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Involvement of Caspase Activation and Mitochondrial Stress in Trichostatin A-Induced Apoptosis of Burkitt's Lymphoma Cell Line, Akata

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Abstract Epstein–Barr virus (EBV) infects more than 90% of the human population and has a potential oncogenic nature. Trichostatin A (TSA) has potent antitumor activity, but its exact mechanism on EBV-infected cells is unclear. This study examined the effects of TSA on proliferation and apoptosis of the Burkitt's lymphoma cell line, Akata. TSA treatment inhibited cell growth and induced cytotoxicity in both the EBV-negative and -positive Akata cells. TSA sensitively induced apoptosis in both cells, as demonstrated by the increased number of positively stained cells in the TUNEL assay, the migration of many cells to sub-G_1 phase by flow cytometric analysis, and the formation of DNA ladders. This suggests that EBV has no effect on the sensitivity to TSA. Western blot analysis showed that the cleavage of PARP and Bid and the activation of caspases are closely related to the TSA-induced apoptosis of the cells. The reduction in mitochondrial transition potential and the release of apoptosis-inducing factor from mitochondria to cytosol was also observed after the TSA treatment, but was suppressed by treating the cells with a cathepsin B inhibitor. Overall, these findings suggest that besides the caspase-dependent pathway, mitochondrial events are also associated with the TSA-induced apoptosis of Akata cells. J. Cell. Biochem. 99: 1420–1430, 2006.

Key words: trichostatin A; Akata cells; Epstein–Barr virus; apoptosis

Epstein–Barr virus (EBV) is a human gammaherpes virus that infects most of the adult population worldwide. In the some cases of EBV infections, the virus immortalizes the host cells, but is maintained under latent condition via the cytotoxic T lymphocyte-mediated host immune responses [Chang and Liu, 2000]. In addition, EBV is associated with several malignant diseases, such as Burkitt's lymphoma (BL), nasopharyngeal carcinoma, Hodgkin's disease and lymphoproliferative disorders in immunodeficient individuals [Epstein et al., 1964; Davies et al., 1991; Miller et al., 1994; Westphal et al., 2000].

Until the isolation of EBV-negative cell clones from the EBV-positive BL cell line, Akata, it was

Abbreviations used: AIF, apoptosis-inducing factor; EBV, Epstein–Barr virus; HDAC, histone deacetylase; TSA, trichostatin A; BL, Burkitt's lymphoma; PI, propidium iodide; PARP, poly (ADP ribose) polymerase.

Young-Ok Son and Ki-Choon Choi contributed equally to this work.

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difficult to isolate EBV-negative cells from originally EBV-positive BL cell lines [Rowe et al., 1987]. Only EBV-negative clones cannot grow in low-serum conditions. In addition, EBV-positive clones can form colonies on soft agar and tumor masses in nude mice, while EBV-negative clones cannot [Shimizu et al., 1994]. Therefore, it was suggested that the malignant phenotype of Akata is dependent on the presence of EBV. This suggestion is clearly supported by a report showing that BL cells with a type I latency such as EBV-positive Akata cells are resistant to apoptosis [Komano et al., 1998].

Histone deacetylase (HDAC) inhibitors have received considerable interest as possible therapeutics for the treatment of cancer. HDAC inhibitors appear to induce cell-cycle arrest and apoptosis via the p53-independent upregulation of cell-cycle inhibitors such as p21 and p27 [Kwon et al., 1998; Gray et al., 1999; Sambucetti et al., 1999]. Among the HDAC inhibitors, trichostatin A (TSA) was originally used as an antifungal agent and is one of the most potent inhibitors of HDAC activity [Kyrylenko et al., 2003; Rahman et al., 2003]. Increasing interest has been focused on the regulatory effects of TSA on the cell-cycle progression, differentiation, and apoptosis, such that TSA actively induces cell-cycle arrest and apoptosis in various cancer cells even at nanomolar concentrations [Ailenberg and Silverman, 2003; Donadelli et al., 2003; Roh et al., 2004].

A recent study examined the effects of TSA on the proliferation and apoptosis of EBV-infected Akata cells and showed that the cells were more sensitive to the TSA-induced inhibition of proliferation and viability than lymphoma cell lines BJAB and Jurkat, as well as NIH3T3 cells [Kook et al., 2005]. In addition, the TSA-induced apoptosis of the cells was almost completely inhibited by a pancaspase inhibitor, z-VAD-fmk, indicating that caspase-dependent pathway might be involved in the TSA-induced apoptosis of the cells. However, there was no dramatic inhibition of TSA-induced apoptosis in the Akata cells treated with either z-IETD-fmk or z-LEHD-fmk. This suggests that besides the common activation of caspase such as caspase-8, -9, and -3, other caspases or caspase-independent pathway(s) are also involved in the TSA-induced apoptosis of EBV-infected Akata cells.

Therefore, this study examined the precise mechanism(s) involved in the TSA-mediated apoptosis of Akata cells and tried to determine the effects of EBV on the sensitivity to TSA using EBV-negative and -positive Akata cells.

MATERIALS AND METHODS

Chemicals and Laboratory Wares

Unless otherwise specified, all chemicals and laboratory wares were obtained from Sigma Chemical Co. (St. Louis, MO) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ), respectively. Trichostatin A (TSA), pancaspase inhibitor (z-VAD-fmk), and cathepsin B inhibitor (z-FA-fmk) were dissolved in dimethylsulfoxide (DMSO) immediately before use, and the final DMSO concentration did not exceed 0.5% (v/v) throughout the experiments.

Cell Culture

Both the EBV-negative and -positive Akata cell lines of a BL origin were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT) and antibiotics. The cultures were maintained at 37°C with a gas mixture of 5% CO_2/95% air, and 5×10^5 cells per milliliter were resuspended in either 5 ml or 250 μl media for spreading onto either 6-well or 96-well flat-bottomed plates, respectively. The cells were pretreated with caspase inhibitors prior to adding various TSA concentrations (1–300 nM). At various times (0–36 h), the cells were processed for analyzing the proliferation and apoptosis.

Measurement of DNA Synthesis

The level of DNA synthesis by Akata cells after the treatment with TSA and/or caspase inhibitors was measured by adding 1 μCi of [methyl-^{3}H] thymidine deoxyribose (TdR; Amersham Pharmacia Biotech, Inc., Piscataway, NJ) to each well for additional 12 h of culture periods. The cells were then collected using a cell harvester (Inotech Inc., Switzerland), and the tritium content was measured using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

Determination of Cytotoxicity

Cellular cytotoxicity induced by TSA was measured using a trypan blue exclusion assay. Briefly, the cells were cultured in RPMI-1640

supplemented with 10% FBS and various concentrations (1–300 nM) of TSA in the presence or absence of caspase inhibitors. After incubation, the cells were stained with 0.4% trypan blue and approximately 100 cells were counted for each treatment. The cytotoxicity was calculated as follows: % cytotoxicity = [(total cells−viable cells)/total cells] × 100.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick-End Labeling (TUNEL) Assay

The cells exposed to TSA in the presence of caspase inhibitor for various times were fixed with 1% buffered formaldehyde (pH 7.5) on ice for 30 min. After washing with PBS, the cells were resuspended in 70% ice-cold ethanol and stored at −20°C for 1 h. The cells were rehydrated with PBS and incubated in a TdT buffer containing 30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM $CoCl_2$, 0.05 mg/ml BSA, 0.1 mM DTT, 7.5 U/ml TdT, and 0.4 nM/ml FITC-5-dUTP. After 30 min of incubation at 37°C, the reaction was blocked by transferring the cells to a buffer containing 300 mM sodium chloride, 30 mM sodium citrate, and 2% bovine serum albumin for 30 min. The cells were then washed with PBS and observed under a fluorescence microscopy (Axioskop 2, Carl Zeiss, Germany).

Propidium Iodide (PI) Staining

The cells treated with TSA and/or caspase inhibitors were fixed with 80% ethanol at 4°C for 24 h, and incubated overnight at 4°C with 1 ml of a PI staining mixture (250 μl of PBS, 250 μl of 1 mg/ml RNase in 1.12% sodium citrate, and 500 μl of 50 μg/ml PI in 1.12% sodium citrate). After staining, 1×10^4 cells were analyzed by flow cytometry using the FACS Calibur® system (Becton Dickinson, San Jose, CA).

DNA Fragmentation Assay

After exposing Akata cells to TSA in the presence of caspase inhibitor for various times, the cells were collected and incubated with a lysis buffer (1% NP-40 and 1% SDS in 50-mM Tris-HCl, pH 8.0) at 65°C for 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol and aqueous phase was precipitated with two volumes of ethanol overnight at −20°C. The pellet was air-dried and resuspended in a TE buffer (10 mM Tris-Cl, pH 8.0, and 1 mM EDTA), and the degree of fragmentation was analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining.

Western Blot Analysis

Cell lysates were made in an NP-40 lysis buffer (30 mM Tris-Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% NP-40) and protein content was measured using the Bradford [1976] method. Equal amounts of protein (30 μg/sample) were separated electrophoretically by 15% SDS–PAGE and blotted onto PVDF membranes (Bio-Rad). The membranes were blocked for at least 1 h with 50 mM Tris (pH 7.5) containing 500 mM NaCl, 1% BSA, and 5% non-fat dried milk, and the blots were probed with primary antibodies for 2 h at room temperature or overnight at 4°C. The membranes were washed three times with a blocking buffer and incubated with horseradish peroxidase-conjugated anti-IgG in blocking buffer for 1 h. After a vigorous washing, the blots were developed with enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Buckingham, UK) and exposed to X-ray film (Eastman-Kodak, Rochester, NY). Polyclonal antibodies specific against poly (ADP ribose) polymerase (PARP, SC-7150), caspase-3 (SC-7148), caspase-8 (SC-6134), and apoptosis-inducing factor (AIF; SC-9417), and monoclonal antibodies specific against caspase-9 (SC-17784) and cytochrome c (SC-13156), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody against Bid (550-365) and monoclonal antibodies specific against α-tubulin and actin were obtained from BD Bioscience (Pharmingen, CA) and Sigma Chemical Co., respectively. Polyclonal antibody against caspase-1 (#2225) was purchased from Cell Signaling Technology (Beverly, MA).

Measurement of Mitochondrial Transition Potential (MTP)

Akata cells were incubated with 300 nM TSA in the presence of 50 μM z-FA for 36 h. After incubation, the cells were collected and resuspended in PBS, and then stained with 50 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Eugene, OR) for 20 min at 37°C. The fluorescence related to the mitochondrial membrane potential was measured using the FACS Calibur® system.

Statistical Analysis

All the data is expressed as a mean ± standard error (SE). Scheffe's multiple range test was used for multiple comparisons using SPSS version 10.0 software. A P-value <0.05 was considered significant.

RESULTS

TSA Actively Inhibits Proliferation of Akata Cells

The effect of TSA on cell proliferation was determined by tritium incorporation using two types of BL cells; EBV-negative and -positive Akata cells. As shown in Figure 1, the addition of TSA to the cultured Akata cells inhibited tritium incorporation by the cells. In particular, EBV-positive cells were more sensitive to the TSA-mediated inhibition of tritium uptake by the cells. When Akata cells were treated with 300 nM TSA for 36 h, the level of tritium uptake by EBV-negative and -positive cells decreased to 31.3% and 16.2% of the untreated cells, respectively (Fig. 1A). In addition, TSA-mediated inhibition of DNA synthesis in the cells was time-dependent (Fig. 1B).

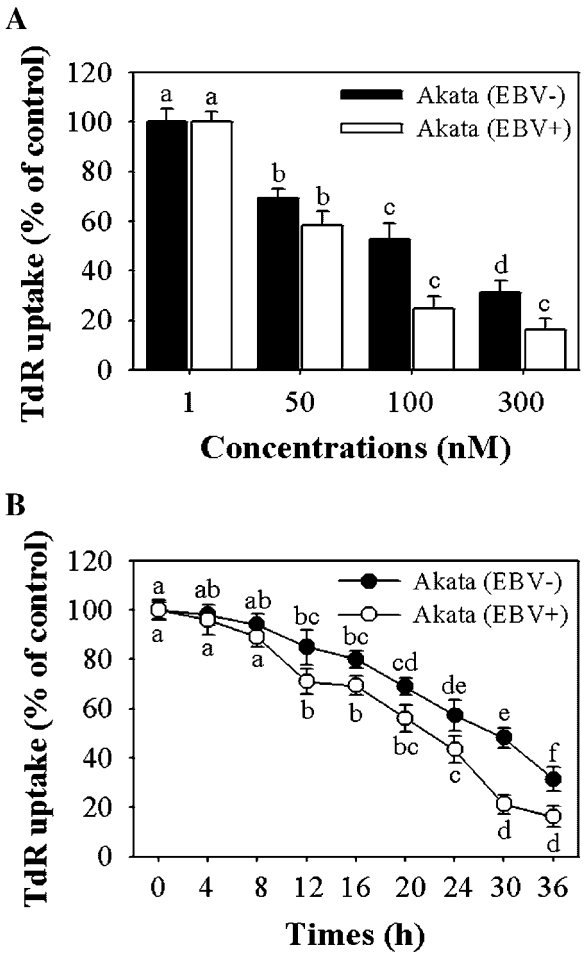

Fig. 1. Effect of TSA on DNA synthesis in Akata cells. **A**: EBV-negative and -positive Akata cells were treated with the indicated doses of TSA for 36 h and incubated with [methyl-^{3}H] TdR for the final 12 h of the incubation period. **B**: Akata cells were treated with 300 nM TSA for the indicated times and incubated with [methyl-^{3}H] TdR for an additional 12 h of the incubation periods. The results represent the mean ± SE of triplicate experiments. Different superscripts represent significant differences ($P<0.05$) among groups by Scheffe's multiple range test.

TSA Reduces the Viability of Akata Cells

In order to determine how TSA inhibited cell proliferation, the experiment was focused on ascertaining if TSA exerted a cytotoxic effect on Akata cells by monitoring trypan blue exclusion (Fig. 2). TSA indeed had a cytotoxic

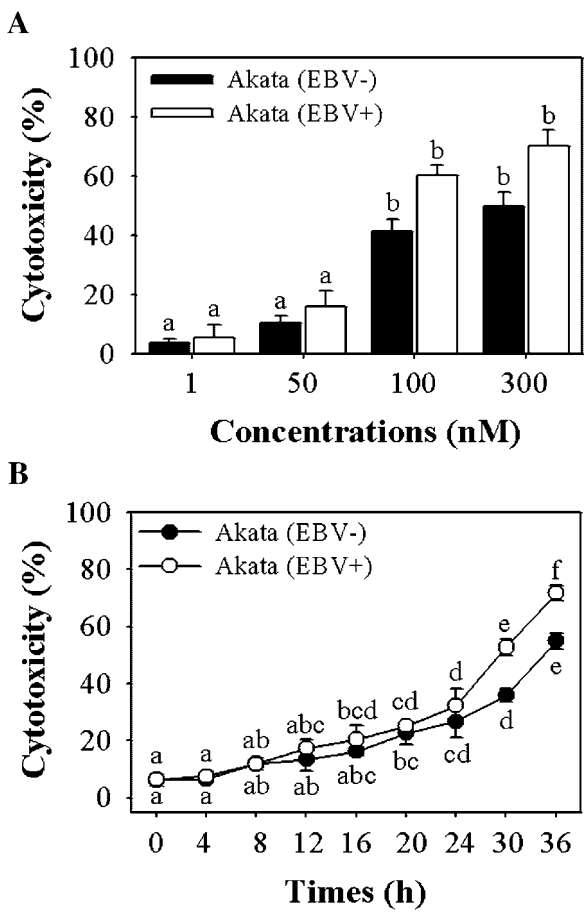

Fig. 2. Cytotoxic effect of TSA in Akata cells. EBV-negative and -positive Akata cells were treated with the indicated doses of TSA for 36 h (**A**) or with 300 nM TSA for the indicated times (**B**), and then processed for trypan blue staining. The results represent the mean ± SE of three separate experiments. Different superscripts represent significant differences ($P<0.05$) among groups by Scheffe's multiple range test.

effect on EBV-negative Akata cells and the cytotoxicity induced after incubating the cells with 300 nM TSA for 36 h was 49.9% (Fig. 2A). In contrast, more than 70% of EBV-positive Akata cells positively stained with trypan blue when treated with 300 nM TSA for 36 h. A time-course study also revealed that EBV-positive Akata cells were more sensitive to the TSA-mediated cytotoxicity than EBV-negative cells (Fig. 2B).

TSA-Induced Reduction of Cell Viability in Akata Cells Results From Apoptosis

In order to understand the nature of TSA-induced cytotoxicity in Akata cells, the cells were subjected to apoptosis assays, including a TUNEL assay and cell-cycle analysis after PI staining (Fig. 3). Initially, a time-dependent increase in the number of positively stained Akata cells was observed after TSA treatment in both the EBV-negative and -positive Akata cells (Fig. 3A). TSA-mediated apoptosis was further confirmed by examining the cell cycle after PI staining. The results showed that after TSA treatment, there was a dose-dependent increase in cell populations in the sub-G_0/G_1 phase of cell cycles in both the EBV-negative and -positive Akata cells (Fig. 3B).

Caspase-8 Activation, and PARP and Bid Cleavage Are Closely Associated With TSA-Mediated Apoptosis in Akata Cells

Bid is a proapoptotic member of the Bcl-2 family and a substrate of caspase-8, which reacts sensitively to Fas or TNF receptor-mediated death signaling. In addition, the cleavage of PARP is an important marker in the apoptotic process. Therefore, this study investigated whether or not PARP, Bid, and caspase-8 are associated with TSA-induced apoptosis using Western blot analyses (Fig. 4). TSA treatment resulted in the time-dependent degradation of procaspase-8 in EBV-negative Akata cells. After 8-h exposure to 300 nM TSA, caspase 55/54 kDa protein degradation was detected, and the protein bands almost completely disappeared when the cells were exposed to 300 nM TSA for 20 h. In addition, the TSA treatment caused a clear reduction in the level of 24 kDa Bid protein in the cells. Furthermore, a time-dependent increase in the PARP 85 kDa cleavage products with the corresponding degradation of PARP 116 kDa protein was observed in the cells after TSA treatment. PARP cleavage started from 8 h of treatment and only the 85 kDa cleaved proteins were present when the cells were exposed to 300 nM TSA for 30 h.

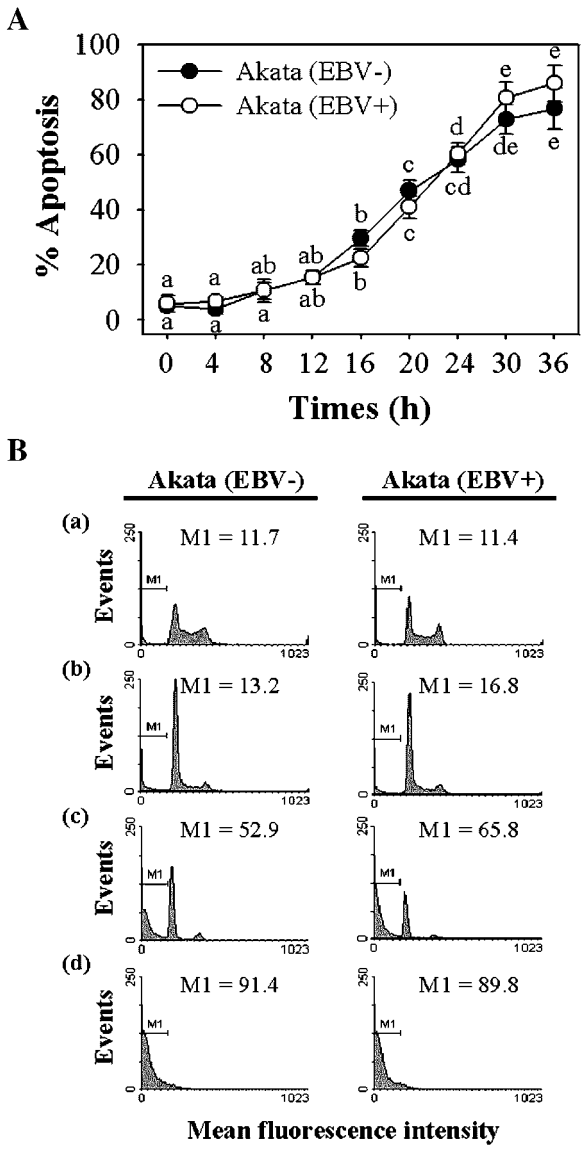

Fig. 3. TSA-mediated apoptosis induction of Akata cells. **A**: TUNEL assay of Akata cells. EBV-negative and -positive Akata cells were treated with 300 nM TSA for the indicated times. After incubation, the cells were stained with FITC-conjugated dUTP and the degree of apoptosis was assessed. The data show the mean ± SE of three separate experiments. Different superscripts represent significant differences ($P < 0.05$) among groups by Scheffe's multiple range test. **B**: Dose-dependent increase in apoptosis in the TSA-treated Akata cells. The cells were either untreated (a) or treated with 50 (b), 100 (c), and 300 nM TSA (d) for 36 h. After incubation, the cells were stained with PI and analyzed by flow cytometry. The figure shows a representative staining profile for 10,000 cells per experiment. M1 is the cell population defined as apoptotic.

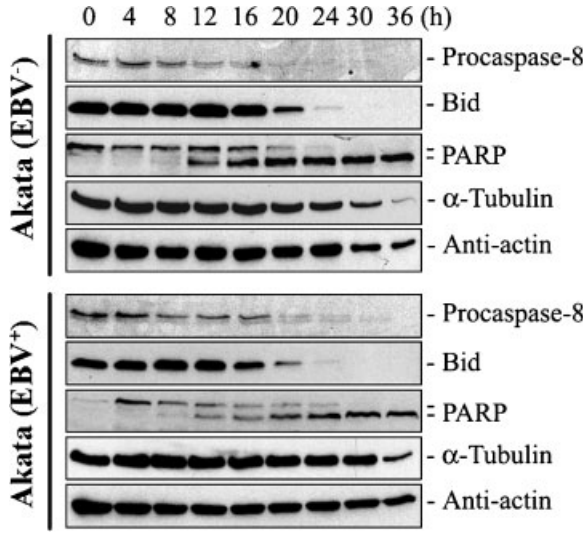

Fig. 4. Western blot analysis of procaspase-8, Bid, and PARP in Akata cells. EBV-negative and -positive Akata cells were incubated in the presence of 300 nM TSA for various times (0–36 h). The cell lysates were analyzed by 10–15% SDS–PAGE followed by immunoblot analysis. A representative result from three independent experiments is shown. α-Tubulin and actin were used as the internal markers.

TSA-mediated activation of caspase-8 and cleavage of Bid and PARP proteins were also observed in the EBV-positive Akata cells.

Caspase-Dependent Pathway is Involved in the TSA-Induced Apoptosis

In order to further examine the involvement of caspase-dependent pathway in TSA-induced apoptotic death, Akata cells were treated with 300 nM TSA for 36 h in the presence or absence of either 50 μM z-VAD-fmk or 50 μM z-FA-fmk. Both the pancaspases and cathepsin B inhibitor significantly inhibited the TSA-induced cytotoxicity in the cells (Fig. 5A). In addition, the preventive effect of them on TSA-induced cytotoxicity was higher in that z-VAD-fmk, compared to z-FA-fmk, was added to the cells. This indicates that TSA-induced cell death in Akata cells is caspase-dependent, which is supported by the results obtained from the apoptosis assays. The TUNEL assay showed that the numbers of TSA-induced positively stained cells were significantly reduced after the z-VAD-fmk treatment in both the EBV-negative and -positive Akata cells (Fig. 5B). When TSA-exposed Akata cells were treated with 50 μM z-VAD-fmk, the apoptotic cells decreased to the levels similar to those of EBV-negative (11.8%) or -positive untreated Akata cells (8.2%). In

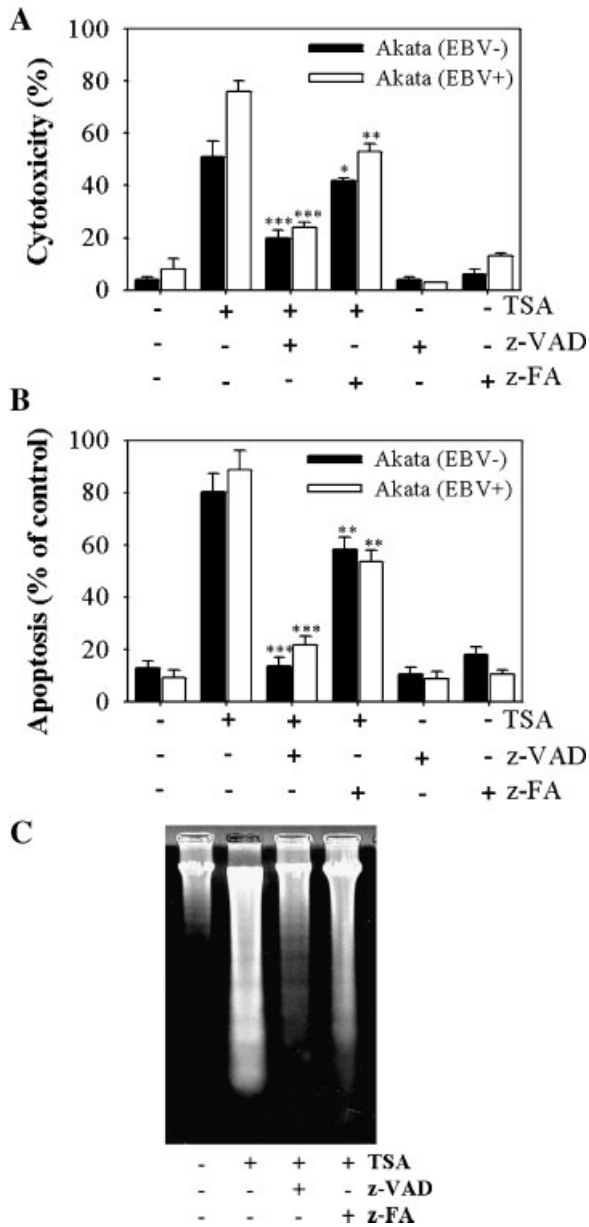

Fig. 5. Effect of caspase inhibitors on TSA-induced cytotoxicity and apoptosis of Akata cells. EBV-negative and -positive Akata cells were untreated or treated with 50 μM z-VAD-fmk or 50 μM z-FA-fmk 1 h before exposing them to 300 nM TSA and then incubated for 36 h. After incubation, the cells were processed for trypan blue staining (**A**) and TUNEL staining (**B**). Each bar shows the mean ± SE of three separate experiments and $^{**}P < 0.01$ and $^{***}P < 0.001$ represent significant differences between the experimental and positive control values (TSA treatment alone). **C**: Analysis of DNA fragmentation using agarose gel electrophoresis. EBV-positive Akata cells were incubated in the presence of z-VAD-fmk or z-FA-fmk and then incubated with 300 nM TSA for 36 h. Genomic DNA was prepared and analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining. A representative result from three independent experiments is shown.

addition, the presence of z-VAD-fmk clearly inhibited the TSA-induced formation of apoptotic ladders of genomic DNA in EBV-positive Akata cells (Fig. 5C). The inhibitory effects of caspase inhibitors on TSA-induced apoptosis were also observed by analyzing the cell cycle after PI staining (Fig. 6). When the EBV-positive Akata cells were treated with 300 nM TSA for 36 h 90.6% of the cell population was in the sub-G_0/G_1 phase of the cell cycle, while the percentage of apoptotic cells decreased to 20.8% after treatment with 50 μM z-VAD-fmk (Fig. 6B).

Bands (24 kDa) of the Bid protein in both the EBV-negative and -positive Akata cells disappeared completely after treating the cells with 300 nM TSA for 36 h (Fig. 7). However, the TSA-induced disappearance of Bid protein was clearly suppressed by the treatment with 50 μM z-VAD-fmk, suggesting the involvement of caspase cascades in the TSA-induced apoptosis. TSA-induced increase in the 85 kDa cleaved PARP protein in Akata cells was also more clearly inhibited by z-VAD-fmk than by z-FA-fmk.

Mitochondrial Stress Is Also Related to the TSA-Induced Apoptosis

The cleavage of specific substrates for each protease (caspase-1, -3, -9) involved in the execution of apoptosis was investigated to further confirm that TSA-induced apoptosis of Akata cells is caspase-dependent (Fig. 8). As shown in the figure, there was a dramatic increase in the activity of caspase-1 (20 kDa), caspase-3 (32 kDa), and caspase-9 (46 kDa) after the TSA treatment compared with the control. Incubating the cells with a cathepsin B inhibitor, z-FA-fmk, inhibited the TSA-induced activation of caspase-1 but had no effect on the caspase-3 activity.

Treating the Akata cells with 300 nM TSA resulted in the release of cytochrome c and AIF from mitochondria to cytosol. However, treating the EBV-infected Akata cells with z-FA-fmk resulted in some limited inhibition of this

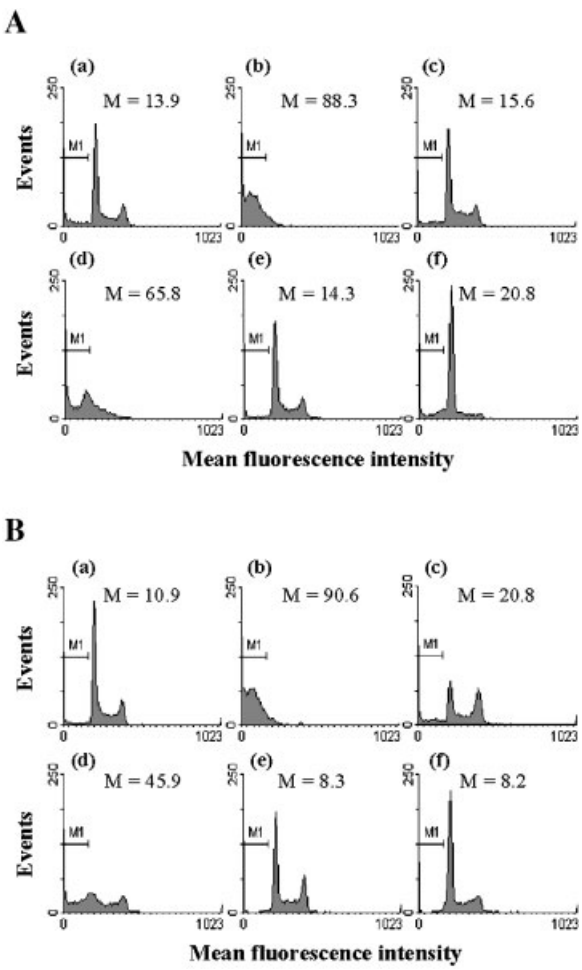

Fig. 6. Flow cytometric analysis of Akata cells after PI staining. EBV-negative (**A**) and -positive Akata cells (**B**) were incubated without (a) or with 300 nM TSA in the absence (b) and the presence of either 50 μM z-VAD-fmk (c) or 50 μM z-FA-fmk (d) for 36 h. (e) and (f) show the results from the cells treated with 50 μM z-VAD-fmk or 50 μM z-FA-fmk alone. The figures show a representative PI staining profile for 10,000 cells per experiment. M1 is the cell population defined as apoptotic.

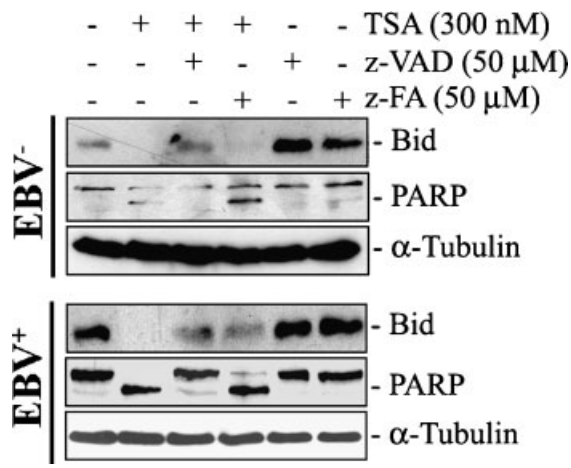

Fig. 7. Effects of caspase inhibitors on the TSA-mediated cleavage of Bid and PARP proteins in Akata cells. EBV-negative and -positive Akata cells were incubated with 300 nM TSA for 36 h in the presence or absence of z-VAD-fmk or z-FA-fmk. The cell lysates were analyzed by 15% SDS–PAGE followed by immunoblot analysis. A representative result from three independent experiments is shown. α-Tubulin was used as the internal standard for loading control.

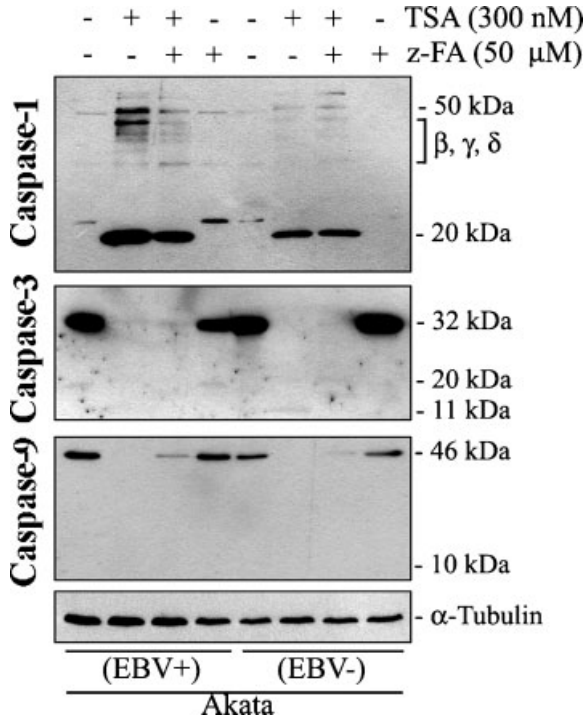

Fig. 8. Effects of z-FA-fmk on caspase activation in Akata cells. EBV-negative and -positive Akata cells were incubated with 300 nM TSA for 36 h in the presence or absence of z-FA-fmk. The cell lysates were analyzed by 15% SDS–PAGE followed by immunoblot analysis. A representative result from three independent experiments is shown.

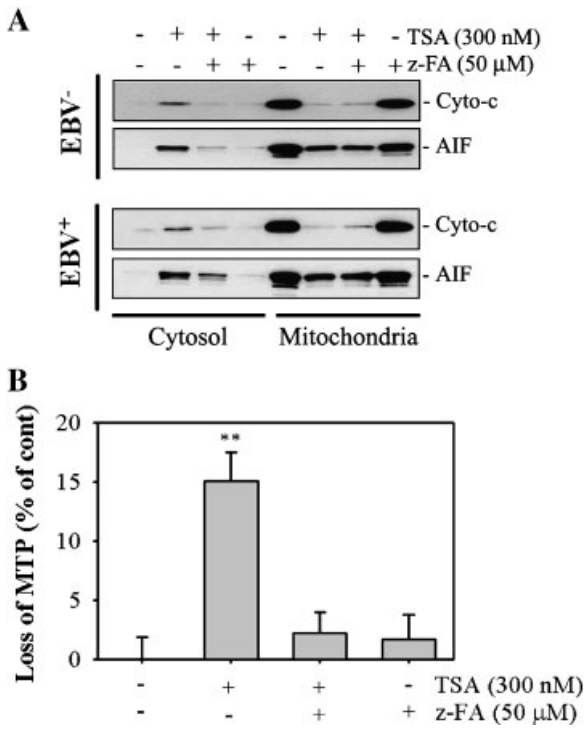

Fig. 9. Effects of TSA on mitochondrial events in Akata cells. **A**: EBV-negative and -positive Akata cells were incubated with 300 nM TSA for 36 h in the presence or absence of z-FA-fmk and analyzed by immunoblot analysis. A representative result from three independent experiments is shown. **B**: Mitochondrial transition potential (MTO) of EBV-positive Akata cells was examined by $DiOC_6$ staining followed by flow cytometry analysis. The figures show the relative percentage of MTP reduction. Each bar shows the mean ± SE of three separate experiments and $^{**}P < 0.01$ shows the significant difference between the experimental and control values.

release (Fig. 9A). This is similar to that observed in EBV-negative Akata cells. Therefore, the effects of TSA on a classic apoptotic event, MTP, were examined because the increased cytoplasmic levels of calcium alter mitochondrial homeostasis (Fig. 9B). The MTP was significantly reduced in EBV-infected Akata cells after the TSA treatment. When the cells were treated with 300 nM TSA for 36 h, the level of MTP was measured to be 15.1% of that observed in the untreated control cells. However, treating the cells with 50 μM z-FA-fmk gave almost complete protection against the TSA-mediated loss of MTP. This protection was also observed in the case where z-VAD-fmk was added to the cell cultures (data not shown).

DISCUSSION

It has been suggested that the malignant phenotype of Akata cells is dependent on the presence of EBV [Shimizu et al., 1994] and EBV-positive Akata cells are more resistant to apoptosis than EBV-negative Akata cells [Komano et al., 1998]. In contrast, this study showed that EBV-positive Akata cells are more sensitive to the TSA-mediated inhibition of proliferation and viability than EBV-negative Akata cells. This suggests that the presence of EBV is closely related to the sensitivity to TSA. However, several apoptosis assays revealed that TSA induces apoptosis in both the EBV-negative and -positive Akata cells, as evidenced by the increased number of positively stained cells in the TUNEL assay, the migration of a substantial population of cells to the sub-G_1 phase and the formation of apoptotic DNA ladders. Therefore, it is believed that the presence of EBV does not affect the sensitivity to TSA even though the inhibitory effect of TSA on the proliferation of EBV-positive Akata cells was higher than EBV-negative Akata cells.

Apoptosis induction through cell-cycle arrest is believed to be a common pathway of

TSA-mediated antitumor activity because TSA blocks growth arrest at a different cell-cycle phase according to the cell types studied and induces apoptosis in various cancer cells [Wharton et al., 2000; Herold et al., 2002; Ailenberg and Silverman, 2003; Papeleu et al., 2003; Roh et al., 2004]. However, cell-cycle analysis through PI staining of the cells did not show any arrest of cell-cycle progression. This suggests that the TSA-induced apoptosis of Akata cells is independent on cyclin-dependent kinase (CDK) inhibitors such as p21 and p27.

Biochemical changes such as the activation of caspases or endonucleases, the cleavage of PARP, and the eventual fragmentation of genomic DNA, are important characteristics in the apoptotic process [Arends et al., 1990; Patel et al., 1996]. Bid is a proapoptotic member of the Bcl-2 family and is cleaved by caspase-8. This activates cytochrome c releasing factor, Bax, in Fas or TNF-induced apoptosis [Kluck et al., 1997; Liu et al., 2004]. In particular, caspase-3, which is activated by proteolytic cleavages such as caspase-8 and caspase-9 cleaves an essential DNA repair enzyme, PARP and the cleaved PARP then activates a calcium/magnesium-dependent endonuclease, resulting in internucleosomal DNA fragmentation [Yakovlev et al., 2000; Annunziato et al., 2003]. Therefore, a caspase cascade such as caspase-8, -9, and -3 activation is believed to play a key role in the execution of apoptosis by TSA. This was supported by the observation that TSA-induced apoptosis occurs through caspase-8 and -9 activation in EBV-positive Akata cells which was inhibited by treating the cells with z-VAD-fmk [Kook et al., 2005]. In addition, this study showed that the cleavage of PARP and Bid, and the activation of caspase 8 are closely associated with TSA-induced apoptosis. This suggests that the TSA-mediated apoptosis of Akata cells is caspase dependent. This was confirmed by showing that the z-VAD-fmk treatment offered almost complete protection against TSA-induced growth inhibition and apoptosis. This led us to postulate that TSA induces apoptosis through a caspase-dependent pathway in Akata cells. However, our previous results showed that the caspase-8 or caspase-9 specific inhibitor-mediated reduction of TSA-induced apoptosis was quite weak, even though a z-VAD-fmk treatment caused a dramatic decrease in TSA-induced cytotoxicity [Kook et al., 2005]. In addition, the cathepsin B inhibitor appeared to significantly inhibit the TSA-induced apoptosis of Akata cells. This suggests that other pathways are involved in the TSA-induced apoptosis of Akata cells. Additional experiments showed that caspase-1 is partially involved in the TSA-induced apoptosis of Akata cells. Furthermore, the release of AIF and cytochrome c from the mitochondria to cytosol appears to play an important role in the TSA-mediated apoptosis of these cells. This highlights the involvement of mitochondrial stress in the TSA-induced apoptosis of Akata cells, which was further supported by the reduction in MTP by TSA and its protection by the cathepsin B inhibitor. In addition, many reports suggest that mitochondrial stress, cytochrome c release, and changes in the expression of various Bcl-2 family members are another processes in the apoptotic response to HDACs [Medina et al., 1997; Ruefli et al., 2001; Zhu et al., 2001].

In summary, TSA affects the expression of a small subset of genes regulating progression of the cell cycle such as cyclin A and $p21^{cip/waf}$ [Eickhoff et al., 2000] and modulates the apoptosis regulators including caspases and some proteases [Henderson et al., 2003]. This study showed that in the Burkitt's lymphoma, Akata cells, TSA induces caspase-dependent apoptosis through the activation of caspase-1, -3, -8, and -9, as well as caspase-independent apoptosis through the reduction of mitochondrial membrane potential and the release of AIF regardless of the presence of EBV. However, further detailed studies will be needed to determine how TSA is connected to the mitochondrial pathway.

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