ERK1/2 Is Highly Phosphorylated in Melanoma Metastases and Protects Melanoma Cells from Cisplatin-Mediated Apoptosis

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Activation (phosphorylation) of mitogen-activated protein kinase (MAPK) signal transduction through BRAF and RAS causes a variety of functional effects including cell survival and cell death. In this study, we observed high extracellular signal-regulated kinase (ERK)1/2 phosphorylation levels in clinical melanoma metastases and various melanoma cell lines. Treatment of melanoma cell lines with cisplatin, a potent antitumor agent, increased the level of phosphorylated-ERK (P-ERK)1/2 and enhanced chemoresistance through activation of the cell survival protein 90-kDa ribosomal S6 kinase (RSK)1. The mitogen-activated protein kinase kinase (MEK) inhibitor (U0126) was able to block this effect and reduced cell viability and sensitized cells to cisplatin-induced apoptosis, as shown by PARP cleavage, caspase 3 expression, and annexin-V staining. In conclusion, the MAP kinase–ERK pathway is activated in melanoma and reduces the sensitivity of melanoma to cisplatin. Thus, inhibition of ERK1/2 in combination with selected chemotherapeutic agents may hold promise for more effective therapy of melanoma.

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INTRODUCTION

Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase (MAPK) family, which regulates essential cellular functions like proliferation, differentiation, cell survival, and cell death (Cowley *et al.*, 1994; Hoshino *et al.*, 1999). ERK is activated by numerous extracellular agents such as growth factors, cytokines, hormones, and tumor promotors (Johnson and Vaillancourt, 1994; Robinson and Cobb, 1997; He *et al.*, 1999).

Constitutive activation of the ERK pathway has been described in cell lines derived from pancreas, colon, lung, ovary, and kidney cancer (Hoshino *et al.*, 1999). In melanoma, activation of ERK1/2 was observed by immunohistochemistry in 54% of primary and 33% of metastastic melanomas, respectively (Jorgensen *et al.*, 2003). One reason for this activation seems to be a somatic missense mutation of the *BRAF* gene, which was reported to occur within the kinase domain in 66% of malignant melanomas and shown to cause elevated kinase activity from RAS to mitogen-activated protein kinase kinase (MEK)1/2 and ERK1/2 (Davies *et al.*, 2002). However, this mutation was also observed in 82% of nevi, suggesting that the RAS/RAF/MAPK pathway is a critical step in the initiation of melanocyte neoplasias but that by itself seems insufficient to cause melanomagenesis (Pollock *et al.*, 2003). Furthermore, the identification of factors that modulate the dysbalance in favor of ERK may provide additional targets for effective therapy. In melanoma, epidermal growth factor (EGF) is one of the growth factors that can shift the balance in favor of ERK and may eventually cause tumor progression (Mirmohammadsadegh *et al.*, 2005).

To answer questions related to ERK regulation during chemotherapy against melanoma, we chose cisplatin, one of the most potent antitumor agents, with clinical activity against a variety of solid tumors (Siddik, 2003). Cisplatin interacts with DNA to form DNA adducts and activates several signal pathways like MAPK, p53, AKT, and p73 (Siddik, 2003). It has been reported that cisplatin is able to activate p38 MAPK in different human cell lines and that its inhibition causes resistance to chemotherapy (Losa *et al.*, 2003). A recent study demonstrated that cisplatin-induced apoptosis occurred through the p38 MAPK and not through the MEK1/2–ERK1/2 pathway (Wu *et al.*, 2005). Other studies suggested that ERK1/2 activation by cisplatin induced cell death (Wang *et al.*, 2000; Arany *et al.*, 2004; Choi *et al.*, 2004). In contrast, the inhibition of ERK enhanced the sensitivity to cisplatin in ovarian cancer by accumulation of p53 (Persons *et al.*, 1999). These conflicting findings of pro- and antiapoptotic functions may reflect differences in the

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Abbreviations: BrdU, 5-bromodeoxyuridine; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; NHM, normal human melanocytes; P-ERK, phosphorylated-ERK; rEGF, recombinant human EGF; RSK, 90-kDa ribosomal S6 kinase

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cellular context and target proteins regulated by ERK such as p53 and 90-kDa ribosomal S6 kinase (RSK) that are involved in apoptosis and cell survival. The RSK family of serine/threonine kinases regulates gene expression by phosphorylating a number of transcription factors (Chen *et al.*, 1993). In humans, the RSK family consists of four isoforms (RSK1–4) and two structurally related proteins, called RSK-like protein kinase (RLPK/MSK1)-A and RSK-B (MSK2). RSK family members are unusual among serine/threonine kinases in that they contain two distinct kinase domains, both of which are catalytically functional (Fisher and Blenis, 1996).

Activated RSK has both cytoplasmic and nuclear substrates. RSK plays an active role in nuclear signaling by phosphorylating the cyclic AMP response element-binding protein (Xing *et al.*, 1996), c-Fos (Chen *et al.*, 1993), and IκB (Schouten *et al.*, 1997). Phosphorylation of Bad (Bonni *et al.*, 1999; Shimamura *et al.*, 2000) and C/EBP*β* (Buck *et al.*, 2001) by RSK can protect cells from apoptosis. Moreover, RSK phosphorylates histone H3 (Sassone-Corsi *et al.*, 1999), suggesting that RSK may regulate chromatin remodeling.

In this study, we examined the importance of P-ERK in malignant melanoma metastases as well as its modulation after treatment with recombinant human EGF (rEGF), cisplatin, and the MEK inhibitor U0126. The manipulation of ERK activity by specific inhibitors provided evidence that its antiapoptotic function in melanoma is mediated through the activation of the survival protein RSK1.

RESULTS

High P-ERK in melanoma metastases

ERK1/2 was constitutively expressed in normal human melanocytes (NHM) and different melanoma cell lines as well as in most (27 of 31; 87%) investigated melanoma metastases of patients before receiving chemotherapy (Figure 1). In contrast, phosphorylated ERK1/2 levels were low in NHM, upregulated in melanoma cell lines, and highly abundant in 25 of 31 (81%) melanoma metastases (Figure 1). Immunohistochemically, ERK and activated ERK could be demonstrated in the cytoplasm and nucleus of melanoma cells, respectively (Figure 2b and d). In a recent study, we have demonstrated high levels of EGFR on NHM as well as on melanoma metastases (Mirmohammadsadegh *et al.*, 2005) and have suggested EGF as one important growth factor responsible for ERK phosphorylation in melanoma. Therefore, we confirmed the EGFR expression on the melanoma cell lines used in this study (Figure 3). Western blot experiments demonstrated high EGFR levels on NHM and A375 cells and HaCaT (immortalized epithelial cell line as positive control) and revealed low levels on BLM and MV3 cells, respectively (Figure 3).

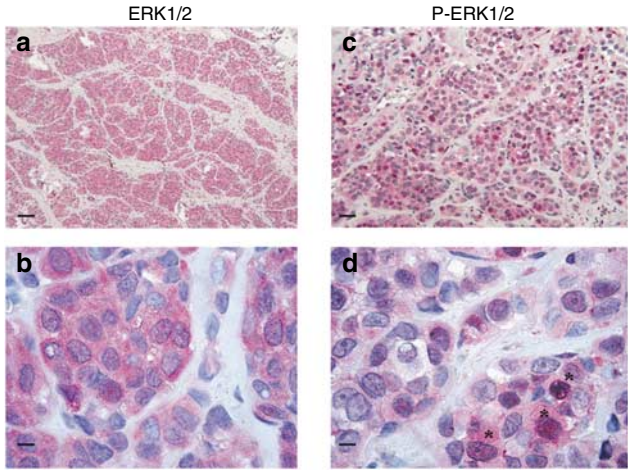

Figure 2. Immunohistochemistry of activated ERK1/2. (**a** and **b**) A representative sample of a metastastic melanoma showed, upon higher magnification, predominant cytoplasmatic ERK1/2 staining. (**c**) A corresponding section showed intense nuclear staining with P-ERK1/2. (*__d__) Upon higher magnification, most cells contained cytoplasmatic and pronounced nuclear staining. Of note, some tumor cells were negative for P-ERK1/2. The appropriate isotype controls showed no staining (data not shown). Bar = 2 μM in (**a**), 4 μM in (**c**), and 10 μM in (**b**) and (**d**), respectively.

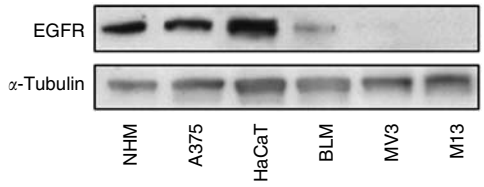

Figure 3. Expression of EGFR. NHM and various melanoma cell lines (A375, BLM, MV3, and M13) were analyzed by Western blot analysis. NHM and A375 cells showed the highest EGFR levels, whereas BLM cells had significantly lower amounts of EGFR. HaCaT cells (immortalized epithelial cell line) were used as positive control.

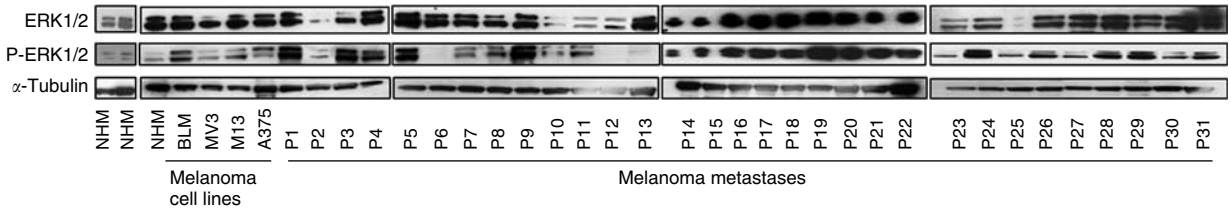

Figure 1. Western blot analysis of ERK1/2 and P-ERK1/2. ERK1/2 was constitutively expressed in nearly all investigated samples. ERK1/2 phosphorylation was low in NHM, showed basal levels in melanoma cell lines (BLM, MV3, M13, and A375) and was high in most of the melanoma metastases. Equal loading was confirmed by α-tubulin.

Phosphorylation of ERK by rEGF

Next, we investigated the effect of rEGF on ERK phosphorylation of NHM and A375 cells, known to contain high levels of EGFR. rEGF was able to induce expression and activation of ERK1/2 in NHM and A375 cells as early as after 5 minutes (Figure 4). This finding correlated with the effect of EGF on proliferation of NHM, where proliferation has been shown 24 hours after treatment with rEGF (Mirmohammadsadegh *et al.*, 2005).

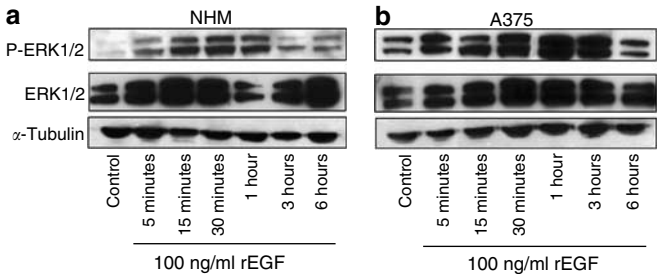

Figure 4. Effect of rEGF on NHM and A375 cells. rEGF induced expression and activation of ERK1/2 in (**a**) NHM and (**b**) A375. Note that the effect lasted for up to 24 hours, probably owing to the limited stability of EGF. Equal loading was confirmed by α-tubulin.

Effect of cisplatin and U0126 on melanoma cell viability and apoptosis

Next we analyzed, if treatment with cisplatin, a commonly used anticancer drug as well as specific inhibition of the MAPK pathway, were able to reduce viability of melanoma cell lines and attempted to explore how these drugs affected ERK activation *in vitro* (Figure S1).

Different melanoma cell lines, A375, BLM, MV3, and M13 were used to investigate the effect of cisplatin in combination with the MEK1/2 inhibitor U0126 with regard to cell viability, as measured by conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, and cell proliferation/cells in S-phase, as measured by 5-bromodeoxyuridine (BrdU) incorporation. We observed that most melanoma cell lines treated with cisplatin and U0126 showed a significant decrease in cell survival (Figure 5a) and proliferation (Figure 5b) over cisplatin treatment alone, in particular at lower concentrations (10 and 20 μM), whereas MV3 and BLM cells were less sensitive to different concentrations (Figure 5a and b). Collectively, these results strongly indicate that cisplatin-mediated inhibition of survival and proliferation of melanoma cells is enhanced in the presence of the MEK inhibitor

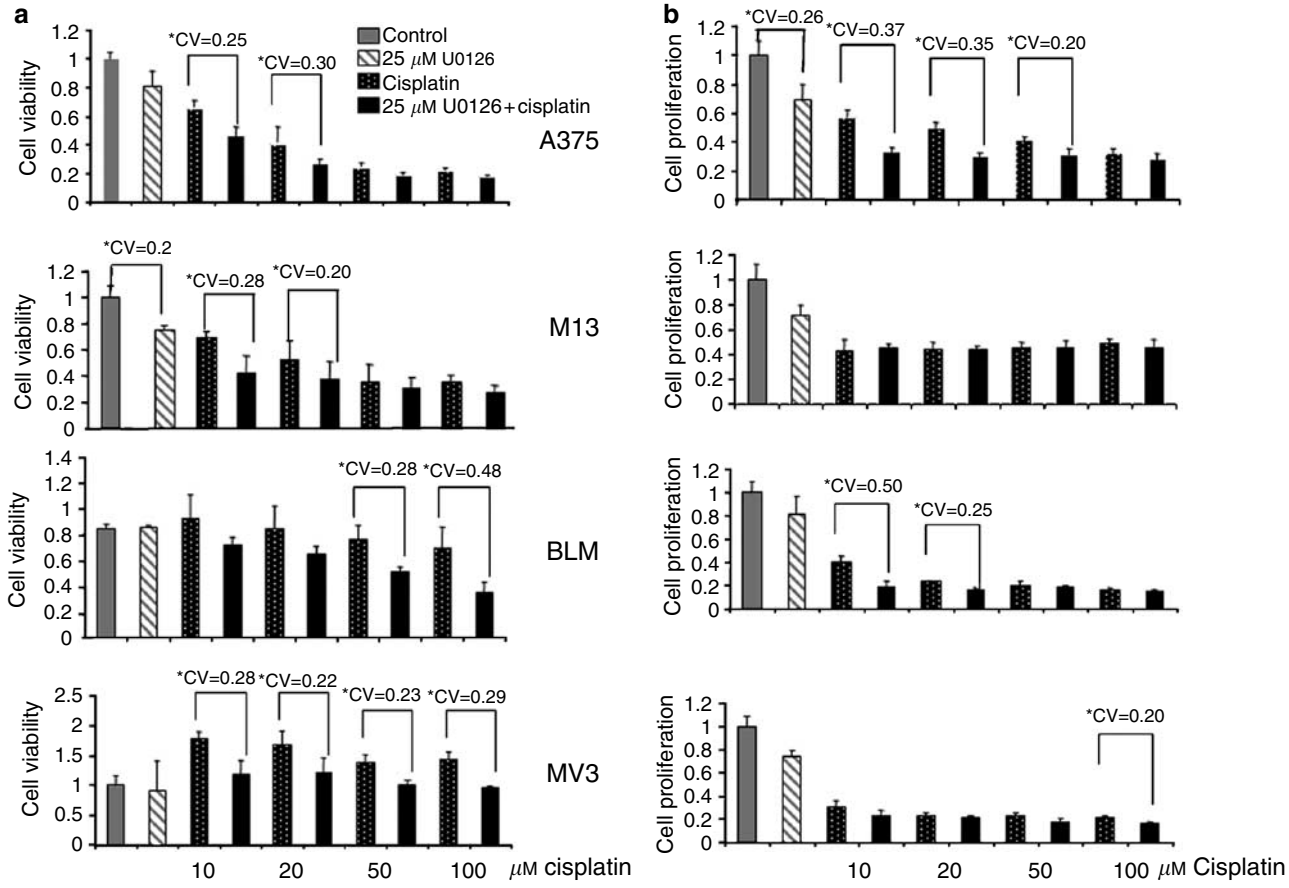

Figure 5. MTT and BrdU assays. (**a**) The effects of U0126 in combination with cisplatin on viability of different melanoma cell lines are shown. Melanoma cell lines were pretreated with 25 μM U0126 for 3 hours and co-incubated with increasing concentrations of cisplatin for 24 hours. The co-treatment of cisplatin and U0126 exhibited greater reduction in (**a**) cell viability and (**b**) proliferation than cisplatin monotherapy in most of the investigated melanoma cell lines. The variation was considered significant when the coefficient of variation (CV) was greater than 0.2*.

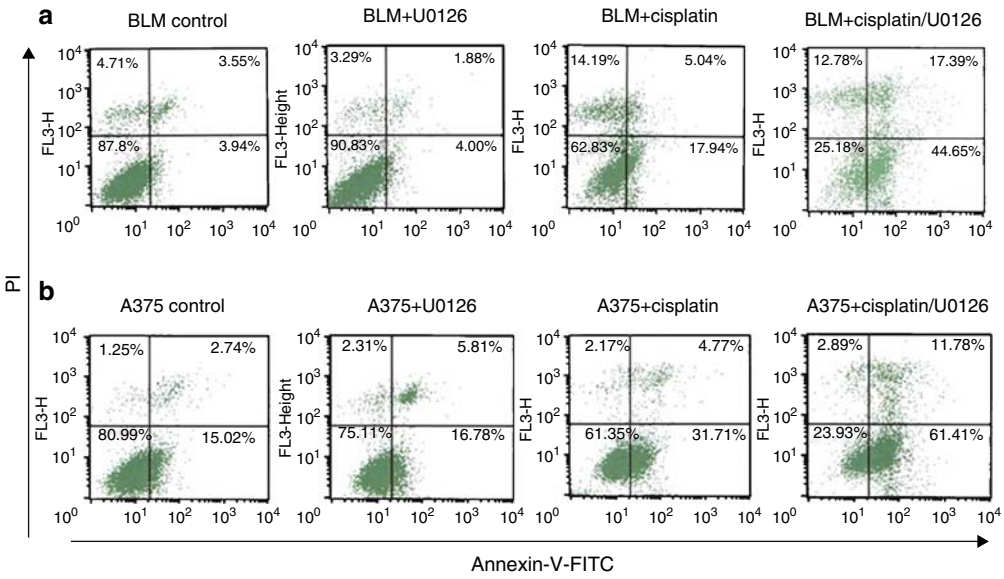

Figure 6. Annexin-V staining was performed on melanoma cell lines (a) BLM and (b) A375 treated with 25 μM U0126, 100 μM cisplatin alone, and in combination for 24 hours. U0126 single treatment did not induce apoptosis in (**a**) BLM and (**b**) A375 cells lines. (**a** and **b**) Cisplatin increased the rate of annexin-V-positive cells in both cell lines. Upon cisplatin/U0126 co-treatment, a significant percentage of (**a**) BLM and (**b**) A375 cells became annexin-V positive. Cisplatin/U0126 co-treatment induced higher rates of apoptosis than individual treatment alone.

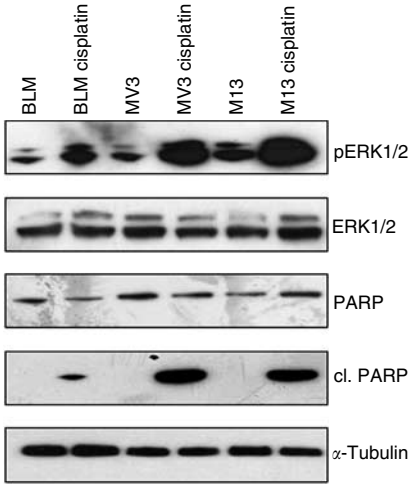

Figure 7. Effect of cisplatin on ERK1/2 phosphorylation. Different melanoma cell lines were treated with 100 μM cisplatin for 24 hours. Cisplatin induced the phosphorylation of ERK1/2 and the cleavage of PARP in A375, BLM, MV3, and M13 cells. The level of ERK1/2 expression was unaffected. α-Tubulin was used as internal control.

U0126 and that these inhibitory effects are more significant at lower cisplatin concentrations for most melanoma cell lines investigated.

Furthermore, following combination therapy, 2.48 times more BLM cells and 1.93 times more A375 cells became annexin-V-positive, respectively (Figure 6a and b), when compared with cells treated with cisplatin only. The MEK inhibitor U0126 alone had no effect on apoptosis (Figure 6a and b).

Effect of cisplatin on ERK1/2 regulation

Given that ERK1/2 was phosphorylated in melanoma metastases and cisplatin being a potent anticancer drug, it was important to study the effect of cisplatin with regard to the phosphorylation status on ERK1/2. We observed increasing phosphorylation of ERK1/2 after treatment of different melanoma cell lines with 100 μM cisplatin (Figure 7). This upregulation correlated with a high expression of cleaved poly (ADP-ribose) polymerase (PARP), an apoptosis marker (Figure 7). Next, the role of ERK1/2 in the regulation of apoptosis was investigated in more detail.

Regulation of apoptosis-related proteins by cisplatin and U0126

Next, the time course of cisplatin monotherapy and cisplatin/U0126 combination therapy was analyzed with regard to P-ERK1/2 activation (Figure 8). Cisplatin induced a time-dependent phosphorylation of ERK1/2 in A375 cells with a maximum at 12 hours (Figure 8a). The proapoptotic proteins cleaved PARP and caspase 3 were detected between 12 and 24 hours, which confirmed the occurrence of apoptosis (Figure 8a). Furthermore, the activation of the tumor suppressor protein p53 on different serine residues (P-serine at position 6, 9, 15, 20, 37, and 392) was investigated using phosphorylation-specific anti-serine antibodies. Only serine at position 6 was phosphorylated by cisplatin at 12 hours, being consistent with ongoing apoptosis (Figure 8a). Interestingly, at the same time point, the cell-survival protein RSK1 was also strongly activated by cisplatin in A375 cells (Figure 8a) and BLM cells (Figure 9a), suggesting that cisplatin-treated cells initially tried to avoid apoptosis by activation of cell-survival proteins, but ultimately cell death occurred. Furthermore, the activation of the cell survival protein P-RSK1 was inhibited by U0126 (Figures 8b and 9b).

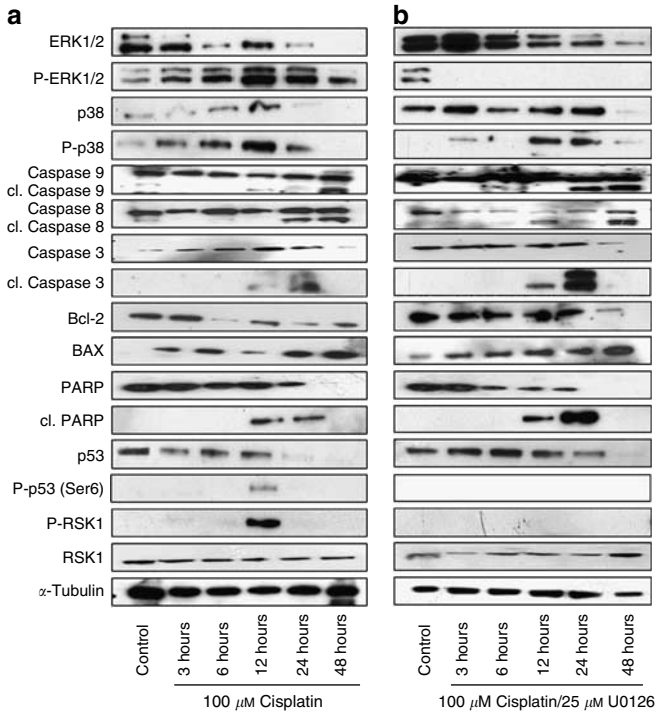

Figure 8. Effect of cisplatin and U0126 on the expression of ERK1/2, p38, and apoptosis-related proteins in A375 cells. (**a**) Western blot analysis of A375 after treatment with 100 μM cisplatin. Cisplatin-induced phosphorylation of ERK1/2 and p38 with a maximum at 12 hours. Cleavage of caspases 8 and 9 was observed after 24 and 48 hours, respectively. Cleaved PARP and caspase 3 were induced between 12 and 24 hours. Bcl-2 decreased, whereas Bax increased with time. Cisplatin-induced serine phosphorylation of p53 at amino acid position 6 and activation of the survival protein RSK1 were detected at 12 hours. (**b**) A375 cells were pre-incubated with 25 μM U0126 for 3 hours before treatment with 100 μM cisplatin. Every 6 hours U0126 was replaced due to limited stability. U0126 strongly inhibited the activation of ERK1/2, whereas it had no effect on cisplatin-induced P-p38 and p38, suggesting a different regulatory pathway. Caspases 8 and 9 were cleaved earlier and stronger upon combination treatment. The combination of cisplatin and U0126 induced stronger cleavage of PARP and caspase 3 as cisplatin alone. α-tubulin confirmed equal loading and quality of protein extract.

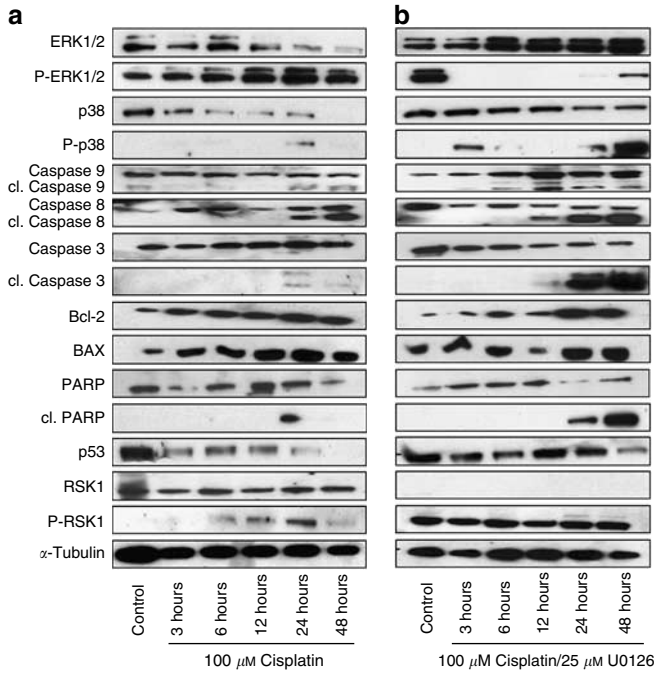

Figure 9. Effect of cisplatin and U0126 on expression of ERK1/2, p38, and apoptosis-related proteins in BLM cells. (**a**) Cisplatin-induced activation of ERK1/2 showed a maximum at 24 hours. P38 was phosphorylated by cisplatin after 24 hours, although weaker than in A375 cells. Cleavage of caspases 8 and 9 occurred after 24 hours. Time-dependent upregulation of Bcl-2 and Bax was detectable. Cleavage of PARP and caspase 3 was also induced by cisplatin with a maximum at 24 hours. Cisplatin-induced activation of RSK1 occurred at 6 hours with a maximum at 24 hours. (**b**) Upon co-incubation with U0126 (25 μM) and cisplatin (100 μM), the activation of ERK1/2 was inhibited by U0126 and cisplatin was unable to revert this effect. Constant levels of p38 but an upregulation of P-p38 expression were observed at 48 hours. Like in A375 cells, the cleavage of PARP and caspase 3 was strongly induced and persisted between 24 and 48 hours. α-tubulin confirmed equal loading and quality of protein extract.

To elucidate the molecular mechanism of cisplatin-mediated apoptosis in melanoma cells and its enhancement upon inhibition of MEK/ERK pathway, we compared the regulation of apoptosis-related proteins post-cisplatin treatment to co-treatment of cisplatin and U0126 in A375 and BLM melanoma cells. First, we demonstrated that cisplatin-induced time-dependent phosphorylation of ERK1/2 in A375 and BLM cells was abolished upon co-treatment of cisplatin and U0126 (Figures 8a and b and 9a and b), whereas the pattern of cisplatin-induced phosphorylation of p38 remained unchanged upon co-treatment of cisplatin and U0126 (Figure 8a and b). We also observed an induction of proteins related to apoptosis from both intrinsic (Bcl-2 and Bax) and extrinsic apoptotic pathways (caspases 9, 8, and 3) as well as a more pronounced PARP cleavage upon co-treatment of cisplatin/U0126 in both A375 and BLM cells (Figures 8a and b and 9a and b).

These data provide evidence that activated ERK1/2 seems to phosphorylate different antiapoptotic proteins that aim to rescue cells from death conditions.

Cisplatin-activated ERK1/2 interacts with p53 and RSK1 in A375 cells but only with RSK1 in BLM cells

As described in the literature, activated ERK1/2 may exert opposing functions such as cell death and cell survival (Persons *et al.*, 1999; Wang *et al.*, 2000; Arany *et al.*, 2004; Choi *et al.*, 2004; Wei *et al.*, 2004). Therefore, to analyze the role of activated ERK1/2 in melanoma was anti- or proapoptotic, A375 and BLM melanoma cell lines were treated with cisplatin alone or in combination with U0126, and the interaction of P-ERK1/2 with either P-p53 at serine position 6 or P-RSK1 was investigated using immunoprecipitation and Western blot techniques (Figure 10). In A375 cells, cisplatin-activated ERK1/2 interacted with p53 as well as with RSK1 (Figure 10a). Inhibition of ERK1/2 phosphorylation by U0126 and treatment with cisplatin prevented this interaction (Figure 10a), suggesting that cisplatin-activated

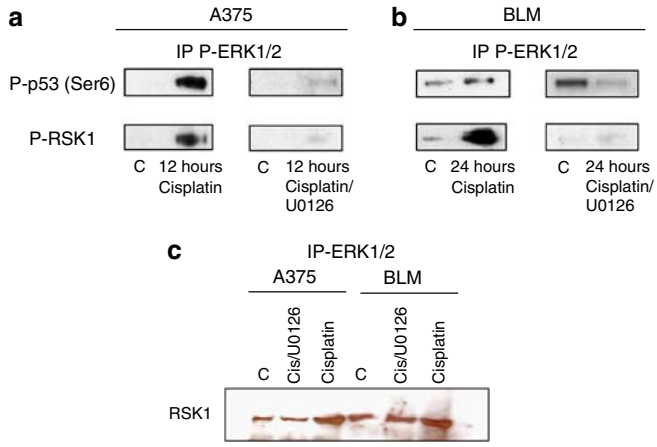

Figure 10. Interaction of P-ERK1/2 and ERK1/2 with p53 and RSK1. A375 and BLM cells were treated with 100 μM cisplatin alone and in combination with 25 μM U0126. Immunoprecipitation (IP) with a P-ERK1/2 antibody and Western blot analysis with P-p53(ser 6) and P-RSK1 were performed. (**a**) Cisplatin induced the interaction of P-ERK1/2 with P-p53 (Ser6) as well as with P-RSK1 in A375 cells. Inhibition of ERK1/2 activation strongly reduced the cisplatin-induced interaction between P-ERK1/2 and P-p53 (Ser6). (**b**) Treatment of BLM with cisplatin had no effect on the interaction between P-ERK1/2 and P-p53 (Ser6). In contrast, cisplatin again induced the interaction between P-ERK1/2 and P-RSK1. (**c**) Cisplatin induced the interaction of ERK1/2 with RSK1. c, untreated cells.

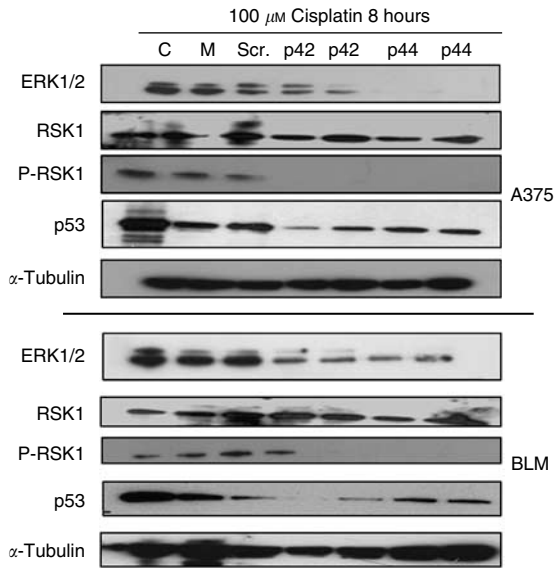

Figure 11. Inhibition of ERK1/2 blocks the activation of RSK1. SiRNA against ERK1/2 (p42 and p44) was found to block the activation of P-RSK1 in A375 cells and showed a strong reduction in BLM cells. SiRNA mediated inhibition of ERK1/2 had no effect on RSK1 and p53 expression. C, untreated cells; M, Mock transfected cells; Scr, scrambled siRNA; p42 and p44, the two subunits of ERK1/2, respectively.

ERK1/2 directly interacts with p53 and RSK1 to phosphorylate these proteins at serine position 6 (p53) and at threonine/serine positions 359 and 363, respectively (Figure 10a). In contrast, there was no significant interaction between P-ERK1/2 and p53 in BLM cells irrespective of treatment

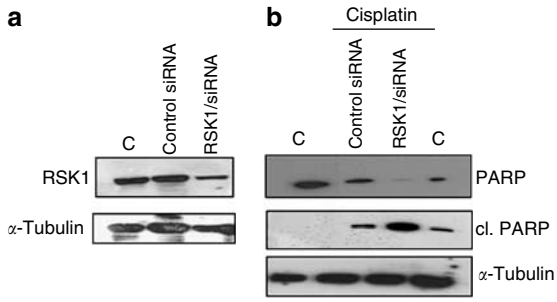

Figure 12. SiRNA mediated decrease of RSK1. A375 and BLM were treated with 10 nmol siRNA for 72 hours. (**a**) SiRNA mediated reduction of RSK1 expression in A375 and BLM cells. (**b**) Cisplatin treatment following siRNA-mediated reduction of RSK1 showed increased PARP cleavage in comparison with cisplatin treatment in control siRNA-treated cells.

with cisplatin or U0126 (Figure 10b). The strong activation of RSK1 under apoptotic conditions in BLM cells may be the reason for their lower sensitivity to cisplatin and the delayed activation of proapoptotic proteins like PARP and caspase 3 in comparison with A375 cells (Figures 8a and b, 9a and b, and 10b). Furthermore, we observed a weaker interaction between total ERK1/2 and RSK1 after cisplatin treatment in comparison with P-ERK1/2 interacting with RSK1 in A375 and BLM cells (Figure 10c). In the same experiment, we could not detect such an interaction with p53 (data not shown). These data demonstrate that for an effective interaction with RSK1 and p53, the activated form of ERK1/2 is imperative. Importantly, melanoma cell lines and four metastasis samples were also analyzed with regard to their P-ERK1/2 and P-RSK1 status (Figure S2). Both melanoma cell lines and four metastasis patient samples showed elevated P-ERK1/2. The BLM melanoma cell line and two out of the four melanoma metastasis patients that had elevated P-ERK1/2 also showed elevated P-RSK1, suggesting a correlation.

To corroborate these findings, we used siRNA against ERK1/2 to determine its effects on active RSK1 in the presence of cisplatin (Figure 11). While at least the p44 band of ERK was completely blocked by specific siRNA, P-RSK1 became undetectable in A375 cells and was strongly reduced in BLM cells, demonstrating their regulation by ERK1/2 (Figure 11).

To investigate the effect of RSK1 on cell death, the expression of RSK1 was reduced by specific siRNA (Figure 12a) before incubation of the cells with cisplatin for 24 hours (Figure 12b). A375 cells with reduced levels of RSK1 were more sensititve to cisplatin-mediated cell death in comparison with cells exposed to cisplatin only, as demonstrated by cleaved PARP (Figure 12b).

In summary, we provide evidence for a crosstalk between proliferation/cell survival pathways activated by the ERK cascade and the growth arrest/apoptosis function of the tumor suppressor protein p53 and the survival protein RSK1. It seemed that cisplatin-mediated activation of ERK1/2 led to phosphorylation of two proteins with opposing functions, that is anti- and proapoptotic; survival was triggered through the activation of RSK1, with cell death eventually emerging through the activation of p53.

DISCUSSION

The MAPK signaling pathway is involved in the regulation of cell growth and development, with a dysbalance leading to abnormal proliferation and eventual tumorigenicity. Activation of ERK appears to be required for many cells to pass the G_1 restriction point and to enter the S-phase, during which cellular DNA replication takes place (Lenormand *et al.*, 1993).

Our data on ERK1/2 expression in melanoma metastasis samples revealed high ERK activity, which caused disturbed apoptosis and increased tumor cell proliferation. In melanoma, one reason for the high P-ERK1/2 levels may result from the mutation of the *BRAF* gene as a constitutive activator in RAS–Raf signaling (Davies *et al.*, 2002), but another cause seems to be the high production of growth factors like EGF. The expression of the EGFR on NHM and its importance for melanoma development has been discussed controversially (Shahbazi *et al.*, 2002; Stove *et al.*, 2003; Grahn and Isseroff, 2004). In a recent study, we showed high EGFR expression in melanoma metastases and NHM (Mirmohammadsadegh *et al.*, 2005). This permitted us to investigate whether EGF was responsible for the phosphorylation of ERK1/2 in melanoma. Indeed, rEGF induced JAK-1 and SRC-mediated activation of ERK1/2 in NHM and in melanoma cell lines A375 and BLM, respectively, and was thus likely contributing to the P-ERK1/2 dysbalance in melanoma. In addition, EGF was shown to be involved in melanocyte proliferation through EGFR-mediated activation of STAT3 and STAT5 (Mirmohammadsadegh *et al.*, 2006).

For treatment of malignant melanoma, it is important to know if a reduction in ERK1/2 phosphorylation will permit more successful therapy. Treatment with cisplatin, one of the most potent antitumor agents, did not decrease ERK1/2 phosphorylation *in vitro*; to the contrary, it increased the activation of ERK1/2.

The role of cisplatin-activated ERK1/2 with regard to apoptosis has also been discussed controversially. Depending on the type of cancer, cisplatin-induced ERK1/2 activation has shown both pro- or antiapoptotic functions (Persons *et al.*, 1999; Wang *et al.*, 2000; Wei *et al.*, 2004). This discrepancy, however, needs further study to define the role of cisplatin-activated ERK1/2 in apoptosis of melanoma. Inhibition of ERK1/2 activation by specific MEK inhibitors and co-treatment with cisplatin induced strong caspase 3- and PARP-mediated apoptosis in our study as in ovarian cancer (Persons *et al.*, 1999). To our surprise, cisplatin-activated ERK1/2 was able to activate the cell survival protein RSK1 as well as the tumor suppressor protein p53 in a cell type-specific manner (Woessmann *et al.*, 2002). ERK1/2 has been shown to phosphorylate the members of the RSK family by interaction through a docking site located near the C-terminus (Roux *et al.*, 2003). RSK plays an active role in nuclear signaling by phosphorylating the cyclic AMP response element-binding protein (Xing *et al.*, 1996), cFos (Chen *et al.*, 1993), and IκB (Schouten *et al.*, 1997), leading to cell survival and proliferation. The role of p53 induced by cytotoxic drugs and ERK signaling has been partially studied in ovarian cancer, where accumulation and phosphorylation of p53 has been demonstrated (Persons *et al.*, 1999). Phosphorylation of p53 in response to chemotherapy usually occurs at serine 15 and less frequently at other sites (Eisenmann *et al.*, 2003).

Our findings are in concert with a previous study, which supported a role of ERK signaling in activating RSK1 in melanoma, leading to inactivation of the proapoptotic BAD and to increased survival of melanoma cells (Eisenmann *et al.*, 2003). Under normal conditions, we could not detect RSK1 activation in A375 and BLM cells, which suggests that ERK1/2-activated RSK1 occurs only under cellular stress such as exposure to cytotoxic drugs (e.g., cisplatin).

Our study argues that high ERK1/2 phosphorylation in melanoma supports tumor progression and protects tumor cells from apoptosis. Furthermore, these observations indicate that ERK1/2 activation partially protects cells from cisplatin-mediated apoptosis, through the activation of RSK1. Inhibition of ERK1/2 phosphorylation by specific inhibitors and RSK1 activation by RNAi technology led to earlier and stronger apoptosis as detected by the expression of cleaved caspase 3 and PARP. Therefore, simultaneous treatment with cisplatin and U0126 may result in synergistic therapeutic effects at tolerable doses of cisplatin. Further investigation on the mechanism of how the EGF pathway activates ERK and studying its inhibition in combination with anticancer drugs may be helpful in improving melanoma therapy. The significance of phosphorylated RSK1 deserves further study as a therapeutic marker in clinical specimens.

MATERIALS AND METHODS

Cell culture

NHMs were cultured in PMA-free melanocyte medium (both obtained from PromoCell, Heidelberg, Germany). Cell culture was maintained in a 37°C incubator in a moist atmosphere of 5% CO_2. The human melanoma cell lines BLM, MV3, M13, and A375 were cultured in DMEM (Invitrogen-Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. As a positive control, the HaCaT epithelial cell line was used. Inhibitors of MEK-1/2 (U0126) and cisplatin were obtained from Sigma-Aldrich, Taufkirchen, Germany. rEGF was obtained from BioSource, Solingen, Germany.

Tumor specimens

The melanoma metastasis samples were extracted from several different patients, who signed informed consent. Samples were snap-frozen in liquid nitrogen and homogenized using a dismembrator (Braun-Melsungen, Melsungen, Germany). The powder was immediately transferred into ice-cold lysis buffer containing 25 mM hydroxyethylpiperazine ethanesulfonic acid pH 7.9, 50 mM NaF, 15 mM Triton X-100, 5 mM EDTA, 100 mM NaCl, and 1 tablet protease inhibitor cocktail per 10 ml buffer (Roche Applied Science, Mannheim, Germany) for Western blot analysis. The study was conducted according to the Declaration of Helsinki Principles and was approved by the Institutional Review Board.

Western blot analysis

Cells were washed twice with ice-cold phosphate buffered saline and lysed in lysis buffer (see above). The samples were pretreated with ultrasound (10 pulses on ice; Sonoplus, Bandelin Electric,

Germany) and were centrifuged at 14,000 × *g* for 20 minutes at 4°C. The protein concentration of the cellular extracts was determined using the advanced protein assay reagent (TEBU, Germany). Twenty micrograms of protein extract were electrophoresed on 4–12% NuPage Bis–Tris–Glycine gels (Invitrogen, Germany) for 2 hours at 120 V. Proteins were blotted onto polyvinylidene fluoride membranes (Roth, Karlsruhe, Germany) at 160 mA for 60 minutes using a tank blot system. The membranes were blocked with 1% non-fat dry milk powder in 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20 (TBST buffer) for 1 hour at 4°C and washed three times with TBST. Immunostaining was performed using antibodies against ERK1/2, P-ERK1/2, cleaved PARP, PARP, cleaved caspase 3, caspase 3, caspase 8, caspase 9, p53 and P-RSK1, Bcl-2 (each Cell Signaling, New England Biolabs, Frankfurt, Germany). Bax and RSK1 antibodies were obtained from Becton Dickinson Biosciences, Heidelberg, Germany. α-tubulin antibody was purchased from Oncogene Research Products, Calbiochem, Darmstadt, Germany. Bound antibody was visualized using the chemiluminescence detection system (Pierce, Rockford, IL) following the supplier's instructions.

Immunoprecipitation

Cells were lysed in Western blot lysis buffer (see above) and incubated with P-ERK1/2, ERK1/2 (Cell Signaling, New England Biolabs, Germany) antibodies overnight at 4°C with constant rotation followed by incubation with immobilized protein A/G (Pierce) for 2 hours. Immunoprecipitates were washed five times with lysis buffer and then resuspended in Laemmli buffer, boiled, and analyzed by Western blotting using anti-p53, anti-P-p53-serine 6, anti-RSK1, and anti-P-RSK1 (threonine/serine positions 359/363).

RNA interference

SiRNAs against p42 and p44 (ERK1/2) were provided by Dharmacon (Solingen, Germany). Cells (2×10^5) were incubated with 100 nM siRNA transfection solution without fetal calf serum and antibiotics for 4 hours. At 24 and 48 hours, cells were detached with trypsin and centrifuged for 5 minutes at 500 × *g* before protein extraction as described above. SiRNA against RSK1 was obtainted from Qiagen (Germany). The transfections were performed in six-well plates according to the manufacturer's recommended protocol.

FACS analysis using the Annexin-V/PI method

The appearance of phosphatidyleserine on the extracellular side of membrane was evaluated with annexin-V/PI method. The melanoma cell lines A375 and BLM were exposed to U0126 and cisplatin, trypsinized, and washed twice in ice-cold phosphate-buffered saline before resuspension in 1 × binding buffer (Invitrogen). Thereafter, 5 μl of Annexin V-FITC (Vybrant; Invitrogen) and 5 μl propidium iodide (100 μg/ml) were added to 100 μl of cell suspension and incubated for 15 minutes at room temperature protected from light. Finally, 400 μl of binding buffer were added to the samples and handled ice-cold until analysis. The fluorescent signals of FITC and PI was detected by FL1 at 518 nm and FL2 at 620 nm, respectively, on a FACSCalibur (Becton Dickinson Bioscences) and apoptotic cells (annexin-V-positive/PI-negative) were quantified.

MTT and BrdU assay

Melanoma cells were exposed to U0126 and cisplatin in a 96-well plate for 24, 48, and 72 hours cells before incubation with 10 μl of 5 mg/ml stock of MTT (Sigma) for 2 hours at 37°C. Upon solubilization, the number of surviving cells was measured at $A_{540\,nm}$. BrdU assays were performed according to the supplier's instructions (Roche Applied Science).

Statistical analysis

All data were analyzed by coefficient of variation. A coefficient of variation value greater than 0.2 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Figure S1. U0126-mediated reduction of P-ERK1/2.

Figure S2. Detection of RSK1 and P-RSK1 in melanoma metastases by Western blotting.

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