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p38 protects human melanoma cells from UV-induced apoptosis through down-regulation of NF-κB activity and Fas expression

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Identifying mechanisms that underlie the resistance of human melanoma to radiation and chemotherapy is expected to assist in developing new strategies for the treatment of this tumor type. We recently demonstrated that through up-regulation of TNFα, ATF2 increases the resistance of late stage melanoma cells to apoptosis induced by UV-irradiation. In elucidating the role of ATF2 kinases, we now demonstrate that ASK1/MKK6/p38 elicits suppression of Fas expression. ASK1/p38 downregulates the expression of a Fas via NF-κB/SP1 site on the Fas promoter. Deletion or mutation of NF-κB/SP1 within the Fas promoter abrogates p38 effect. ASK1/p38 silences the Fas promoter by inhibition of IκBα phosphorylation – thereby limiting NF-κB activity. Forced expression of a dominant negative form of p38 (p38-ASP) or treatment with p38 pharmacological inhibitor, SB203580, increases NF-κB activity, Fas expression and the levels of UVC-induced apoptosis in late stage melanoma cells. Inhibition of p38 activity also restored NF-κB activity and Fas expression in early-phase melanoma cells, suggesting that p38 elicited suppression of Fas expression is not restricted to late phase melanoma. Identifying p38-mediated down-regulation of Fas expression illustrates a novel regulatory pathway by which ASK1/MKK6/p38 alters the degree and nature of the UV-induced apoptosis of melanoma cells. *Oncogene* (2000) **19,** 3003–3012.

Keywords: p38; stress kinases; NF-κB; UV-irradiation; radiation resistance; TNF

Introduction

Malignant melanoma, a tumor whose incidence maintains a constant increase in the last decade, responds poorly to chemotherapy and irradiation. The molecular mechanism of melanoma progression and the regulation of apoptosis remains largely unknown (Meier *et al*., 1998). In earlier studies we demonstrated the role of the transcription factor ATF2 in the radiation resistance of late-stage LU1205 human melanoma cells (Ronai *et al*., 1998; Ivanov and Ronai, 1999). Forced expression of an inactive form of ATF2, in which the amino-terminal transactivation domain was deleted, reduced radiation resistance and augmented UV-induced apoptosis through the silencing of TNFα expression and the increase in the fraction of cells that underwent UV-induced Fas-mediated apoptosis. Conversely, over-expression of full-length ATF2 in the presence of its upstream kinase p38, increased TNFα expression and protection of melanoma cells from UV-irradiation (Ivanov and Ronai, 1999).

ATF2 is activated both by JNK and by p38 stress kinases, which phosphorylate this transcription factor on residues 69 and 71 (Minden and Karin, 1997; Ip and Davis, 1998). Common to regulation by p38 and JNK, in response to stress and DNA damage, is activation of the upstream components of kinase cascades, ASK1 (Chen *et al*., 1999; Ichijo *et al*., 1997) and MEKK1 (Minden and Karin, 1997; Ip and Davis, 1998), both of which are coordinated by TRAF2 (Yuasa *et al*., 1998). TRAF2 is also capable of eliciting negative regulatory signals as illustrated for TNFα expression in TRAF2 null cells (Nguyen *et al*., 1999), possibly due to the nature of its interaction with multiple signaling components including NIK, ASK1, GCK and MEKK1.

Compelling evidence supports both a protective and a promoting role of p38 in the regulation of cell death, depending on the cell type and the nature of the stimuli administered, although the mechanisms underlying these changes are not well understood (Xia *et al*., 1995). For example, p38 elicits a pro-apoptotic signal in response to UV-B irradiation of human keratinocytes through upregulation of caspases (Shimizu *et al*., 1999). This signal can be attenuated by the PI3K/AKT pathway (Berra *et al*., 1998). Along the same line, the anti-proliferative effect of IL-1 is mediated by p38 in the A375 melanoma cells (Itoh *et al*., 1999). An example of an anti-apoptotic effect of p38 is provided by Jurkat cells, where SB202190, which inhibits p38 and JNK, potentiated Fas (APO-1)- or UV-induced apoptosis (Nemoto *et al*., 1998). Furthermore, protection of cells from TNF-mediated cytotoxicity requires early activation of p38 (Roulston *et al*., 1998). Like p38, JNK was shown to elicit either an anti- or a pro-apoptotic signal, depending on the type of cells and damage. For example, JNK can elicit a pro-apoptotic signal via upregulation of Fas ligand expression (Faris *et al*., 1998a,b) in response to various stimuli, including cisplatin (Sanchez-Perez and Perona, 1999) and UV-irradiation (Chen *et al*., 1996).

The nature of these opposing signals may relate to their divergent effects on the same cellular targets. For example, JNK phosphorylates E2F and thus inhibits its activity whereas p38 reverses Rb-mediated repression of E2F1 (Wang *et al*., 1999). In other cases, stress kinases activate the same subset of substrates. UV-irradiation, which efficiently induces both JNK and p38, leads to phosphorylation and transcriptional activation of pro- and anti-apoptotic components

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including ATF2, c-Myc, Bcl2, c-Jun and p53 (Hsu *et al*., 1999; Fuchs *et al*., 1998; Yamamoto *et al*., 1999; Noguchi *et al*., 1999). Investigation of mechanisms underlying the regulation of apoptosis has revealed the role of death receptors, including Fas, TNFR1, DR-3, TRAIL-R1 and TRAIL-R2, whose activity is selectively initiated upon interaction with their respective ligands (Ashkenazi and Dixit, 1998; Nagata, 1997). Through their c-Jun and ATF2 substrates, p38 and JNK have been implicated in the regulation of major death-signaling cascades including Fas Ligand (FasL) transcription (Faris *et al*., 1998a,b; Kasibhatla *et al*., 1998), as in the regulation of TNFα expression (Minden and Karin, 1997; Rhoades *et al*., 1992; Tsai *et al*., 1996), suggesting that the balance between the stress kinases and their effectors is the key to understanding the regulation of the degree and nature of the apoptotic cascade. Since numerous observations point to a delicate balance between the stress-signaling components that are coordinated in a cell-type specific fashion, we further elucidated the regulation of UV-induced melanoma apoptosis by stress kinases.

In the present study we show that in melanoma cells p38 negatively regulates the expression of Fas. We demonstrate that this negative regulation is mediated via suppression of NF-κB transcriptional activity by the ASK1/p38 signaling pathway. The role ASK1/p38 plays in regulating the apoptotic pathway induced by UV-irradiation in melanoma cells through positive regulation of TNFα and negative regulation of Fas is discussed.

Results

MKK6/p38 protects whereas MKK7/JNK promotes cell death of UV-treated melanoma cells

As a first step in assessing the role of ATF2 kinases in melanoma resistance to irradiation, we used upstream components of the JNK and p38 kinases, MKK7 and MKK6, respectively. Forced expression of these constructs in the melanoma LU1205 cells led to efficient phosphorylation of p38 (Figure 1a) and JNK (not shown) and to a corresponding increase in the phosphorylation of the respective substrates c-Jun and ATF2 (not shown). Increase in phosphorylation of c-Jun and ATF2 is reflected in higher transcriptional activity directed by the 5xJun2tk-Luc reporter (Figure 1b), which is activated by Jun/ATF2 heterodimers (van Dam *et al*., 1998). By contrast, p38-ASP, a dominant negative form of p38, efficiently suppressed UVC-induced activity of 5xJun2tk-Luc (Figure 1b). Co-transfection of MKK6-DD, a constitutive active form of MKK6, with GFP, as marker for transfected cells, allowed us to monitor the degree of death in the transfected cells.

Under these conditions, MKK6-DD (compared to an empty vector) caused a twofold decrease in the basal level of cell death and more than a 2.5-fold decrease in UV-treated cells (Figure 1c), suggesting that MKK6 elicits protection of melanoma cells from UV-induced death. Indeed, forced expression of the dominant negative form of p38, p38-ASP led to more

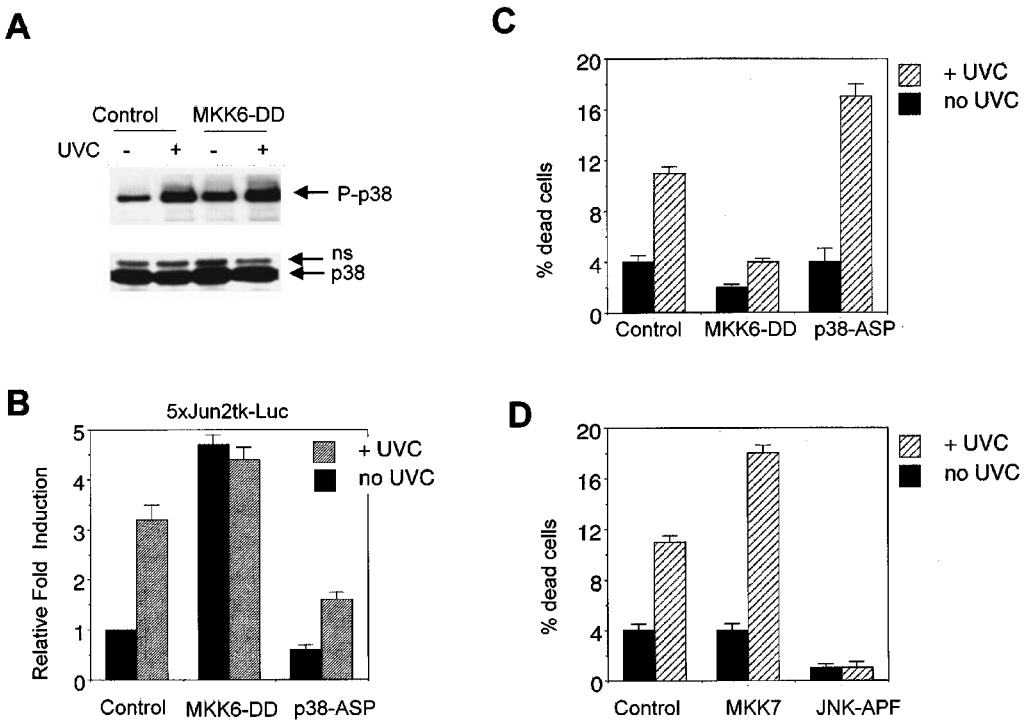

Figure 1 Effects of MKK6/p38 and MKK7/JNK1 signaling on UVC-induced death of LU1205 cells. (**a**) Western blot analysis of phospho-p38 levels after transient transfection of MKK6-DD into LU1205 cells. (**b**) Effect of MKK6-DD and p38-ASP on 5xJun2tk-Luc reporter construct. 5×10^5 cells were transiently transfected with 0.5 μg of reporter construct plus 1.0 μg of MKK6-DD or p38-ASP in the presence of 0.25 μg of pCMV-β-gal. Twenty-four hours after transfection cells were irradiated with UVC (60 J/m^2). After an additional 18 h, irradiated and untreated cells were analysed for luciferase and β-galactosidase activity. The normalized ratio of luciferase activity to β-galactosidase is shown. (**c** and **d**) UVC-induced death levels of LU1205 cells, which had been transiently transfected with GFP and MKK6-DD (0.25 and 1 μg, respectively) or GFP and p38-ASP, were determined using PI staining and flow-cytometric analysis. The ratio of GFP$^+$ PI$^+$ cells to the total number of GFP$^+$ cells indicates cell death levels for transfected cells

than a 50% increase in the level of UV-induced cell death (Figure 1c). In contrast to the effect of MKK6/p38, MKK7 caused an 80% increase in the degree of cell death following UV-irradiation, whereas the dominant negative form of JNK, JNK-APF, abolished UV-mediated apoptosis (from 11 to 1.5%; Figure 1d). These observations suggest that whereas MKK6 protects, MKK7 promotes death of UV-treated melanoma cells. These findings are in line with our former studies revealing the role of transcriptionally active ATF2 in protection of melanoma cells from apoptosis and radiation resistance (Ivanov and Ronai, 1999).

p38-elicited protection from cell death coincides with decreased Fas expression

To further elucidate the nature of p38-elicited protection of melanoma cells from cell death, we used the pharmacological inhibitor of p38 catalytic activity, SB203580 (Young *et al*., 1997). Addition of the inhibitor 1 h before UV-irradiation of LU1205 cells decreased ATF2 phosphorylation (not shown) and transcriptional activity mediated by 5xJun2tk-Luc construct (Figure 2a). Under the same conditions, inhibition of p38 led to a dose-dependent increase of up to fourfold in transcriptional activities mediated by NF-κB, about 3.5-fold in activation of Fas promoter and fivefold in transcriptional activities mediated by FasL promoter (Figure 2a). These results suggest that while suppressing ATF2 activities, inhibition of p38 increases NF-κB activities, Fas and FasL transcription, before as well as after UV-irradiation.

Similar to the effect of p38-ASP, the pharmacological inhibitor of p38 caused a dose-dependent increase (from 10 to 24%) in the fraction of cells that underwent apoptosis in response to UV-irradiation (Figure 2b). Increase in UV-induced apoptosis coincided with an increase in surface Fas expression (Figure 2c), which is the receptor of the prevailing apoptotic pathway upon UV-treatment of these melanoma cells (Ivanov and Ronai, 1999). Surface expression of Fas, measured by mean fluorescence intensity (MFI) representative of antigenic density on a per-cell basis, increased (from 95 to 121 MFI on a log scale) in SB203580-treated cells before (Figure 2c) and after (not shown) UV-treatment. These results suggest that p38 down-regulates Fas expression in a manner that coincides with protection of these melanoma cells from UV-induced death.

p38 suppression of Fas expression is mediated via the NF-κB/SP1 site on the Fas promoter

To further delineate the effect of p38 on Fas expression, we used nested deletions of the Fas promoter (Chan *et al*., 1999). Co-expression of the -1.7 kb Fas promoter-luciferase with MKK6-DD, a constitutively active form

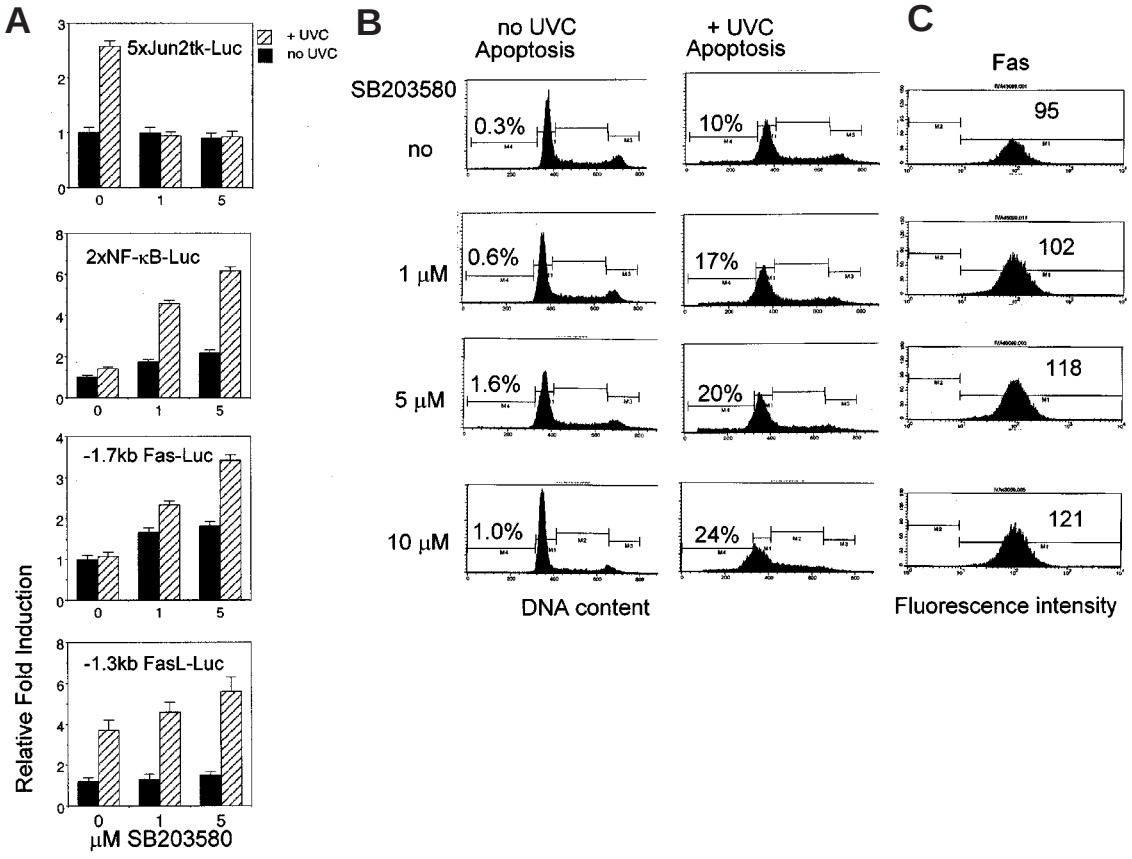

Figure 2 Inhibition of p38 activity by SB203580 increased UVC-induced apoptosis of LU1205 cells. (**a**) SB203580 decreased 5xJun2tk-Luc but increased 2xNF-κB-Luc and the Fas and FasL promoter activity 16 h after UVC irradiation of LU12105 cells. Transient transfection and luciferase analysis were performed as described in the legend to Figure 1. (**b**) DNA fragmentation analysis of LU1205 cells treated with indicated concentrations of SB203580 and irradiated with UVC. Per cent apoptotic cells is indicated. (**c**) Effect of SB203580 on surface expression levels of Fas determined by PE-conjugated anti-Fas antibodies analysed by flow cytometry. Medium fluorescence intensity is indicated

of p38 upstream kinase, resulted in almost complete suppression of Fas promoter activity. Conversely, expression of a dominant negative form of p38 led to an increased level of Fas-Luc activity (Figure 3a). MKK6-DD's ability to down-regulate Fas promoter activity was also seen with nested deletion of the Fas promoter (−306), but not with the −259 or −240 fragments (Figure 3a). This suggests that a certain degree of suppression may be mediated through response elements that are located within the −306/−259 domain which contain one of the three NF-κB sites within the Fas promoter (Chan *et al*., 1999). Forced expression of dominant negative p38 caused 2–3-fold increase in Fas-mediated transactivation using the −240 or −259 Fas-promoter-derived fragment, yet, a super-induction of Fas promoter activity was seen only with the −306 fragment, which resulted in an over ninefold increase in Fas-Luc activity (Figure 3a). These findings suggest that the sequence within the −306 fragment serves as the primary target for mediating suppression of Fas promoter activity. The latter domain contained the NF-κB/SP1 site (Chan *et al*., 1999), suggesting that p38-mediated suppression is elicited through one of these target sequences.

The ability of dominant negative p38 to increase transactivation mediated by the Fas promoter (Figure 3a) is in agreement with the observation that p38 inhibition by SB203580 increased Fas expression (Figure 2a,c).

To further elucidate the effect of p38 on NF-κB/SP1 target sequence, we used a reporter construct containing 6xNF-κB/SP1 sites obtained from Fas promoter (Chan *et al*., 1999). Forced expression of p38-ASP caused a fourfold increase in 6xNF-κB/SP1-Luc activity, which further increased (eightfold) after UV-irradiation (Figure 3b). Mutation of the NF-κB/SP1 site of the Fas promoter (Chan *et al*., 1999) substantially decreased the promoter activity and abrogated the effect of p38-ASP (Figure 3c).

Similar to the Fas promoter, the FasL promoter contained an SP1/NF-κB site, which is crucial for its activity (McClure *et al*., 1999; Hsu *et al*., 1999). Forced expression of p38-ASP led to a marked increase in FasL-Luc activity, which was abrogated upon mutation in the SP1/NFAT/NF-κB sites at position −288/−267 (Figure 3d) but not in the AP1/CREB site at position −336/−330 (not shown). These observations suggest that p38 elicits suppression of FasL transcription through the NFAT/SP1/NF-κB site.

Inhibition of NF-κB down-regulates Fas and FasL expression and Fas-mediated apoptosis of UV-treated melanoma cells

To determine the relationship between p38's ability to suppress NF-κB to the silencing of Fas expression, we established LU1205 melanoma cells that stably express the super-repressor IκBΔN (Figure 4a).

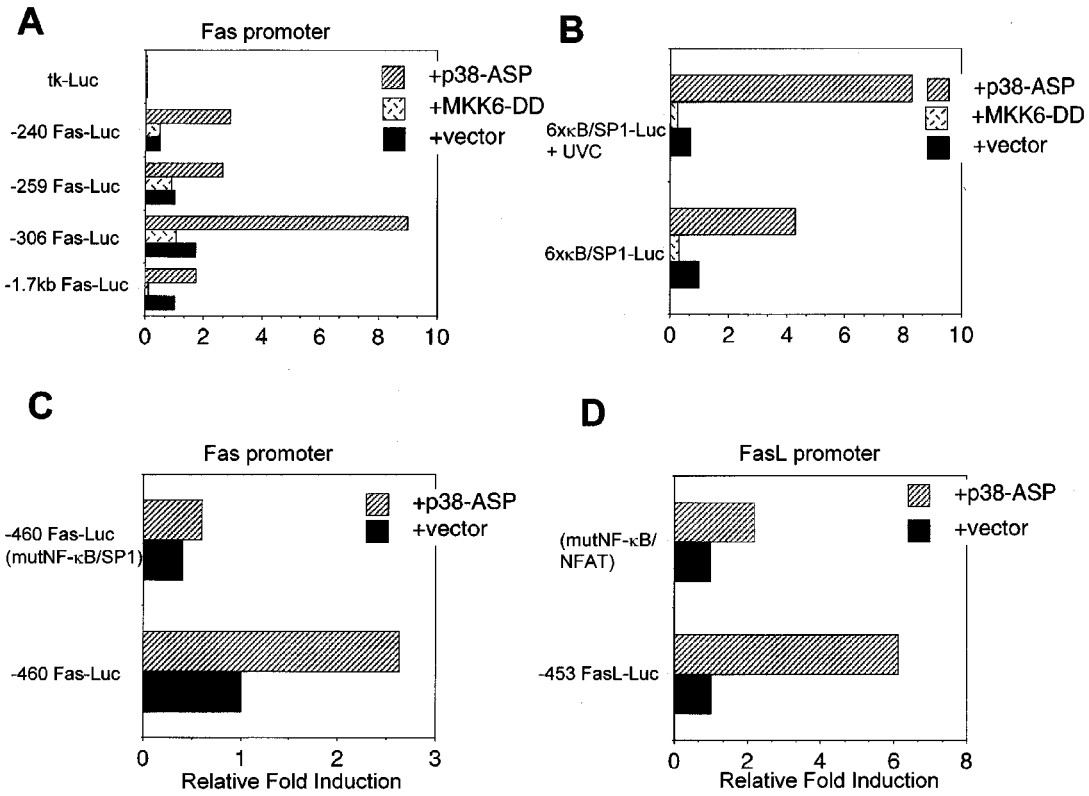

Figure 3 Effect of p38 signaling on Fas expression before and after UVC treatment. (**a**) LU1205 cells were transiently transfected with the nested deletion constructs (0.5 μg) of the 5′ Fas promoter-Luc in the presence of empty vector pcDNA3 or p38-ASP and MKK6-DD expression constructs (1 μg) and 0.25 μg of pCMV-β-gal. After an additional 18 h, cells were analysed for luciferase and β-galactosidase activity. The normalized ratio of luciferase activity to β-galactosidase is shown. (**b**) 6xNF-κB/SP1-Luc construct, which contained six repeats of the NF-κB/SP1 site from the Fas promoter, was transiently co-transfected into LU1205 cells together with empty vector or with MKK6-DD and p38-ASP. (**c**) −460 Fas-Luc construct was used with the wild type or mutated NF-κB/SP1 sites. (**d**) −453 FasL-Luc construct (0.5 μg) or its mutated variant was transfected into LU1205 cells together with empty vector or p38-ASP (1 μg). Analysis of luciferase activity was carried out as indicated above

Expression of the IκBΔN reduced NF-κB (p65–p50 heterodimer) DNA binding activity as monitored by gel-shift (Figure 4b) and luciferase assay (Figure 4c). Inhibition of NF-κB coincided with a 3–5-fold decrease in Fas and FasL promoter activities (Figure 4c), which was also seen at the levels of Fas and FasL expression (not shown). This finding suggests that NF-κB positively regulates Fas expression and further establishes the relationship between NF-κB and Fas/FasL expression. Antagonistic antibodies to Fas reduced apoptosis of the parent but not of IκBΔN transfected cells, suggesting that suppression of Fas expression by IκBΔN abolished Fas-mediated death in response to UV-treatment. Conversely, pretreatment of IκBΔN expressing cells with antagonistic mAbs to TNFR1 caused a decrease in the degree of UV-induced cell death (Figure 4d). Along the same line, treatment with TNFα and cycloheximide induced apoptosis in the IκBΔN cells but not in the parent LU1205 cultures (Figure 4e). This observation suggests that attenuating NF-κB activity sensitized LU1205 cells to TNFα mediated cell death. Further, antagonistic antibodies to TNFα also elicited a clear decrease in the level of UV-induced cell death in IκBΔN but not in the parent LU1205 cells (not shown). These observations suggest that reduced NF-κB activity affected the death signaling cascade and point to a delicate balance between TNFR- and Fas-signaling pathways, which affects the nature and degree of UV-mediated apoptosis of these melanoma cells.

ASK1/p38 elicits suppression of Fas expression via downregulation of NF-κB activities

To further study p38/NF-κB relation to the regulation of Fas expression, we used the constitutively active forms of p38 upstream kinases MKK6-DD and ASK1ΔN. Forced expression of ASK1ΔN increased p38 phosphorylation (Figure 5a) and led to a marked (13-fold) decrease in NF-κB promoter activities (Figure 5b). Whereas JNK-APF, a dominant negative form of JNK, did not block ASK1-mediated inhibition of NF-κB transcriptional activities, p38-ASP was capable of attenuating some of this inhibition (from 13 to sixfold).

Similar to ASK1, MKK6-DD was also efficient in eliciting inhibition of NF-κB activities (>sevenfold), which were partially attenuated upon co-expression of p38-ASP (down to fourfold). Forced expression of p38-ASP efficiently increased the level of NF-κB transcriptional activity (>threefold over the high basal level in these cells; Figure 5b). Furthermore, forced expression of ASK1ΔN caused

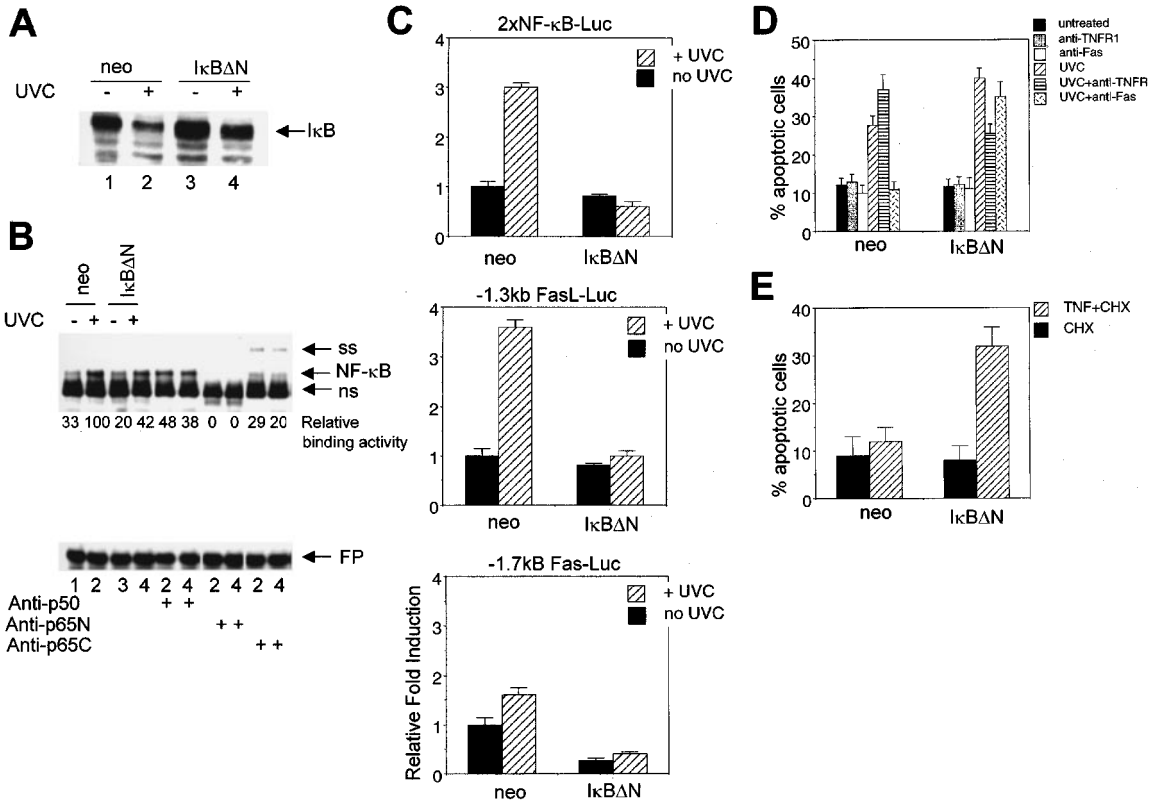

Figure 4 Suppression of NF-κB activity by IκBΔN confers sensitivity to TNFα-dependent apoptosis. (**a**) LU1205 cells were stably transfected with pCMV4-IκBΔN plus pcDNA3-neo or with pCMV4 plus pcDNA3-neo constructs (control). Levels of IκB were detected in nontreated or UVC-treated transfected cells 18 h after treatment by Western blotting with an anti IκB (C-terminal) Ab. (**b**) EMSA of nuclear extracts of control and IκBΔN-transfected cells 3 h after UVC irradiation (lanes 1–4). UVC-treated nuclear extracts of control (lane 2) and IκBΔN-transfected cells (lane 4) were pretreated before EMSA with antibodies against NF-κB p50 or p65 subunits (N-terminal and C-terminal) as indicated. Position of NF-κB DNA-binding complexes is indicated by arrows; ss – super-shifted and ns – nonspecific bands. (**c**) The Fas, FasL, and NF-κB promoter activity in control or IκBΔN-transfected cells. (**d**) Apoptosis analysis of LU1205-neo and LU1205/IκBΔN cells in the presence of antagonistic Abs against Fas and TNFR1 was performed as indicated in Materials and methods. (**e**) Analysis of apoptosis of LU1205-neo and LU1205/IκBΔN cells 30 h after treatment with TNFα (10 ng/ml) and CHX (10 μg/ml)

a dose-dependent decrease in promoter activities of Fas (Figure 5c). Down-regulation of the Fas promoter could be attenuated upon forced expression of p38-ASP (Figure 5c), further confirming that suppression of the Fas promoter by ASK1 is mediated by p38.

To reveal mechanisms that underlie ASK1/MKK6 inhibition of NF-κB activities, we monitored the level of IκB phosphorylation, which directly reflects the availability of active NF-κB. Forced expression of ASK1ΔN reduced the level of IκB phosphorylation, which could be partially restored upon expression of p38-ASP (data not shown). Similarly, inhibition of p38 by the pharmacological inhibitor, or treatment with UV-irradiation, led to a twofold increase in the phosphorylation of IκB, monitored in solid phase kinase reaction. A further increase in phosphorylation of IκB of more than fourfold was observed upon co-treatment of UV and SB203580 (Figure 5d, upper panel). Increased IκB phosphorylation inversely correlated with the level of IκB expression, most likely due to increased degradation of phosphorylated IκB (Figure 5d, lower panel).

Low NF-κB activity and poor Fas expression coincides with high p38 activity in early-stage melanoma WM1552 cells

The relationship demonstrated in our studies for ASK1/p38 dependent NF-κB activity and Fas/FasL expression is exemplified in the human melanoma tumor WM1552-derived cells. This early-phase melanoma cell line exhibits low activity of JNK and NF-κB which were shown to be attributed to poor GCK expression (Ivanov *et al*., 2000). The WM1552 cells possess poor NF-κB and Fas activities as reflected in the analysis of the respective promoters (not shown). Corresponding to the low levels of NF-κB and Fas promoter there is a relatively low expression of Fas cell surface protein, which is increased upon UV irradiation and furthermore (39 to 53 MFI) upon treatment with the pharmacological inhibitor of p38 (Figure 6a). Similarly, expression of FasL is elevated in cells treated with the SB203580 inhibitor (Figure 6b). Increase in Fas and FasL expression coincides with an increased level of cell death from 20 to 35% in UV-irradiated and SB203580-treated cells (Figure 6c). Similarly,

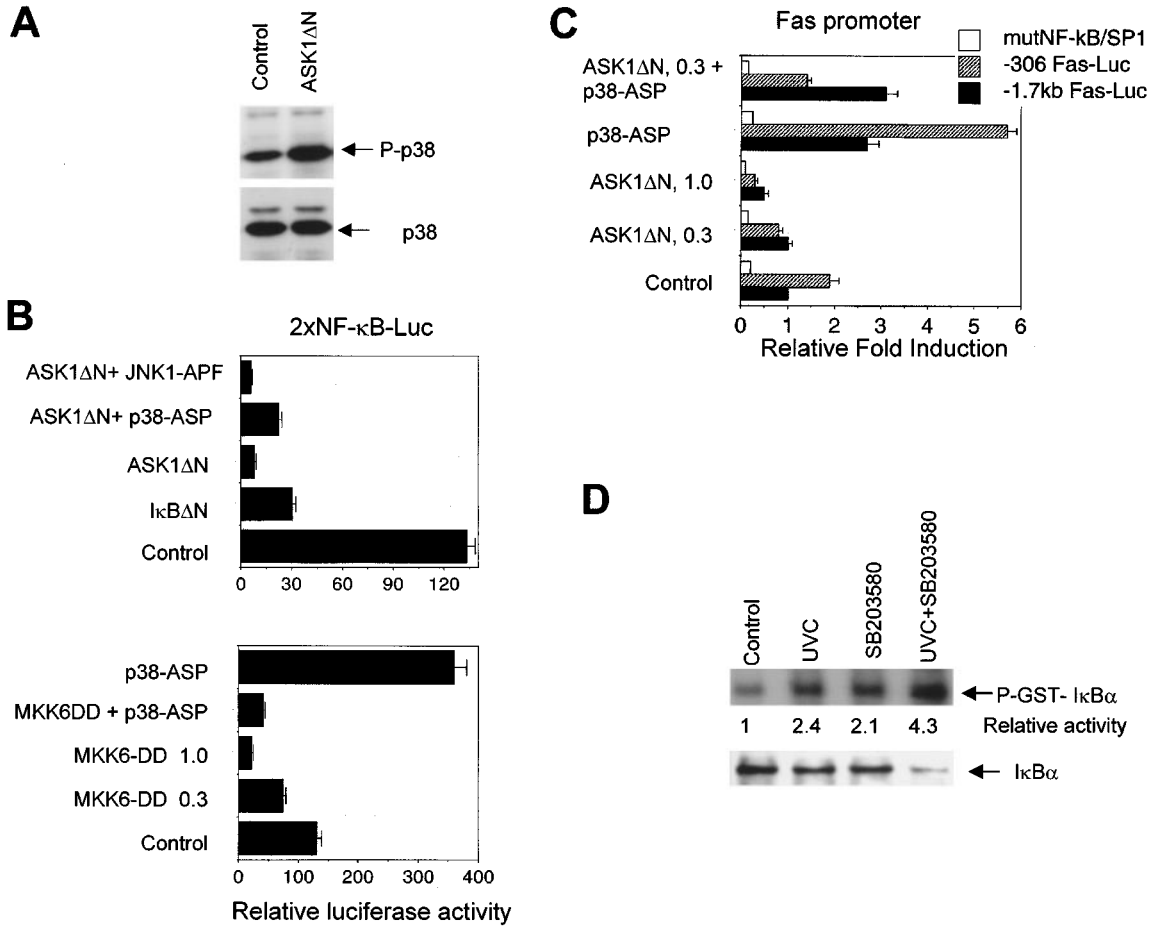

Figure 5 Effect of ASK1/p38 signaling on NF-κB and Fas promoter activities. (**a**) LU1205 cells were transiently transfected with empty vector pcDNA3 or ASK1ΔN construct. Western blot analysis was performed for detection of phospho-p38 and p38 levels. (**b**) LU1205 cells were transiently transfectd with 2xNF-κB-Luc (0.5 μg) plus ASK1ΔN (0.3 μg), or MKK6-DD (0.3, 1.0 μg), IκBΔN (0.3 μg), p38-ASP or JNK-APF (1 μg) constructs (control cells were transfected 2xNF-κB plus pcDNA3). Relative luciferase activity was detected 36 h after transfection. (**c**) LU1205 cells were transiently transfected with Fas-promoter-Luc constructs (0.5 μg) and ASK1ΔN (0.3 or 1 μg as indicated). p38-ASP (1 μg) was transfected alone or in combination with ASK1ΔN. (**d**) Solid-phase kinase assay with GST-IκB as substrate was also performed for control, UVC-treated, SB203580 (5 μM) treated, UVC + SB203580-treated cells. Relative radioactivity of GST-IκB is indicated. Western blot analysis was performed for detection of total IκBα levels (lower panel)

forced expression of p38 dominant negative construct increased level of apoptotic cells from 23 to 35% after UV-irradiation (Figure 6d). This data provides an example for a relevant scenario of melanoma cells in which altered regulation of NF-κB and Fas could be attributed to p38 and is reflected in the sensitivity of these melanoma cells to undergo apoptosis in response to UV-irradiation.

Discussion

Unveiling mechanisms that underlie the resistance of melanoma to radiation and chemotherapy is important to the identification of a means to better overcome this major clinical obstacle. In this study we identify a new regulatory pathway, which plays a key role in the regulation of Fas expression. Our data demonstrates that p38 elicits suppression of Fas expression in human melanoma cells, which is mediated by its inhibition of IκB phosphorylation and silencing of NF-κB. Weak activity of NF-κB and low expression of Fas are found to be ASK1/p38-dependent since inhibition of p38 activities was sufficient to increase NF-κB activity, Fas/FasL expression and the respective degree of apoptosis in response to UV-irradiation. Our studies also identify WM1552 early-phase melanoma cell line where this mechanism prevails, suggesting that p38-elicited down-regulation of Fas expression is not restricted to late-phase melanoma cells. Through its suppression of NF-κB activity and Fas expression, p38 augments the up-regulation of TNFα, which is mediated by its ATF2 effector. Given that TNFα and Fas comprise the balanced regulation of programmed cell death in human melanoma, up-regulation of TNFα in accordance with down-regulation of Fas, is expected to have a major impact on melanoma sensitivity to undergo apoptosis in response to external stimuli. Indeed, melanoma cells in which the Fas pathway represent the major death signaling and where TNFα elicits a survival signal, the combined effects of p38 through ATF2 and NF-κB is expected to decrease the degree of damage-induced death and thus confer resistance to such treatments. Our data directly supports this model (Figure 7) which suggests that p38 plays a critical role in regulating melanoma's resistance to irradiation, and identifies the mechanism by which p38 mediates this regulation. Although in the present study we have

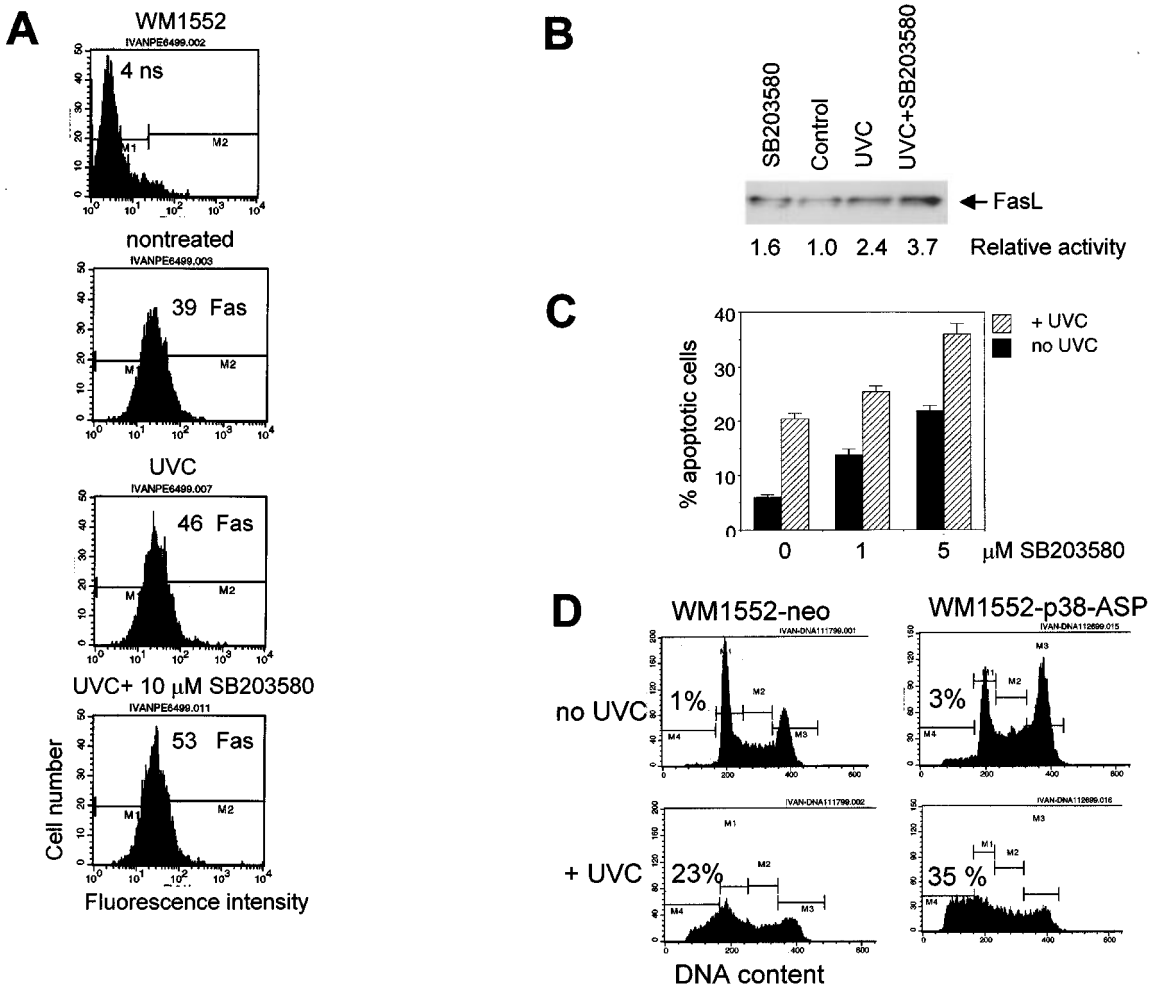

Figure 6 Inhibition of p38 activity by SB203580 increased, Fas/FasL expression and levels of UVC-induced apoptosis in early-phase WM1552 cells. (**a**) Surface expression of Fas was determined using flow cytometric analysis of cells that were subjected to UV-irradiation, SB203580 treatment, UV + SB203580 or sham-treated as indicated. (**b**) Western blot analysis of FasL levels after indicated treatments of WM1552 cells. (**c**) Apoptotic levels were determined after indicated treatment of cells with p38 inhibitor SB203580 using Annexin-V-FITC staining and flow cytometric analysis. (**d**) Level of apoptosis (DNA fragmentation analysis) in WM1552 cells that stably express p38-ASP or Neo control constructs was performed before and after treatment with UVC irradiation

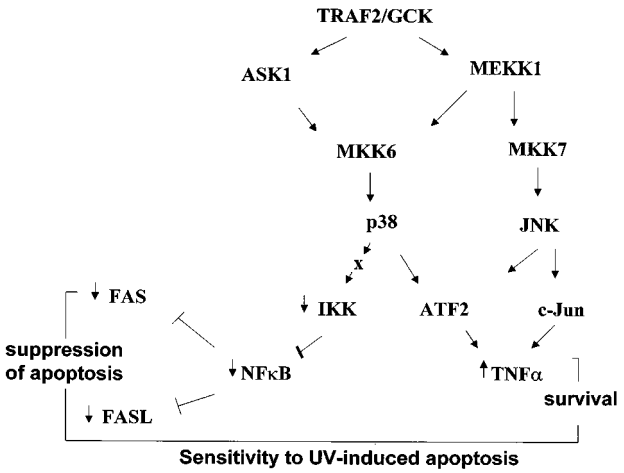

Figure 7 Model for the role of p38 in the regulation of apoptosis to UV-treated melanoma cells. Our previous studies have demonstrated the changes in TRAF2/GCK expression and activities during melanoma progression. Through the regulation of both p38 and JNK signaling TRAF2/GCK and their effectors p38/JNK kinases and ATF2/c-Jun transcription factors were implicated in melanoma sensitivity to radiation (Ivanov *et al*., 2000; Ivanov and Ronai, 1999; Ronai *et al*., 1998). Data shown in the present study identifies another layer of regulation, namely, the suppression of Fas expression upon p38 activation. This suppression is mediated via down-regulation of NF-κB activities that are p38-dependent. According to these observations activated p38 decreases the phosphorylation of IKK and NF-κB inhibitor. Whereas the mechanism underlying this decrease is yet to be elucidated, it results in the inhibition of NF-κB activity. Being important positive factor in the regulation of Fas and FasL expression, such suppression yields the decrease in Fas and FasL expression. In concordant with this suppression the p38 pathway effectively increases the activity of ATF2 and c-Jun which were implicated as positive factors in the regulation of TNFα expression. Given the antiapoptotic role of TNFα and the proapoptotic signal of Fas the change in Fas *vs* TNFα expression results in the increased sensitivity of the melanoma cells to radiation. It is expected that similar changes would take place in tumors where Fas and TNFα elicit negative and positive apoptotic signals, respectively

focused on two melanoma-derived cell lines, representing early and late phases in melanoma development, it is expected that p38 suppression of Fas via NF-κB will be also found in other melanomas as well as in different tumor types.

The finding that p38 can suppress NF-κB is supported by studies of p38's role in apoptosis of hematopoietic cells, in which the ability of the pharmacological inhibitor of p38 to block p38 mediated protection from apoptosis was associated with increased NF-κB activity (Birkenkamp *et al*., 1999). Compounds that are known to stimulate p38, including sorbitol or sodium salicylate, effectively suppressed NF-κB activity, which could be reversed upon the expression of dominant negative p38 constructs or the addition of pharmacological inhibitors of p38 (Schwenger *et al*., 1997, 1998). More recent studies point to the inhibition of IκB phosphorylation by p38, although the nature of this inhibition is not known (Alpert *et al*., 1999). Here we demonstrate that ASK1, an upstream regulator of the p38 pathway, mediates efficient suppression of Fas expression via inhibition of NF-κB activity. Further support for the relationship between UV-irradiation and NF-κB-dependent apoptosis is provided by finding that UVA-irradiation decreases NF-κB activity in human keratinocytes (Djavaheri-Mergny *et al*., 1999), and that increased apoptosis in skin requires inhibition of NF-κB activity (Van Hogerlinden *et al*., 1999).

In addition, the current studies determine that p38 also silences FasL expression. Suppresion of FasL is mediated through the SP1/NFAT/NF-κB site. Although we cannot exclude NFAT phosphorylation as a result of an indirect effect of p38 activity (Chow *et al*., 1997), it is likely that reduced FasL promoter activity is also mediated via p38-mediated suppression of NF-κB activity. Coordinated NF-κB-dependent suppression of Fas and FasL is expected to cause efficient attenuation of Fas-mediated apoptosis. Indeed, suppression of p38 caused an increase in UV-induced, Fas-mediated, apoptosis. JNK may attenuate the suppressive effects of p38, as supported by the finding that JNK positively contributes to the expression of FasL (Faris *et al*., 1998a,b), thereby antagonizing the effect of p38.

Previously reported data from other laboratories – as well as from ours – points to the role of p38/ATF2 in positively regulating TNFα expression (Ivanov and Ronai, 1999; Schafer *et al*., 1999; Cain *et al*., 1999). The concordant roles of p38/NF-κB in the regulation of Fas *vs* TNF signaling is further illustrated by the observation that blocking NF-κB shifted UV-induced apoptosis from the Fas-mediated-cascade to the TNF-mediated-cascade. Accordingly, through its inhibition of Fas/FasL and activation of TNF, ASK1/p38 and its transcription factor effectors emerge as an important regulator of apoptotic signaling in human melanoma cells (Figure 7).

Other cellular regulatory pathways contribute to NF-κB activity, some of which may involve phosphorylation-independent degradation of IκB (Li and Karin, 1998). Among other regulatory cascades is AKT/PKB, which was implicated in the regulation of IκB (Romashkova and Makarov, 1999; Ozes *et al*., 1999). Analysis of PKB/AKT phosphorylation in the melanoma cells studied here did not reveal changes after UV-treatment. Other IKK regulatory components include NIK, TRIP or IRAK, which mediate TNF/TRAF2- or IL1-inducing signals (Maniatis, 1999). While expected to be cell type dependent, the nature of converged IκB regulation by multiple signaling pathways is a subject for intense investigation.

In summary, our findings position p38 as a key regulator of TNF and Fas/FasL signaling in melanoma cells. These observations provide new understanding of the cellular determinants involved in the choice between TNF- and Fas-mediated cell death, and identify ASK1/p38 as a key regulatory component in dictating the nature and degree of UV-irradiation-induced apoptosis in this tumor type.

Materials and methods

Cell lines

Human melanoma LU1205 cells were maintained in MCDB153/L 15 medium (4 : 1) supplemented with 5% fetal bovine serum (FBS), L-glutamine and antibiotics. WM1552 are early-phase melanoma derived cells, which were maintained in the same medium supplemented with insulin (5 μg/ml). LU1205/neo, and LU1205/IκBΔN cell lines were maintained in the same medium supplemented with G418 (200 μg/ml). Cells were grown at 37°C with 5% CO_2.

Stable transfection and selection

The expression vector pCMV4-IκBΔN (Brockman *et al*., 1995) plus pcDNA3-neo was also co-transfected by electroporation (230 V, 1050 μF) into LU1205 cells as previously described (Ronai *et al*., 1998). In parallel, pcDNA3-neo vector (Invitrogen, Carlsbad, CA, USA) was transfected to generate control LU1205-neo cells. LU1205/neo and LU1205/IκBΔN cell lines were created as a mixed population of G418-resistant clones.

Transient transfection and luciferase assay

Transient transfection of different reporter constructs (0.25–0.5 μg) together with quantities of expression vectors and pCMV-βgal (0.5 μg) into 5×10^5 LU1205 melanoma cells was performed using Lipofectamine (Life Technologies-BRL). The reporter constructs used were: 2xNF-κB-Luc (Zandi *et al*., 1997), 5xJun2tk-Luc, 5xTRE-tk-Luc and vector tk-Luc (van Dam *et al*., 1998), -615 TNF-Luc and -36 TNF-Luc (Rhoades *et al*., 1992), -1.3 kb FasL-Luc (Kasibhatla *et al*., 1998), -453 FasL-Luc, -318 FasL-Luc and their mutated variants (Holtz-Heppelmann *et al*., 1998; McClure *et al*., 1999), the Fas promoter constructs: 1.7 kb FPR1-Luc, Δ5, Δ5.7 Δ5.8, Δ6 and their mutated variants (Chan *et al*., 1999). The expression constructs, pcDNA3-FLAG-MKK7β1 (Tournier *et al*., 1999), pcDNA3-JNK1-APF (Butterfield *et al*., 1997) p38-ASP (Enslen *et al*., 1998) MKK6-DD (Han *et al*., 1996), and ASK1ΔN (Saitoh *et al*., 1998) were also used in these experiments. Luciferase activity was determined using the Luciferase assay system from Promega (Madison, WI, USA) and normalized based on β-galactosidase levels in transfected cells.

Transient transfection and GFP assay

Melanoma cells (5×10^5) were transiently co-transfected with expression vectors together with marker plasmid encoding Green Fluorescent Protein (pGFP; 1 μg and 0.25 μg, respectively) using Lipofectamine (Life Technologies-BRL). Forty hours after transfection, cells were irradiated with UVC (60 J/m^2) and 18 h later were stained with propidium iodide (PI) and analysed by flow cytometry. The ratio of GFP^+PI^+/total GFP^+ cells was used to measure levels of cell death of transfected cells.

Treatment and apoptosis studies

Cells were exposed to UVC at 60 J/m^2 as previously described (2). Cycloheximide (10 μg/ml; Sigma, St. Louis, MO, USA) SB203580 (1–10 μM; Calbiochem, San Diego, CA, USA) and recombinant TNFα (1–10 ng/ml) (Pharmingen, San Diego, CA, USA) were used to treat melanoma cells. Antagonistic monospecific antibodies against Fas (clone G254–274), against TNFα (clone Mab1 from Pharmingen, San Diego, CA, USA), and against TNFR1 (clone 16803.1 from R&D Systems, Minneapolis, MN, USA) were added as indicated at final concentrations of 1–5 μg/ml.

Annexin-V-FITC staining in the presence of PI was performed for detection of apoptosis levels using an Annexin-V-FITC kit (Pharmingen). Flow-cytometric analysis was performed on a FACScan or FACS Calibur flow cytometer (Becton Dickinson) using the Lysis II or CellQuest program, respectively. DNA fragmentation analysis was performed as described previously (Nicoletti *et al*., 1991). Cells were pelleted and resuspended in 0.5 ml of hypotonic buffer with 0.1% Triton X-100 containing PI (40 μg/ml) and DNase-free RNase A (1 mg/ml). Cells were incubated at 37°C for 30 min and analysed on a flow cytometer (Becton Dickinson). The percentage of cells to the left of the diploid $G_{0/1}$ peak, characteristic of hypodiploid cells that have lost DNA, was calculated as the percentage of apoptotic cells. Analysis was performed without light scatter gating. Surface expression of Fas was determined using anti-Fas-PE Ab (Pharmingen) and flow-cytometric analysis.

Oligonucleotides and Electrophoretic Mobility Shift Assay (EMSA)

Double-stranded oligonucleotides used in this study as specific probes for the transcription factor NF-κB, and the method used for the EMSA were described previously (Ivanov *et al*., 1995). DNA-binding activities were quantified using the BioRad Molecular Imaging system (model G5-250), equipped with Molecular Analysis software (BioRad, Hercules, CA, USA).

Western blot analysis

Cell lysates (50–100 μg protein) were resolved on 10% SDS–PAGE, transferred to nitrocellulose and processed according to the standard manual. The polyclonal Abs used were Phospho-MKK3/6 (Ser 189/207); Phospho-p38 (Thr180/Tyr182); Phospho-ATF2 (Thr71) from New England Bio-Labs. Polyclonal anti-IκB (C-terminal) Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-FasL Abs were obtained from Transduction Laboratories (Lexington, KY, USA). The primary Abs were used at dilutions 1 : 1000–1 : 3000. The secondary Abs were anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (dilution 1 : 5000). Signals were detected using the ECL system (Amersham, Arlington Heights, IL, USA).

Kinase assay

Protein kinase assays were carried out using fusion proteins, GST-IκBα, as a substrate as previously described (Adler *et al*., 1996). Briefly, the GST-IκBα fusion protein (0.5 μg/assay) was bound to glutathione-sepharose beads after addition of melanoma proteins as the source of IKK (10 μg/assay) in the presence of kinase buffer (20 mM HEPES, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM NaF, 1 mM $NaVO_3$, 50 mM NaCl) at room temperature for 15 min. The beads were pelleted and washed extensively with PBST (150 mM NaCl, 16 mM sodium phosphate, pH 7.5, 1% Triton X-100, 2 mM EDTA, 0.1% β-MeOH, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine) before incubation with [γ-^{32}P]ATP (50 c.p.m./fmol; Amersham) in the presence of kinase buffer. Following extensive washing, the phosphorylated GST-IκBα was boiled in SDS sample buffer and the eluted proteins were run on a 15% SDS-polyacrylamide gel. The gel was dried and phosphorylation of the IκB substrate was determined by autoradiography, followed by quantification with a phosphorimager (BioRad).

Abbreviations

AP1, activator protein-1; ASK1, apoptosis signal-regulating kinase 1; ATF2, activating transcription factor 2; FasL, Fas ligand; NF-κB, nuclear factor kappa B; PI, propidium iodide; TNFα, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor; TRAF2, tumor necrosis factor receptor associated factor 2; GFP, green fluorescent protein; IκB, inhibitor NF-κB; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; MFI, medium fluorescence intensity; MKK, MAPK kinase; CHX, cycloheximide; NFAT, nuclear factor of activated T lymphocytes.

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