Berberine induces mitochondrial apoptosis of EBV-transformed B cells through p53-mediated regulation of XAF1 and GADD45α

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Abstract. Berberine exhibits antiproliferative or cytotoxic effects against various cancers. ROS and wild-type p53 play a critical role in berberine-induced cytotoxic effects. In this study, we investigated the correlation between XAF1 and functional p53 in EBV-transformed B cells or cancerous B cells after treatment with berberine. Berberine decreased cell viability and induced apoptosis through a mitochondria-dependent pathway in EBV-transformed B cells and cancerous B cells, but not in normal peripheral blood mononuclear cells. Activated p53 and its downstream targets XAF1 and GADD45α interacted with PUMA, Bax, and Bim in mitochondria after treatment with berberine. Blocking phosphorylation of p38/JNK MAPK and treatment with PFT-α, a selective p53 inhibitor, effectively prevented apoptosis and the upregulation of phosphorylated p53, XAF1, and GADD45α. NAC, a ROS scavenger, also suppressed berberine-induced mitochondria disruption and the whole apoptotic process via restoration of p53-related proteins and proapoptotic Bcl-2 family proteins. Taken together, our results suggest that ROS generation might be a predisposing event in berberine-induced mitochondrial apoptosis in EBV-transformed B cells through the upregulation of XAF1 and GADD45α expression by MAPK and functional p53.

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Abbreviations: EBV, Epstein-Barr virus; GADD45α, growth arrest and DNA damage inducible alpha; MDM2, mouse double minute 2 homolog; NAC, N-acetylcysteine; PUMA, p53 upregulated modulator of apoptosis; XAF1, X-linked inhibitor of apoptosis protein-associated factor 1; XIAP, X-linked inhibitor of apoptosis protein

Key words: berberine, EBV-transformed B cells, XAF1, GADD45α, p53

Introduction

Berberine, an alkaloid found in many plant species [e.g., *Berberis aquifolium* (Oregon grape) and *Hydrastis canadensis* (goldenseal)], exhibits antiproliferative or cytotoxic effects against cancerous cells of different origins (1-3). Berberine inhibits the proliferation of MCF-7 breast cancer cells through upregulation of p53 and a mitochondria-dependent apoptotic pathway (4). Berberine also strongly induces cytotoxicity and apoptosis in ALL cells that express p53 and MDM2 oncoprotein (5). However, previous studies did not clearly identify the target of activated p53 protein involved in inducing or controlling the apoptosis pathway after treatment with berberine.

It has long been recognized that p53 is the most commonly mutated tumor suppressor gene and that loss of p53 expression or function is associated with an increased risk of tumor development (6). XAF1, a novel target gene of p53, is downregulated by wild-type p53, but not by mutant p53, at both mRNA and protein levels (7). XAF1 was previously identified as a binding partner of XIAP that directly interacts with endogenous XIAP (8). XAF1 was reported to antagonize the anticaspase activity of recombinant XIAP and reverse the protective effect of XIAP overexpression in various cancer cell lines (9). Epigenetic silencing of XAF1 by aberrant promoter methylation is associated with cancer development and progression (10). Restoration of XAF1 expression induces cancer cell apoptosis *in vitro* and *in vivo* (11,12).

p53 binds to the core promoter of the XAF1 gene with high affinity. p53 knockdown or abrogation of this element by site-specific mutation inhibits this binding and abolishes the inhibitory effect of p53 on XAF1 transcription (12). Overexpression of XAF1 leads to activation of wild-type p53 via phosphorylation of gastric or colon cancer cells, resulting in nuclear accumulation of p53 and enhanced p53-dependent apoptosis (7). However, the exact role of activated p53 in XAF1 expression and function remains unclear, especially in hematologic cancers.

GADD45α gene is one of the numerous downstream targets of p53 (13). The role of GADD45α in the regulation of apoptosis is controversial and may depend on cell type and the nature of the environmental stimulus that triggers apoptosis. GADD45 family members are able to activate the p38/JNK MAPK pathway (14,15), and p38/JNK can contribute to the activation of p53 (16,17). Moreover, the p53-responsive genes GADD45 and $p21^{WAF1}$ are significantly induced in adult T-cell leukemia/lymphoma cells after treatment with ionizing radiation (18). Based on these reports, we predicted that GADD45α might play some role in the regulation of XAF1 by p53 in berberine-induced apoptosis of EBV-transformed B cells.

In this study we examined whether the functional p53 status of EBV-transformed B cells or cancerous B cells affects their response to berberine. We also explored the interactions between MAPK-activated p53 and XAF1 in the induction of mitochondrial apoptosis signaling after treatment with berberine.

Materials and methods

Preparation of EBV-infectious supernatant and generation of EBV-transformed B cells. Preparation of cell-free EBV virions and generation of EBV-transformed B cells were carried out as described previously (19). Human Burkitt's lymphoma Raji and Daudi cells and multiple myeloma IM-9 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% FBS (Hyclone) and antibiotics under a humidified atmosphere with 5% CO_2. This study was approved by the Institutional Bioethics Review Board at the Medical College of Inje University, and all donors gave informed consent for the study.

Analysis of apoptotic cells via flow cytometry. The percentage of cells undergoing apoptosis in human EBV-transformed B cells (4 weeks, $2.5x10^5$ cells/ml) and normal PBMCs was determined via flow cytometry using FITC-conjugated Annexin V (BD Biosciences, San Diego, CA, USA) and 7-AAD (BD Biosciences). To determine optimal conditions, experiments were performed using different concentrations (0, 10, 25, 50, 75 and 100 μM) of berberine (Sigma-Aldrich, St. Louis, MO, USA) and different periods of incubation (2, 4, 8, 16 and 24 h). DMSO was used as a vehicle control. To examine the role of caspases, cells were pretreated with the caspase-3 inhibitor z-DEVD-fmk (N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone, 20 μM in DMSO; Calbiochem, San Diego, CA, USA), the caspase-9 inhibitor z-LEHD-fmk (z-Leu-Glu(OMe)-His-Asp-(OMe)-fluoromethylketone, 20 μM; Calbiochem), or the broad spectrum caspase inhibitor z-VAD (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, 20 μM in DMSO; Calbiochem) for 2 h before treatment with berberine. To inhibit the production of ROS, cells were pretreated with the antioxidant NAC (10 mM; Sigma-Aldrich) for 1 h. To block activation of p38-MAPK or JNK, cells were pretreated with SB203580 (10 μM, Calbiochem) or SP600125 (25 μM, Calbiochem) respectively, for 2 h. To block activation of p53, cells were pretreated with PFTα (50 μM, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. Cells were harvested, washed in PBS, and incubated with Annexin V and 7-AAD in Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at room temperature for 15 min in the dark. The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) equipped with CellQuest Pro software (BD Biosciences).

Measurement of mitochondria membrane potential ($\Delta\Psi_m$) and intracellular reactive oxygen species production. Changes in mitochondrial membrane potential were measured using $DiOC_6$ (3, 3'-dihexyloxacarboxyanine iodide; Molecular Probes, Eugene, OR, USA). Cells were treated with berberine or DMSO for 24 h, harvested, washed twice in PBS, resuspended in PBS supplemented with $DiOC_6$ (20 nM), incubated at 37˚C for 15 min in the dark, and immediately analyzed with flow cytometry. Intracellular accumulation of ROS was monitored via flow cytometry after staining with the fluorescent probe, DCFH-DA (10 μM, 2', 7'-dichlorodihydro-fluorescein diacetate; Molecular Probes). DCFH-DA is deacetylated in cells by esterase to a non-fluorescent compound, DCFH, which remains trapped within the cell and is cleaved and oxidized by ROS in the presence of endogenous peroxidase to a highly fluorescent compound, DCF (2', 7'-dichlorofluorescein). Briefly, cells were preincubated with 10 μM DCFH-DA for 30 min at 37˚C. The cells were seeded in 6-well plates ($5x10^5$ cells/ml) and treated with or without berberine. Cells were washed, resuspended in PBS, and ROS levels were determined using a FACSCalibur flow cytometer.

Western blot analysis. After drug treatment, cells were harvested and lysed in NP-40 buffer (Elpis Biotech, Daejeon, Korea) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). To address phosphorylation events, a set of phosphatase inhibitors (Cocktail II, Sigma-Aldrich) was added to the NP-40 buffer. Protein concentration was determined using a Pierce BCA assay kit (Thermo Scientific, Rockford, IL, USA). An equal volume of 2X Laemmli sample buffer (Elpis Biotech) was added to each sample of lysate containing 10 μg protein and immediately boiled for 5 min at 100˚C. The insoluble material was spun down at 13,000 rpm. Total cell lysates (~$5x10^6$ cells/sample) were subjected to SDS-PAGE on 10-15% (w/v) acrylamide gels under reducing conditions. Separated proteins were transferred to nitrocellulose membranes (Millipore Corp., Billerica, MA, USA), the membranes were blocked with 5% skim milk and western blot analysis was performed using a commercial kit. Chemiluminescence was detected using an ECL kit (Advansta Corp., Menlo Park, CA, USA) and the multiple Gel DOC system (Fujifilm, Tokyo, Japan). Primary antibodies against the following proteins were used: caspase-3, caspase-9, PARP, β-actin, Bcl-2, Bax, phospho-Bim (Ser^{69}), PUMA, XIAP, phospho-p53 (Ser^{15}), p53, phospho-JNK (Thr^{183}/Tyr^{185}), JNK, phospho-p38-MAPK (Thr^{180}/Tyr^{182}), p38-MAPK, phospho-ERK1/2 (Thr^{202}/Tyr^{204}), ERK1/2, phospho-Akt (Ser^{473}), and Akt (Cell Signaling Technology, Beverly, MA, USA); XAF1 (Abcam, Cambridge, UK); GADD45α, Ref-1, and COX-IV (Santa Cruz Biotechnology); phospho-Bax (Ser^{184}) (Bioss Inc., Woburn, MA, USA); Bim and β-tubulin (BD Biosciences).

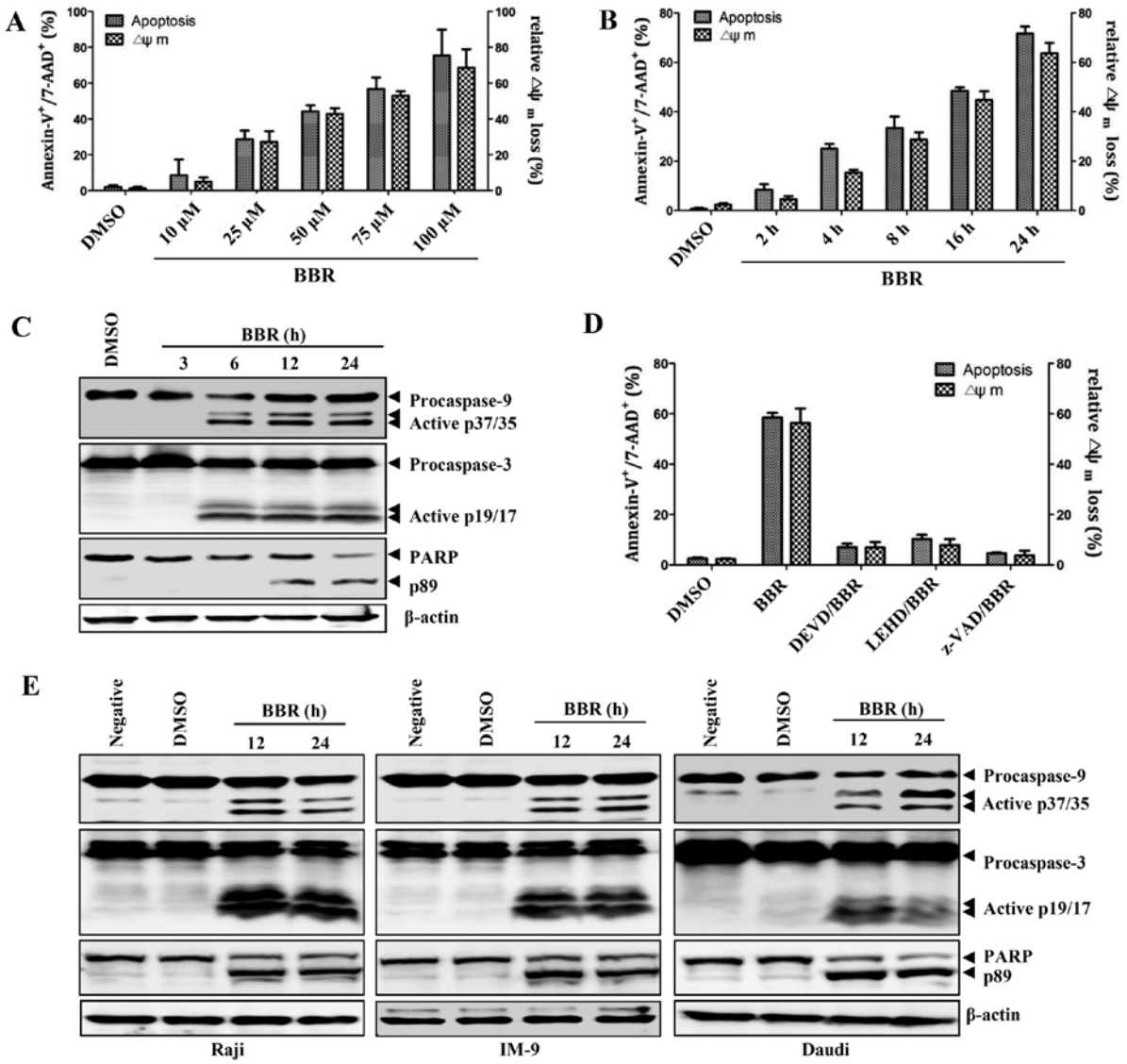

Figure 1. Berberine induces caspase-dependent apoptosis in EBV+ B lymphoma cells. (A) EBV-transformed B cells ($2.5x10^5$ cells/ml) were cultured in 6-well plates and treated with 10, 25, 50, 75 and 100 μM berberine overnight. (B) EBV-transformed B cells ($2.5x10^5$ cells/ml) were treated with 50 μM berberine for 2, 4, 8, 16 and 24 h. To detect the degree of apoptosis, cells were stained with Annexin V-FITC and 7-AAD and analyzed with flow cytometry. The percentage of apoptotic cells is determined by Annexin $V^+/7\text{-}AAD^+$ staining. To measure disruption of $\Delta\Psi_m$, the cells were stained with $DiOC_6$; diminished $DiOC_6$ fluorescence (%) indicates $\Delta\Psi_m$ disruption. Each value was expressed as the mean ± SD of three determinations. (C) EBV-transformed B cells were treated with 50 μM berberine for the indicated times. Western blot analysis of active caspase-9, -3 or PARP cleavage was performed to characterize the apoptotic response. β-actin was used to normalize protein content. (D) EBV-transformed B cells were pre-incubated with z-LEHD-fmk (20 μM), z-DEVD-fmk (20 μM) or z-VAD-fmk (20 μM) for 2 h and then treated with 50 μM berberine for 24 h. The number of apoptotic cells (Annexin $V^+/7\text{-}AAD^+$) and $\Delta\Psi_m$ ($DiOC_6$) were determined as described in Materials and methods. Each value was expressed as the mean ± SD of three determinations. (E) Raji, IM-9 or Daudi cells were exposed to 50 μM berberine for the indicated times. Western blot analysis of active caspase-9, -3 or PARP cleavage was performed to characterize the apoptotic response. Results are representative of three independent experiments.

Detection of translocated GADD45α, XAF1, Bax, Bim, and Puma. Mitochondrial and cytosol cellular fractions were prepared using a Cytosol/Mitochondria Fractionation kit (Calbiochem). Untreated or berberine-treated cells ($1x10^7$ cells) were harvested by centrifugation at 600 x g for 5 min at 4˚C, washed twice with cold PBS, and resuspended in 250 μl Cytosol Extraction buffer containing a protease inhibitor cocktail and 1 mM dithiothreitol (DTT). After incubation on ice for 10 min, the cells were homogenized on ice using a Dounce tissue homogenizer and centrifuged at 700 x g for 10 min at 4˚C. The supernatants were collected and centrifuged again at 10,000 x g for 30 min at 4˚C. The resulting supernatants were harvested as cytosolic fractions and the pellets were resuspended in 50 μl Mitochondria Extraction buffer containing a protease inhibitor cocktail and 1 mM DTT and designated the mitochondrial fractions. The nuclear fraction was prepared using a Nuclear/Cytosol Fractionation kit (Biovision, Mountain View, CA, USA). Briefly, $2x10^6$ cells were harvested and resuspended in 200 μl cytosol extraction buffer A. After incubation on ice for 10 min, the cell suspension was added to cytosol extraction buffer B and incubated on ice for 1 min. After centrifugation the pellets were resuspended in 100 μl nuclear extraction buffer mix and designated the nuclear fraction.

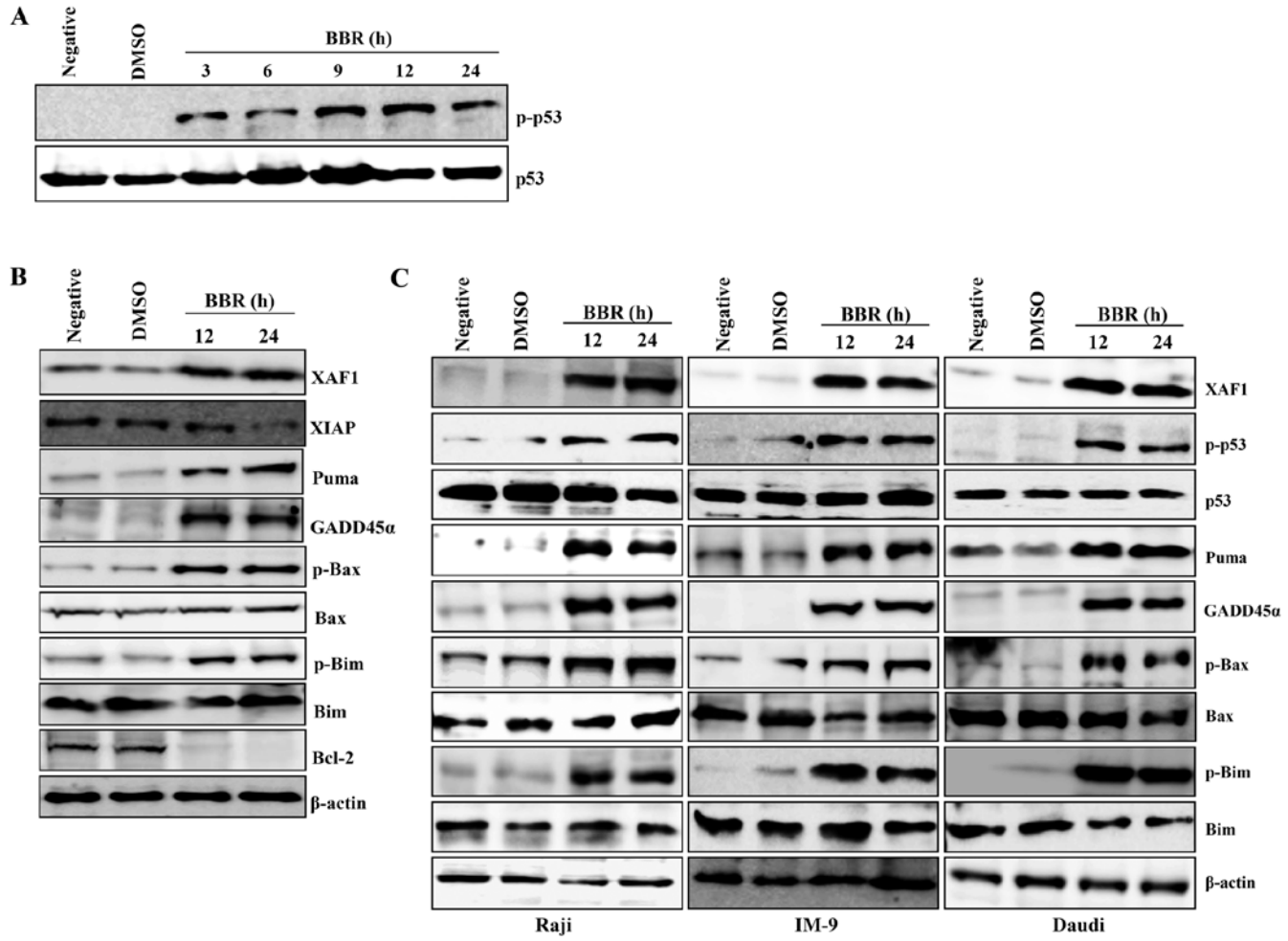

Figure 2. Berberine-induced apoptosis targets a p53-related signal pathway in EBV$^+$ B lymphoma cells. (A and B) EBV-transformed B cells were treated with 50 μM berberine for the indicated times. Western blot analysis of phospho-p53 and p53 (A), and XAF1, XIAP, Puma, GADD45α, phospho-Bax, Bax, phospho-Bim, Bim, and Bcl-2 (B) protein. β-actin was used to normalize protein content. (C) Cells were treated with 50 μM berberine for the indicated times. Results are representative of three independent experiments.

Co-immunoprecipitation (co-IP) assay. For the protein binding assay, cells were treated with berberine for 24 h. Cells were harvested (5x10^6 cells/sample) and lysed in NP-40 buffer containing a protease inhibitor cocktail. To reduce non-specific binding of protein, we performed pre-clearing on equal amounts of cell lysates by incubating the samples with washed protein G PLUS-agarose beads (Santa Cruz Biotechnology). For IP, pre-cleared lysate was incubated with the optimal amount of antibody against XAF1, Puma, GADD45α, or p53 antibody at 4˚C for 2 h on a rotator. The immunoprecipitates were harvested using protein G PLUS-agarose beads and incubated at 4˚C for 2 h under rotary agitation. The supernatant was removed and the beads were washed four times in lysis buffer. Finally, the immunoprecipitates were eluted by boiling the beads in Laemmli sample buffer for 10 min and characterized by western blotting with the appropriate antibodies.

Results

Berberine induces apoptosis in EBV-transformed B cells through the disruption of mitochondria. The viability of EBV-transformed B cells was significantly decreased in a dose-dependent manner after treatment with different concentrations of berberine. However, inhibition of proliferation and induction of apoptosis was not observed at the same doses in normal PBMCs (data not shown). To confirm that berberine induced apoptosis in EBV-transformed B cells we used a 7-AAD and Annexin V staining method. Treatment with different concentrations of berberine resulted in time- and dose-dependent induction of apoptosis and altered mitochondria membrane potential in EBV-transformed B cells (Fig. 1A and B). Treatment with berberine induced activation of capase-3 and -9, which was detected by generation of the cleaved products. Appearance of cleaved PARP, a target of caspase-3, was also observed 12 h after treatment of EBV-transformed B cells or cancerous B cells with berberine (Fig. 1C and E). EBV-transformed B cells were pretreated with several caspase inhibitors including z-VAD (pan-caspase inhibitor), z-DEVD (caspase-3 inhibitor), and z-LEHD (caspase-9 inhibitor) 2 h before treatment with berberine. These inhibitors reduced caspase activity and effectively blocked berberine-induced apoptosis in EBV-transformed B cells (Fig. 1D). These data suggest that berberine induces apoptosis in EBV-transformed B cells or cancerous B cells, but in not normal PBMCs, through a mitochondria-dependent pathway.

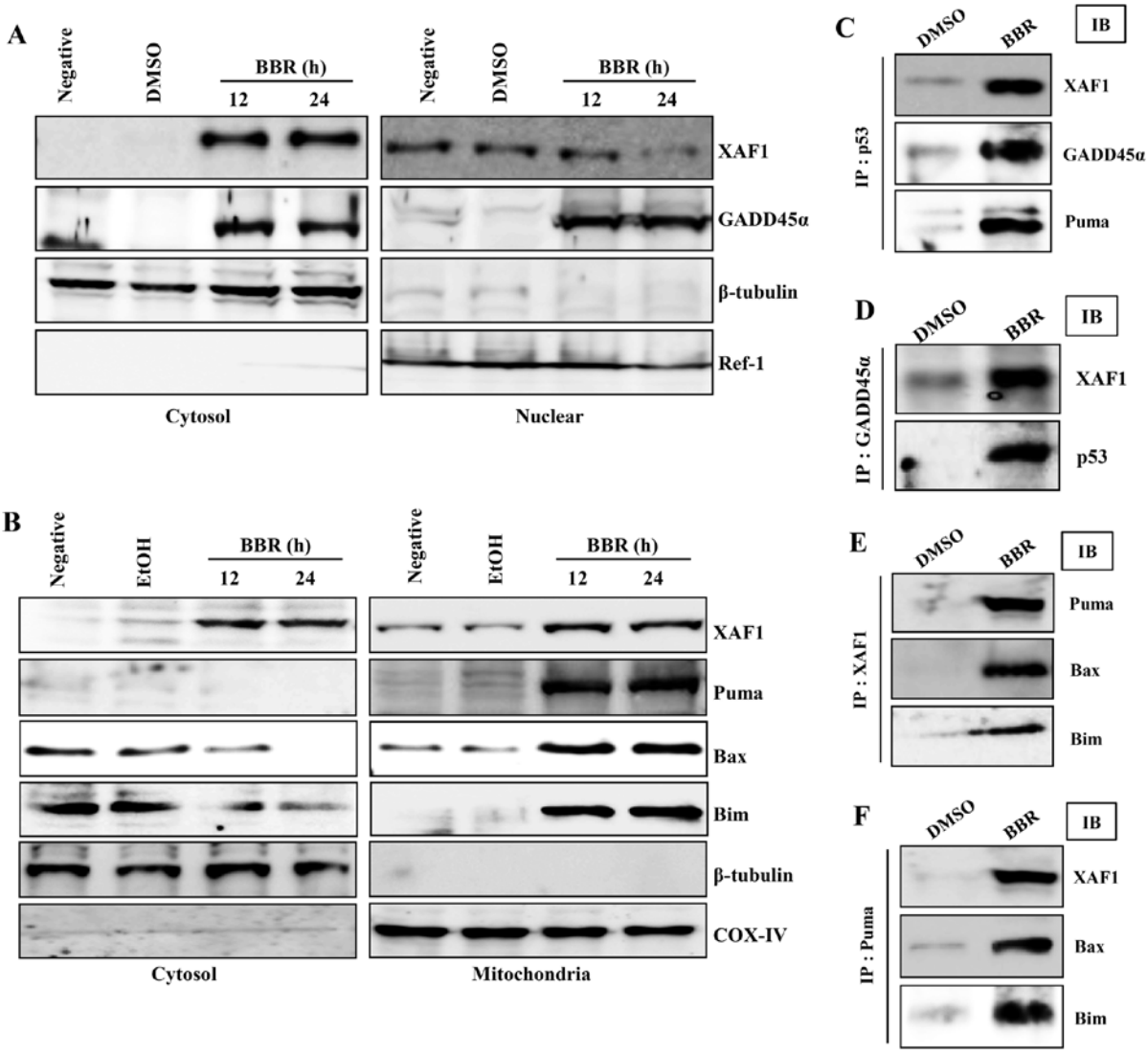

Figure 3. Berberine-induced p53 is involved in GADD45α translocation to the nucleus and XAF1 and Puma translocation to mitochondria. (A and B) EBV-transformed B cells were treated with 50 μM berberine for the indicated times. Cells were harvested and the amount of XAF1 and GADD45α in cytosolic and nuclear fractions (A), and the amount of XAF1, Puma, Bax and Bim in cytosolic and mitochondrial fractions (B) was determined. Fractionation was performed as described in Materials and methods and the mitochondria marker, COX-IV, the cytosol marker, β-tubulin, and the nuclear marker, Ref-1, were used to verify the purity of each fraction. (C-F) For the binding assay, p53 (C), GADD45α (D), Puma (E) or XAF1 (F) was immunoprecipitated using specific antibody followed by immunodetection of XAF1, GADD45α, Puma, p53, Bax and Bim in the immunoprecipitate as detailed in Materials and methods. Results are representative of three independent experiments. IP, immunoprecipitation; IB, immunoblotting.

Berberine-induced apoptosis mainly targets the p53-related signaling pathway in EBV-transformed B cells. As berberine-induced apoptosis is associated with activation of p53 in breast cancer (MCF-7) cells (4,20), we next examined whether XAF1 (an antagonist of XIAP) and GADD45α, both downstream targets of p53, and the Bcl-2 protein family are associated with mitochondrial apoptosis signaling after treatment with berberine in EBV-transformed B cells or cancerous B cells. Berberine increased the level of phosphorylated p53 in a time-dependent manner (Fig. 2A). Expression of XAF1 and GADD45α were induced in EBV-transformed B cells or cancerous B cells. However, expression of XIAP and the antiapoptotic protein Bcl-2 significantly decreased to almost undetectable levels 24 h post-treatment in EBV-transformed B cells. Similar to GADD45α and XAF1, we observed induction of PUMA, phosphorylated Bax, and phosphorylated Bim after berberine treatment in EBV-transformed B cells and cancerous B cells (Fig. 2B and C). These data suggest that berberine-induced apoptosis in EBV-transformed B cells or cancerous B cells is not only related to phosphorylated p53 and its downstream targets, XAF1 and GADD45, but also involves apoptotic members of the Bcl-2 protein family disrupting the mitochondria membrane.

Re-located XAF1 in the cytosol binds to p53 and GADD45α to regulate berberine-induced mitochondrial apoptosis signaling. Next, we further examined the interaction between XAF1 and apoptosis-related proteins to elucidate the role of XAF1 in regulating the apoptotic signaling induced by berberine. XAF1 was not detected in cytosol before treatment with berberine, whereas, XAF1 in the nuclear fraction disappeared after treatment with berberine. However, GADD45α was observed in both nuclear and cytosolic fractions of EBV-transformed B cells treated with berberine (Fig. 3A). Interestingly, XAF1 in

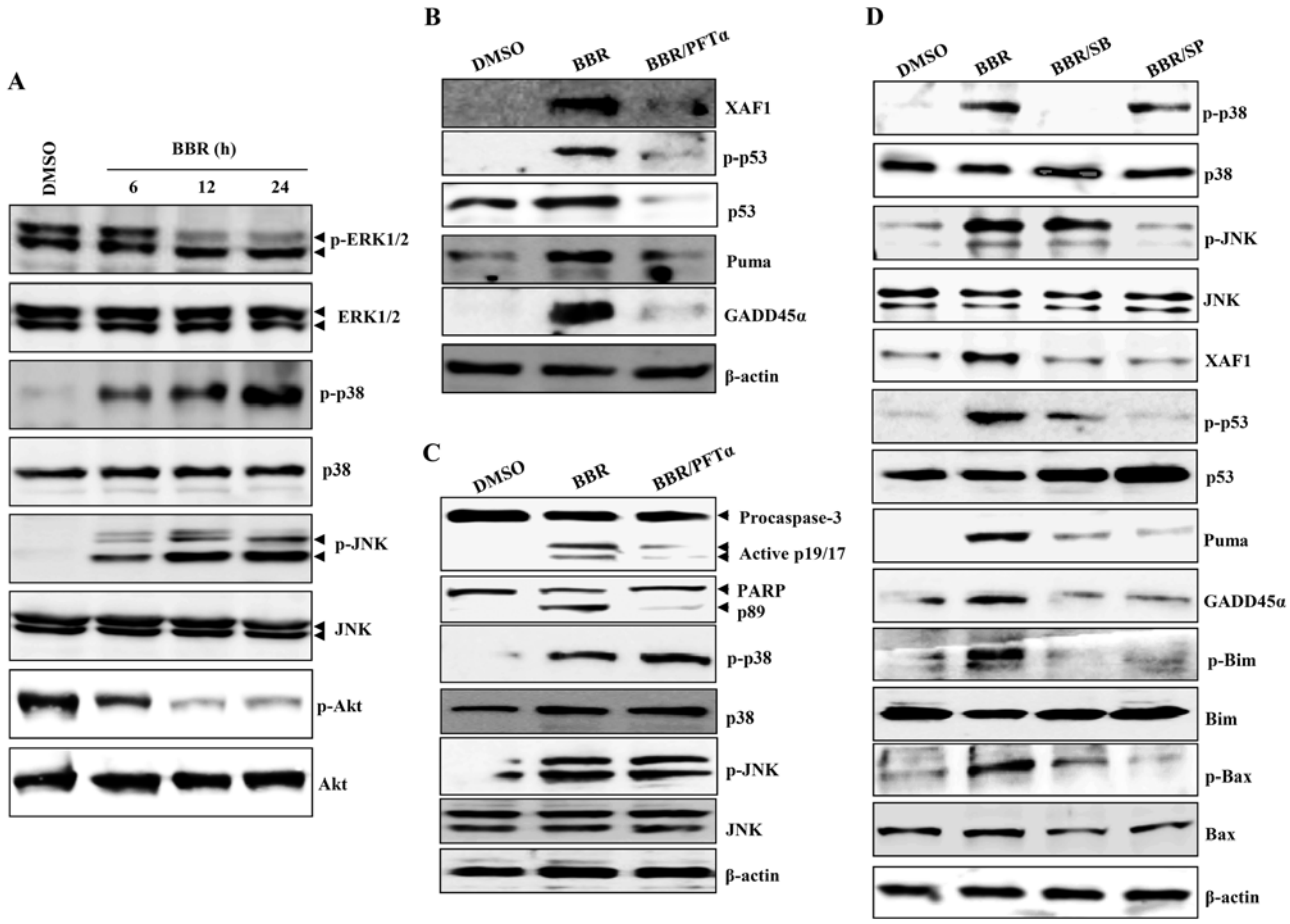

Figure 4. p38-MAPK and JNK-mediated p53 activation regulates XAF1-mediated mitochondrial apoptosis signaling. (A) EBV-transformed B cells were treated with 50 μM berberine for the indicated times prior to cell lysis. Western blot analysis of phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, JNK, phospho-Akt, and Akt was performed. (B and C) EBV-transformed B cells were pre-incubated with PFTα (50 μM) for 1 h and then treated with 50 μM berberine for 24 h. Western blot analysis of the indicated proteins was performed. (D) To inhibit p38-MAPK or JNK phosphorylation, EBV-transformed B cells were pretreated with SB203580 (10 μM) or SP600125 (25 μM) for 2 h. Western blot analysis of the indicated proteins was performed. β-actin was used to normalize protein content. Results are representative of three independent experiments.

the mitochondrial fraction were increased in berberine-treated EBV-transformed B cells. Bax and Bim disappeared from the cytosol and were accumulated in mitochondria after treatment with berberine; furthermore pro-apoptotic protein, PUMA was detected in the mitochondria (Fig. 3B). To further characterize the relationship between p53 and XAF1 and/or GADD45α, we investigated the potential interactions of these proteins using immunoprecipitation and immunoblotting. After treatment of EBV-transformed B cells with berberine, XAF1 translocated from the nucleus and interacted with p53 and GADD45α in the cytosol in addition relocated XAF bound to PUMA, Bax, and Bim in mitochondria (Fig. 3C-F). These data suggest that re-localization of XAF1 from the nucleus to the cytoplasm might be one of the key processes in the induction of apoptosis through mitochondrial signaling in cancerous B cells after treatment with berberine.

p38/JNK-mediated p53 activation regulates XAF1-mediated mitochondrial apoptosis signaling. Death receptors and the mitogen-activated protein kinase (MAPK) pathway promote apoptosis in HeLa cells after treatment with 300 μM berberine (20,21). GADD family proteins are known to be upstream activators of p38/JNK MAPK (15,22). Next, we examined whether berberine-induced apoptosis involves activation of these signaling pathways. Levels of the phosphorylated forms of JNK and p38 increased in a time-dependent manner after treatment of EBV-transformed B cells and cancerous B cells with berberine, whereas expression of the phosphorylated forms of ERK1/2 and Akt decreased (Figs. 4A and 6A). PFT-α inhibits p53 apoptotic signaling by interfering with its nuclear translocation and the transcriptional activation of p53 inducible genes (23,24). PFT-α blocked the berberine-induced upregulation of phosphorylated p53, PUMA, GADD45α, and XAF1 in EBV-transformed B cells (Fig. 4B). PFT-α also abrogated the cleavage and activation of caspase-3 and -9, but did not prevent the phosphorylation of JNK and p38-MAPK (Fig. 4C). To investigate the role of p38/JNK MAPK in p53-mediated apoptosis after treatment with berberine, EBV-transformed B cells were pretreated with SB203580 (p38-MAPK inhibitor) or SP600125 (JNK MAPK inhibitor) 2 h before treatment with berberine. These inhibitors effectively prevented the upregulation of phosphorylated p53, XAF1, GADD45α, and proapoptotic Bcl-1 family proteins including PUMA, phosphorylated Bim, and phosphorylated Bax (Fig. 4D). These data

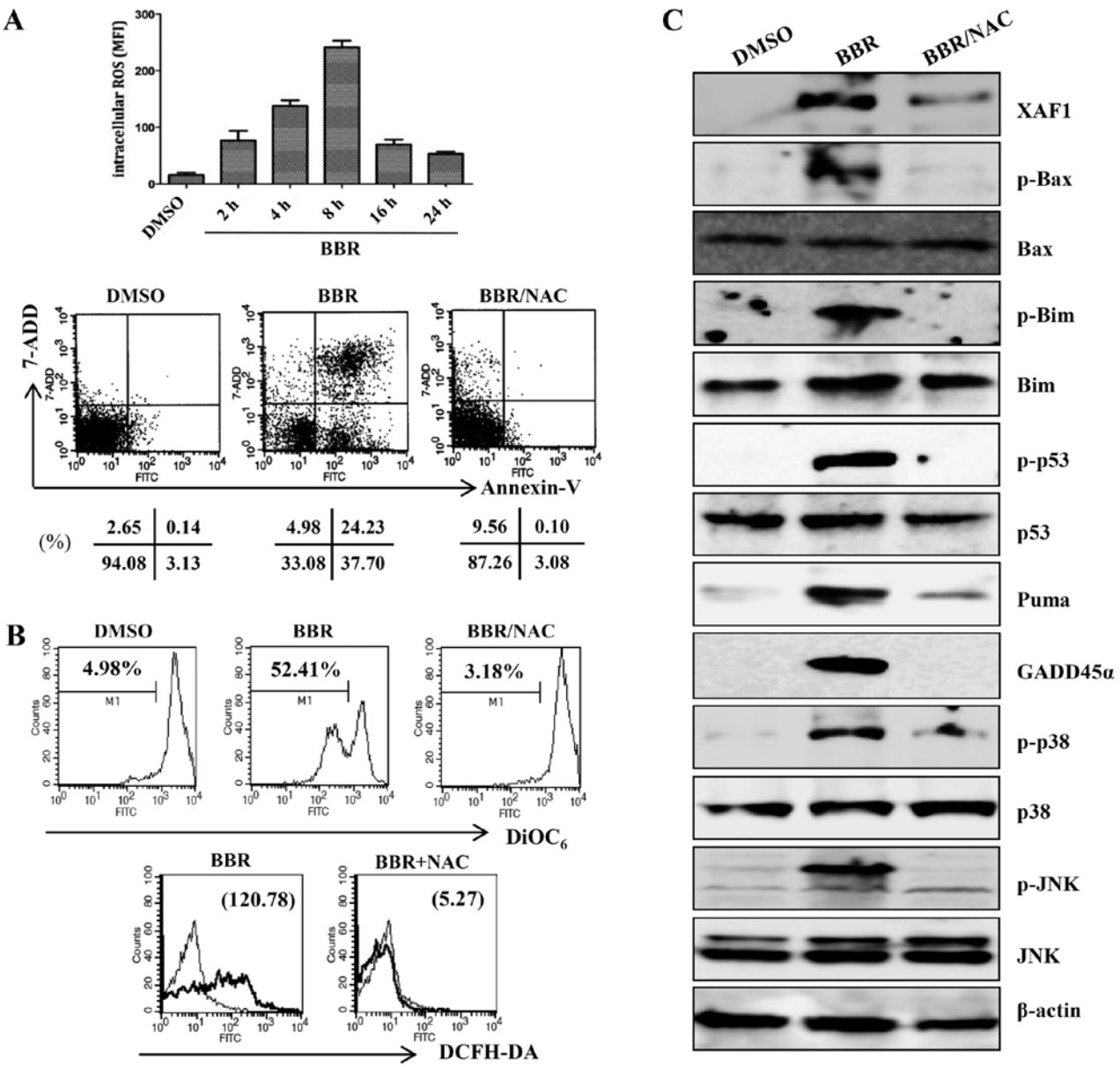

Figure 5. ROS production is an initial step in berberine-induced mitochondrial apoptosis signaling via the MAPK/p53/XAF1 pathway. (A) EBV-transformed B cells were pretreated with 10 μM DCFH-DA for 30 min and then treated with 50 μM berberine for the indicated times. Results are presented as the mean fluorescence intensity (MFI) of DCF. The bar chart represents statistical analysis of the rate of intracellular ROS production. Each value is expressed as the mean ± SD of three independent determinations. (B) EBV-transformed B cells ($2.5x10^5$ cells/ml) were treated with 50 μM berberine for 24 h. To detect the degree of apoptosis, cells were stained with Annexin V-FITC and 7-AAD and analyzed with flow cytometry. The percentage of apoptotic cells is determined by the percentage of Annexin $V^+/7\text{-}AAD^+$ cells. To measure disruption of $\Delta\psi m$, the cells were stained with $DiOC_6$. Diminished $DiOC_6$ fluorescence (%) indicates $\Delta\Psi_m$ disruption. Each value was expressed as the mean ± SD of three determinations. To measure ROS production, cells were pretreated with 10 μM DCFH-DA for 30 min and then treated with 50 μM berberine for the indicated time periods. Cells were analyzed with flow cytometry. Numbers in the DCF histograms show the mean fluorescence intensity (MFI). Thin-line and thick-line histograms represent the ROS levels of DMSO-treated control cells and berberine- or NAC/berberine-treated cells, respectively. (C) Effect of ROS inhibitor on berberine-induced JNK/p38-MAPK phosphorylation and p53, Bax, Bim, Puma, and XAF1 expression. To inhibit ROS production, EBV-transformed B cells were pretreated with NAC (10 mM) for 2 h and western blot analysis of the indicated proteins was performed. β-actin was used to normalize protein content. Results are representative of three independent experiments.

suggest that functional p53 is not only required for berberine-induced apoptosis through regulation of XAF1-GADD45α, but is also regulated by the p38/JNK MAPK signaling pathway.

Generation of ROS is an initial step in berberine-induced mitochondrial apoptosis signaling via the MAPK/p53/XAF1 pathway. ROS are associated with the activation of MAPKs and caspases in apoptosis (25). To investigate whether ROS were involved in the MAPK/p53/XAF1-mediated apoptosis induced by treatment with berberine, EBV-transformed B cells were treated with berberine for the indicated times and the intracellular ROS level was measured by addition of DCFH-DA. The ROS level was significantly higher in the berberine-treated EBV-transformed B cells than in the control group at 2 h after treatment and peaked at 8 h after treatment, after which ROS levels were downregulated. N-acetylcysteine (NAC), a ROS quencher, effectively blocked berberine-induced apoptosis (Fig. 5A). NAC also suppressed ROS production by

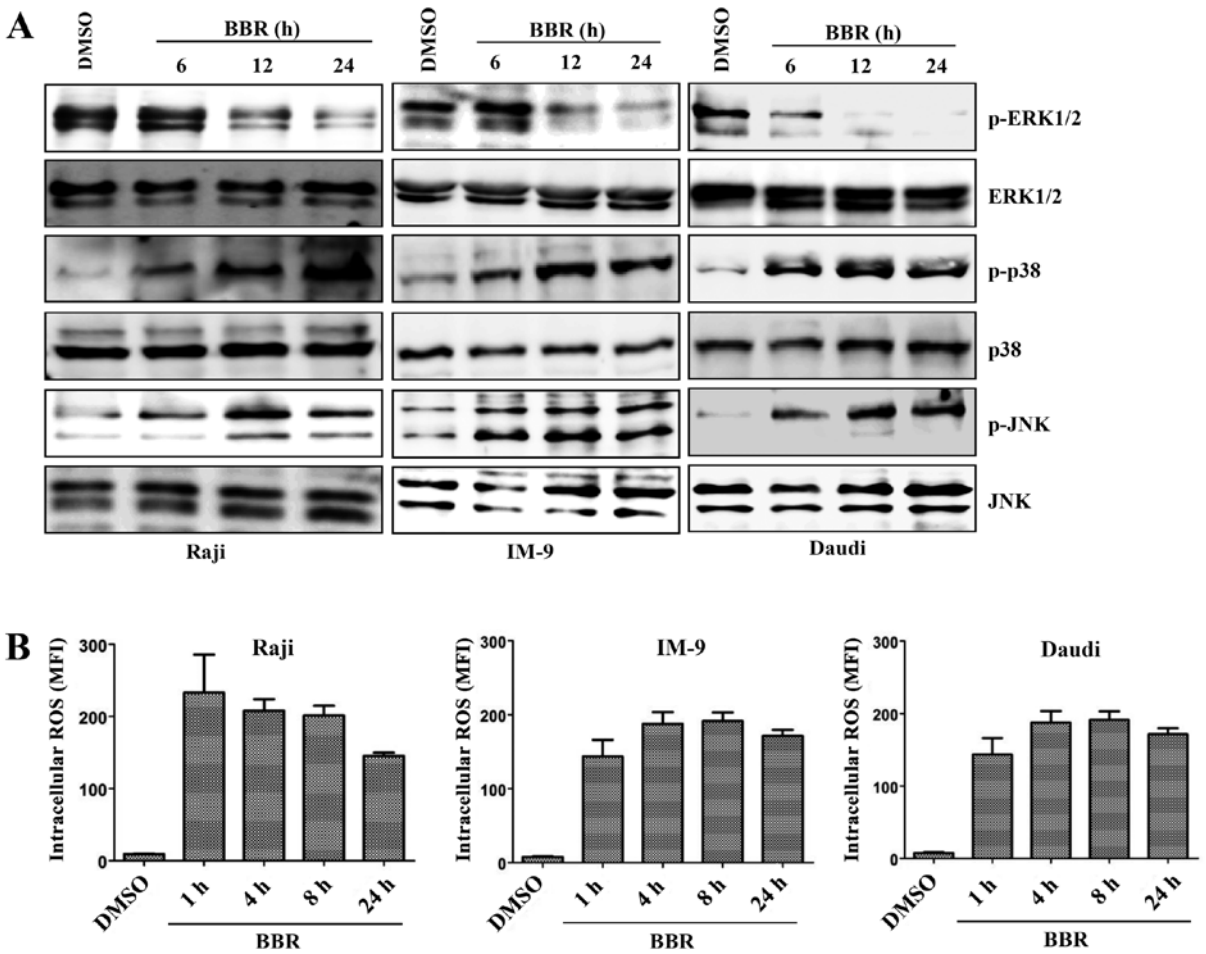

Figure 6. Berberine induces p38-MAPK/JNK phosphorylation and triggers rapid generation of ROS. (A) Raji, IM-9, and Daudi cells were treated with 50 μM berberine for the indicated times prior to cell lysis. Western blot analysis of phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, and JNK was performed. β-actin was used to normalize protein content. (B) Raji, IM-9, and Daudi cells were pretreated with 10 μM DCFH-DA for 30 min and then treated with 50 μM berberine for the indicated times. Cells were analyzed using flow cytometry. Results are presented as the mean fluorescence intensity (MFI) of DCF. A bar chart representing the statistical analysis of rate of intracellular ROS production based on three independent experiments. Each value is expressed as the mean ± SD of three determinations.

berberine and the berberine-induced mitochondria disruption (Fig. 5B). Pre-incubation with NAC almost completely blocked the activation of p38/JNK MAPK and the change in mitochondria-related apoptotic proteins (Bax, Bim, and PUMA). The phosphorylation of p53 and expression levels of its downstream targets (XAF1, GADD45α) were restored to control levels after preincubation of EBV-transformed B cells with NAC (Fig. 5C). ROS level was also significantly higher in the berberine-treated EBV-positive cancerous B cells (Raji, IM-9, Daudi cells) than in the DMSO-treated control cells at even 1 h after treatment and was maintained up at 24 h after treatment (Fig. 6B). These data suggest that ROS generation might be one of the predisposing events promoting mitochondria-mediated apoptosis through MAPK/p53 signaling after treatment of EBV-transformed B cells with berberine.

Discussion

Berberine has various pharmacological activities against infection and inflammation, and even antiarrhythmic activities (26). Although berberine has been reported to inhibit the cell growth and induce apoptosis of various cancer cells (3,27,28), there is insufficient evidence for its anticancer effects, especially in lymphoma. Berberine induces apoptosis in human promonocytic U937 cells through a mitochondrial/caspase pathway (27). Our results suggest that activation of the MAPK-p53 signaling pathway by intracellular ROS might be the key event inducing berberine-mediated apoptosis in EBV-transformed B cells through disruption of the mitochondrial potential. Moreover, berberine-induced apoptosis involves activation of the p53 target proteins XAF1 and GADD45α (Fig. 7).

p53 mutations are the most common genetic changes found in human cancers (29). Mutations of p53 often contribute to aggressive tumor behavior and therapeutic resistance (30,31). Some studies have shown that the expression of wild-type p53 is necessary for the cytotoxic response to chemotherapeutic agents (31,32); however, others have reported opposite results (33,34). Berberine induces apoptosis in human epidermoid carcinoma cells associated with increased expression of Bax, reduced expression of Bcl-2 and Bcl-xL, and disruption of the mitochondrial membrane potential (3). Transcriptional activation of proapoptotic Bax and Bak by functional p53 enhances the permeability of the mitochondrial membrane, which in turn results in repression of antiapoptotic members of this

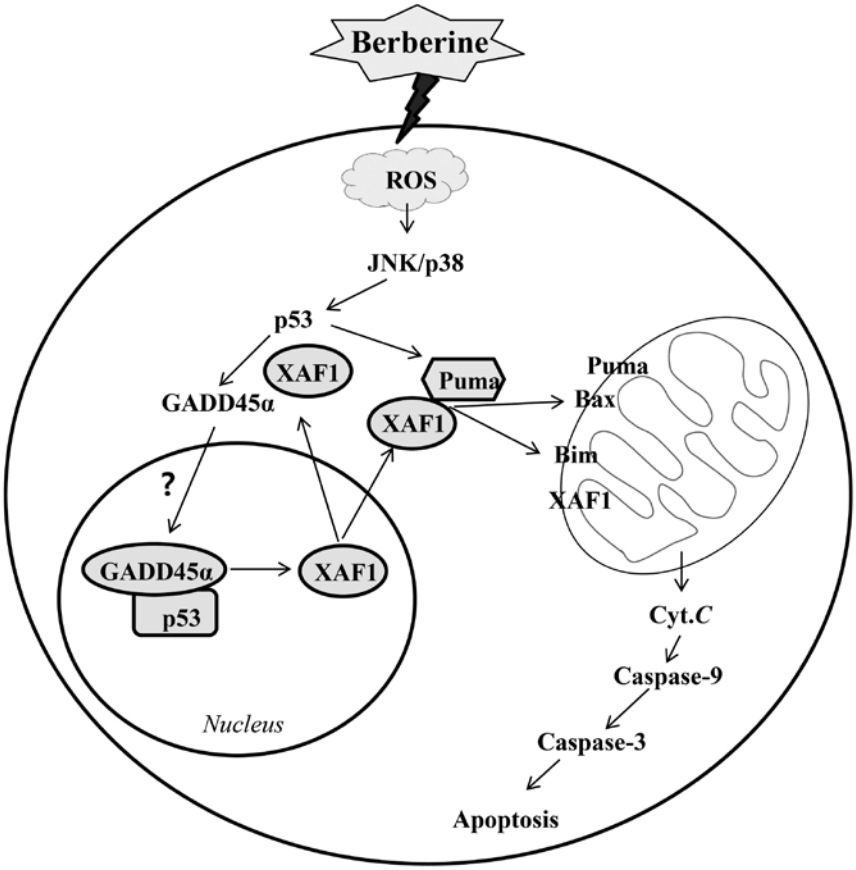

Figure 7. Schematic diagram of the intracellular signaling mechanism during berberine-induced apoptosis in EBV+ B lymphoma cells. Berberine treatment increases intracellular ROS production and leads to activation of JNK and p38-MAPK, resulting in progressive upregulation of p53. p53 then translocates GADD45α into the nucleus and XAF1 into the cytosol, and upregulates Puma. XAF1 and Puma mediate translocation of Bax and Bim into the mitochondria. Ultimately, berberine induces $\Delta\Psi_m$ disruption and triggers caspase-dependent apoptosis.

family including Bcl-2 and Bcl-x (35,36). These findings are consistent with the results of our study. We first showed that the activity of proapoptotic Bcl-2 family members, including Bax, Bim, and PUMA, was regulated by interaction with the XAF1-GADD45α complex during berberine-induced apoptosis of EBV-transformed B cells. We also observed that XAF1 co-existed with Bax, Bim, and PUMA proteins in the mitochondrial membrane fraction by co-immunoprecipitation analyses.

XIAPs belong to an important family of antiapoptotic proteins involved in resistance to cancer therapy (37). XIAPs can bind and inactivate key caspases involved in the initiation (caspase-9) and execution (caspase-3 and -7) of the apoptosis cascade (38,39). Thus, XIAPs represent critical regulatory factors in mitochondrial apoptosis signaling. XAF1 was first defined as a XIAP interacting protein that antagonized the effect of XIAP in inhibiting caspase activation (9). Low expression of XAF1 correlates with promoter hypermethylation and is strongly associated with tumor stage (40,41). Restoration of XAF1 expression induces cancer cell apoptosis and inhibits tumor growth in multiple types of cancers, including gastric, colon, liver, pancreatic, and prostate cancers (11,42,43). Byun *et al* first reported that 7 of 9 (78%) primary tumors with reduced XAF1 expression carried wild-type p53 and 13 of 15 (87%) primary tumors with mutant p53 showed normal expression of XAF1 (12). Once activated, p53 translocates and accumulates in the nucleus, where it can either cause cell cycle arrest or induce cell apoptosis (44). Berberine was shown to induce p53-independent, XIAP-mediated apoptotic cell death in p53-null leukemia cells (45). However, the functions of XAF1 as a tumor suppressor in lymphoma are not fully understood. XAF1 can activate p53-mediated apoptosis in cancer cells through post-translational modification (7) although the exact mechanism of the interaction between XAF1 and functional p53 in the control of apoptosis is still unclear. XAF1 may enhance the protein stability of phosphorylated p53, thus leading to prolonged activation of p53 and expression of its target gene p21 under stress condition (41). p53 provokes cell cycle arrest in G0/G1 and G2/M phases by upregulating p21 and downregulating cyclin B1 (46). Adeno-XAF1 transduction similarly induces cell cycle G2/M arrest and upregulates the expression of p21 and downregulates the expression of cyclin B1 and cdc2 (42). Exposure to 5-aza-cytidine induces the upregulation of p53 and its downstream effectors $p21^{WAF1}$ and GADD45 (47).

p53-dependent and -independent mechanisms have been reported to play a role in the regulation of GADD45α expression (48). However, the expression of GADD45α for induction of apoptosis did not change after translocation of XAF1 into the cytosol following treatment with berberine. Using immunoprecipitation and immunoblotting analysis we showed that XAF1 formed a complex with p53 and GADD45α. Although direct interaction between GADD45α and p53 or XAF1 was detected in the present study, we still need to investigate how the

p53-XAF1-GADD45α complex is formed in the cytosol or is translocated into nucleus to determine its exact role in apoptosis.

In this study, co-incubation with the selective p53 inhibitor PFT-α not only blocked phosphorylation of p53, but also restored expression of XAF1, GADD45, and Puma, and cleavage of caspase-3 and PARP to control levels. This result is consistent with previous reports that PFT-α blocks apoptosome-mediated processing and activation of caspase-9 and -3 and inhibits the induction of p53, p21, and Bax (49,50). GADD45α induces apoptosis through induction of the G2/M phase arrest in response to nerve growth factor stimulation independent of the p38 and JNK pathways (51,52), although GADD45α also plays some role in the regulation of p53 through p38/JNK (16,17). ROS act as an early signal activator to provoke apoptosis in vinca alkaloid-treated lung adenocarcinoma cells (53). In our previous report, we proposed that ROS generation might be the initiating event in the translocation of XAF1 and phosphorylation of ERK1/2 in dexamethasone-induced apoptosis of EBV-transformed B cells (54). Likewise, NAC, p38 inhibitor, and JNK inhibitors significantly inhibited berberine-induced apoptosis by restoration of proapoptotic proteins, including PUMA, phosphorylated Bax, and phosphorylated Bim to control levels and completely blocked the upregulation of XAF1, GADD45α, and phosphorylation of p53.

Based on our findings, the interaction of XAF1 with functional p53 appears to have novel and important clinical potential in chemotherapy against cancers originating from B cells. Although the exact mechanism underlying the regulation of p53 by XAF1 is still unclear, our data suggest that the role of XAF1 as a promoter of the mitochondrial apoptosis pathway might provide a novel target for cancer therapy, particularly for cancers with expression of wild-type p53. The induction of XAF1 translocation and GADD45α activation by phosphorylated p53 can be considered a new target pathway, as exemplified by berberine-induced mitochondrial apoptosis signaling in EBV-transformed B cells and cancerous B cells.

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