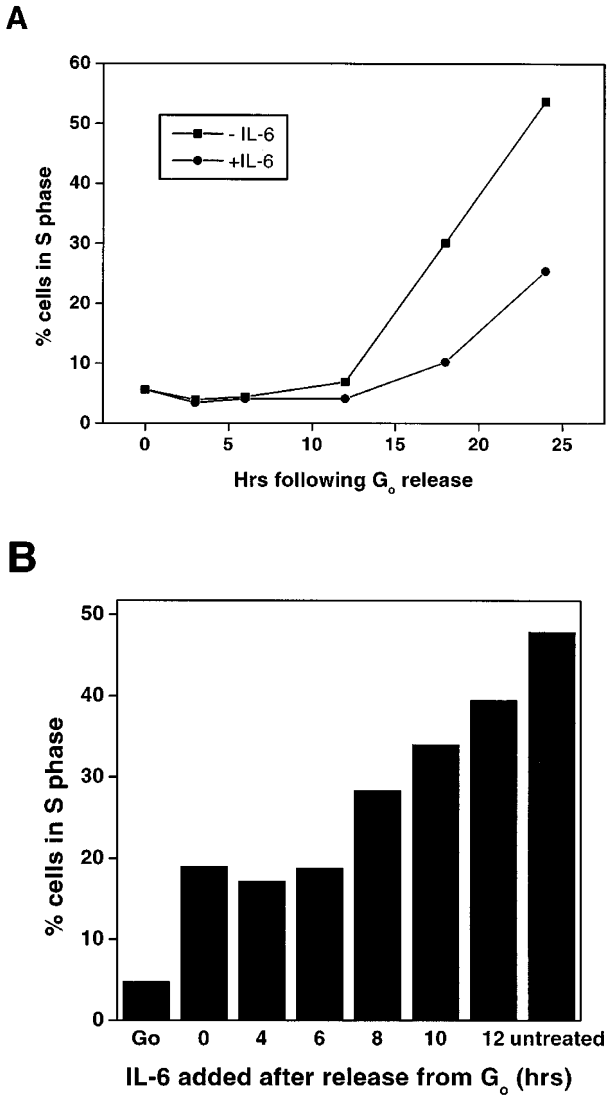

Figure 1 The effect of IL-6 on cell cycle distribution in a growth synchronized population of melanoma cells. (**a**) WM35 cells were synchronized in G_0 as described in Materials and methods and thereafter released from quiescence at time zero without (■) or with (●) 10 ng/ml IL-6 and harvested at the indicated time points. (**b**) WM35 cells were synchronized as in (**a**) and 10 ng/ml IL-6 was added to the cultures at different time points following release; the cells were harvested at 24 h. FACScan analysis was performed to estimate the percentage of cells entering S phase

IL-6 inhibits S-phase entry in a synchronized population of melanoma cells by blocking downregulation of p21WAF1/CIP1 and p27Kip1

To further define how $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ might contribute to G_1 arrest following IL-6 treatment of early-stage IL-6 sensitive human melanoma cells, WM35 cells were synchronized and then released from G_0 either with or without addition of IL-6. The total level of $p21^{WAF1/CIP1}$ was, as we have shown previously (Flørenes *et al*., 1996), relatively low in G_0 arrested WM35 cells, whereas $p27^{Kip1}$ was highly expressed in quiescent cells (Figure 3a). Following release from G_0, a significant induction of $p21^{WAF1/CIP1}$ was observed in both IL-6 treated and untreated cells, peaking around 6 h later. As cells progressed through G_1 there was a rapid decline in $p21^{WAF1/CIP1}$ noticeable after 12 h of release. Loss of $p21^{WAF1/CIP1}$ from both cyclin D1/cdk4 and cyclin E/cdk2 complexes coincided with kinase activation and with the onset of pRb phosphorylation (Figure 3b, c and data not shown). The latter changes were clearly inhibited by addition of IL-6, suggesting that inhibition of cell progression through G_1 phase is related to a block in $p21^{WAF1/CIP1}$ downregulation. In contrast to $p21^{WAF1/CIP1}$, total cellular $p27^{Kip1}$ protein, as well as its abundance in cyclin E/cdk2 complexes were maximal in G_0, and remained relatively constant during early to mid G_1 phase. A decline in both total $p27^{Kip1}$ and in its association with cyclin E/cdk2 was first observed by 18 h as the cells moved into S phase (Figure 3a and c). Following release from quiescence, the amount of $p27^{Kip1}$ in cyclin D1/cdk4 complexes increased, peaking by 12 h and declined thereafter as cells progressed into S phase (Figure 3b). Loss of $p27^{Kip1}$ from cyclin D1/cdk4 and from cyclin E/cdk2 complexes was inhibited in IL-6 treated cells, suggesting that $p27^{Kip1}$ may also contribute to G_1 arrest induced by IL-6.

Resistant melanoma cell lines fail to upregulate p21WAF1/CIP1 and p27Kip1 in response to IL-6

Results obtained with WM35, suggest that the kinase inhibitors $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ are important mediators of growth arrest following IL-6 treatment of early stage melanomas. In order to examine whether $p21^{WAF1/CIP1}$ and/or $p27^{Kip1}$ play a general role in determining the response of human molanomas to IL-6, additional IL-6 sensitive cell lines WM902B and WM1341B as well as the IL-6 resistant lines WM983A, WM239, WM9, WM 45.1 and MeWo were treated with 10 ng/ml IL-6 for 12 and 24 h, and the levels of KIP inhibitors were evaluated (Figure 4). It should be mentioned that WM902B is the only one of these cell lines that expresses the cdk-inhibitor $p16^{INK4a}$ and none of the examined cell lines express $p15^{INK4b}$ (data not shown). As was observed for WM35, both WM1341B and WM902B cell lines expressed moderate levels of $p21^{WAF1/CIP1}$ protein which significantly increased after addition of IL-6 to asynchronously growing cultures (Figure 4). Of the five IL-6 resistant cell lines tested, only two (WM239 and WM9) showed detectable levels of $p21^{WAF1/CIP1}$ protein. $p21^{WAF1/CIP1}$ protein levels did not increase following IL-6 treatment in any of the five IL-6 resistant cell lines. While $p27^{Kip1}$ was detectable in all melanoma cell lines regardless of their IL-6 sensitivity, a rise in $p27^{Kip1}$ levels was seen only in the sensitive lines following IL-6 treatment. In one cell line, WM1341B upregulation of $p27^{Kip1}$ was modest and transient. This cell line did, however, show the strongest upregulation of $p21^{WAF1/CIP1}$ after addition of IL-6 (Figure 4).

We next examined whether the effects of IL-6 on $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ protein levels were reflected at the mRNA level. Northern analysis showed that $p21^{WAF1/CIP1}$ mRNA is upregulated by IL-6 in all three sensitive cell lines (WM35, WM902B, WM1341B). Two patterns of $p21^{WAF1/CIP1}$ expression were noted in the resistant lines. In two resistant cell lines, WM9 and WM239, $p21^{WAF1/CIP1}$ mRNA was expressed constitutively in asynchronously growing cultures, but it was not induced by IL-6. In contrast, $p21^{WAF1/CIP1}$ mRNA was barely detectable in the three other IL-6 resistant cell lines, WM983A, WM45.1 and

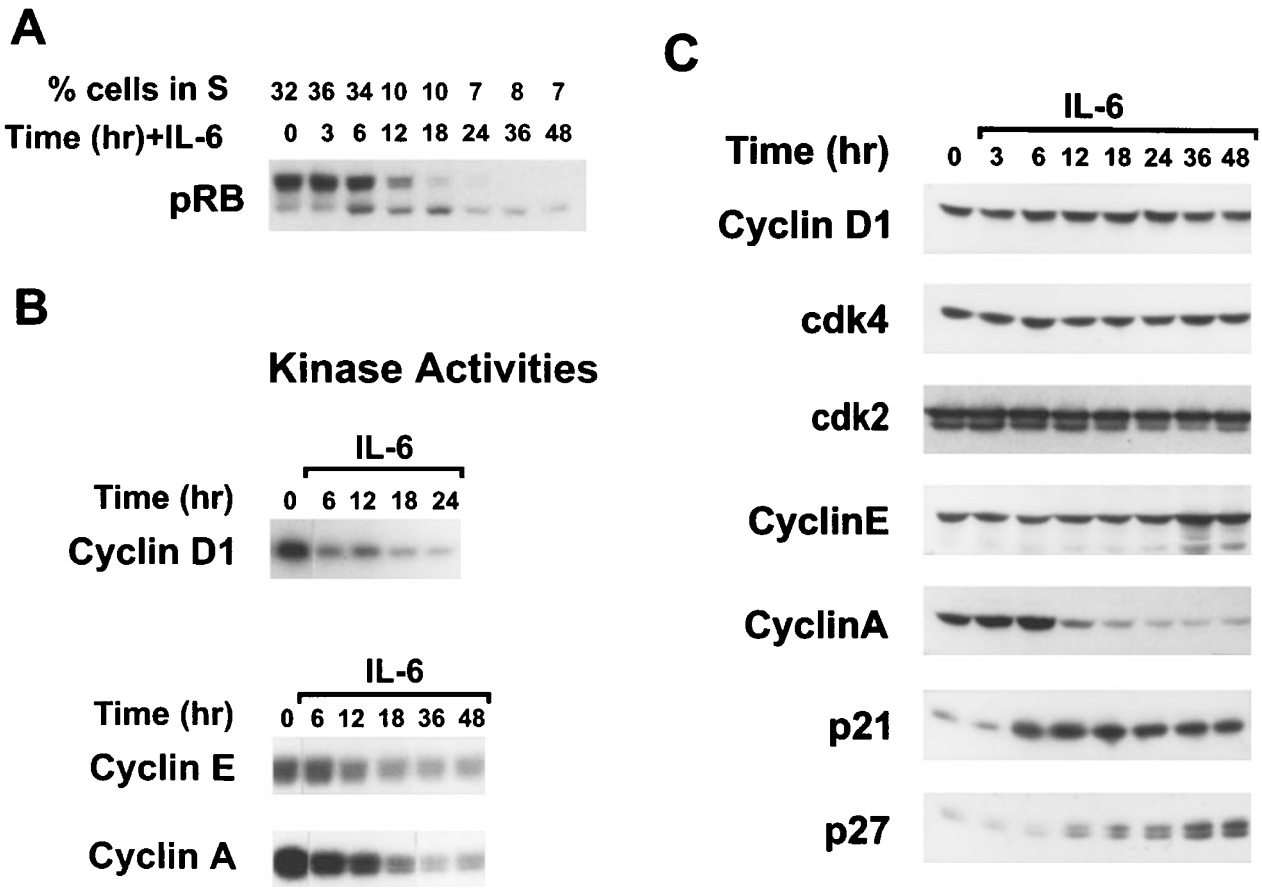

Figure 2 The effect of IL-6 on G_1 associated cell cycle regulators and cyclin/cdk associated kinase activities. (**a**) A proliferating population of WM35 cells was treated with IL-6 and the phosphorylation status of the retinoblastoma protein was examined. Corresponding DNA profiles were determined by FACscan analysis and the percentage of cells in S phase is indicated for each time point. (**b**) Cyclin/cdk activities. For cyclin D1 associated kinase activity cyclin D1 was immunoprecipitated with DCS-11 antibody from untreated cells (0) and from cells treated with IL-6 for the length of time indicated. The reaction was carried out using recombinant retinoblastoma protein as substrate. For cyclin E and cyclin A associated kinase activities the complexes were immunoprecipitated using antibodies to cyclin E and cyclin A, respectively from cells treated with IL-6 and histone H1 was used as substrate in this case. (**c**) Total cell lysates were analysed by Western blotting as described in (**a**) for the expression of cyclin D1, cyclin E, cyclin A, cdk4, cdk2 and the kinase inhibitors $p21^{WAF1}$ and $P27^{Kip1}$

MeWo, all of which also express little or no $p27^{WAF1/CIP1}$ protein, and fail to upregulate $p21^{WAF1/CIP1}$ mRNA in the presence of IL-6. Examples of these expression patterns are shown in Figure 5. The level of $p21^{Kip1}$ mRNA did not change following IL-6 treatment in either IL-6 sensitive or resistant melanoma cell lines (data not shown).

IL-6 treatment leads to binding of STAT1 and STAT3 transcription factors to the $p21^{WAF1/CIP1}$ promoter in both IL-6 sensitive and resistant melanoma cell lines

Binding of IL-6 to its receptor has been shown to trigger intracellular signals through the JAK/STAT pathway, particularly involving STAT1 and STAT3 transcription factors (Kishimoto *et al*., 1995). Both STAT1 and STAT3 have been shown to bind and activate the $p21^{WAF1/CIP1}$ promoter (Chin *et al*., 1996). We speculated that IL-6 mediated growth inhibition in melanoma cells might involve STAT1 and STAT3 action on $p21^{WAF1/CIP1}$. We sought to determine whether IL-6 could induce STAT binding to the $p21^{WAF1/CIP1}$ promoter sequence in IL-6 sensitive melanoma cell lines, and whether loss of such STAT activation and DNA binding might explain the failure to upregulate $p21^{WAF1/CIP1}$ in the IL-6 resistant cell lines.

Both IL-6 sensitive and resistant melanoma cell lines showed comparable levels of STAT1 and STAT3 proteins (Figure 6a). However, STAT protein levels were slightly and reproducibly increased by IL-6 treatment in the sensitive but not in the resistant lines. An electrophoretic mobility shift assay (EMSA) was performed to examine whether IL-6 can induce binding of STAT1 and STAT3 to the $p21^{WAF1/CIP1}$ promoter. Despite the difference in the ability to upregulate $p21^{WAF1/CIP1}$ following IL-6 treatment, both sensitive and resistant cell lines showed equal IL-6 dependent STAT1 and STAT3 binding to the $p21^{WAF1/CIP1}$ promoter. The only exception to this pattern was noted in the case of MeWo cells, where IL-6 resistance was paralleled by attenuation of activation of STAT in the presence of the cytokine (see Figure 6b). A supershift analysis showed that in these cells only STAT3 was able to bind to the $p21^{WAF1}$promoter.

The results of EMSA experiments suggested that STAT binding to the $p21^{WAF1/CIP1}$ promoter could be activated in both the IL-6 sensitive and resistant cell

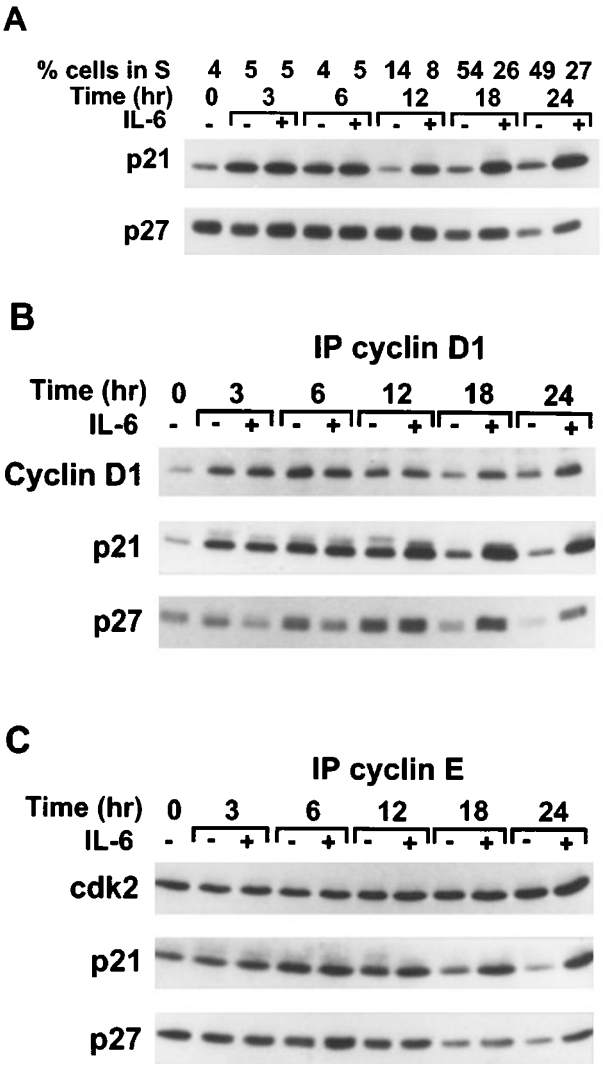

Figure 3 The effect of IL-6 on levels of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ and their association with cyclin/cdks following release from G_0 in WM35 cells. (**a**) Western blot of total lysates showing the profile of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ during the G_1 to S phase transition in cells incubated without (−) or treated with (+) IL-6. Corresponding DNA profiles were determined by FACscan analysis and the percentage of cells in S phase is indicated for each time point. Cyclin D1 (**b**) and Cyclin E (**c**) were immunoprecipitated from the same lysate as shown in (**a**) and associated $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ inhibitors were visualized. Cyclin E could not be visualized in Cyclin E immunoprecipitates because it co-migrates with the heavy chain of the mAbE172 antibody

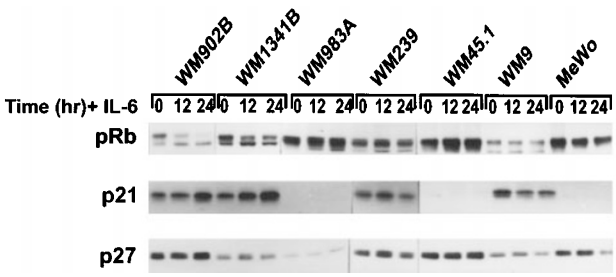

Figure 4 Western blot analysis of IL-6 influence on pRb, $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ in the panel of melanoma cell lines. The phosphorylation status of pRb, and the total protein levels of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ were analysed following treatment of asynchronously growing IL-6 sensitive melanoma cell lines WM902B, WM1341B, and the IL-6 resistant cell lines WM983A, WM239, WM45.1, WM9 and MeWo with 10 ng/ml IL-6 for 12 and 24 h

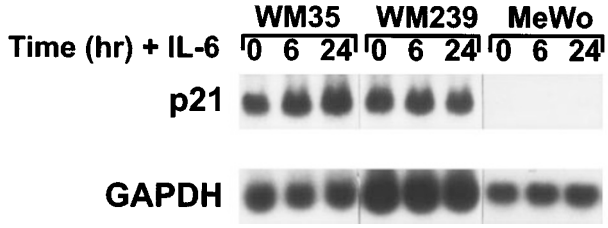

Figure 5 Northern blot analysis of $p21^{WAF1/CIP1}$ following treatment of melanoma cells with IL-6. The early stage melanoma cell line WM35, and the metastatic cell lines WM239 and MeWo were cultured with 10 ng/ml IL-6 for 6 and 24 h. Ten micrograms of total RNA in each lane was hybridized with a $p21^{WAF1/CIP1}$ cDNA probe and, as control, with a GAPDH cDNA probe as described in Materials and methods

lines, even though only the former cells upregulated $p21^{WAF1/CIP1}$ mRNA and protein. This indicated that the acquisition of IL-6 resistance was not due to a simple loss-of-function defect at the receptor or STAT activation level. In fact, it is conceivable that transduction of the growth inhibitory signal may be compromised by a gain-of-function event associated with melanoma progression. To test this hypothesis, we took advantage of a panel of somatic hybrids generated between IL-6 sensitive and resistant melanoma cell lines (MacDougall *et al*., 1995). As a control, we used a hybrid made between hygromycin and neomycin resistant variants of WM35. This WM35 × WM35 hybrid remained sensitive to IL-6 and showed the same type of accumulation of $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ in response to IL-6 treatment as was seen in the parental WM35 line (Figure 7). However, hybrids between the IL-6 resistant cell line WM239 and either of the sensitive lines failed to upregulate $p21^{WAF1/CIP1}$ or $p27^{Kip1}$ and remained resistant to IL-6 mediated growth inhibition. This result suggests that inability to upregulate both CKIs by IL-6 resistant melanoma cell lines is indeed a dominant trait.

Discussion

Our previous work has demonstrated that early stage human melanoma cell lines are growth arrested by IL-6, whereas this effect is lost in cell lines derived from more advanced stage melanoma lesions (Lu and Kerbel, 1993). The pattern of growth response to IL-6 correlates well with the stage of progression of the tumor of origin and with the tumorigenic properties of the respective cell lines in nude mice. IL-6 is of particular relevance to melanoma biology since this cytokine is produced by cells present in the cutaneous micromillieau such as fibroblasts, keratinocytes, endothelial cells and inflammatory cells. Thus, it is possible that IL-6 produced by such cells could be responsible, at least in part, for maintaining normal melanocytes in a quiescent state (Rak *et al*., 1996). It is also possible that the growth inhibitory effects of IL-6 may contribute to the very slow growth, or dormancy,

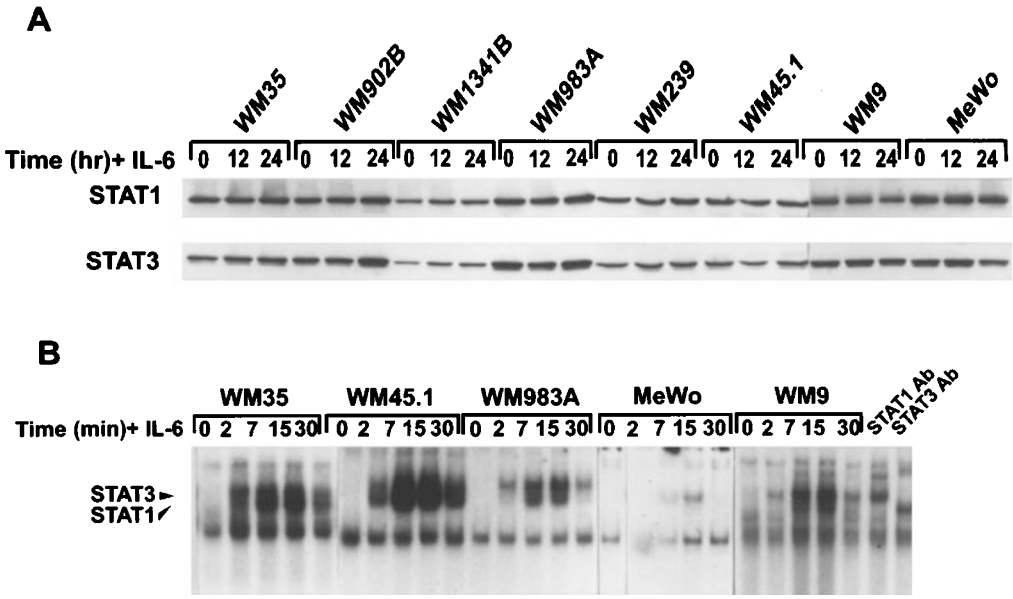

Figure 6 The effect of IL-6 on STAT protein levels and binding to the p21$^{WAF1/CIP1}$ promoter. (**a**) Western blot analysis shows the protein levels of STAT1 and STAT3 following treatment of early stage (WM35, WM902B, WM1341B) and advanced stage melanoma cell lines (WM983A, WM239, WM45.1, WM9, MeWo) with 10 ng/ml IL-6 for 12 and 24 h. (**b**) Representative DNA mobility shift assay showing binding of STAT1 and STAT3 to the p21$^{WAF1/CIP1}$ promoter. Nuclear extracts made from WM35, WM45.1, WM983A, MeWo and WM9 treated with 50 ng/ml IL-6 for 2, 7, 15 and 30 min and sujbected to DNA Electrophoretic Mobility Shift Assay (EMSA) using a γ^{32}P-ATP labeled oligonucleotide probe recognizing the SIE site in the p21$^{WAF1/CIP1}$ promoter as described in Materials and methods. A supershift assay was performed to confirm the identity of STAT binding to the p21$^{WAF1/CIP1}$ promoter by adding anti-STAT1 or anti-STAT3 antibodies to the reaction mixture before adding the probe (shown for WM9 cells)

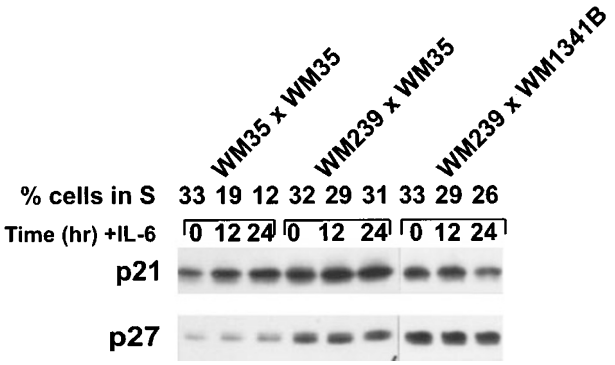

Figure 7 The effect of IL-6 on levels of p21$^{WAF1/CIP1}$ and p27^{Kip1} in somatic hybrids between IL-6 resistant and sensitive cell lines. Hybrids were made between WM239 and WM35 or WM1341B respectively, and the cells were treated for 12 and 24 h with 10 ng/ml IL-6 and p21$^{WAF1/CIP1}$ and p27^{Kip1} levels were assayed by Western blot. A hybrid made between hygromycin or neomycin selected WM35 cells was included as a control. DNA profiles were determined by FACScan analysis and the percentage of cells in S phase is indicated for each time point

of early stage primary melanoma lesions *in vivo*. In this respect, it is interesting to note that ingrowth of IL-6 producing endothelial cells (via tumor angiogenesis) into such early stage lesions is sometimes associated with histological regression, followed by rapid outgrowth of more malignant and presumably IL-6 resistant tumors (Rak *et al*., 1996; Barnhill and Levy, 1993). Endothelial cell derived IL-6 may participate in a positive paracrine feedback loop where melanoma cells exposed to IL-6 would upregulate the production of angiogenic factors such as bFGF or VEGF that would stimulate directed migration of more IL-6 producing endothelial cells (Rak *et al*., 1996). Our observation that VEGF can be upregulated by IL-6, even in melanoma cell lines which are not growth inhibited by this cytokine, may suggest an indirect role of IL-6 in melanoma angiogenesis and help explain why expression of IL-6 receptors is retained during tumor progression (Rak *et al*., 1996).

At the mechanistic level our results suggest that altered regulation of p21$^{WAF1/CIP1}$ and p27^{Kip1} expression may play a crucial role in development of IL-6 mediated growth resistance and possibly in melanoma progression in general. IL-6 treatment of early stage melanoma cell lines such as WM35, WM902B and WM1341B was found to induce G_1 arrest and hypophosphorylation of the pRb protein, through inhibition of cyclin D1/cdk4 and cyclin E/cdk2 activities. Our data suggests that this inhibition in cdk activities results from the increase in the levels p21$^{WAF1/CIP1}$ and p27^{Kip1} and the increased association of both inhibitors with cyclin/cdk complexes. These observations support an important role for p21$^{WAF1/CIP1}$ and p27^{Kip1} in IL-6 mediated growth arrest.

Further support for an important role for KIP inhibitors in IL-6 mediated growth arrest in early stage melanomas came from recent findings (Maelandsmo *et al*., 1996) demonstrating a correlation between loss of p21$^{WAF1/CIP1}$ expression and increased tumorigenic potential of human melanoma cell lines. Similarly, a decrease in both p21$^{WAF1/CIP1}$ and p27^{Kip1} proteins in metastatic specimens as compared to primary human melanoma lesions was shown in our recent immunohistochemical studies (Maelandsmo *et al*., 1996; Flørenes *et al*., 1998). Moreover, in the present work we have demonstrated that expression of p21$^{WAF1/CIP1}$ and p27^{Kip1} can be increased by IL-6 in sensitive lines but neither was increased in any of the IL-6 resistant lines.

The level of p21$^{WAF1/CIP1}$ protein in melanoma cells is subject to important regulation at the mRNA level (Maelandsmo *et al*., 1996; Vidal *et al*., 1995). A number of studies have demonstrated both p53-dependent and independent regulation of this kinase inhibitor (Sherr and Roberts, 1995). Our recent clinical study showed no correlation between the loss of p21$^{WAF1/CIP1}$ and the status of p53 in human melanoma specimens (Maelandsmo *et al*., 1996). In the present study, p21$^{WAF1/CIP1}$ induction by IL-6 was not preceded by an increase in p53 in early stage melanoma cell lines (data not shown). Interestingly, melanoma cell lines derived from late stage primary lesions or metastases displayed either very low constitutive levels of p21$^{WAF1/CIP1}$, or if the protein was expressed, its level could not be further increased by IL-6 treatment. In both cases, this was associated with resistance to IL-6 mediated growth arrest. The reason for loss of p21$^{WAF1/CIP1}$ expression in three of the advanced melanomas is not entirely clear. However, several studies have shown that mutations of the p21$^{WAF1/CIP1}$ gene are rare in human tumors, including melanoma, and hence aberrant regulation of this CKI is a likely possibility (Vidal *et al*., 1995; Shiohara *et al*., 1994).

Members of the STAT pathway have been shown recently to be important regulators of p21$^{WAF1/CIP1}$ expression (Chin *et al*., 1996; Matsumura *et al*., 1997). We wished to explore the possible involvement of this pathway in the patterns of p21$^{WAF1/CIP1}$ expression we observed in early *versus* advanced stage lesions, and in the presence or absence of IL-6. A variety of growth inhibitory cytokines such as IFN-γ, high (inhibitory) concentrations of EGF or thrombopoietin have been shown to induce STAT1, STAT3 (Chin *et al*., 1996) and STAT5 (Matsumura *et al*., 1997) binding to and activation of the p21$^{WAF1/CIP1}$ promoter. Here we show that in human melanoma cells, IL-6 can also induce STAT1 and STAT3 binding to p21$^{WAF1/CIP1}$ promoter, surprisingly, in both IL-6 sensitive and resistant lines. The lack of IL-6 mediated induction of p21$^{WAF1/CIP1}$ in the resistant WM239 and WM9 melanoma cell lines, which express p21$^{WAF1/CIP1}$ constitutively is somewhat surprising since in both cases the interaction of STAT1 and STAT3 with the p21$^{WAF1/CIP1}$ promoter appears intact. This failure to upregulate p21$^{WAF1/CIP1}$ likely reflects a defect in the IL-6-dependent signaling rather than a general block in p21$^{WAF1/CIP1}$ expression, since in WM9 cells, both p21$^{WAF1/CIP1}$ accumulation and growth inhibition can be readily induced by TGF-β, a cytokine whose response does not involve STAT activation (data not shown). Thus, based on our EMSA and expression data, it can be speculated that the defect in IL-6 dependent regulation of p21$^{WAF1/CIP1}$ in advanced melanoma cells involves events downstream of STAT binding to the p21$^{WAF1/CIP1}$ promoter. It is also possible, although less likely, that in melanoma cells IL-6 may regulate p21$^{WAF1/CIP1}$ levels in a manner not entirely dependent on STAT1/3 activation.

One possible explanation for the inability of IL-6 to activate the p21$^{WAF1/CIP1}$ expression in IL-6 resistant melanoma cell lines is that additional regulatory signals may exist in highly malignant tumor cells which would obliterate the IL-6 signaling in a dominant fashion. Our results with somatic melanoma cell hybrids seem to support such a possibility. Neither growth arrest nor induction of p21$^{WAF1/CIP1}$ by IL-6 could be rescued in WM239 (IL-6 resistant) cells when these cells were fused with the IL-6 sensitive WM35 or WM1341B cell lines.

In conclusion, our results suggest that p21$^{WAF1/CIP1}$ is regulated by IL-6 in early stage melanoma cells and this contributes importantly to G_1 arrest induced by this cytokine. The loss of IL-6 dependent growth inhibition in advanced stage melanoma cell lines is associated with loss of induction of p21$^{WAF1/CIP1}$, with failure to increase p27^{Kip1} protein and consequent loss of KIP inhibitor association with target cdks. Furthermore, this phenotype cannot be rescued in somatic cell hybrids sugggesting a dominant alteration associated with melanoma progression. It is conceivable therefore that putative oncogenes or loss of tumor suppressor genes driving progression of human melanoma may be closely related to pathways of IL-6 signal transduction and regulation of p21$^{WAF1/CIP1}$ in this particular type of tumor.

Materials and methods

Cell cultures

All human melanoma cell lines used in this study were routinely cultured in RPMI 1640 medium (Gibco) supplemented with 5% fetal bovine serum (FBS) (Hyclone Labs, Logan, NY, USA). The cell lines of the WM series were kindly provided by Dr Meenhard Herlyn (Wistar Institute, Philadelphia, PA, USA) and have been described in detail elsewhere (MacDougall *et al*., 1993; Cornil *et al*., 1991). The MeWo cell line was derived from a lymph node metastasis and its properties have been described elsewhere (Ishikawa *et al*., 1988a,b). The somatic hybrid cell lines WM35 $\times$ WM35, WM35 $\times$ WM239, and WM1341B $\times$ WM239 were established as described by MacDougall *et al*. (1995). For treatment of asynchronous growing cells with IL-6, 7×10^5 cells were plated overnight in 100 mm culture dishes in RPMI 1640 containing 5% FBS. The medium was then changed to RPMI 1640 medium containing 1% FBS and 10 ng/ml IL-6. The cells were harvested at different times following addition of IL-6 and washed twice with PBS prior to preparation of protein lysates, RNA and samples for flow cytometry.

WM35 cells were synchronized in G_0 by growth to confluence in RPMI 1640 medium supplemented with 5% FBS followed by serum starvation (1% FBS) for two additional days. The cells were then trypsinized, washed twice with PBS and plated at 7×10^5 cells per 100 mm petri dish in complete medium, with or without 10 ng/ml IL-6. The cells were harvested at different intervals after release from G_0 as described above. Alternatively, 10 ng/ml IL-6 was added to the cultures at different times following release, and harvested after 24 h.

Flow cytometric analysis

Cells were harvested for flow cytometric analysis, and fixed in 70% ethanol for 1 h at 4°C. The cells were washed twice with PBS, resuspended in a solution of 50 μg/ml propidium iodide and 10 μg/ml RNAse in PBS (PI solution) and incubated for at least 15 min at room temperature in the dark before data acquisition and subsequent analysis on a Becton Dickinson FACScan, using CellFit software.

Antibodies

Antibody to the retinoblastoma protein (pRb) was obtained from Pharmingen (San Diego, CA, USA) and

antibodies to cyclin D1, cyclin A, cdk4, $p21^{WAF1/CIP1}$, STAT1 (C-24) and STAT3 (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cdk2 was from Upstate Biotechnology (Lake Placid, NY, USA) and $p27^{Kip1}$ and anti-ISGF3 (STAT3) antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Cyclin D1 antibody DCS-11 was a gift from J Bartek (Danish Cancer Society, Denmark). Cyclin A mAbE67 was provided by J Gannon and T Hunt (ICRF, UK) and cyclin E mAbE172 was from E Lees and E Harlow (MGH, Boston, MA, USA). A monoclonal antibody, JC-6 that recognizes both $p16^{INK4a}$ and $p15^{INK4b}$ was provided by J Koh and E Harlow (MGH, Boston, MA, USA).

Immunoblotting

Cells were lysed in ice-cold NP-40 lysis buffer (1% NP40, 10% glycerol, 20 mM Tris HCl pH 7.5, 137 mM NaCl, 100 mM sodium vanadate, 1 mM phenylmethyl sulphonyl fluoride (PMSF) and 0.02 mg/ml each of aprotinin, leupeptin and pepstatin). Lysates were sonicated and clarified by centrifugation. Protein quantitation was done by Bradford analysis and 30 μg protein/lane was resolved by SDS polyacrylamide gel electrophoresis (SDS–PAGE). Transfer and hybridization were as described (Dulic *et al*., 1992). The relative amounts of proteins were quantitated, and where indicated, by scanning several ECL-exposures using an Ultrascan XL Laser Densitometer (LKB, Bromma, Sweden). For detection of cyclin D1 and cyclin E associated proteins, cyclin D1 and cyclin E were immunoprecipitated from 200 μg total protein followed by SDS–PAGE and Western blot analysis.

Cyclin-dependent kinase assays

Cyclin D1 associated cdk4 kinase assay was performed using the method of Matsushimi *et al*. (1994). Briefly, cyclin D1/cdk4 complexes were immunoprecipitated with DCS-11 antibody. Precipitates were collected on protein A sepharose beads, washed extensively, and reacted with [γ^{32}-P]ATP and recombinant retinoblastoma protein substrate (expression vector provided by Y Zhao, Lab. of E Harlow, Massachusetts General Hospital, MA, USA). Cyclin E and cyclin A associated cdk2 activities were determined by immunoprecipitating cyclin E or cyclin A and using histone H1 as substrate (Boehringer Mannheim, Quebec, Canada).

Northern blot analysis

Total cellular RNA was extracted by Trizol reagent as described by the manufacturer (Gibco–BRL, Grand Island, NY, USA). Northern blot analysis was performed as previously described (Maelandsmo *et al*., 1996). The filters were hybridized with a $p21^{WAF1/CIP1}$ cDNA probe kindly provided by Dr Bert Vogelstein, The John Hopkins Oncology Centre, Baltimore, MA, USA. The hybridizations were carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM sodium-EDTA at 65°C over night as described by Church and Gilbert (1984). For multiple hybridizations, the bound probe was removed by incubating the filters twice for 5 min in 0.1× standard saline citrate (SSC), 0.1% SDS at 95–100°C. To correct for uneven amount of RNA loaded in each lane, the filters were rehybridized with a GAPDH cDNA probe.

DNA electrophonetic mobility shift assay (EMSA)

The ability of STAT1 and STAT3 to bind to the $p21^{WAF1/CIP1}$ promoter upon IL-6 treatment was examined by DNA electrophonetic mobility shift assay. Briefly, nuclear extract was prepared as described (Andrews and Faller, 1991) from 2×10^6 untreated cells or from cells treated with 50 ng/ml IL-6 for 2–30 min. Gel mobility assays were performed according to the protocol of Chin *et al*. (1996) except that nuclear extract was incubated with radiolabeled probe for 60 min at room temperature. The double-stranded oligonucleotide $p21^{WAF1/CIP1}$-SIE1 (5′-GATCTCCTTCCCG-GAAGCA-3′) containing a $p21^{WAF1/CIP1}$ binding site was used as a probe (Chin *et al*., 1996). In the supershift assays, anti-STAT1 (C-24, Santa Cruz, CA, USA) or anti-STAT3 (C-20, Santa Cruz) were added to the reaction mixture before adding the probe.

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References

Andrews NC and Faller DV. (1991). *Nucleic Acids. Res.,* **19,** 2499.

Barnhill RL and Levy MA. (1993). *Am. J. Pathol.,* **143,** 99–104.

Borsellino N, Belldegrum A and Bonavida B. (1995). *Cancer Res.,* **55,** 4633–4639.

Caldas C, Hahn SA, da Costa LT, Redston MS, Schutte M, Seymour AB, Weinstein CL, Hruban RH, Yeo CJ and Kern SE. (1994). *Nat. Genet.,* **8,** 27–32.

Catzavelos C, Bhattacharya N, Ung YC, Wilson JA, Roncari L, Sandhu C, Shaw P, Yeger H, Morava-Protzner I, Kapusta L, Franssen E, Pritchard KI and Slingerland JM. (1997). *Nat. Med.* **3,** 227–230.

Chin YE, Kitagawa M, Su W-CS, You Z-H, Iwamoto Y and Fuk X-Y. (1996). *Science,* **272,** 719–722.

Chiu JJ, Sgagias MK and Cowan KH. (1996). *Clin. Cancer Res.,* **2,** 215–221.

Church GM and Gilbert W. (1984). *Proc. Natl. Acad. Sci. USA,* **81,** 1991–1995.

Cornil I, Theodorescu D, Man S, Herlyn M, Jambrosic J and Kerbel RS. (1991). *Proc. Natl. Acad. Sci. USA,* **88,** 6028–6032.

Di Leonardo A, Linke SP, Clarkin K and Wahl GM. (1994). *Genes Dev.,* **8,** 2540–2551.

Dulic V, Lees E and Reed SI. (1992). *Science,* **257,** 1958–1961.

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B. (1993). *Cell,* **75,** 817–825.

Eustace D, Han X, Gooding R, Rowbottom A, Riches P and Heyderman E. (1993). *Gyne. Oncol.,* **50,** 15–19.
Flørenes VA, Bhattacharya N, Bani MR, Ben-David Y, Kerbel RS and Slingerland JM. (1996). *Oncogene,* **13,** 2447–2457.
Flørenes VA, Maelandsmo GM, Kerbel RS, Slingerland JM, Nesland JM and Holm R. (1998). *Am. J. Pathol.,* **153,** 305–312.
Flores JF, Walker GJ, Glendening JM, Haluska FG, Castresana JS, Rubio M-P, Pastorfide GC, Boyer LA, Kao WH, Bulyk ML, Barnhill RL, Hayward NK, Housman DE and Fountain JW. (1996). *Cancer Res.,* **56,** 5023–5032.
Fynan TM andReiss M. (1993). *Crit. Rev. Oncog.,* **4,** 493–540.
Hannon GJ and Beach D. (1994). *Nature,* **371,** 257–261.
Hengst L and Reed SI. (1996). *Science,* **271,** 1861–1864.
Hirama T and Koeffler HP. (1995). *Blood,* **86,** 841–854.
Ishikawa M, Dennis JW, Man S and Kerbel RS. (1988a). *Cancer Res.,* **48,** 665–670.
Ishikawa M, Fernandez B and Kerbel RS. (1988b). *Cancer Res.,* **48,** 4897–4903.
Jennings MT, Maciunas RJ, Carver R, Bascome CC, Juneau P, Misulis K and Moses HL. (1991). *Int. J. Cancer,* **49,** 129–139.
Jiang H, Lin J, Su Z, Herlyn M, Kerbel RS, Weissman BE, Welch DR and Fisher PB. (1995). *Oncogene,* **10,** 1855–1864.
Jiang H, Lin J, Su ZZ, Collart FR, Huberman and Fisher PB. (1994). *Oncogene,* **9,** 3397–3406.
Kamb A, Gruis NA, Weaver-feldhaus J, Liu Q, Harshman K, Tavtigan SV, Stockert E, Day III RS, Johnson BE and Skolnick MH. (1994a). *Science,* **264,** 436–440.
Kamb A, Shattuck-Eidens D, Eeles R, Liu Q, Gruis NA, Ding W, Hussey C, Tran T, Miki Y and Weaver-feldhaus J. (1994b). *Nat. Genet.,* **8,** 23–26.
Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K, Asaku H, Tang B, Tanabe O, Tanada H, Kuramoto A and Kishimoto T. (1988). *Nature,* **332,** 83–85.
Kishimoto T. (1989). *Blood,* **74,** 1–10.
Kishimoto T, Akira S, Narazaki M and Taga T. (1995). *Blood,* **86,** 1243–1254.
Koff A, Masahiko O, Polyak K, Roberts JM and Massague J. (1993). *Science,* **260,** 536–539.
Laiho M, Weis FMB and Massague J. (1990). *J. Biol. Chem.,* **265,** 18518–18524.
Loda M, Cukor B, Tam SW, Lavin P, Fiorentino M, Draetta GF, Jessup JM and Pagano M. (1997). *Nat. Med.* **3,** 231–234.
Lu C and Kerbel RS. (1993). *J. Cell Biol.,* **120,** 1281–1288.
Lu C, Vickers MF and Kerbel RS. (1992). *Proc. Natl. Acad. Sci. USA,* **89,** 9215–9219.
MacDougall JR, Kobayashi H and Kerbel RS. (1993). *Mol. Cell. Different.,* **1,** 21–40.
MacDougall JR, Lin Y, Rak J and Kerbel RS. (1995). *Cancer Res.,* **55/18,** 4174–4181.
Maelandsmo GM, Flørenes VA, Hovig E, Oyjord T, Engebraaten O, Holm R, Borresten A-L and Fodstad O. (1996). *Br. J. Cancer,* **73,** 909–916.
Maelandsmo GM, Holm R, Fodstad O, Kerbel RS and Flørenes VA. (1996). *Am. J. Pathol.,* **149,** 1813–1822.
Martin-Castellanos C and Moreno S. (1997). *Trends in Cell Biol.,* **7,** 95–98.
Matsumura I, Ishikawa J, Nakajima K, Oritani K, Tomiyama Y, Miyagawa J, Kato T, Miyazaki H, Matsuzawa Y and Kanakura Y. (1997). *Mol. Cell Biol.,* **17,** 2933–2943.
Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ and Kato JY. (1994). *Mol. Cell Biol.,* **14,** 2066–2076.
Miki S, Iswano M, Miki Y, Yamamoto M, Tang B, Yokokawa K, Sonoda T, Hirano T and Kishimoto T. (1989). *Febs. Lett.,* **250,** 607–610.
Mitzutani Y, Bonavida B, Koishihara Y, Akamatsu K, Ohsugi Y and Yoshida O. (1995). *Cancer Res.,* **55,** 590–596.
Morgan DO. (1995). *Nature,* **374,** 131–134.
Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K and Carson DA. (1994). *Nature,* **368,** 753–756.
Noda A, Ning Y, Venable SF, Pereira-Smith OM and Smith JR. (1994). *Exp. Cell Res.,* **211,** 90–98.
Oh JW, Katz A, Harroch S, Eisenbach L, Revel M and Chebath J. (1997). *Oncogene,* **15,** 569–577.
Okamoto M, Lee C and Oyasu R. (1997). *Cancer Res.,* **57,** 141–146.
Otsuki T, Clark HM, Wellmann A, Jaffe ES and Raffeld M. (1995). *Cancer Res.,* **55,** 1436–1440.
Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF and Rolfe M. (1995). *Science,* **269,** 682–685.
Parry D, Bates S, Mann DJ and Peters G. (1995). *EMBO J.,* **14,** 503–511.
Polyak K, Kato J-Y, Solomon MJ, Sherr CJ, Massague J, Roberts JM and Koff A. (1994a). *Genes & Develop.,* **8,** 9–22.
Polyak K, Lee M-H, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P and Massague J. (1994b). *Cell,* **78,** 59–66.
Porter PL, Malone KE, Heagerty PJ, Alexander GM, Gatti LA, Firpo EJ, Daling JR and Roberts JM. (1997). *Nat. Med.* **3,** 222–225.
Rak J, Filmus J and Kerbel RS. (1996). *Eur. J. Cancer,* **32A,** 2438–2450.
Reed JA, Loganzo F, Shea CR, Walker GJ, Fores JF, Glendening JM, Bogdany JK, Shiel MJ, Haluska FG, Fountain JW and Albino AP. (1995). *Cancer Res.,* **55,** 2713–2718.
Resnitzky D, Tiefenbrun N, Berissi H and Kimchi A. (1992). *Proc. Natl. Acad. Sci. USA,* **89,** 402–406.
Reynisdottir I, Polyak K, Iavarone A and Massague J. (1995). *Genes & Develop.,* **9,** 1831–1845.
Sandhu C, Garbe J, Bhattacharya N, Daksis J, Pan CH, Yaswen P, Koh J, Slingerland JM and Stampfer MR. (1997). *Mol. Cell Biol.,* **17,** 2458–2467.
Shao ZM, Dawson MI, Li XS, Rishi AK, Sheikh MS, Han QX, Ordonez JV, Shroot B and Fontana JA. (1995). *Oncogene,* **11,** 493–504.
Sherr CJ. (1996). *Science,* **274,** 1672–1677.
Sherr CJ and Roberts JM. (1995). *Genes & Develop.,* **9,** 1149–1163.
Shiohara M, El-Deiry WS, Wada M, Nakamaki T, Takeuchi S, Yang R, Chen D-L, Vogelstein B and Koeffler HP. (1994). *Blood,* **84,** 3781–3784.
Slingerland JM, Hengst L, Pan C-H, Alexander D, Stampfer MR and Reed SI. (1994). *Mol. Cell. Biol.,* **14,** 3683–3694.
Spruck CH, 3rd, Gonzalez-Zulueta M, Shibata A, Simoneau AR, Lin MF, Gonzales F, Tsai YC and Jones PA. (1994). *Nature,* **370,** 183–184.
Steinman RA, Hoffman B, Iro A, Guillouf C, Liebermann DA and el-Houseini ME. (1994). *Oncogene,* **9,** 3389–3396.
Swope VB, Abdel-Malek Z, Kassem LM and Nordlund JJ. (1991). *J. Invest. Dermatol.,* **96,** 180–185.
Tam SW, Theodoras AM, Shay JW, Draetta GF and Pagano M. (1994). *Oncogene,* **9,** 2663–2674.
Vidal MJ, Loganzo F, de Oliverira AR, Hayward NK and Albino AP. (1995). *Melanoma Res.,* **5,** 243–250.
Welch DR and Riber M. (1996). *Mol. Cell. Different.,* **4,** 91–111.
Zeng YX and El-Deiry WS. (1996). *Oncogene,* **12,** 1557–1564.
Zhang XG, Klein B and Bataille R. (1989). *Blood,* **74,** 11–13.