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Melphalan-Induced Apoptosis of EBV-Transformed B Cells through Upregulation of TAp73 and XAF1 and Nuclear Import of XPA

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Melphalan (Mel) is widely used to treat patients with hematologic cancer, including multiple myeloma, but its mechanism of action in EBV-transformed B cells is poorly described. In this study, we demonstrate a novel mechanism by which transcriptionally active p73 (TAp73) induces translocation of X-linked inhibitor of apoptosis protein–associated factor 1 (XAF1) and xeroderma pigmentosum group A (XPA) during apoptosis caused by Mel treatment. We observed that Mel induced significant generation of reactive oxygen species (ROS) and subsequent apoptosis, as well as an early phosphorylation of p38 MAPK that preceded expression of the mitochondria membrane potential disruption–related molecules and the cleavage of caspases. In particular, Mel led to upregulation of TAp73, XAF1, and Puma and induced XPA nuclear import and translocation of Bax into mitochondria. Mel-induced apoptosis was inhibited by pretreatment with the ROS scavenger 4-amino-2,4-pyrrolidine-dicarboxylic acid (APDC) and the p38 MAPK inhibitor SB203580. We supposed that ROS generation might be the first event in Mel-induced apoptosis, because APDC blocked the increase in ROS, p38 MAPK, and TAp73, but SB203580 did not block ROS generation. Moreover, Mel elicited activation of ATR, and APDC inhibited phosphorylation of ATR but not SB203580. APDC and SB203580 completely blocked XPA and Bax translocation. We conclude that Mel promotes TAp73-mediated XAF1 and Puma expression via ROS generation and ATR/p38 MAPK pathway activation, thereby triggering apoptosis. Our results provide evidence of a novel alternate regulatory mechanism of TAp73 and reveal that Mel may be a therapeutic drug for curing EBV-related malignancies. The Journal of Immunology, 2013, 191: 000–000.
overexpression of XAF1 induces cancer cell death and inhibits tumor growth in many types of cancers, including colon, gastric, prostate, and pancreatic cancers (12–15). Although XAF1 is thought to be a proapoptotic nuclear protein (16), after relocalization to mitochondria it promotes translocation of Bax to mitochondria and Cyt. c release from mitochondria (17). However, the role of XAF1 in apoptosis of EBV-transformed B cells and its putative correlation with the reactive oxygen species (ROS), p38 MAPK, and TP53 pathway have not been studied.

Melphanal (Mel) is a well-known alkylating drug used in cancer chemotherapy. This cytotoxic drug exerts its pharmacologic effect by causing interstrand cross-links in the main groove of DNA (18). Initially, Mel was only used to treat multiple myeloma, but it was shown to be effective in the treatment of several other tumors, including ovarian, melanoma, and breast cancer. In humans, bulky DNA lesions produced by DNA-damaging agents can be removed only by nucleotide excision repair (NER) (19). Among the NER proteins, xeroderma pigmentosum group A (XPA) plays a unique role in DNA damage recognition.

EBV is a ubiquitous human herpesvirus 4 associated with several diseases, including infectious mononucleosis, Hodgkin’s lymphoma, Burkitt’s lymphoma, nasopharyngeal carcinoma, immunoblastic B lymphoma associated with HIV, and some gastric carcinomas, as well as autoimmune diseases, such as multiple sclerosis, Sjögren’s syndrome, and rheumatoid arthritis (20). In this study, we aimed to assess the effect of TP53 on the cellular response to Mel in EBV-transformed B cells, as well as the underlying molecular mechanisms. We were interested in whether TP53 has any regulatory role in other apoptotic pathways, such as the XAF1- or XPA-signaling pathway, upon Mel treatment. In this article, we report a study on Mel-induced apoptosis in EBV-transformed B cells demonstrating that TP53 is induced by ROS production and p38 MAPK activation, and it mediates both the induction of apoptosis and the activation and translocation of XAF1 to the cytosol. We show that, in cells undergoing TP53-dependent apoptosis, TP53 displays a nuclear-localization pattern and induces translocation of XPA to the nucleus, whereas Bax translocates from the cytosol to mitochondria, causing Cyt. c release. Therefore, Mel could be a therapeutic drug for curing EBV-related malignancies and influencing future hematological cancer therapy.

Materials and Methods
Preparation of EBV infectious culture supernatant
Cell-free EBV supernatant stock was prepared from an EBV-transformed B95-8 marmoset cell line (American Type Culture Collection, Manassas, VA). To establish EBV infection of B cells from normal PBMCs, we isolated PBMCs from whole blood by Ficoll-Paque (Amer sham Life Science, Buckinghamshire, U.K.) gradient centrifugation. PBMCs were added to EBV stock supernatant in a culture flask, and after 2 h of incubation at 37°C, RPMI 1640 culture medium (HyClone, Logan, UT) and 1 mg/ml AGT GTG TAG AGG AGA CAG GAA TC-3

Analysis of apoptotic cells by flow cytometry
To examine the percentage of cells undergoing apoptosis in human EBV-transformed B cells (4 wk, 5 × 10^6 cells/ml) and normal PBMCs was determined by flow cytometry using FITC-labeled Annexin-V and 7-aminoactinomycin D (7-AAD) (both from BD Biosciences San Diego, CA). To determine optimal conditions, experiments were performed using different concentrations of Mel (0, 1, 5, 10, 50, 100 μM) and different incubation times (2, 4, 8, 12, 16, 24 h). Ethanol (EOH; 0.01%) was used as a vehicle control. To examine the role of caspases, cells were treated with z-Leu-Glu(Ome)-His-Asp-(Ome)-fluoromethylketone (z-LEHD-fmk; 20 μM, caspase-3 inhibitor), N-benzoyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEV-Fmk; 20 μM in DMSO, caspase-3 inhibitor), or N-benzoyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (z-IETD-fmk; 20 μM in DMSO, caspase-8 inhibitor; all from Calbiochem, San Diego, CA) for 2 h before Mel treatment. To inhibit production of ROS, cells were pre-treated with 4-amino-2,4-pyrrolinedicarboxylic acid (APDC; 100 μM, antioxidant) or N-acetylcysteine (NAC; 10 mM, antioxidant; both from Sigma-Aldrich) for 2 h. To inhibit activation of p38 MAPK, cells were pre-treated with SB203580 (10 μM; Calbiochem) for 2 h. Cells were then harvested, rinsed with PBS, and resuspended in 100 μl 1× Annexin-V binding buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM CaCl2). Then, 3 μl Annexin-V–FITC and 3 μl 7-AAD (both from BD Biosciences) were added, and cells were incubated at room temperature for 15 min in the dark with gentle vortexing. The stained cells were analyzed using a FACSCalibur flow cytometer equipped with CellQuest Pro software (both from BD Biosciences).

Measurement of mitochondria membrane potential and intracellular ROS production
The changes in mitochondrial membrane potential (Δψm) were checked using 3,3′-dihexyloxycarbostyryl iodide (DiOC6; Molecular Probes, Eugene, OR). Cells were treated with EtOH or Mel for 24 h, harvested, washed twice in PBS, resuspended in PBS supplemented with DiOC6 (20 μM), incubated at 37°C for 15 min in the dark, and analyzed immediately with a flow cytometer using the FL-1 filter. The intracellular accumulation of ROS was monitored by flow cytometry after being stained with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA; 10 μM; Molecular Probes), as previously described (21) with slight modifications. DCF-DA was deacetylated in cells by esterase to a nonfluorescent compound, DCFH, which remains trapped within the cell and is cleared and oxidized by ROS in the presence of endogenous peroxidase to a highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). EBV-transformed B cells were preincubated with 10 μM DCFH-DA for 30 min at 37°C, seeded in six-well plates (5 × 10^5 cells/ml), and treated or not with Mel for 24 h. Cells were washed and resuspended in PBS, and ROS levels were determined using a FACSCalibur flow cytometer.

Quantitative real-time PCR
Total cellular RNA was extracted using an RNeasy Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer’s protocol. cDNA was made from 2 μg total RNA using oligo(dT) and reverse transcriptase (both from Bioneer, Daejeon, Korea). Quantitation of mRNA levels was performed using an Eco real-time PCR system (Ilumina, San Diego, CA) and a SYBR Green Master Mix kit (Takara, Tokyo, Japan) following the specific primer sets: XAF1 (upstream primer, 5′-TGCA TCC TAA GGA GGA AA-3′; downstream primer, 5′-CTC AGC TTG ACT TGG AA-3′), XIAP (upstream primer, 5′-GTTG CCA CGC AGT CTA CAA ACTT CTG G-3′; downstream primer, 5′-CGT CTT CAA TAA TCT GCC ATG GAT GC-3′), Bcl-2 (upstream primer, 5′-GGATT TTG GCT CTC TCT TTG AG-3′; downstream primer, 5′-CAG CCA GGA AAT AAA CAT-3′), Bax (upstream primer, 5′-CCA AGA AGA TGG GCC AGT GT-3′; downstream primer, 5′-CAG CCC ATG ATG GCT AT-3′), Noxa (upstream primer, 5′-AGG ACT GTT CTT GAT CTC-3′; downstream primer, 5′-GTC CAC CTC CTG AAA GAA CTC-3′), and Puma (upstream primer, 5′-GTTG TAG AGG CAG AGA GAA GAA TC-3′; downstream primer, 5′-GCT CTT GCT GAT GGA GAG AAG CTC-3′). A specific primer set for β-actin (upstream primer, 5′-ATG CAC GAA ACT ACC TTC AA-3′; downstream primer, 5′-ATC CAC AGG TAC TGG C-3′) was used as a control. The relative mRNA quantitation was calculated using the arithmetic equation 2 ΔΔCq, where ΔCq is the difference between the threshold cycle of a given target cDNA and an endogenous reference cDNA.

Immunoblotting
Western blot was performed as previously described (22). Jurkat cells, treated for 8 h with etoposide (25 μM), were used as a positive control for caspase-3. Jurkat cells treated with 100 ng/ml PMA for 10 min were used as a positive control for DNA-ERK1/2. Briefly, after treatment, cells were harvested and lysed in Nonident P-40 (NP-40) buffer (Elsip Biotech, Daejeon, Korea) supplemented with a protease inhibitor mixture (Sigma-Aldrich). To address phosphorylation events, an additional set of phosphatase inhibitors (Cocktail II; Sigma-Aldrich) was added to NP-40 buffer. Protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL). The membrane was first incubated in the solution of 2× Laemmli sample buffer (Elsip Biotech) was added to each lysate, and each protein (10 μg/sample) was immediately boiled for 5 min at 100°C. The insoluble material was spun down at 13,000 rpm (22). Total-cell lysates (5 × 10^6 cells/sample)
were subjected to SDS-PAGE on gel containing 15% (w/v) acrylamide under reducing conditions. Separated proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA), the membranes were blocked with 5% skim milk, and commercial Western blot analysis was performed. Chemiluminescence was detected using an ECL kit (Advanta, Menlo Park, CA) and the multiple Gel DOC system (Fujifilm, Tokyo, Japan). The following primary Abs were used: caspase-8, caspase-3, caspase-9, poly (ADP-ribose) polymerase (PARP), β-actin, Bc-2, Bax, phospho-Bad (Ser112), Bad, phospho-Bad (Ser155), Bax, phospho-p53 (Ser15), p53, phospho-JNK (Thr183/Tyr185), JNK, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-ATR (Ser428), and β-tubulin (BD Biosciences).

Detection of XPA, XAF1, TAp73, Bax, and Puma translocation
Mitochondrial and cytosol cellular fractions were prepared using a Cytosol/ Mitochondria Fractionation kit (Calbiochem). A total of 1 × 10^6 cells, with or without several treatments, were harvested by centrifugation at 600 × g for 5 min at 4°C and washed twice with cold PBS. Subsequently, the cells were resuspended in 250 μl Cytosol Extraction buffer containing a protease inhibitor mixture and 1 mM DTT. After incubation on ice for 10 min, cells were homogenized on ice using a Dounce tissue homogenizer. Homogenized cells were centrifuged at 700 × g for 10 min at 4°C, and supernatants were collected. Supernatants were centrifuged again at 10,000 × g for 30 min at 4°C. The resulting supernatants were harvested and designated as cytosolic fractions; the pellets were resuspended in 250 μl Mitochondria Extraction buffer containing a protease inhibitor mixture and 1 mM DTT and designated as mitochondrial fractions. The nuclear cellular fraction was prepared using a Nuclear/Cytosol Fractionation kit (Biovision, Mountain View, CA). A total of 2 × 10^6 cells, with or without various treatments, was harvested and suspended in 200 μl Cytosol Extraction buffer after incubation on ice for 10 min. The cell suspension was added to a cytosol extraction buffer B and incubated on ice for 1 min. Obtained pellets were resuspended in 100 μl Nuclear Extraction buffer mix and designed as the nuclear fractions.

Coimmunoprecipitation assay
For XAF1–Bax binding, Puma–Bax binding, TAp73–XAF1 binding, TAp73–XPA binding, XAF1–XPA binding, or TAp73–Puma binding assays, cells were treated with Mel for 24 h. Cells (5 × 10^6 cells/sample) were harvested and lysed in NP-40 buffer (Elpis Biotech) containing a protease inhibitor mixture (Sigma–Aldrich). To reduce nonspecific binding of protein, we performed preclearing on equal amounts of cell lysates by incubating cell samples with washed hein G PLUS–agarose beads (Santa Cruz Biotechnology). For immunoprecipitation (IP), precleared lysisate plus the optimal amount of anti-XAF1, anti-Puma, or anti-Tap73 Abs was incubated at 4°C for 2 h on arotator. The immunoprecipitates were harvested by protein G PLUS–agarose beads (Santa Cruz Biotechnology) and incubated at 4°C for 2 h under rotary agitation. Following incubation, the supematant was removed, and the beads were washed in lysis buffer four times. Finally, immunoprecipitates were eluted by boiling the beads in SDS-PAGE sample buffer for 5 min and then characterized by Western blotting with appropriate Abs.

Small interfering RNA transfection
Experimentally verified human ATR–small interfering RNA (siRNA) duplex, TAp73–siRNA duplex, and negative control–siRNA were obtained from Bioneer. Cells were transiently transfected by electroporation under optimized conditions. Briefly, cells (5 × 10^6/sample) were electroporated with 300 nM siRNA using Ingenio Electroporation Solution (Mirus Bio, Madison, WI) and the Bio-Rad Gene Pulser Xcell system (Bio-Rad, Hercules, CA), according to the manufacturers’ protocol.

Results
Mel induces apoptosis in EBV-transformed B cells but not in normal PBMCs
To assess the chemosensitivity of Mel on EBV-transformed B cells, we first examined whether Mel could modulate the proliferation of EBV-transformed B cells. Cells were treated with different concentrations of Mel (1, 5, 10, 50, 100 μM) for 24 h, and its effect on cell proliferation was analyzed by AlamarBlue assay. Mel inhibited the cell proliferation of EBV-transformed B cells in a dose-dependent manner, suggesting potential multitarget activity (Supplemental Fig. 1A). Mel exhibited ~50% proliferation inhibition at a concentration of 10 μM. Therefore, we examined whether the cytotoxicity of Mel was due primarily to apoptosis. Cells were treated with different concentrations of Mel (1, 5, 10, 50, 100 μM) for 24 h, and apoptotic cells were measured by Annexin-V/7-AAD staining. Fig. 1A shows that different concentrations of Mel have apoptotic-inducing effects on cells and that 1.60 ± 0.97% of EtOH-treated cells were Annexin-V+ and 7-AAD+, whereas 8.29 ± 1.11, 27.59 ± 8.09, 48.97 ± 0.97, 57.22 ± 0.39, or 62.21 ± 0.41% of cells were apoptotic after 24 h of treatment with 1, 5, 10, 50, or 100 μM of Mel, respectively. As shown in Fig. 1B, cells were exposed to Mel for various times: 2.26 ± 1.11, 2.65 ± 1.44, 14.48 ± 5.60, 38.42 ± 6.98, 63.56 ± 8.72, or 65.54 ± 7.17% of cells were Annexin-V+ and 7-AAD+ after treatment with Mel for 2, 4, 8, 12, 16, or 24 h, respectively. Because the optimal concentration and duration of Mel treatment were 10 μM and 24 h, we chose these conditions to evaluate protein alterations in Mel-induced apoptosis. We checked for significant cell death in normal human PBMCs after Mel treatment (Supplemental Fig. 1B) as a method of determining whether Mel has any cytotoxic effect on normal human PBMCs. We observed no significant cell death, implying that Mel selectively induces the cytotoxic effect on cancerous EBV-transformed B cells rather than on normal human PBMCs. Numerous anticancer drugs cause apoptosis of tumor cells via activation of mitochondrial apoptotic pathways. One of the major factors affecting death induction through these pathways is a rapid and early disintegration of Δψm. Thus, we examined the integrity of mitochondrial function after Mel treatment

![Figure 1](http://www.jimmunol.org/)
to investigate whether Mel-induced apoptosis was involved in a breakdown of $\Delta \psi_{\text{m}}$. Mel significantly induced $\Delta \psi_{\text{m}}$ disruption, especially between 8 and 24 h of treatment (Fig. 1B; from $31.88 \pm 1.38\%$ to $68.36 \pm 5.95\%$).

**Mel induces activation of caspase-9 and caspase-3 but not caspase-8**

Based on the preliminary assays in which a strong apoptotic effect of Mel was elicited in EBV-transformed B cells, we further scrutinized the possible molecular mechanism underlying Mel-induced apoptosis. We first examined proteolytic processing of caspases by Western blot, because caspase activation was reported to play a role in apoptosis mediated by various stimuli. The activities of caspase-9 and caspase-3 increased significantly in a time-dependent manner, correlating with cleavage of PARP (Fig. 2A) and leading to the release of Cyt. c from the mitochondria (Fig. 3D). However, cleavage of procaspase-8 into the characteristic 18-kDa active fragments did not appear significantly until 24 h as well as at an early time (2–6 h, data not shown) after Mel treatment. As shown in Fig. 2B, inactivation of caspase-9 or caspase-3 by z-LEHD-fmk (caspase-9 inhibitor) or z-DEVD-fmk (caspase-3 inhibitor) effectively blocked Mel-mediated apoptosis ($62.94 \pm 2.83\%$ to $8.25 \pm 0.67\%$; $z$-LEHD-fmk, $7.50 \pm 1.16\%$; $z$-DEVD-fmk, respectively) and $\Delta \psi_{\text{m}}$ disruption ($63.98 \pm 10.46\%$ to $4.98 \pm 0.81\%$; $z$-LEHD-fmk, $5.90 \pm 1.38\%$; $z$-DEVD-fmk, respectively). However, the caspase-8 inhibitor $z$-IETD-fmk did not block Mel-induced apoptosis (Fig. 2B), nor did it inhibit activation of downstream signals, including caspase-9, caspase-3, and PARP (Fig. 2C). These results suggest that caspase-9 and caspase-3 are associated closely with Mel-induced apoptosis.

**XAF1 and Puma directly interact and promote Bax translocation during Mel-induced apoptosis**

The Bcl-2 and IAP families are closely associated with mitochondria-related events. They can inhibit or activate the release of downstream factors that lead to the activation of caspase-3 and PARP in the execution of apoptosis by $\Delta \psi_{\text{m}}$ disruption. To investigate the apoptosis-related proteins, antiapoptotic (Bcl-2, Bcl-xL, XIAP, Livin, and Survivin) and proapoptotic (Bax, Bad, Bim, Puma, Noxa, and XAF1) proteins were detected by real-time PCR and Western blot after cells were treated with 10 $\mu$M Mel for different times. We found that Mel significantly mitigated the expression of Bcl-2, XIAP, c-IAP1, c-IAP2, and Livin and slightly reduced the expression of Survivin after 24 h of treatment (Fig. 3A–C, Supplemental Fig. 2A). In contrast, Mel greatly increased the expression of XAF1 and Puma but had little effect on the protein levels of Bax, Bad, Bim, Bcl-xL, and Noxa (Fig. 3C, Supplemental Fig. 2A, 2B), although it affected the mRNA levels of Bax and Noxa to some degree (Fig. 3A, 3B). Mel increased the phosphorylation of Bax and Bad but not Bim (Fig. 3C). Bax and Bad translocation provokes the permeabilization of the mitochondrial outer membrane and initiates disintegration of $\Delta \psi_{\text{m}}$ and the release of Cyt. c from

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**FIGURE 2.** Effect of Mel on caspase activation. (A) EBV-transformed B cells were treated with 10 $\mu$M Mel for the indicated times. Western blots to active caspase-8, caspase-9, caspase-3, or PARP cleavage were performed to characterize an apoptotic response. Jurkat cells treated with etoposide (25 $\mu$M, 8 h) were used as a positive control for caspase-8. $\beta$-actin was used to normalized protein contents. Arrowhead indicates a nonspecific band. (B and C) Cells were preincubated with z-LEHD-fmk (20 $\mu$M), z-DEVDFmk (20 $\mu$M), or z-IETD-fmk (20 $\mu$M) for 2 h and then treated with 10 $\mu$M Mel for 24 h. (B) The number of apoptotic cells (Annexin-V+/7-AAD+) and $\Delta \psi_{\text{m}}$ (DiOC6) were obtained as described in Materials and Methods. Each value is the mean $\pm$ SD of three determinations. (C) Western blots for active caspase-9, caspase-3, or PARP cleavage. Results are representative of three independent experiments.
mitochondria (23, 24). We separated the mitochondrial and cytosolic fractions after 12 or 24 h of Mel treatment and determined the Cyt. c level by Western blot. As depicted in Fig. 3D, there was a dramatic increment in the level of Cyt. c released from mitochondria to cytosol and in the levels of Puma, Bax, and Bad translocation from cytosol to mitochondria at 12 and 24 h compared with control. Mitochondrial levels of phospho-Bax and phospho-Bad were also increased in Mel-treated cells. Moreover, under control conditions, XAF1 resided primarily in the nucleus (16), whereas it redistributed from the nucleus to the cytosol and mitochondria after Mel treatment (Fig. 3E). It was reported that Bax is required for XAF1- or Puma-mediated apoptosis and is translocated by XAF1 or Puma activation (17, 25). However, how XAF1 and Puma affect the function of Bax, as well as Bad, after Mel treatment is unclear. To determine whether XAF1 and Puma can affect Bax and Bad directly, the interaction between XAF1 or Puma and Bax, Bad, or Bcl-xL was assessed by co-IP analysis. The results of co-IP show that the amount of Bax and Bcl-xL binding to XAF1 and Puma increased significantly after Mel treatment (Fig. 3F, 3G) and that the amount of Puma binding to XAF1 was enhanced after Mel treatment, suggesting that both XAF1 and Puma could interact with both Bax and Bcl-xL.

**Mel-induced apoptosis requires TAp73 expression and XPA translocation but not p53 activation**

After Mel treatment, TAp73 mRNA levels remained unchanged, indicating that TAp73 protein is upregulated by posttranscriptional mechanisms (data not shown). TAp73 protein levels were undetectable in untreated cells, whereas its expression was enhanced at 4 h and induced in a time-dependent manner following treatment with Mel. p53 activation was not induced by Mel treatment, and total p53 protein level was decreased in a time-dependent manner. Expression of the TAp73 downstream targets, XAF1 and Puma, increased at 8 h and was maintained until 24 h following Mel treatment. In contrast, XPA expression remained unchanged until 12 h after Mel treatment, although its expression was diminished at 24 h (Fig. 4A). To investigate the effect of Mel on TAp73 nuclear redistribution, we used fractionation procedures to separate the nuclear and cytosol fractions. Interestingly, following exposure to Mel, a significant increase in the nuclear fraction, and the XPA level was coincidently enhanced in the nuclear fraction (Fig. 4B). To validate whether TAp73 plays a role during Mel-induced apoptosis, we checked the interaction...
between TAp73 and XAF1 or XPA by co-IP analysis. Compared with EtOH-treated controls, treatment with Mel improved the binding of XAF1 and XPA to TAp73 (Fig. 4C). Meanwhile, Mel treatment did not affect the binding of XAF1 to XPA (Fig. 4D). These results suggest that TAp73 may play an important mechanistic role as a connecting molecule between XPA and XAF1 in the Mel-induced apoptosis of EBV-transformed B cells.

**p38 MAPK mediates the upregulation of TAp73 and XAF1 in Mel-induced apoptosis**

MAPK signaling is involved in several events of cellular stress-induced cell apoptosis and TAp73 upregulation (7, 8). Therefore, we tested whether certain MAPKs could act as inducers of TAp73 expression after Mel treatment. First, to examine the possibility that MAPKs mediate TAp73 expression, cells were treated with Mel and analyzed for the activity of various MAPKs, including ERK1/2, JNK, and p38 MAPK. Fig. 5A shows that Mel apparently induced an activation of p38 MAPK and the phosphorylation level exhibited in a time-dependent manner, whereas it had no effect on JNK or ERK1/2, although JNK was transiently activated slightly at 4 h. These results suggest that p38 MAPK is a potential inducer of Mel-induced TAp73 expression and translocation. To corroborate the role of p38 MAPK in Mel-induced apoptosis, cells were treated with Mel in the presence or absence of the p38 MAPK inhibitor, SB203580. The results show that SB203580 inhibited Mel-induced TAp73, XAF1, and Puma expression and restored the

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Mel-induced TAp73 is closely involved in XPA translocation to the nucleus and XAF1 and Puma translocation to mitochondria. Cells were treated with 10 μM Mel for the indicated times. (A) Total cell extracts were collected at the indicated time after treatment with 10 μM Mel, and Western blots to TAp73, XPA, XAF1, Puma, phospho-p53, and p53 were carried out. (B) Cells were harvested, and the amounts of TAp73, XAF1, and XPA in cytosol and nuclear fractions were detected. The nuclear marker, Ref-1, and the cytosol marker, β-tubulin, were used to verify the purity of each fraction; fractionation was performed as described in Materials and Methods. To perform binding assays, TAp73 (C) or XPA (D) was immunoprecipitated using a specific Ab, followed by immunodetection of XPA, XAF1, Puma, and TAp73 in the immunoprecipitate, as detailed in Materials and Methods. Results are representative of three independent experiments. IB, Immunoblotting.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Mel causes rapid activation of p38 MAPK and leads to upregulation of TAp73 through p38 MAPK activation in EBV-transformed B cells. (A) Cells were treated with 10 μM Mel for the indicated times prior to cell lysis. Total protein was subjected to Western blot analysis and successively immunoblotted against MAPKs. PMA-treated Jurkat cells (100 ng/ml PMA for 10 min) were used as a positive control for phospho-ERK1/2. (B) Effect of p38 MAPK inhibitor on Mel-induced mitochondrial-related protein activation and TAp73 expression. To inhibit p38 MAPK phosphorylation, cells were pretreated with SB203580 (10 μM) for 2 h. Western blots for TAp73, XAF1, Puma, Bcl-2, phospho-p38, p38, and β-actin protein were performed. Results are representative of three independent experiments.
Mel-induced decrease in Bcl-2 levels (Fig. 5B). Moreover, Mel suppressed the activation of PI3K/Akt and mTOR in a time-dependent manner (Supplemental Fig. 3). Collectively, these observations substantiate the role of p38 MAPK in TAp73, XAF1, and Puma upregulation.

**ROS is a critical upstream signal for p38 MAPK activation and mitochondrial events by Mel**

In apoptosis regulation, ROS is directly involved in the protease cascade, such as the induction of caspases and MAPKs (26). Moreover, ROS is an early signal that mediates apoptosis (27) and is an important acting system of many chemotherapeutic agents. To assess the impact of Mel on ROS, cells were treated with 10 μM Mel for 30 min or for 1, 2, 4, 8, or 24 h, followed by the addition of DCFH-DA to measure intracellular ROS levels. We found that Mel induced a marked increase in DCF fluorescence within 30 min, and the Mel-induced ROS level was maintained for 24 h (Fig. 6A, 6B). To clarify the possible link between Mel-induced ROS generation and p38 MAPK phosphorylation during apoptosis, we treated cells with APDC or SB203580, inhibitors of ROS and p38 MAPK, respectively. As illustrated in Fig. 6C, all inhibitors blocked Mel-induced phosphatidylserine exposure and

**FIGURE 6.** Mel induces p38 MAPK phosphorylation through ROS generation. (A) EBV-transformed B cells were treated with 10 μM DCFH-DA for 30 min and then treated with 10 μM Mel for the indicated times. Results are mean fluorescence intensity (MFI) of DCF oxidation. (B) Bar graph showing the rate of intracellular ROS production based on three independent experiments. Data are mean ± SD of three determinations. (C) Effects of APDC and SB203580 on Mel-induced ROS production. Cells were treated with the indicated inhibitors (APDC [100 μM], NAC [10 mM], or SB203580 [10 μM]), as described in Materials and Methods, before being treated with 10 μM Mel or EtOH. (D) Effect of ROS inhibitor on Mel-induced p38 MAPK phosphorylation, TAp73, and XAF1. To inhibit ROS production, cells were pretreated with APDC (100 μM) or SB203580 (10 μM), respectively. Cells were harvested, and the amount of XAF1, PUMA, and Bax in cytosol and mitochondria fractions was determined. (F) Cells were harvested, and the amount of TAp73, XAF1, and XPA in cytosol and nuclear fractions was determined. To block ROS generation or p38 MAPK activation, cells were pretreated with APDC (100 μM) or SB203580 (10 μM), respectively. The cytosol marker, β-tubulin, the nuclear marker, Ref-1, and the mitochondrial marker, COX-IV, were used; also, fractionation was performed as described in Materials and Methods. Results are representative of three independent experiments.
\[ \Delta \psi_{m} \] disruption remarkably. However, APDC was the only inhibitor that significantly diminished Mel-induced ROS production. These results were confirmed by the observation that another ROS scavenger, NAC, also inhibited Mel-induced apoptosis, \[ \Delta \psi_{m} \] disruption, and ROS production (Fig. 6C). Meanwhile, z-DEVD-fmk, z-LEHD-fmk, or z-IETD-fmk pretreatment did not block Mel-induced ROS generation (data not shown). APDC inhibited phosphorylation of p38 MAPK by Mel treatment and blocked expression of TAp73 and XAF1 (Fig. 6D). Both APDC and SB203580 prevented translocation of XAF1, Puma, and Bax to the mitochondria (Fig. 6E) and impeded translocation of TAp73 and XPA to the nucleus and translocation of XAF1 to the cytosol (Fig. 6F). These results suggest that ROS are generated prior to p38 MAPK and play a major role as an upstream regulatory molecule in Mel-induced apoptosis.

**p38 MAPK activation and TAp73 upregulation in response to Mel are triggered by ATR activation**

Generally, cellular responses to genotoxic stress are triggered by the activation of DNA damage sensors, such as ATM or ATR (28). Accordingly, we investigated whether these sensors were related to p38 MAPK activation or TAp73 expression. As illustrated in Fig. 7A, Mel induced activation of ATR at an early time point (1 h) and increased phospho-ATR levels in a time-dependent manner. However, contrary to our expectations, Mel-induced apoptosis did not require phospho-ATM (Supplemental Fig. 4). To verify the activation of DNA damage sensors, such as ATM or ATR (28).

**FIGURE 7.** The activation of p38 MAPK and the expression of TAp73 in Mel-induced apoptosis are dependent upon the activity of ATR. (A) Cells were treated with 10 \( \mu \)M Mel for the indicated times. Phosphorylation of ATR was examined by Western blot analysis. (B) Cells were preincubated with APDC (100 \( \mu \)M) or SB203580 (10 \( \mu \)M) for 2 h and then treated with 100 \( \mu \)M Mel for 2 h. Activation of ATR was detected by Western blot analysis. (C) To perform binding assays, ATR was immunoprecipitated using a specific Ab, followed by immunodetection of phospho-p38 MAPK and TAp73 in the immunoprecipitate, as detailed in Materials and Methods. Results are representative of three independent experiments. IB, Immunoblotting.
enhance ROS generation while eliminating tumor cells by the production of ROS directly in tumor cells or by blocking the intracellular antioxidative proteins of tumor cells. Consistent with this finding, Mel provoked aberrant ROS production at an early time (Fig. 6A). ROS production was reported to occur following mitochondrial outer membrane permeabilization. However, our results showed that Mel-induced ROS production preceded \( \Delta \psi_{\text{m}} \) disruption (Figs. 1A, 6B, 6C) and was not generated from mitochondria alone. These results suggest that Mel probably is involved in the induction of ROS generation throughout the whole cells. Therefore, ROS may act upstream of mitochondrial membrane permeabilization and mediate mitochondrial dysfunction in Mel-induced apoptosis (Fig. 6C). Our study also showed that Mel-induced apoptosis was attenuated by the ROS scavenger, APDC, indicating that ROS are linked closely to Mel-induced apoptosis in EBV-transformed B cells (Fig. 6C).

Oxidative stress–activated MAPKs are important for cell death. In general, p38 MAPK and JNK are associated with apoptosis, whereas ERK1/2 is involved in cell proliferation. Particularly, p38 MAPK is crucial in transmitting apoptotic signals (33). That p38 MAPK activity was necessary for caspase activation suggested that p38 MAPK may regulate some aspect of the mitochondrial pathway, because the release of Cyt. \( c \) from damaged mitochondria shows an important signal for activating caspases. Bax translocation to the mitochondria was shown to diminish \( \Delta \psi_{\text{m}} \) and elicit Cyt. \( c \) release from the mitochondria, resulting in caspase activation. Bax activation may be provoked by phosphorylation of p38 MAPK and JNK or by alteration of intracellular pH. Mel produced a prominent increase in the phosphorylation of p38 MAPK in EBV-transformed B cells (Fig. 5A). Moreover, the p38 MAPK inhibitor SB203580 strongly blocked Bax translocation and imparted significant protection against Mel-induced apoptosis (Fig. 6E). Treatment of the APDC also suppressed p38 MAPK activation, resulting in reduced levels of apoptosis. It is noteworthy that suppression of ROS levels ameliorated the effect of Mel on p38 MAPK phosphorylation, suggesting that Mel produces ROS, which subsequently activates p38 MAPK (34).

p38 MAPK signaling can induce expression of TAp73 (35). Suppression of p38 MAPK activation by SB203580 in these cells blocked TAp73-mediated apoptosis. These results implied that activation of p38 MAPK contributed to the induction of TAp73-mediated apoptosis in EBV-transformed B cells in response to Mel. To further investigate the underlying mechanisms by which Mel enhances TAp73-mediated apoptosis, we turned our attention to XAF1, which is a key mediator of apoptosis and plays an important role in the induction of cell death (15, 17). A previous report (36) showed that activation of the JNK pathway was strongly implicated in the induction of XAF1-mediated apoptosis. Our
study indicates that the expression of XAF1 was upregulated and translocated into mitochondria in Mel-exposed cells. To our knowledge, this is the first report that p38 MAPK and Tap73 function upstream of XAF1 to induce cell apoptosis (Figs. 3, 4, 6). Interestingly, we observed that Mel-induced XAF1 was expressed in the nucleus, the cytoplasm, and the mitochondria, as previously described (17). This may be because each cell has a different drug sensitivity and drug-response rate. Puma is a downstream gene of Tap73 that can be induced by DNA-damage drugs, and it is important in the induction of apoptosis (4). Tap73 can induce mitochondria-mediated apoptosis by directly transactivating the promoters of both Bax and Puma (4). Our study indicated that the Tap73-induced XAF1 with Puma promoted Bax translocation into the mitochondria in Mel-exposed cells (Figs. 3, 4). Bcl-xL also interacted with both XAF1 and Puma. The involvement of Bcl-xL in Puma-mediated apoptosis also was demonstrated in a previous study (37) in which Puma was found to interact with Bcl-xL and induce XAF1 with Puma promoted Bax translocation into the mitochondria in Mel-exposed cells (Figs. 3, 4). Bcl-xL also interacted with both Bax and Puma (4). Our study indicated that the TAp73-mediated apoptosis also was demonstrated in a previous study (37) in which Puma was found to interact with Bcl-xL and activate the XAF1 apoptotic pathway under stressful circumstances.

Collectively, our results clearly support a novel pathway of Tap73-mediated cellular sensitivity to Mel in EBV-transformed B cells. We demonstrated that Tap73 induced the apoptotic response through the nuclear import of XPA into the nucleus after UV exposure (38). Previous reports (9, 39) showed that ATR-mediated p38 MAPK activation induced apoptosis and the ATM/ATR-p38 MAPK pathway promoted G1–S phase accumulation. Mel significantly increased phosphorylated ATR, as well as total ATR, in EBV-transformed B cells (Fig. 7A). Moreover, APDC, but not the p38 MAPK inhibitor SB203580, suppressed the phosphorylation of ATR, and the binding of phospho-p38 MAPK and Tap73 to ATR increased (Fig. 7B, 7C). These results indicate that Mel-induced p38 MAPK activation and Tap73 upregulation were ATR dependent.

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DISCLOSURES

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REFERENCES


