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Molecules and Cells

Betulinic Acid Induces Bax/Bak-Independent Cytochrome c Release in Human Nasopharyngeal Carcinoma Cells

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Betulinic acid (BetA) is an effective and potential anti-cancer chemical derived from plants. BetA can kill a broad range of tumor cell lines, but has no effect on untransformed cells. The chemical also kills melanoma, leukemia, lung, colon, breast, prostate and ovarian cancer cells via induction of apoptosis, which depends on caspase activation. However, no reports are yet available about the effects of BetA on nasopharyngeal carcinoma (NPC), a widely spread malignancy in the world, especially in East Asia. In this study, we first showed that BetA can effectively kill CNE2 cells, a cell line derived from NPC. BetA-induced CNE2 apoptosis was characterized by typical apoptosis hallmarks: caspase activation, DNA fragmentation, and cytochrome c release. Overexpression of Bcl-2 and Bcl-xL could partially prevent apoptosis caused by BetA. Moreover, Bax was not activated during the induction of apoptosis. Bax/Bak knockdown and wild-type CNE2 cells showed the same kinetics of cytochrome c release. We then showed that BetA may impair mitochondrial permeability transition pores (mPTPs), which may partially contribute to cytochrome c release. These observations suggest that BetA may serve as a potent and effective anti-cancer agent in NPC treatment. Further exploration of the mechanism of action of BetA could yield novel breakthroughs in anti-cancer drug discovery.

INTRODUCTION

Cancer, including nasopharyngeal carcinoma (NPC), has become increasingly threatening to the human population (Ellis and Reardon, 2009). NPC, a common malignancy in the world, is especially popular in East Asia (Li et al., 2010). It is derived from epithelial cells and has no specific treatment. At present, the most commonly used treatment strategies for NPC are radiotherapy and chemotherapy (Ali and al-Sarraf, 2000). Betulinic acid (BetA) is a plant-derived pentacyclic triterpenoid that is toxic to cancer cells but has no effect on untransformed cells (Pisha et al., 1995; Zuco et al., 2002). BetA was first found to kill melanoma cells, after which it was reported to kill many other cancer cell lines, such as leukemia, lung, colon, breast, prostate, and ovarian cancer (Chintharlapalli et al., 2007; Ehrhardt et al., 2004; Fulda et al., 1999a; 1999b; Jung et al., 2007; Kessler et al., 2007; Wu et al., 2010). However, no report about the effect of BetA on NPC is available. Our interest in this work is the use of BetA as a treatment strategy for NPC, given that BetA seems to be a powerful drug that could potentially offer a cure for NPC (Eiznhamer and Xu, 2004).

BetA kills cancer cells *via* induction of apoptosis (Pisha et al., 1995). Apoptosis is a significant event in body development and cell-mediated tumor clearance (Meier et al., 2000). Two pathways have been found to execute apoptosis: extrinsic and intrinsic pathways (Fulda and Debatin, 2006). In the intrinsic pathway, mitochondria are suspected to play an important role in BetA-caused apoptosis, a process in which the extrinsic pathway is not involved (Fulda et al., 1997). The mitochondrial pathway is normally dependent on caspase cascade and can be inhibited by Bcl-2/Bcl-xL overexpression (Kroemer et al., 2007; Roberts et al., 2003). An important event that occurs via the intrinsic pathway is cytochrome c (cyt c) release from mitochondria to the cytosol (Liu et al., 1996), hence the term "mitochondrial pathway." Once apoptosis is initiated, cyt c is released from the mitochondria into the cytosol, binds to caspase-9, forms an apoptosome, causes caspase cascade, and finally induces cell apoptosis. During this process, anti-apoptotic members of the Bcl-2 family of proteins, such as Bcl-2, Bcl-xL, and Mcl-1, can inhibit cyt c release or apoptosis, whereas pro-apoptotic members such as Bax, Bak, and Bid, are often activated (Brooks et al., 2007; Strasser, 2005). These proteins are often highly expressed in tumors and are associated with tumor resistance to death-inducing stimuli.

In this study, we found that BetA could effectively kill CNE2 cells, a commonly used NPC cell line. We also found that BetA-induced CNE2 cell death is caspase-dependent and exhibits caspase activation, DNA ladder formation, and Bcl-2/xL inhibition. We detected cyt c release during BetA induction to further investigate the mechanism of BetA-induced CNE2 apoptosis and found that cyt c is released in a Bax/Bak-independent manner. Moreover, the mPTP inhibitors cyclosporine A (CsA) and bongkrekic acid (BKA) could partially prevent BetA-induced CNE2 apoptosis and cyt c release. mPTPs may contribute to BetA-induced CNE2 apoptosis, causing cyt c release.

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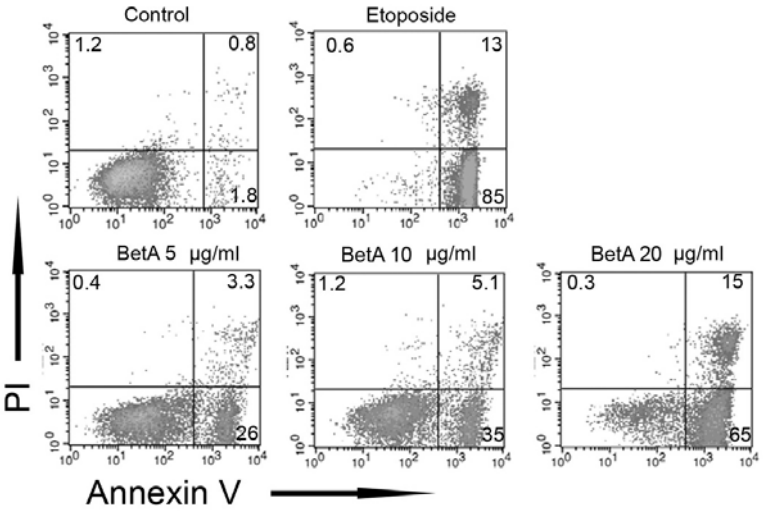

Fig. 1. BetA induces apoptosis in CNE2 cells. CNE2 cells were treated with indicated BetA concentrations. After 48 h of treatment, CNE2 cells were collected and subjected to AV/PI staining and FACS analysis. BetA induced CNE2 cell death in a dose-dependent manner. Etoposide was used as a positive control to confirm the validity of our system.

MATERIALS AND METHODS

Reagents, antibody, and cells

Human NPC CNE2 cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/mL streptomycin. Caspase fluorogenic substrates Ac-DEVD-AFC and Ac-LEHD-AFC were purchased from Calbiochem. Alexa Fluor 488 conjugated donkey anti-mouse IgG (Molecular Probes) and HRP-conjugated secondary antibodies (Santa Cruz) were also used. Other antibodies used were mouse mAb against β-actin (Sigma-Aldrich), cyt c (BD Pharmingen), activated Bax (MAb 6A7, BD Pharmingen), rabbit anti-COX IV (Abcam), anti-caspase 3 (Cell Signaling Technology, Inc.).

Western blot

CNE2 cells or isolated cell fractions were resuspended in PBS with loading buffer. A volume of 18 μl total protein was loaded per lane for SDS-PAGE and blot analysis. Blocking was performed with 5% low fat milk powder at 37°C for 1 h. The primary antibody was incubated with blots overnight at 4°C, and the secondary antibody was incubated at 37°C for 1 h.

DNA fragmentation

CNE2 cells (5×10^5) were treated with different doses of BetA as indicated. Genomic DNA was extracted by DNA extraction buffer (100 mM Tris-Cl, pH 8.0, 5 mM EDTA, 0.2 M NaCl, 0.5% SDS, and 0.2 μg/μl proteinase K) for 2 h at 52°C. DNA was precipitated according to standard ethanol precipitation procedures, resuspended in Tris buffer, and visualized by 2% agarose gel electrophoresis.

Cell death analysis

CNE2 cells were treated, harvested, and stained with Annexin V (PV) and propidium iodide (PI), after which they were analyzed using a FACS Calibur flow cytometer (BD Biosciences).

Cytochrome c release

Cyt c release from the mitochondria into the cytosol was detected by Western blot. CNE2 cells (2×10^5) were treated with BetA at indicated concentrations. Cells were harvested and resuspended in 15 μl digitonin lysis buffer for 5 min (Shi et al., 2009). Supernatants and pellets were collected, loaded onto a 12% SDS-PAGE system, and then probed with cyt c or COX IV antibodies. Quantitative measurement of cyt c release was detected using an InnoCyte Flow Cytometric Cytochrome c Release Kit (Calbiochem, Merk Biosciences).

RNA interference knockdown

Specific shRNA sequences that efficiently knocked down the Bax or Bak expression were applied in this study. The target sequence for Bax was 5′-GGUGCCGGAACUGAUCAGA-3′; that for Bak was 5′-GGAUUCAGCUAUUCUGGAA-3′ (Kim et al., 2008; Zhang and Armstrong, 2007). These sequences were synthesized by Invitrogen and were cloned into pSUPER vector. Recombinant vectors were transfected into CNE2 cells using Lipofectamine 2000 (Invitrogen), and stable cell lines were screened with G418.

Over-expression of Bcl-2 and Bcl-xL

Bcl-2 and Bcl-xL coding sequences were cloned into pcDNA3.1 vector and transfected into CNE2 cells. Stable cell lines were selected with G418.

Laser scanning microscopy

CNE2 cells were treated with BetA, harvested, and fixed with 4% paraformaldehyde for 30 min at room temperature, after which they were permeabilized with 0.2% Triton X-100 for 10 min and incubated at room temperature for 1 h with anti-Bax antibody. Images were visualized by laser scanning confocal microscopy.

Caspase activity assay

CNE2 cells (2×10^5) were treated as indicated, washed, and lysed with 50 μl of lysis buffer. Cell lysates were harvested and incubated with 30 μM of caspase substrates at 37°C. Fluorescence was measured using a fluorescence spectrophotometer.

Cleavage assay

CNE2 cells were treated with BetA, harvested, lysed with 0.5% NP-40 lysis buffer, and probed with anti-caspase-3 antibodies for Western blot analysis. Once activated, capase-3 can be cleaved into 17 kDa to 19 kDa bands.

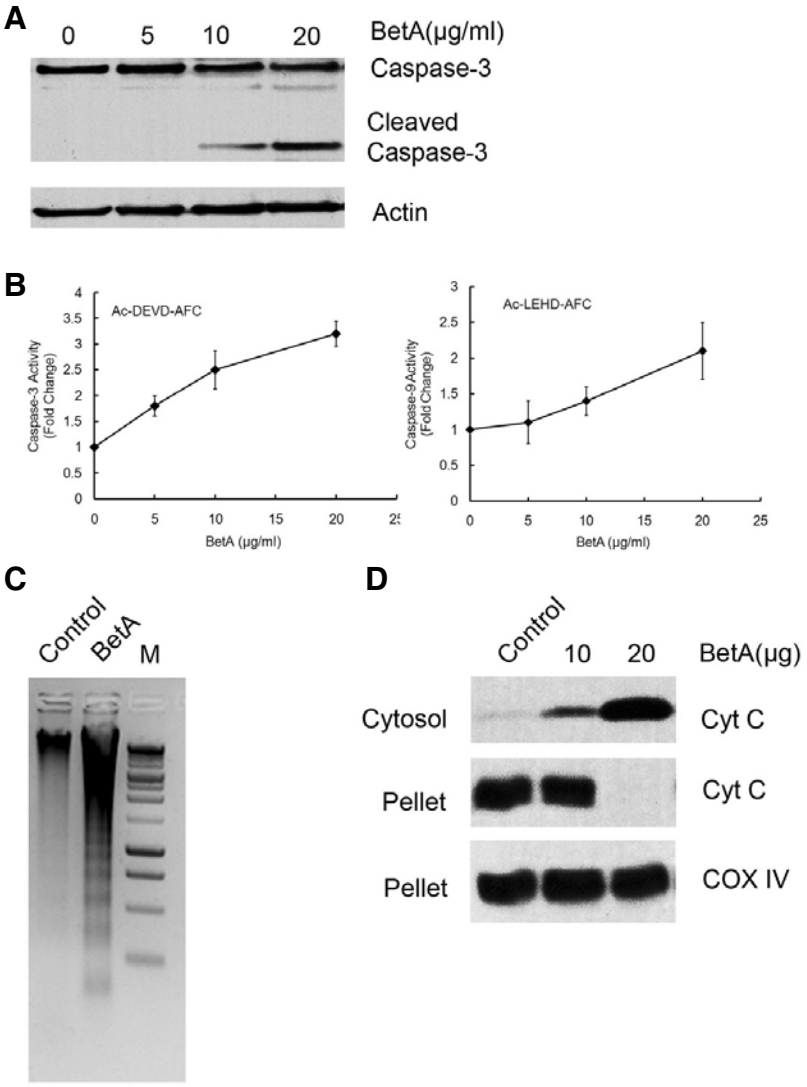

Fig. 2. BetA-induced CNE2 cell apoptosis depends on caspase pathway. (A) CNE2 cells were treated at indicated BetA concentrations. After 24 h of treatment, CNE2 cells were subjected to Western blot analysis to determine caspase-3 activation (upper panel). The cleaved caspase-3 indicates caspase-3 activation. Actin was probed as a loading control (lower panel). (B) BetA induced caspase-3/9 activation in CNE2 cells. CNE2 cells were treated as in (A) and collected for caspase-3/9 activity analysis. Ac-DEVD-AFC is a specific fluorescent substrate for caspase-3 and Ac-LEHD-AFC is a specific fluorescent substrate for caspase-9. Data were calculated from three experiments and are shown as mean ± SD. (C) BetA induced DNA ladder formation in CNE2 cells. CNE2 cells were treated with 20 μg/ml of BetA for 48 h. Cells were then collected and subjected to DNA purification and electrophoresis in 2% agarose gel. M, DNA Marker. (D) BetA induced cyt c release in a dose-dependent manner. CNE2 cells were treated at indicated BetA concentrations and analyzed for cyt c release. The same pellet blot was stripped and probed with anti-COX IV antibodies as a loading control. Pellet, mitochondrial fraction; Cyt c, cytochrome c.

RESULTS

BetA-induced CNE2 cell death

A previous study showed that BetA can specifically kill melanoma cells. Later reports indicated that many other cancer cell lines can be killed by BetA, such as leukemia cell line K562 and Jurkat cells. However, whether or not BetA can kill NPC has yet to be reported. A typical NPC cell line, CNE2, was used in our system to determine the effect of BetA on NPC. CNE2 cells were incubated with 5, 10, or 20 μg/ml BetA for 48 h and observed by microscopy and typhoon blue staining. CNE2 cells exhibited obvious cell death, and could be stained by typhoon blue (data not shown).

Phosphatidylserine (PS) externalization is a significant hallmark of apoptosis (Kerr et al., 1972). CNE2 cells were treated, stained with AV and PI, and analyzed by FACS to confirm further death induced by BetA. At varying concentrations of 5, 10, and 20 μg/ml, BetA was able to induce 29.7, 41.3, or 80.3% CNE2 cell death (AV/PI double-positive), respectively (Fig. 1). Etoposide-treated cells were used as a typical apoptosis control. These results point out that BetA can efficiently kill the NPC cell line CNE2. Interestingly, death was characterized by AV single-positive cells (87.5, 84.7, and 80.9%, respectively). This observation suggests that the BetA-induced CNE2 cell death is apoptotic in nature.

BetA-induced CNE2 cell death is caspase-dependent

Caspases are often activated during apoptosis (Miura et al., 1993). We detected whether or not caspase was activated after BetA treatment to confirm the type of BetA-induced CNE2 cell death. Caspase-3 was cleaved into its 19 kDa activated form in a dose-dependent manner (Fig. 2A). Actin was used as a loading control. We also measured the activities of caspases in BetA-treated CNE2 cells with Ac-DEVD-AFC (caspase-3) and Ac-LEHD-AFC (caspase-9) to verify caspase activation. BetA was able to activate caspase at very low concentrations in a dose-dependent manner (Fig. 2B).

Genomic DNA is degraded into 200 bp fragments during typical apoptosis, which is called DNA fragmentation or DNA ladder (Enari et al., 1998). DNA fragmentation analysis was conducted to detect whether or not BetA causes the formation of a DNA ladder. Genomic DNA was extracted from treated CNE2 cells and run on agarose gel. Clearly, BetA caused the formation of a DNA ladder (Fig. 2C).

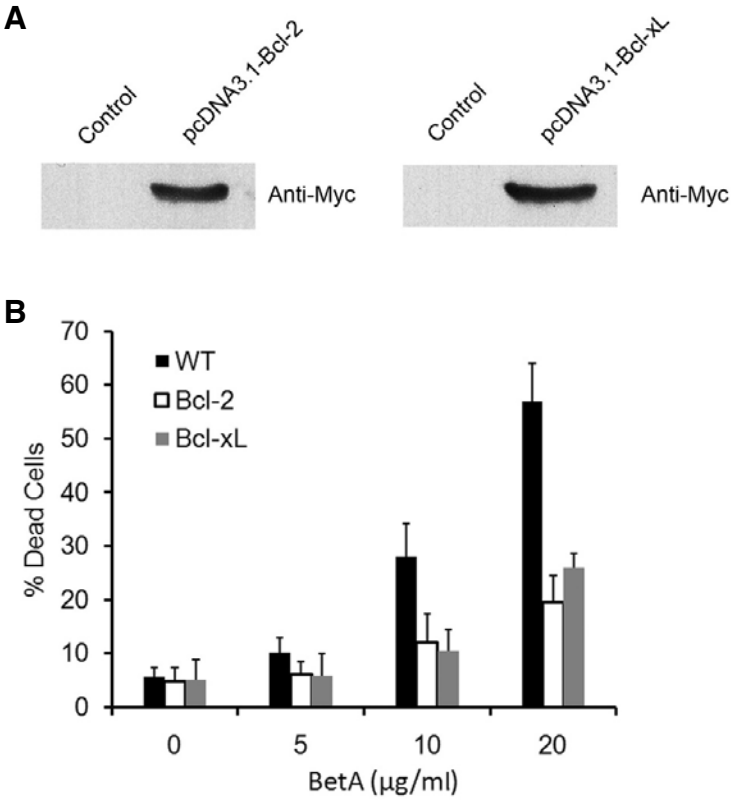

Fig. 3. Bcl-2 and Bcl-xL overexpression prevents BetA-induced apoptosis and cytochrome c release. (A) CNE2 cells were transfected with pcDNA3.1-Bcl-2 or pcDNA3.1-Bcl-xL plasmids, and stable cell lines were screened with G418. Bcl-2 (left panel) and Bcl-xL (right panel). Overexpression of the stable CNE2 cell line was confirmed with anti-myc antibodies. (B) Bcl-2 and Bcl-xL overexpression prevented BetA induced-apoptosis. Different CNE2 cells were treated with indicated BetA concentrations, stained with AV/PI, and then subjected to FACS analysis. Dead cells were calculated by plus PI positive, AV positive and AV/PI double-positive. Data were calculated from three experiments and are shown as mean ± SD.

Cyt c release is a critical step of apoptosis. Once stimulated, cyt c is released from the mitochondria, binds with capsase-9, and forms an apoptosome, leading to caspase activation. Blocking cyt c release causes apoptosis failure (Kim et al., 2005). We assessed whether or not BetA induces the cyt c release from CNE2 cells and found that, upon BetA stimulation, cyt c is released from the pellet (mitochondrial fraction) into the cytosol (Fig. 2D). Here, COX IV was used as a loading control. These data indicate that BetA-induced CNE2 cell death is caspase-dependent and exhibits a typical apoptosis.

Bcl-2 and Bcl-xL prevents BetA-induced CNE2 apoptosis

The mitochondrial pathway of apoptosis is usually regulated by proteins of Bcl-2 family members, such as Bcl-2, Bcl-xL, or Mcl-1. Bcl-2 and Bcl-xL are anti-apoptotic proteins and can efficiently suppress apoptosis (Schwartz and Hockenbery, 2006; Strasser, 2005). We overexpressed Bcl-2 and Bcl-xL in CNE2 cells and analyzed cell death induced by BetA to investigate the molecular mechanism of BetA-induced CNE2 apoptosis. Stable cell lines were selected with G418 and verified by Western blot (Fig. 3A). We then compared the three types of cell death and found that Bcl-2 or Bcl-xL overexpression can partially prevent BetA-induced CNE2 cell apoptosis (Fig. 3B).

Bax is not activated during BetA-induced CNE2 apoptosis

Cyt c release is involved in the Bax/Bak pathway. Upon apoptosis stimulation, Bax and Bak oligomerization occur, forming a pore on the mitochondrial membrane and leading to the release of cyt c (Goping et al., 1998). We used a special antibody that can specifically recognize activated Bax but not inactivated Bax to investigate whether or not BetA induces Bax and/or Bak activation. As shown in Fig. 4, BetA cannot activate Bax. Instead, etoposide, which was used as a positive control to confirm our system, can successfully activate Bax.

BetA-induced cytochrome c release is independent of Bax/Bak

We knocked down Bax and Bak expression in CNE2 cells to investigate the involvement of Bax/Bak in BetA-induced apoptosis. Bax was partially downregulated while Bak was almost completely knocked down in the cancer cells (Fig. 5A). We compared the cell apoptosis of wild-type (WT) CNE2 cells with that of Bax/Bak double-knockdown cells by AV/PI staining (Fig. 5B). At different concentrations of BetA (5, 10, and 20 μg/ml), Bax/Bak double-knockdown cells showed reduced numbers of dead cells compared with WT cells, although the difference was not significant. These results suggest that Bax/ Bak may be partially involved in BetA-induced CNE2 apoptosis but does not significantly contribute to apoptosis. We detected cyt c release using the Western blot to assess the role of Bax/Bak in apoptosis. We found that cyt c is still released in Bax/Bak knockdown CNE2 cells and that the release kinetics in such cells was nearly identical to that in WT cells (Fig. 5C). This finding indicates that BetA-induced cyt c release of CNE2 cells is Bax/Bak-independent. All the observations thus far suggest that Bax and Bak are partially involved in CNE2 apoptosis but do not contribute to cyt c release.

BetA-induced cytochrome c release is partially prevented by mPTP inhibitor

How cyt c is release during BetA-induced CNE2 apoptosis in a Bax/Bak independent manner remains unknown. We wanted to determine whether or not mPTPs are involved in BetA-induced Bax/Bak-independent cyt c release of CNE2 cells because PTP has been suggested to play a role in apoptotic cyt c release. We used CsA, an mPTP inhibitor, in our system. First, we as-

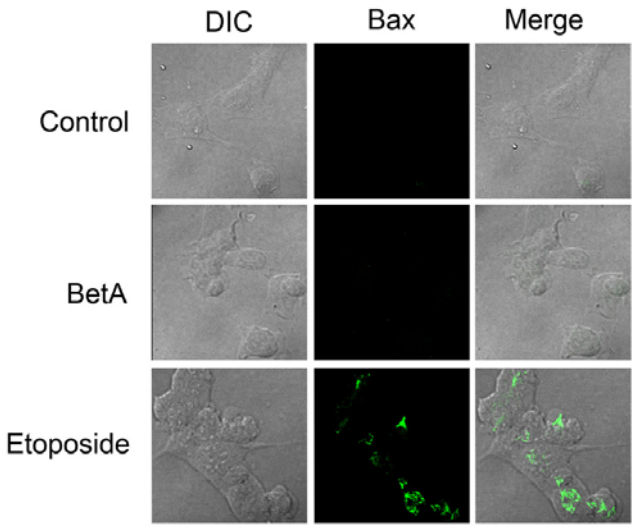

Fig. 4. Bax is not activated during BetA-induced CNE2 apoptosis. BetA-induced CNE2 apoptosis did not involve Bax activation. CNE2 cells were treated with 20 μg/ml of BetA for 48 h and then subjected to immunofluorescent staining. Cells were fixed, permeabilized, and stained with a specific anti-Bax antibody that can specifically recognize activated Bax. BetA cannot activate Bax (middle panel). Etoposide-induced Bax activation served as a positive control. DIC, differential interference contrast.

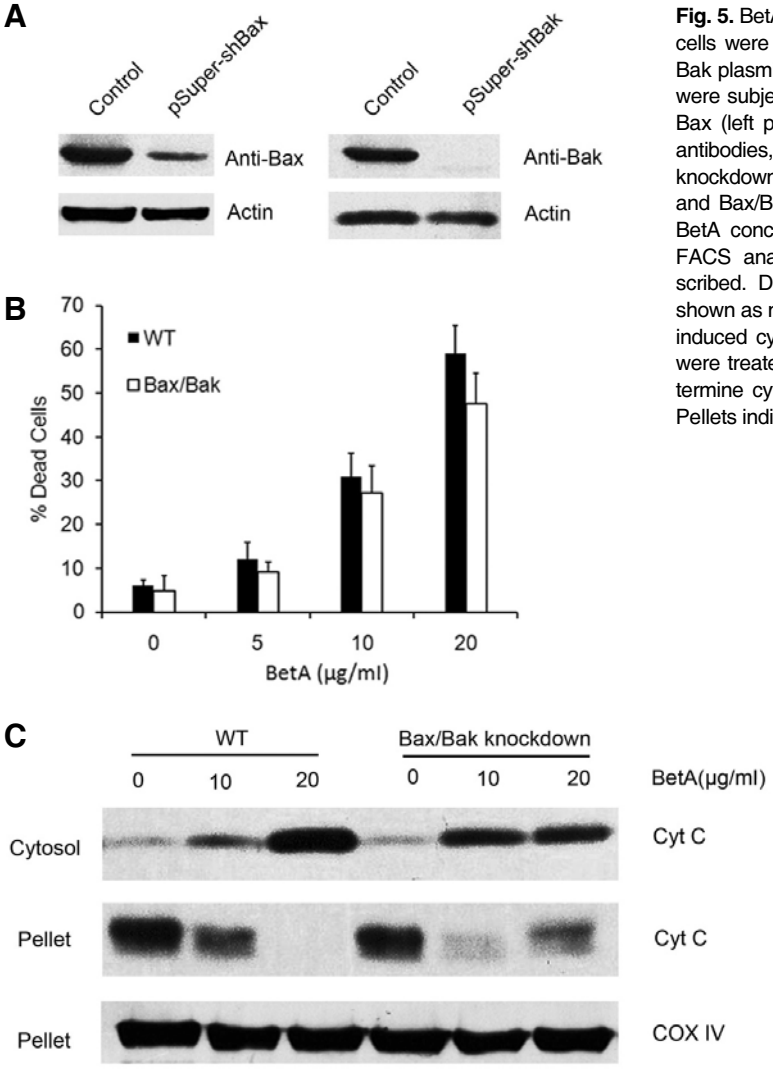

Fig. 5. BetA induces Bax/Bak-independent cyt c release. (A) CNE2 cells were transfected sequentially with pSuper-Bax and pSuper-Bak plasmid, and stable cell lines were screened with G418. Cells were subjected to Western blot analysis for knockdown efficiency. Bax (left panel) and Bak (right panel) were probed with specific antibodies, and actin served as the loading control. (B) Bax/Bak knockdown did not affect BetA-induced apoptosis. Wild-type (WT) and Bax/Bak knockdown CNE2 cells were treated with indicated BetA concentrations, stained with AV/PI, and then subjected to FACS analysis. Dead cells were calculated as previously described. Data were calculated from three experiments and are shown as mean ± SD. (C) Bax/Bak knockdown did not affect BetA-induced cyt c release. WT and Bax/Bak knockdown CNE2 cells were treated as (B) and subjected to Western blot analysis to determine cyt c release. COX IV was used as the loading control. Pellets indicate mitochondrial fractions.

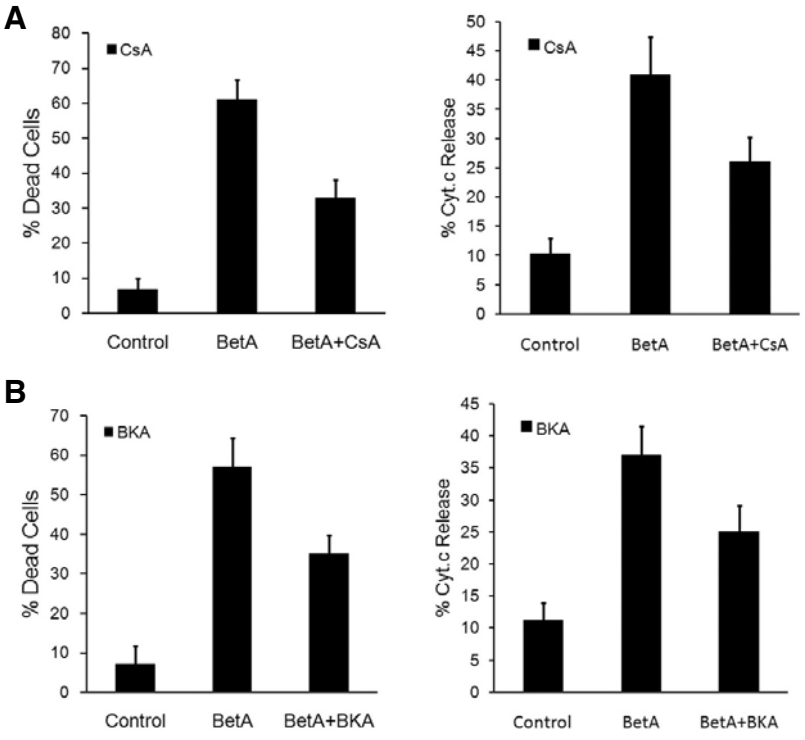

Fig. 6. mPTPs are involved in BetA-induced apoptosis and cytochrome c release. (A) CNE2 cells were pre-treated with or without 5 μg/ml of CsA for 1 h and then treated with 20 μg/ml BetA. After 24 h of treatment, cells were collected and subjected to FACS analysis (left panel) to determine the dead cell count. Cyt c release (right panel) was measured using the InnoCyte Flow Cytometric Cytochrome c Release Kit. The same treatment and analysis were applied in (B), except that CsA was replaced with BKA. Data were calculated from three experiments and are shown as mean ± SD.

sessed the induction of apoptosis and found that CsA partially inhibits BetA-caused CNE2 apoptosis (Fig. 6A, left panel). These results indicate that cyt c release is also partially prevented by CsA treatment (Fig. 6A, right panel). We used another mPTP inhibitor, BKA, in repeat experiments to confirm our findings. As shown in Fig. 6B, BKA also partially prevents BetA-induced cyt c release. These observations indicate that CsA/BKA-sensitive mPTPs are involved in Bax/Bak-independent cyt c release of CNE2 cells induced by BetA.

DISCUSSION

Human NPC is the most common cancer in East Asia. However, the cause of the increased risk for NPC in this region is not clear. Our laboratory is focused on the search for new chemotherapy drugs for the treatment of NPC. BetA, a naturally occurring pentacyclic triterpenoid with the potential to kill melanoma cells (Pisha et al., 1995), has been shown to induce apoptosis in many cancer cell lines, including human melanoma cells, ovarian carcinomas, and leukemia HL-60 cells (Eiznhamer and Xu, 2004; Mukherjee et al., 2004). However, reports on whether or not BetA can kill NPC cell lines are not available.

In this study, we found that BetA can efficiently kill CNE2 cells, a typical NPC cell line. Our data demonstrate that BetA-induced CNE2 cell death occurs through typical apoptosis and that the induction of apoptosis is caspase-dependent. Induction of apoptosis is characterized by caspase activation, formation of a DNA ladder, and cyt c release. Bcl-2 and Bcl-xL overexpression can partially prevent BetA-induced CNE2 apoptosis. Furthermore, we found that Bax is not activated during BetA-induced apoptosis and that BetA-induced cyt c release is Bax/Bak-independent. Our data also indicate that mPTPs contribute to cyt c release induced by BetA.

BetA-induced cell death is characterized by AV single staining, a hallmark of apoptosis. The CNE2 cell membrane PS is externalized and releases an “eat-me” signal (Depraetere, 2000). This agrees with cell death caused by etoposide (Boesen-de Cock et al., 1998). Caspases are always activated during apoptosis. The results of our study demonstrate that BetA treatment causes CNE2 cell caspase-3 and caspase-9 activation in a dose-dependent manner. DNA damage is characterized by the formation of a DNA ladder (Enari et al., 1998). We treated CNE2 cell with BetA and detected DNA ladder formation. These observations indicate that CNE2 cell death induced by BetA is a typical example of apoptosis.

Mitochondria play a central role in caspase-dependent apoptosis (Green and Reed, 1998). They contain a number of pro-apoptotic proteins, such as cyt c, which is released from the mitochondria into the cytosol during apoptosis (Kim et al., 2005). BetA caused mitochondrial damage and results in the release of cyt c in a dose-dependent manner. Cyt c release is often triggered by pro-apoptotic members of the Bcl-2 family, including Bax and Bak (Strasser, 2005). Once stimulated, Bax and Bak are translocated from the cytosol to the mitochondria and integrated into the outer mitochondrial membrane (Goping et al., 1998; Wei et al., 2001). Activation of Bax and Bak triggers the release of many pro-apoptotic factors, including cyt c. However, our results indicated that Bax is not activated during BetA-induced apoptosis, as shown in Fig. 4. We knocked down Bax/Bak expression in CNE2 cells using an RNA interference and found that cyt c is still released. These results suggest that Bax and Bak may not be involved in BetA-induced cyt c release.

How then is cyt c released without the involvement of Bax/Bak? Several studies have indicated that some stimuli, such as chelerythrine, gossypol, and A23187/ArA, could cause Bax/Bak-independent cyt c release (Lei et al., 2006; Mizuta et al., 2007; Wan et al., 2008) and suggested that mPTPs may serve as possible targets during cyt c release. The results in our study indicate that when mPTPs are inhibited by CsA or BKA, BetA-induced cyt c release is partially prevented. Although insignifi-

cant, the inhibition clearly confirms that mPTPs partially contribute to BetA-induced Bax/Bak-independent cyt c release. Mullauer et al. recently published findings that show that BetA induces caspase-dependent, Bax/Bak-independent apoptosis and cyt c release (Mullauer et al., 2009); the results of our study partially agreed with their findings and confirmed the BetA pathway in the CNE2 cell line. However, Mullauer et al. found a significant reduction of cyt c release after CsA treatment, in contrast with our system where a difference was found but it was not significant. This disagreement may be caused by the different cell lines used (leukemia cell line Jurkat versus nasopharyngeal carcinoma cell line CNE2). We used another mPTP inhibitor, BKA to confirm our findings and obtained the same results.

BetA has been suggested to have a broad effect on tumor cells but no effect on untransformed cells. Our study identified NPC cells as a new target for BetA and provides further insights into its mechanism of action. We believe that BetA can serve as a potent and effective anti-cancer agent in NPC treatment. Furthermore exploration of the mechanism of action of BetA may yield novel breakthroughs in anti-cancer drug discovery.

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