Biochemical and Biophysical Research Communications 423 (2012) 313–318

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Epstein–Barr Virus latent membrane protein 1 overcomes all-*trans* retinoic acid-induced apoptosis by inhibiting retinoic acid receptor-β_2 expression

Hyehyeon Lee, So Young Seo, Indira Tiwari, Kyung Lib Jang *

Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea

ARTICLE INFO

Article history:
Received 18 May 2012
Available online 30 May 2012

Keywords:
Apoptosis
DNA methylation
Epstein–Barr Virus
LMP1
Retinoic acid receptor-β_2

ABSTRACT

Nasopharyngeal carcinoma is closely associated with infection with Epstein–Barr Virus (EBV); however, the mechanism is still unclear. Here, we report that the EBV oncoprotein, latent membrane protein 1 (LMP1), suppresses apoptotic cell death provoked by all-*trans* retinoic acid (ATRA) in NPC cells. For this purpose, LMP1 downregulated levels of Bak whilst it upregulated levels of Bcl2, lowering the ratio of Bak to Bcl2. In addition, LMP1 suppressed ATRA-mediated activation of Caspase 9, Caspase 3, and PARP but not Caspase 8 in Ad-AH cells, suggesting that LMP1 acts by blocking the activation of intrinsic apoptosis pathway by ATRA. These effects were almost completely abolished when levels of retinoic acid receptor-β_2 (RAR-β_2) in the LMP1-expressing cells were recovered by either exogenous gene expression or treatment with a universal DNMT inhibitor, 5-Aza-2′dC, indicating that LMP1 executes its antiapoptotic effects by downregulating levels of RAR-β_2 via DNA methylation.

1. Introduction

All-*trans* retinoic acid (ATRA), the most biologically active metabolite of vitamin A, can induce apoptosis in cells derived from various human cancers, including acute promyelocytic leukemia (APL), hepatoma, breast cancer, lung cancer, and head and neck cancer [1]. Activation of either intrinsic and/or extrinsic apoptosis pathways have been detected in this process [1]. For example, ATRA upregulates levels of proapoptotic Caspase 9 and Bax in MCF-7 cells [2,3] whilst it downregulates levels of antiapoptotic Bcl-2 and Survivin in other breast cancer cells [4,5]. In addition, apoptosis induced by ATRA is accompanied by downregulation of Bcl2 in neuroblastoma [6], melanoma [7], and myeloblastic leukemia cells [8]. ATRA also activates extrinsic apoptosis pathways through dysregulation of the death ligand TRAIL and death receptor Fas and upregulation of TNFα and Caspase 8 [9–11]. However, the precise mechanism by which ATRA modulates levels of these apoptotic molecules is poorly understood.

Retinoic acid receptor-β (RAR-β) has four isoforms that have different affinities to retinoid and different biological functions [12]. Of these, RAR-β_2 has attracted attention as the major executor of the antitumor potential of retinoid in various types of cancer cells [12]. Loss of its expression during cancer development is associated with tumorigenesis and retinoid resistance [13–15]; induction of its expression, on the other hand, can suppress carcinogenesis [16]. Moreover, introduction of exogenous RAR-β_2 into cervical, breast, and lung cancer cells increases cell responsiveness to growth inhibition and induction of apoptosis by retinoid [17,18], suggesting that RAR-β_2 plays a critical role in ATRA-mediated apoptosis. However, it remains to be clarified whether RAR-β_2 actually mediates the potential of ATRA to induce apoptosis.

Epigenetic alteration through DNA methylation in RAR-β_2 is common in human malignant tumors [19]. Thus, the methylation status of the RAR-β_2 can be used as a biomarker of for the early detection of malignancy [20]. In nasopharyngeal carcinoma (NPC), the incidence of promoter methylation in RAR-β_2 was 80% whereas no methylation of this gene was detected in samples of normal epithelium [21]. In addition, in comparison with other head-and-neck cancers that are not associated with Epstein–Barr Virus (EBV), the frequency of epigenetic changes is much higher in NPC that almost invariably contains EBV genomes [22]. According to our previous report, the oncogenic latent membrane protein 1 (LMP1) of EBV induces promoter hypermethylation of RAR-β_2 gene in NPC cells, resulting in downregulation of its expression and subsequent decrease in susceptibility to ATRA-induced cell cycle arrest at G_1 phase [23]. Therefore, NPC cells with or without LMP1 expression may provide an ideal model system to clarify roles of RAR-β_2 in ATRA-induced apoptosis. In the present study, we definitely show that RAR-β_2 mediates the potential of ATRA to induce apoptosis by modulating levels of apoptotic molecules in NPC cells. In addition, we demonstrate that LMP1 overcomes ATRA-induced apoptosis by repressing RAR-β_2 expression via DNA methylation.

* Corresponding author. Fax: +82 51 514 1778.
E-mail address: kljang@pusan.ac.kr (K.L. Jang).

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http://dx.doi.org/10.1016/j.bbrc.2012.05.118

2. Materials and methods

2.1. Plasmid construction

To construct the RAR-β_2-expressing plasmid pCMV-3×HA1-RAR-β_2, the full-length RAR-β_2 cDNA was amplified with the use of a primer set, RARβ−F (5′-atg gat gtt ctg tca g-3′) and RARβ−R (5′-gga act gaa ggt act g-3′) from total RNA of Ad-AH cells and then was cloned into pCMV-3×HA1, in frame, downstream of three copies of the influenza virus hemagglutinin (HA) epitope [a nonapeptide sequence (YPYDVPDYA)]. The pcDNA3-based LMP1-expression plasmid has been described before [24].

2.2. Cell lines and transfection

Ad-AH, an EBV-negative human nasopharyngeal cell line [25], was cultured in DMEM supplemented with 10% (v/v) FBS and penicillin G (100 unit/ml) and streptomycin (100 μg/ml). For transient expression, 2×10^5 cells per 60-mm dish were transfected with 1 μg of appropriate plasmid(s) with the use of the Fugene™ 6 transfection kit (Roche) or WelFect-EX™PLUS (WelGENE) following the manufacturer's instructions. Both Ad-AH-vector and Ad-AH-LMP1 were established by stable transfection with pcDNA3 and pcDNA3-LMP1, respectively, followed by selection with G418 (500 μg/ml; Life Technologies).

2.3. Western blot assay

Cells were lysed in buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors. Cell extracts were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF, Amersham). Membranes were then incubated with antibodies against RARβ, Caspase 8, Caspase 9, Caspase 3, PARP, Bax, Bcl2, Bak (Santa Cruz Biotechnology), cytochrome C (Pharmingen), LMP1 (DAKO), γ-tubulin (Sigma) for 2 h at room temperature and subsequently with the appropriate horseradish peroxidase-conjugated secondary antibodies: anti-mouse IgG (H + L)-HRP (Bio-Rad) and anti-rabbit IgG (H + L)-HRP (Bio-Rad) for 1 h at room temperature. The chemiluminescent ECL kit (Amersham) was used to visualize protein bands on X-ray films.

2.4. Growth inhibition assay

For the determination of cell number, MTT assay was performed as described before [23]. Briefly, cells were seeded at 3×10^3 per well in 96-well plates and incubated under the indicated conditions. The cells were then treated with 10 μM 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) for 4 h at 37 °C. The MTT-derived formazan developed by cells was dissolved in DMSO and quantified by measuring absorbance at 550 nm.

2.5. Cell viability analysis

Cell viability was determined by trypan blue exclusion assay [26]. Briefly, 1×10^5 cells in 6-well plates were treated with different concentrations of ATRA (Sigma) for 72 h. The cells were collected by trypsinization and 10 μl of the cell suspension was mixed with an equal volume of 0.4% trypan blue (Sigma). The percentage of viable cells were determined by counting the dead (stained) and live (unstained) cells manually under microscope using a hemocytometer.

2.6. Cell cycle analysis

Cell cycle profile was analyzed using flow cytometry. Briefly, 2×10^6 cells were trypsinized, fixed in 80% ethanol, and resuspended in 50 μg/ml propidium iodide (Sigma) containing 125 U/ml RNase A (Sigma). DNA contents were analyzed by flow cytometry using the Cell-FIT software (Becton–Dickinson Instruments).

2.7. Apoptotic DNA fragmentation assay

DNA fragmentation assay was performed as described before [27]. Briefly, 2×10^6 cells per 60-mm dish were treated with an increasing concentration of ATRA for 72 h. Cells were harvested, washed with PBS, and treated with lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5) for 5 min on ice. After centrifugation, the supernatant was treated with RNase A (5 μg/μl) for 2 h at 56 °C followed by digestion with proteinase K (2.5 μg/μl) for 2 h at 37 °C. The DNA was ethanol precipitated and analyzed by agarose gel electrophoresis.

2.8. TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using *In Situ* Cell Death Detection Kit (Roche). Briefly, 2×10^6 cells were fixed in PBS containing 1% formaldehyde for 30 min at room temperature, permeabilized with 70% ethanol for 30 min at room temperature, and then incubated with the TUNEL reaction mixture containing terminal transferase (TdT) and fluorescein-dUTP for 1 h at 37 °C. The incorporated fluorescein in individual cells was visualized by fluorescence microscopy.

2.9. Statistical analysis

The values indicate mean ± S.D. from at least three independent experiments. The difference between the means of the treatment groups and their controls were assessed with the paired two tailed *t* test; A *P* value of <0.05 was considered to be statistically significant.

3. Results

3.1. LMP1 overcomes ATRA-induced apoptotic cell death

Initially, we examined whether ATRA differentially induces apoptosis in NPC cells with or without LMP1 expression. For this purpose, Ad-AH-vector and Ad-AH-LMP1 cells were treated with 5 μM ATRA for up to 6 days, followed by MTT assay to determine their growth rates (Fig. 1A). Treatment with ATRA for 2 days did not completely inhibit but differentially affected their growth. Under this condition, the growth rate of Ad-AH-LMP1 cells was significantly higher, which is consistent to our previous observation [23]. The number of both cell lines thereafter gradually decreased with different rates: Ad-AH-vector and Ad-AH-LMP1 cells exhibited 58.1% decrease and 47.4% increase, respectively, after incubation for 6 days in the presence of ATRA. These results suggest that LMP1 can suppress ATRA-induced cell death in Ad-AH cells. To examine whether ATRA actually induces cell death in Ad-AH cells, trypan blue exclusion assay was performed to determine cell viability (Fig. 1B). As a result, ATRA dramatically decreased the cell viability of Ad-AH-vector cells in a dose-dependent manner whilst the effect was significantly weaker in the LMP1-expressing cells, indicating that LMP1 can suppress the ATRA-induced cell death in Ad-AH cells. Next, we examined whether the cell death observed in Ad-AH cells resulted from apoptosis induced by ATRA. Results

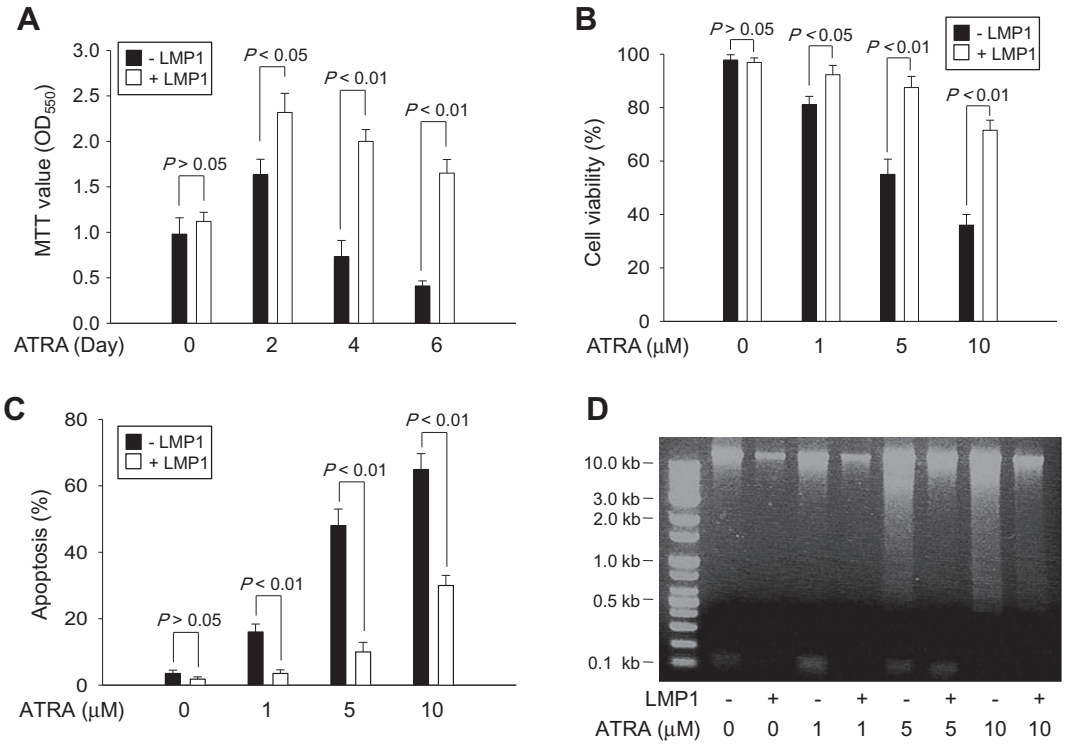

Fig. 1. LMP1 overcomes ATRA-induced apoptotic cell death. Ad-AH-vector and Ad-AH-LMP1 cells were cultivated for the indicated period in the presence of 5 μM ATRA, followed by MTT assay (A). Ad-AH-vector and Ad-AH-LMP1 cells were treated with an increasing concentration of ATRA for 72 h and were subjected to trypan blue exclusion assay (B), TUNEL assay (C), and apoptotic DNA fragmentation assay (D).

from both TUNEL assay (Fig. 1C) and apoptotic DNA fragmentation assay (Fig. 1D) clearly shows that ATRA effectively induces apoptosis of Ad-AH cells in a dose-dependent manner but the effect is much weaker in the LMP1-expressing cells. Taken together, we conclude that LMP1 can suppress ATRA-induced apoptotic cell death in NPC cells.

3.2. LMP1 suppresses activation of intrinsic apoptosis pathway by ATRA

We next investigated the mechanism by which LMP1 overcomes the ATRA-induced apoptosis in Ad-AH cells. For this purpose, we first compared protein levels of both precursor and cleaved forms of key apoptosis molecules in Ad-AH-vector and Ad-AH-LMP1 cells after treatment with an increasing concentration of ATRA (Fig. 2A). Levels of both precursor and active forms of Caspase 8, 9 and 3 in Ad-AH-vector cells were upregulated by ATRA, resulting in cleavage of poly(ADP-ribose) polymerase (PARP), suggesting that both extrinsic and intrinsic apoptosis pathways are involved in the ATRA-induced apoptosis of Ad-AH cells. Interestingly, LMP1 could suppress ATRA-mediated activation of Caspase 8, Caspase 3, and PARP but not Caspase 8 in Ad-AH cells, suggesting that LMP1 suppresses ATRA-induced apoptosis by blocking activation of intrinsic apoptosis pathway.

To investigate whether ATRA differentially affects levels of key regulators in intrinsic apoptosis pathway in the presence or absence of LMP1, we compared levels of proapoptotic Bax and Bak, and antiapoptotic Bcl2 in Ad-AH cells with or without LMP1 expression (Fig. 2B). ATRA upregulated levels of Bak whilst it downregulated levels of Bcl2 in Ad-AH-vector cells. On the other hand, LMP1 downregulated levels of Bak whilst it upregulated levels of Bcl2 both in the presence and absence of ATRA. Levels of proapoptotic Bax were little affected by ATRA in both cell lines. As a consequence, levels of cytochrome C released to the cytosol were much higher in the control cells compared to the LMP1-expressing cells. These results suggest that LMP1 suppresses the ATRA-mediated activation of intrinsic apoptosis pathway at least in part by lowering the ratio of Bak to Bcl2.

To confirm that activation of intrinsic pathway is required for ATRA-induced apoptosis, a specific Caspase 9 inhibitor, Z-LEHD-fmk, was employed to block this pathway. Under the condition, the effects of ATRA on Caspase 9, Caspase 3, and PARP were almost completely abolished in the Ad-AH-vector cells (Fig. 2C). In addition, Z-LEHD-fmk also dramatically lowered the potential of ATRA to induce apoptosis in Ad-AH-vector cells to that in the LMP1-expressing cells (Fig. 2D). Taken together, we conclude that LMP1 suppresses apoptosis by inhibiting the ATRA-mediated activation of intrinsic apoptosis pathway.

3.3. Downregulation of RAR-β_2 by LMP1 is responsible for inhibition of intrinsic apoptotic pathway

Several studies have suggested that RAR-β_2 is the key receptor mediating the antitumor potential of retinoid [12,18]. In addition, our previous report also has demonstrated that LMP1 downregulates levels of RAR-β_2 in NPC cells via DNA methylation [23]. Consistently, ATRA upregulated levels of RAR-β_2 in Ad-AH-vector cells but the potential was severely impaired in the presence of LMP1 (Fig. 3A). Interestingly, levels of RAR-β_2 were directly proportional to those of Bak, both precursor and cleaved forms of Caspase 9 and Caspase 3, and the active form of PARP but inversely to those of Bcl2, suggesting that RAR-β_2 mediates the potential of ATRA to induce apoptosis in Ad-AH-vector cells but is dysfunctional in LMP1-expressing cells. In addition, the effects of LMP1 on these apoptotic molecules were almost completely abolished as levels of RAR-β_2 in LMP1-expressing cells were recovered to those in the control cells

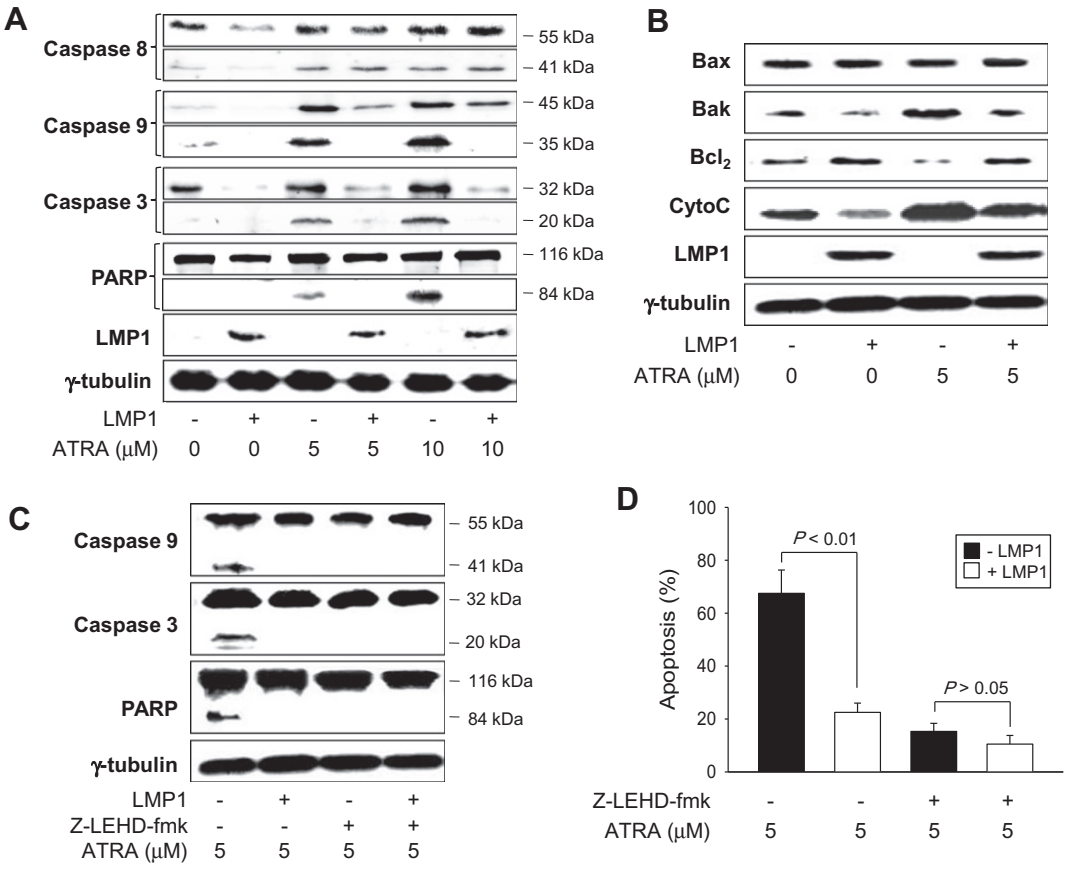

Fig. 2. LMP1 suppresses activation of intrinsic apoptosis pathway by ATRA. (A) Ad-AH-vector and Ad-AH-LMP1 cells were treated with an increasing concentration of ATRA for 72 h. The precursor and cleaved forms of Caspase 8, 9, 3, and PARP were detected from 5 μg and 20 μg of total proteins, respectively, by Western blot analysis. (B) Ad-AH-vector and Ad-AH-LMP1 cells were either mock-treated or treated with 5 μM ATRA for 72 h, followed by Western blot analysis. (C) Ad-AH-vector and Ad-AH-LMP1 cells were treated with 5 μM ATRA for 72 h in the presence or absence of 70 μM Z-LEHD-fmk. (D) Cells prepared as describe in Fig. 2C were subjected to TUNEL assay.

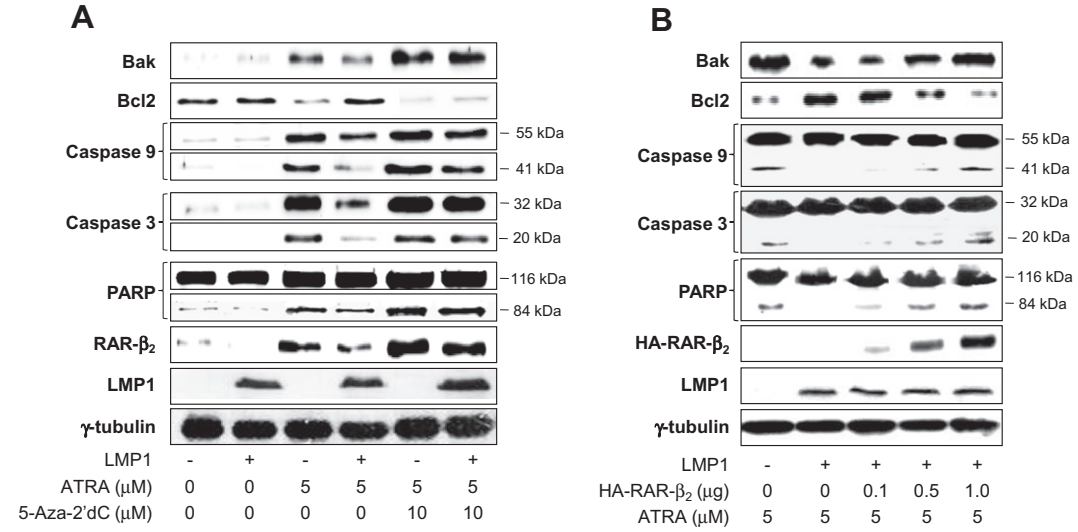

Fig. 3. Downregulation of RAR-β_2 by LMP1 is responsible for inhibition of intrinsic apoptotic pathway. (A) Ad-AH cells with or without LMP1 expression were either mock-treated or treated with 5 μM ATRA in the presence or absence of 10 μM 5-Aza-2′dC. Western blot analysis was performed as described in Fig. 2A. (B) Ad-AH-LMP1 cells were transiently transfected with an increasing amount of RAR-β_2-expression plasmid in the presence of 5 μM ATRA and their protein levels were compared to those in the control cells.

by addition of a universal DNMT inhibitor, 5-Aza-2′dC (Fig. 3A), suggesting that LMP1 executes its antiapoptotic potential by downregulating levels of RAR-β_2 via DNA methylation.

To confirm that RAR-β_2 mediates the potential of ATRA to activate intrinsic apoptosis pathway and its downregulation is responsible for the impaired induction of the pathway in the presence of

LMP1, we attempted to complement RAR-β_2 in the LMP1-expressing cells by exogenous introduction of RAR-β_2-expression plasmid. As RAR-β_2 was complemented in the LMP1-expressing cells, levels of Bak, Bcl2, and active forms of Caspase 9, Caspase 3 and PARP in these cells became similar to those in the control cells (Fig. 3B). Taken together, we conclude that LMP1 inhibits ATRA-mediated activation of intrinsic apoptosis pathway by downregulating levels of RAR-β_2 via DNA methylation.

3.4. LMP1 overcomes ATRA-induced apoptosis by downregulating levels of RAR-β_2 via DNA methylation

Lastly, we investigated whether downregulation of RAR-β_2 via DNA methylation is responsible for the impaired induction of apoptosis by ATRA in the LMP1-expressing cells. Treatment with 5 μM ATRA for 3 days induced drastic morphological changes (Fig. 4A) and increased apoptotic DNA fragmentation (Fig. 4B) and sub G_1 fraction (Fig. 4C) in the control cells. In contrast, these effects were weaker or inappreciable in the LMP1-expressing cells. In the presence of 5-Aza-2′dC, however, the potential of LMP1 to suppress ATRA-induced apoptosis was almost completely abolished, as demonstrated by altered cell morphology (Fig. 4A), increased apoptotic DNA fragmentation (Fig. 4B) and sub G_1 fraction (Fig. 4C) in the LMP1-expressing cells. Therefore, we conclude that LMP1 suppresses ATRA-induced apoptosis by downregulating levels of RAR-β_2 via DNA methylation.

4. Discussion

NPC is a human squamous cell cancer prevalent in Asia and is closely associated with infection with a ubiquitous human herpesvirus, EBV [28]. LMP1 is considered to be the major oncoprotein of EBV. It induces a variety of cellular processes such as morphological changes, proliferation, survival, motility and invasion in epithelial cells, many of which may account for its oncogenic properties [29]. In particular, suppression of the cellular apoptotic program by LMP1 seems to be critical for both the establishment of latent infection and the development of EBV-associated malignancies [30]. The mechanism by which LMP1 protects cells from apoptosis is relatively well defined. LMP1 confers a survival advantage on EBV-infected B cells by activating antiapoptotic genes, such as those encoding A20, Bfl-1, Survivin, and Bcl2 through NF-κB/AP-1-dependent mechanisms [29]. In addition, the PI3-K/Akt pathway activated by LMP 1 phosphorylates and inactivates the proapoptotic Bad and Foxo3a proteins [31]. Consistently, the present study also shows that LMP1 protects cells from apoptosis provoked by ATRA by modulating levels of several proapoptotic and antiapoptotic molecules involved in the intrinsic apoptosis pathway.

Several reports have shown that ATRA induces apoptosis by modulating levels of several proapoptotic and antiapoptotic molecules involved in both extrinsic and intrinsic pathways. It upregulates levels of Caspase 9, the initiator caspase of the intrinsic apoptosis pathway, in MCF-7 mammary carcinoma cells because the promoter of caspase 9 gene contains an RARE and thus serves a direct target for RAR [2]. It has been also demonstrated that ATRA elevates levels of caspase 3, 6, 7, and 9 in keratinocytes [32]. Overexpression of these proteases may trigger apoptosis or increase the susceptibility of cells to apoptosis-inducing agents. ATRA-induced apoptosis is also associated with either upregulation of proapoptotic molecules (e.g. Bax) or downregulation of antiapoptotic proteins (e.g. Bcl-2 and survivin) or both depending on cellular context [3–5,7]. The present study also shows that ATRA not only upregulates levels of Caspase 3, 8, and 9 but also increases their activities in NPC cells. In addition, ATRA elevates the ratio of Bak

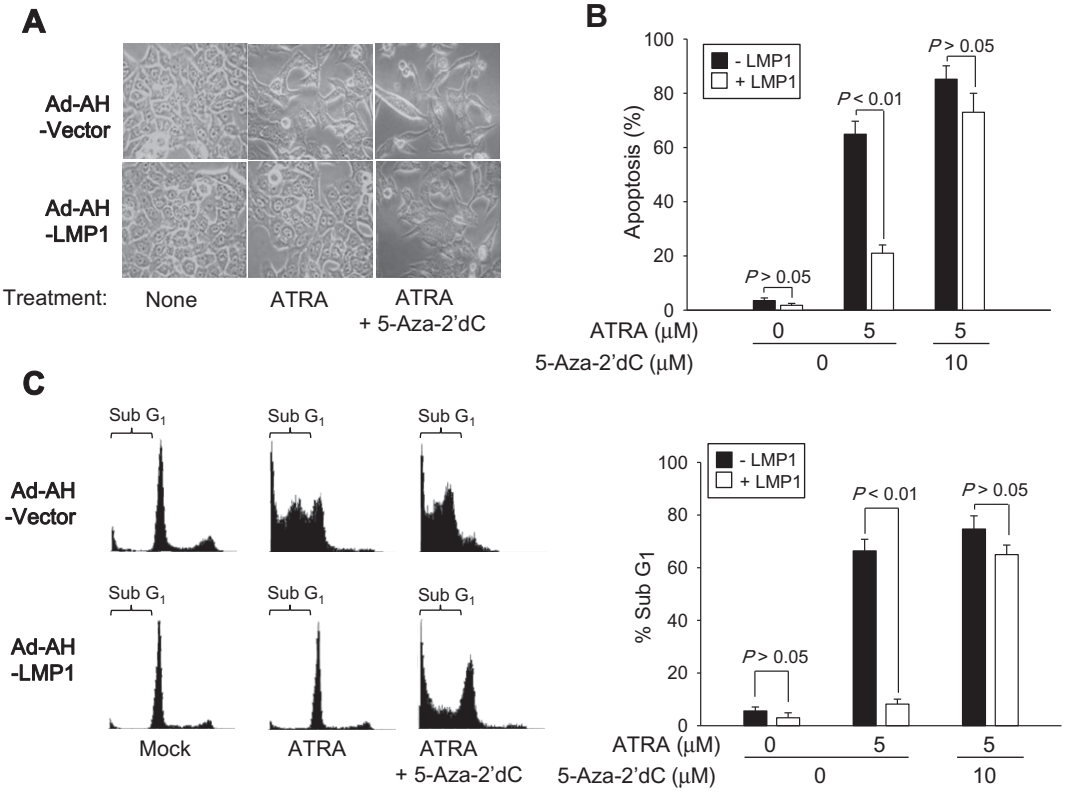

Fig. 4. LMP1 overcomes ATRA-induced apoptosis by downregulating levels of RAR-β_2 via DNA methylation. Cells were prepared as described in Fig. 3A, followed by microscopic observation (A) and TUNEL assay (B). In addition, FACS analysis was performed to analyze the cell cycle profiles (C, left) and Sub G1 fractions (C, right) of these cells.

to Bcl2 by upregulating levels of Bak and downregulating levels of Bcl2. However, levels of Bax were little affected by ATRA, which is inconsistent to the previous report [4]. Therefore, the detailed mechanism by which ATRA induces apoptosis in NPC cells seems to be rather distinct compared to those observed in other cells.

Although both extrinsic and intrinsic pathways seem to be involved in the ATRA-induced apoptosis of Ad-AH cells, the latter pathway appears to be more critical in this process. First, treatment of a Caspase 9 inhibitor, Z-LEHD-fmk, could almost completely abolish the potential of ATRA to induce apoptosis. Second, LMP1 could overcome ATRA-induced apoptosis in NPC cells by blocking activation of intrinsic pathway by ATRA without affecting the extrinsic pathway. For this purpose, LMP1 downregulates levels of Caspase 3 and 9 in the presence of ATRA. In addition, LMP1 downregulates levels of Bak whilst it upregulates levels of Bcl2 and thus lowers the ratio of Bak to Bcl2, leading to inactivation of Caspase 9. However, the extrinsic pathway cannot be completely excluded in the ATRA-induced apoptosis of NPC cells as demonstrated by apoptosis of some LMP1-expressing cells by ATRA in the presence of high doses of ATRA.

RAR-β_2 is considered to be the key mediator of ATRA-induced apoptosis in a various type of cancer cells [1,5,12,16–18]. Consistently, ARTA upregulates levels of RAR-β_2 in NPC cells but not in the presence of LMP1. In addition, complementation of RAR-β_2 in the LMP1-expressing cells via exogenous expression almost completely abolishes the inhibitory effects of LMP1 on the activation of intrinsic apoptosis pathway by ATRA. Therefore, it is possible to assume that RAR-β_2 mediates the potential of ATRA to induce apoptosis in NPC cells and this potential can be suppressed by LMP1 via downregulation of RAR-β_2 expression. The next question is then how LMP1 downregulates expression of RAR-β_2 in NPC cells? Epigenetic alteration through DNA methylation in RAR-β_2 is common in human malignant tumors including NPC [25]. In addition, LMP1 induces the expression and activity of the DNMT 1, 3a, and 3b and downregulates levels of RAR-β_2 via DNA methylation [23,33]. Consistently, treatment with a universal DNMT inhibitor, 5-Aza-2′dC, almost completely abolishes the potentials of LMP1 to modulate levels of apoptotic molecules and thus impairs the ability of LMP1 to suppress ATRA-induced apoptosis in Ad-AH cells. In addition, according to our previous report, LMP1 suppresses the growth-inhibitory effect of ATRA by inhibiting RAR-β_2 expression via DNA methylation [23]. Therefore, it is likely that RAR-β_2 mediates the anti-cancer potentials of ATRA in NPC cells and serves a prime target for LMP1 to overcome these effects.

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