Journal of Biochemistry and Molecular Biology, Vol. 38, No. 6, November 2005, pp. 755-762

JOURNAL OF Biochemistry and Molecular Biology

Epstein-Barr Virus-infected Akata Cells Are Sensitive to Histone Deacetylase Inhibitor TSA-provoked Apoptosis

Sung-Ho Kook[†,‡], Young-Ok Son[†,‡], Seong-Kyu Han[||], Hyung-Soon Lee[†], Beom-Tae Kim[‡], Yong-Suk Jang[‡,§], Ki-Choon Choi[#], Keun-Soo Lee[†,‡], So-Soon Kim[†,‡], Ji-Young Lim[†], Young-Mi Jeon[†], Jong-Ghee Kim[†] and Jeong-Chae Lee[†,‡,*]

[†]Laboratory of Cell Biology in Department of Orthodontics and Institute of Oral Biosciences, Chonbuk National University, Chonju 561-756, Korea

[‡]Research Center of Bioactive Materials, Chonbuk National University, Chonju 561-756, Korea

[||]Department of Oral Physiology and Institute of Oral Biosciences, Chonbuk National University, Chonju 561-756, Korea

[§]Bank for Cytokine Research, Chonbuk National University, Chonju 561-756, Korea

[#]Division of Endocrinology and Metabolism, Department of Internal Medicine, College of Medicine, Korea University, Seoul, Korea

Received 24 June 2005, Accepted 11 October 2005

Epstein-Barr virus (EBV) infects more than 90% of the world's population and has a potential oncogenic nature. A histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), has shown potential ability in cancer chemoprevention and treatment, but its effect on EBV-infected Akata cells has not been examined. This study investigated the effect of TSA on the proliferation and apoptosis of the cells. TSA inhibited cell growth and induced cytotoxicity in the EBV-infected Akata cells. TSA treatment sensitively induced apoptosis in the cell, which was demonstrated by the increased number of positively stained cells in the TUNEL assay, the migration of many cells to the sub-G_0/G_1 phase in flow cytometric analysis, and the ladder formation of genomic DNA. Western blot analysis showed that caspase-dependent pathways are involved in the TSA-induced apoptosis of EBV-infected Akata cells. Overall, this study shows that EBV-infected B lymphomas are quite sensitive to TSA-provoked apoptosis.

Keywords: Akata cells, Apoptosis induction, Epstein-Barr virus, Trichostatin A

Introduction

Epstein-Barr virus (EBV) is a human Herpes virus that infects most of the adult population in the world. The virus has the ability to immortalize host cells, B lymphocytes (Chang and Liu, 2000). EBV is associated with a variety of human malignant diseases, such as Burkitt's lymphoma (BL), nasopharyngeal carcinoma, and Hodgkin's disease, as well as lymphoproliferative disorders in immunodeficient individuals (Epstein *et al*., 1964; Davies *et al*., 1991; Miller *et al*., 1994; Westphal *et al*., 2000).

Akata cells were derived from a Japanese patient, and are unique cells in that they sustain the type I latency, which expresses the EBV-associated nuclear antigens-1, EBV-encoded small nuclear RNAs, and a transcript from the BamHI A region (Farrell, 1995). EBV-positive clones can grow in low-serum conditions and form tumor masses in nude mice, while EBV-negative clones can not (Shimizu *et al*., 1994). In addition, it was reported that BL cells with a type I latency, such as EBV-positive Akata cells, are resistant to apoptosis (Komano *et al*., 1998). Therefore, the malignant phenotype of Akata is dependent on the presence of the EBV.

Histone deacetylase (HDAC) inhibitors have received considerable attention as possible therapeutics for the treatment of cancer (Kim *et al*., 2003). HDAC inhibitors appear to induce cell cycle arrest and apoptosis via the p53-independent upregulation of various 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 identified as an antifungal agent, and is one of the most potent inhibitors of HDAC activity. TSA inhibits the class I and II HDACs by blocking their catalytic reaction with DNA (Kyrylenko *et al*., 2003; Rahman *et al*., 2003). 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).

*To whom correspondence should be addressed.
Tel: +82-63-270-4049; Fax: +82-63-270-4049
E-mail: leejc88@chonbuk.ac.kr

Therefore, TSA regulates transcription and chromatin function, and induces apoptosis in many tumor cells.

Despite the great deal of knowledge regarding the effects of TSA on proliferation, differentiation, and apoptosis in tumor cells, the effect of TSA on EBV-infected cells is unknown. Therefore, this study investigated the effects of TSA on the proliferation and apoptosis of EBV-infected Akata cells.

Materials and Methods

Chemicals and laboratory wares Unless otherwise specified, all chemicals and laboratory wares were purchased from the Sigma Chemical Co. (St. Louis, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, USA), respectively. Trichostatin A (TSA), pancaspase inhibitor (z-VAD-fmk), cathepsin B inhibitor (z-FA-fmk), caspase-8 inhibitor (z-IETD-fmk), and caspase-9 inhibitor (z-LEHD-fmk) were dissolved in dimethylsulfoxide (DMSO) immediately before use and the final concentration of DMSO did not exceed 0.5% (v/v) throughout the experiments.

Cell lines and cell treatment EBV-negative or -infected Akata cells of a BL origin, BJAB, and Jurkat cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, USA) and antibiotics. NIH3T3 cells were cultured in DMEM supplemented with 10% FBS and antibiotics. All cultures were maintained at 37ºC using a gas mixture of 5% CO_2/95% air, and 5×10^5 cells per ml were resuspended in either 5 ml or 250 µl of the media to spread onto either 6-well or 96-well flat-bottomed plates, respectively. Cells were pretreated with the caspase inhibitors prior to adding the various concentrations (1-300 nM) of TSA. The level of proliferation, cytotoxicity, and apoptosis of cells were analyzed at various times (0-36 h).

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

Determination of cytotoxicity The level of cell cytotoxicity induced by TSA was measured using a trypan blue exclusion assay. Briefly, cells were incubated with various TSA concentrations (1 to 300 nM) in the presence or absence of caspase inhibitors. After incubation, cells were stained with 0.4% trypan blue, and approximately 100 cells were counted for each treatment. The level of cytotoxicity was calculated as follows: % cytotoxicity = [(total cells − viable cells)/total cells] × 100.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay Cells were fixed with 1% buffered formaldehyde (pH 7.5) on ice for 30 min. After washing with PBS, cells were resuspended in 70% ice-cold ethanol and kept at −20ºC for 1 h. Cells were rehydrated with PBS and were incubated in a TdT buffer containing 30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM $CoCl_2$, 50 µg/ml BSA, 0.1 mM DTT, 7.5 U/ml TdT, and 0.4 nM/ml FITC-5-dUTP. After incubation at 37ºC for 30 min, the reaction was blocked by transferring cells to a buffer containing 300 mM sodium chloride, 30 mM sodium citrate, and 2% bovine serum albumin. Cells were then washed with PBS and observed using fluorescence microscope (Axioskop 2, Carl Zeiss, Germany).

Propidium iodide (PI) staining Cells were fixed with 80% ethanol at 4ºC for 24 h, and then incubated overnight at 4ºC with 1 ml of the 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 using a FACS Calibur® system (Becton Dickinson, San Jose, USA).

DNA fragmentation assay Cells were incubated with a lysis buffer (1% NP-40 and 1% SDS in 50-mM Tris-HCl, pH 8.0) at 65ºC for 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol, and aqueous phase was precipitated with 2 volumes of ethanol at −20ºC overnight. The pellet was air-dried and resuspended in TE buffer (10 mM Tris-Cl, pH 8.0, and 1 mM EDTA). The level of fragmentation was analyzed by 2% agarose gel electrophoresis, which was followed by ethidium bromide staining.

Western blot analysis Cell lysates were made in a lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, and 1 mg/ml of aprotinin, leupeptin, and pepstatin), and protein content was quantified using the Bradford (1976) method. Equal amounts of protein (30 µg/sample) were separated electrophoretically by 12% SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked with PBS containing 5% non-fat dried milk for at least 1 h, and the blots were probed with primary antibodies for either 2 h at room temperature or overnight at 4ºC. The membranes were washed three times with a washing buffer and incubated with horseradish peroxidase-conjugated anti-IgG in a blocking buffer for 1 h. After washing, the blots were developed with enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, USA) and exposed to X-ray film (Eastman-Kodak, Rochester, USA). Polyclonal antibodies specific to poly (ADP ribose) polymerase (PARP, SC-7150) and caspase-8 (SC-6134) were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Polyclonal antibody against Bid (550-365) and monoclonal antibodies specific against α-tubulin and β-actin were obtained from BD Bioscience (Pharmingen, USA) and Sigma Chemical Co., respectively.

Statistical analysis The results are expressed as mean ± standard error (SE). One-way ANOVA using SPSS ver. 10.0 software was used for multiple comparisons. A value of $p < 0.05$ was considered significant.

Results

TSA sensitively inhibited proliferation of EBV-infected Akata cells The effect of TSA on the proliferation was

Table 1. Dose-dependent antiproliferative effect of TSA

TSA (nM)	Akata (EBV+)	Akata (EBV-)	BJAB	Jurkat	NIH3T3
1	103.1 ± 5.1	102.5 ± 5.3	89.8 ± 2.3	107.6 ± 3.1	110.9 ± 4.4
50	55.2 ± 5.8	68.3 ± 3.8	89.1 ± 5.6	73.4 ± 2.3	104.9 ± 3.1
100	23.4 ± 4.6	51.4 ± 5.1	73.0 ± 4.3	80.8 ± 3.6	79.9 ± 1.9
300	15.4 ± 3.7	32.2 ± 3.2	45.9 ± 1.3	39.5 ± 2.4	79.3 ± 2.4

The cells were treated with the indicated TSA doses for 36 h and then processed for TdR uptake assay. The results are the mean ± SE of triplicate experiments and are given as percentage of TdR uptake in untreated control cells.

Table 2. Dose-dependent cytotoxic effect of TSA

TSA (nM)	Akata (EBV+)	Akata (EBV-)	BJAB	Jurkat	NIH3T3
1	4.1 ± 2.1	3.8 ± 1.4	1.5 ± 1.3	2.3 ± 2.2	3.5 ± 1.9
50	18.3 ± 3.9	10.6 ± 2.4	4.3 ± 1.9	6.1 ± 1.8	19.1 ± 3.3
100	60.3 ± 3.8	43.4 ± 4.8	12.1 ± 3.8	10.7 ± 2.8	22.7 ± 3.5
300	65.4 ± 4.9	50.1 ± 6.2	27.3 ± 3.4	31.3 ± 3.4	26.2 ± 3.6

The cells were treated with the indicated TSA doses for 36 h and then processed for trypan blue staining. The results represent the mean ± SE of triplicate experiments.

determined by level of TdR incorporation of cells. As shown in Table 1, the addition of TSA to the cultured cells resulted in a dose-dependent inhibition of TdR incorporation. When EBV-negative and -infected Akata cells were treated with 300 nM TSA for 36 h, TdR uptake of the cells decreased to 32.2% and 15.4% of the untreated control cells, respectively. In the lymphoma cell lines BJAB and Jurkat, this effect was less pronounced, while the non-lymphoma NIH3T3 cells were nearly not affected. In addition, more than 60% of the EBV-infected Akata cells stained positively with trypan blue when they were treated with 100 nM TSA for 36 h (Table 2). At the same treatment of TSA, the level of cytotoxicity of EBV-negative Akata cells was 43.4%, while it was measured from 12.1 to 22.7% in other cell lines. In the time-course experiments, a sensitive induction of TdR uptake inhibition and trypan blue positive staining in the EBV-infected Akata cells after TSA treatment was also found (data not shown).

TSA induced apoptosis of EBV-infected Akata cells Cells were subjected to apoptosis assays, which included PI staining and agarose gel electrophoresis of genomic DNA in order to understand the nature of TSA-induced cytotoxicity in cells (Fig. 1). After incubation with 300 nM TSA, flow cytometry showed a time-dependent increase of apoptosis in the Akata cell lines such that 24 and 90% for EBV-infected cells vs. 23 and 72% for EBV-negative cells were apoptotic after 16 and 36 h incubation, respectively (Fig. 1A). Results obtained for BJAB, Jurkat, and NIH3T3 cells were also time-dependent but the apoptotic level was not dramatic compared with those of Akata cells. In addition, the TUNEL assay for EBV-infected Akata cells confirmed the results of flow cytometric analysis (data not shown). The induction of TSA-mediated apoptosis was further examined by investigating the ladder formation of genomic DNA after the TSA treatment (Fig. 1B). TSA treatment dose-dependently induced apoptotic laddering of genomic DNA in EBV-infected Akata cells, and the formation of DNA ladders appeared even though 50 nM of TSA had been added to the cells.

Caspase-8 activation, and Bid and PARP cleavage are associated with TSA-mediated aoptosis in EBV-infected Akata cells Western blot analyses were carried out to determine if the well-known apoptosis related factors, PARP, Bid, and caspase-8, are associated with TSA-induced apoptosis (Fig. 2). Adding TSA to EBV-infected Akata cells degraded procaspase-8 in a dose-dependent manner. At 36 h of exposure to 300 nM TSA, the bands of procaspase-8 had almost disappeared. There was also a clear reduction in the level of Bid protein in the cells after TSA treatment, such that the 24 kDa Bid protein was not detected when treated with 300 nM TSA for 36 h. Furthermore, a dose-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. Only 85 kDa cleaved proteins were found after the cells were exposed to 300 nM TSA for 36 h.

TSA induced a caspase-dependent apoptosis in EBV-infected Akata cells In order to further elucidate the involvement of caspase-dependent pathway in TSA-induced apoptotic death, EBV-infected Akata cells were treated with 300 nM TSA for 36 h in the presence and absence of 50 μM z-VAD-fmk or 50 μM z-FA-fmk. Both the pancaspases and cathepsin B inhibitor significantly prevented TSA-induced cytotoxicity and apoptosis in the cells (Fig. 3A). The preventive effect was further higher in that z-VAD-fmk, rather than z-FA-fmk, was added to the cells. This was supported by the results obtained from Western blot analysis (Fig. 3B). As

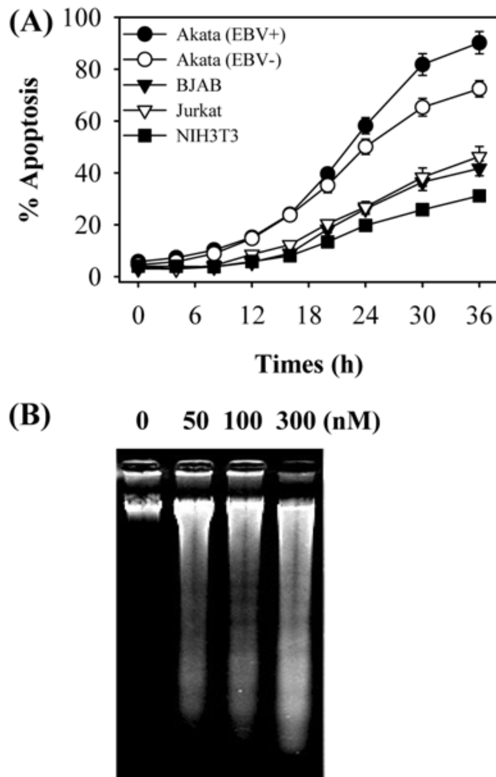

Fig. 1. Time- and dose-dependent induction of apoptosis by TSA. (A) Percent apoptosis quantified by flow cytometry after incubation with 300 nM for the indicated times. A total of 10,000 counts per experiment were analyzed and the percentage of apoptotic cells present in sub-G_1 population is plotted using WinMDI 2.8 program. (B) Analysis of DNA fragmentation using agarose gel electrophoresis. EBV-infected Akata cells were incubated with the indicated TSA doses for 36 h. Genomic DNA was analyzed using 2% agarose gel electrophoresis followed by ethidium bromide staining. A representative result from three independent experiments is shown.

shown in the figure, bands (24 kDa) of Bid protein were completely disappeared by treating the cells with 300 nM TSA for 36 h in the cells. However, the TSA-induced disappearance of Bid protein was suppressed by the treatment of pancaspase or cathepsin B inhibitor on the cells. TSA-induced increase of 85 kDa cleaved PARP protein in cells was also clearly inhibited by z-VAD-fmk more than z-FA-fmk treatment. From these results, we considered that TSA-induced apoptosis of EBV-infected Akata cells was closely related to the caspase-dependent pathway.

TSA-mediated cytotoxicity is inhibited by treating with more caspase-8 than caspase-9 specific inhibitor Both caspase-8 and -9 inhibitors reduced the level of TSA-induced cytotoxicity in the cells (Fig. 4A). However, the suppressive effect of the inhibitors on TSA-induced cytotoxicity was not remarkable, in that more than 50% of the trypan positively stained cells was observed when 20 μM z-IETD-fmk or z-LEHD-fmk had been added to the cells. Meanwhile, z-IETD-fmk, but not z-LEHD-fmk, decreased the number of TSA-induced TUNEL positively stained cells. To further confirm if caspase-8 and/or -9 is associated with the TSA-mediated apoptosis, immunoblot analysis was carried out (Fig. 4B). As shown in the figure, the bands (24 kDa) of Bid protein had completely disappeared after treating the cells with 300 nM TSA for 36 h. The TSA-induced disappearance of Bid protein was weakly suppressed by treating the cells with z-IETD-fmk and z-LEHD-fmk. In contrast, TSA-induced increase of 85 kDa cleaved PARP protein in the cells was inhibited by treating them with more z-IETD-fmk than z-LEHD-fmk.

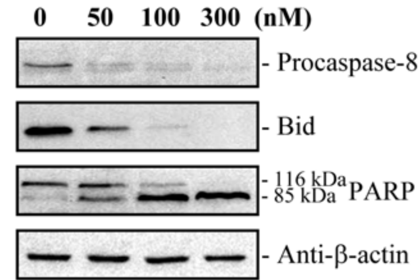

Fig. 2. Western blot analysis of procaspase-8, Bid, and PARP in EBV-infected Akata cells. The cells were incubated in the presence of 0 to 300 nM TSA for 36 h. Cell lysates were analyzed by 12% SDS-PAGE followed by immunoblot analysis. A representative result from three independent experiments is shown. Anti-β-actin was used as the internal marker.

EBV-infected Akata cells were treated with 300 nM TSA for 36 h in the presence of 20 and 40 μM z-IETD-fmk in order to more evaluate the involvement of caspase-8 in TSA-induced apoptosis. Trypan blue exclusion and TUNEL experiments showed that z-IETD-fmk significantly reduced the level of TSA-induced cytotoxicity of the cells in a dose-dependent manner (Fig. 4C). Western blot analysis also showed that the degradation of procaspase-8 and Bid proteins, as well as production of PARP cleaved proteins induced by TSA in the cells were inhibited by z-IETD-fmk in a dose-dependent manner, but this inhibition was not remarkable (Fig. 4D).

Discussion

This study examined the effects of TSA on the proliferation and apoptosis of EBV-infected Akata cells. Akata cells were more sensitive to TSA-mediated inhibition of proliferation than lymphoma cell lines BJAB and Jurkat, as well as NIH3T3 cells. In addition, trypan blue staining experiments showed that the viability of Akata cells was much more susceptible to the reduction by TSA treatment, than that of other cell lines studied was. These results indicate a sensitive influence of TSA on Akata cells.

Because TSA blocks proliferation at different cell cycle phases depending upon the cell types examined and induces apoptosis in a variety of cancer cells (Herold *et al.*, 2002;

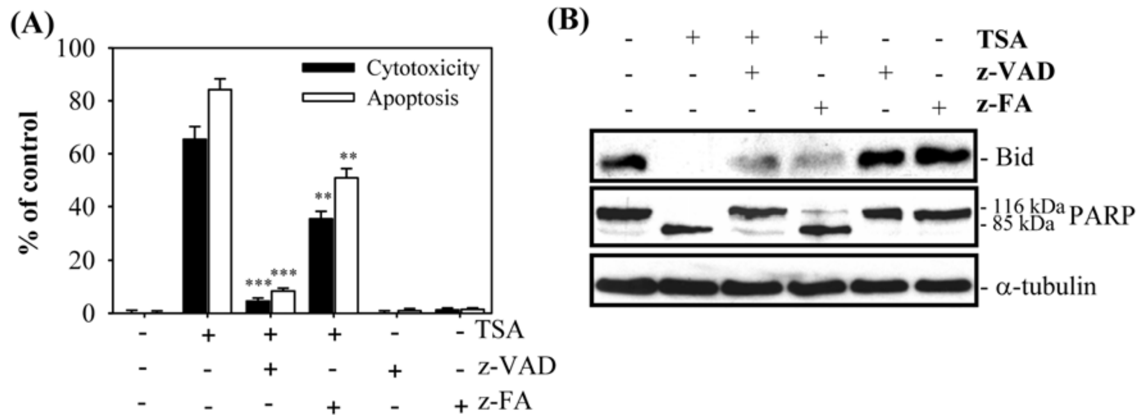

Fig. 3. Effect of pan-caspase inhibitor on TSA-induced cytotoxicity in EBV-infected Akata cells. The cells were pretreated with 50 μM z-VAD-fmk or 50 μM z-FA-fmk 1 h before exposing them to 300 nM TSA, and incubated for an additional 36 h. (A) Trypan blue staining and TUNEL assay. Each bar represents the mean ± SE of triplicate experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ represent significant differences between the experimental and positive control values (TSA treatment alone). (B) Western blot analysis. Cell lysates were analyzed by 12% SDS-PAGE followed by immunoblot analysis. A representative result from three independent experiments is shown.

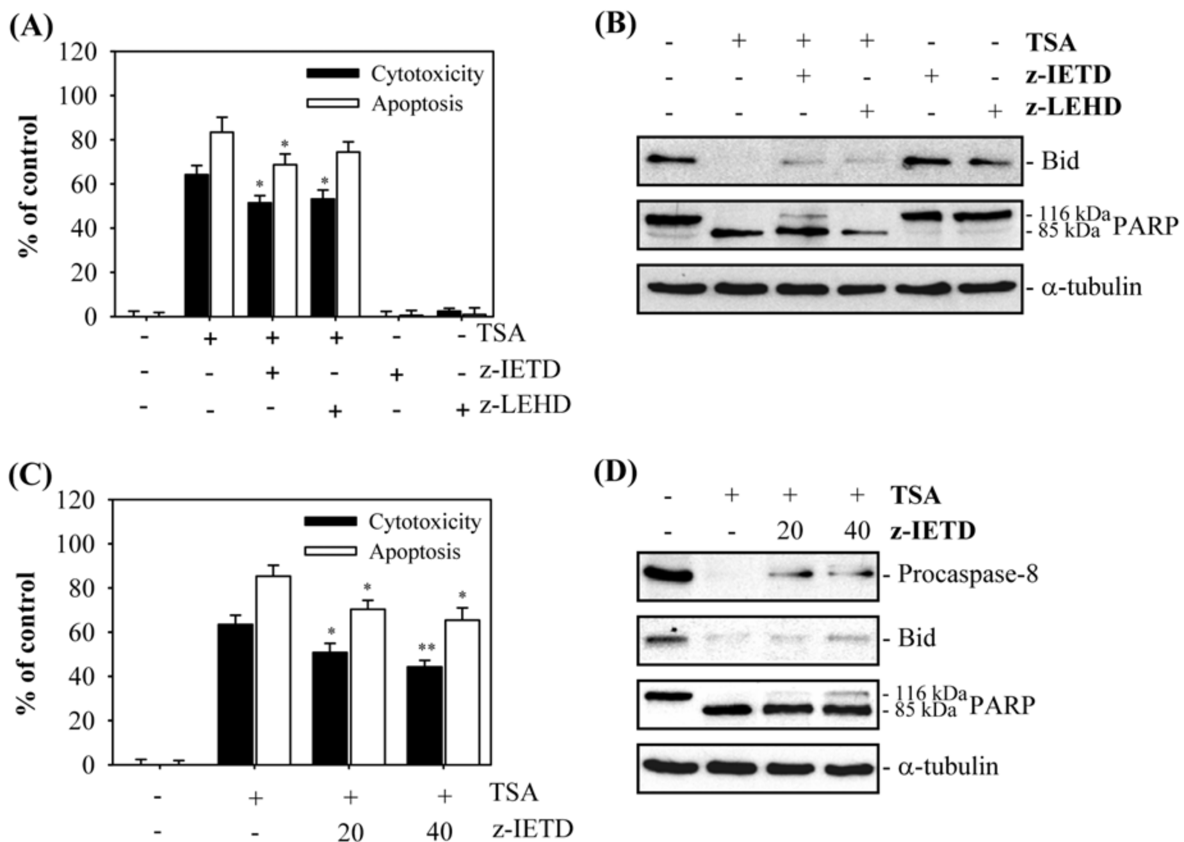

Fig. 4. Effect of caspase-8 and -9 inhibitors on TSA-induced cytotoxicity in EBV-infected Akata cells. The cells were pretreated with 20 μM z-IETD-fmk or 20 μM z-LEHD-fmk 1 h before exposing them to 300 nM TSA, incubated for an additional 36 h, and then processed for trypan blue staining and TUNEL assay (A) or Western blot analysis (B). In addition, the cells were pretreated with the 20 μM or 40 μM z-IETD-fmk 1 h before exposing them to 300 nM TSA and incubated for additional 36 h. Trypan blue staining and TUNEL assay (C) or Western blot analysis (D) were carried out. Each bar represents the mean ± SE of three separate experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ represent significant differences between the experimental and positive control values (TSA treatment alone).

Ailenberg and Silverman, 2003; Papeleu *et al*., 2003; Roh *et al*., 2004), apoptosis induction through cell cycle arrest is believed to be a common pathway for TSA-mediated antitumor activity. It was also reported that apoptosis could be

induced at micromolar to nanomolar concentrations of TSA in various cancer cell lines. For example, 1 μM TSA did not induce apoptosis in hepatoma cell line HepG2, whereas 250 nM TSA seemed to be enough to induce apoptosis in Huh-7 hepatoma cells (Yamashita *et al*., 2003). When human osteosarcoma cell line HOS was treated with 300 nM TSA for 24 h, approximately 40% of the cells was to be apoptotic (Roh *et al*., 2004). However, this study showed that incubation of Akata cells with TSA even at concentrations $\leq$100 nM significantly induced apoptosis of the cells. Although it was known that malignant phenotype of Akata cells is dependent on the presence of EBV (Shimizu *et al*., 1994) and these cells are resistant to apoptosis (Komano *et al*., 1998), our findings suggest that EBV-infected Akata cells are quite sensitive to the TSA-mediated apoptosis. In addition, flow cytometric analysis of Akata cells showed the migration of many cell populations to the sub-G_0/G_1 phase after the TSA treatment without the arrest of G_0/G_1 or G_2/M phase of the cell cycle progression. This suggests that TSA-induced apoptosis of these cells is independent on cyclin-dependent kinase (CDK) inhibitors such as p21 and p27 (Wharton *et al*., 2000). Moreover, a sensitive induction of TSA-mediated apoptosis was seen in both the EBV-negative and -positive Akata cells. In this point, we could not exclude a possibility that the sensitivity of Akata cells on TSA was not dependent on the presence of EBV. To determine the exact mechanism(s) by which TSA induces a selective apoptosis induction in normal versus cancer cells, additional experiments using human primary B lymphocytes, which could be an ideal normal counterpart of EBV-infected tumorigenic cells, should be performed.

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 Bcl-2 family and is cleaved by caspase-8, in which the cytochrome c releasing factor, Bax, is activated in Fas or TNF-induced apoptosis (Kluck *et al*., 1997; Liu *et al*., 2004). Caspase-3 is activated by proteolytic cleavage such as caspase-8 and caspase-9, and believed to play a key role in the execution of apoptosis (Annunziato *et al*., 2003; Qu and Qing, 2004). Moreover, caspase-3 cleaves an essential DNA repair enzyme, PARP, and PARP cleavage activates a calcium/magnesium-dependent endonuclease, which results in internucleosomal DNA fragmentation (Yakovlev *et al*., 2000). Our results showed that the cleavage of PARP and Bid, as well as the activation of caspase-8 are associated with TSA-induced apoptosis in EBV-infected Akata cells, thereby indicating that TSA-mediated apoptosis of these cells might be caspase dependent. This was supported by the results that z-VAD-fmk, rather than z-FA-fmk, markedly inhibited the TSA-induced cytotoxicity and apoptosis of the cells.

Cytochrome c releasing factor, Bid, is a caspase-8 substrate, while the most critical apoptotic protease, caspase-3, is a substrate of caspase-8 or -9. The caspase-8 and -9 specific inhibitors, z-IETD-fmk and z-LEHD-fmk, significantly reduced the TSA-induced cytotoxicity of EBV-infected Akata cells. z-IETD-fmk, but not z-LEHD-fmk, also inhibited the TSA-induced apoptosis of the cells. This inhibition is believed to be associated with the suppression of TSA-mediated cleavage of Bid and PARP proteins. Particularly, however, the z-IETD-fmk-mediated reduction of TSA-induced apoptosis was not dramatic, even though there was a dose-dependent decrease in TSA-induced cytotoxicity by treating an inhibitor to the cells. This means that other caspase-dependent pathways besides caspase-8 and -9 are involved in the TSA-induced apoptosis of EBV-infected Akata cells.

Various antitumor drugs trigger mitochondrial permeabilization, which leads to caspase-9 activation that stimulates the activation of caspase-3 and -7 (Adrain and Martin, 2001). On the other hand, the apoptotic pathway can be also triggered by cell surface death receptor such as the Fas and TNF receptor families. These activate caspase-8 and caspase-10, which are specific activators of mitochondrial permeabilization and apoptosome (Bouillet and Strasser, 2002). Similarly, mitochondrial stress, cytochrome c release, and expressional changes in Bcl-2 family members are another process in the apoptotic response to HDACs (Medina *et al*., 1997; Ruefli *et al*., 2001; Zhu *et al*., 2001). Indeed, TSA induced the cytosolic release of cytochrome c release and caspase-3 activation in both the EBV-negative and -positive Akata cells (data not shown, personal communication with Dr. W.-K. Lee, Myongji University). In addition, TSA arrested cell cycle progression and induced apoptosis of B-cell lymphomas by down-regulating Bcl-2 expression (Duan *et al*., 2005). This suggests the involvement of mitochondrial stress in the TSA-mediated apoptosis of Akata cells. However, the possibility that the activation of caspase-1 and -11 are associated with TSA-induced apoptosis of Akata cells could not be excluded, because the cathepsin B inhibitor, z-FA-fmk, had a mild inhibitory effect on TSA-induced apoptosis of the cells.

In summary, cell growth arrest and apoptosis induction in tumor cells is a prominent indicator of tumor treatment response when employing a bioactive material to reduce and control cancer growth (Steele, 2003). In particular, TSA affects the expression of a small subset of genes regulating the progression of cell cycle, such as cyclin A and $p21^{cip/waf}$ (Eickhoff *et al*., 2000) and modulates the apoptosis regulators, which include caspases and some proteases (Henderson *et al*., 2003). This study showed that TSA actively inhibited growth and induced apoptosis in the EBV-infected Akata cells. However, additional experiments will be needed to determine the precise mechanism(s) involved in the TSA-mediated apoptosis of these cells. In addition, further detailed studies will also be needed to elucidate how caspase activation is achieved by the TSA treatment, and how TSA is connected to the mitochondrial pathway.

Acknowledgment This work was supported by the Grant from Korea Research Foundation endowed to RCBM of Chonbuk National University (KRF-2004-005-F00022).

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