Acta virologica **58**: 76 – 80, 2014 doi:10.4149/av_2014_01_76

The effect of Astragalus polysaccharide on the Epstein-Barr virus lytic cycle

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Received August 13, 2013; accepted January 24, 2014

Summary. – Effects of a polysacharide from Chinese herbal plant *Astragalus membranaceus* (APS) on the expression of Epstein-Barr virus (EBV) immediate early proteins Zta, Rta and EA-D in Raji cells were examined. EBV switch from latent to lytic cycle in Raji cells was induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) and sorbol butyrate (SB) and the effects of APS were examined by immunofluorescence, western blotting and flow cytometry. APS in a non-cytotoxic concentration of 30 μg/ml significantly suppressed the expression of Zta, Rta and EA-D during the EBV lytic cycle. Our observations indicate that APS is potentially useful as an anti-EBV drug.

Keywords: Astragalus polysaccharide; Epstein-Barr virus; anti-EBV drug

Introduction

Epstein-Barr virus (EBV), a human B lymphotropic herpesvirus, infects at least 90% of the world's human population. Primary infection is usually asymptomatic in children, but in adolescents and in young adults EBV can cause infectious mononucleosis, with fever and enlargement of tonsils, lymphnodes, liver and spleen (Henle *et al.,* 1968). EBV is also associated with several hematologic neoplasias, such as Burkitt lymphomas and nonhematologic undifferentiated nasopharyngeal, carcinoma and gastric cancer (Thorley-Lawson, 2001). In immunosuppressed individuals, EBV may lead to lymphoproliferative disorders as non-Hodgkin lymphomas (Stevens *et al.,* 2001; Navarro and Kaplan, 2006). Following primary infection in immunocompetent individuals, the virus generally establishes lifelong persistence in B lymphocytes. Most EBV-infected B cells in the healthy host show a resting phenotype with very limited expression of the viral genome. In other words, the virus is typically latent (Miyashita *et al.,* 1997). However, the virus has to enter a lytic cycle to proliferate. The switch from latent to lytic EBV infection is mediated by expression of the two EBV immediate-early viral proteins, Zta and Rta. Zta and Rta encode transcriptional activators that activate the complete cascade of lytic viral gene expression. In the lytic form of viral infection, many additional viral genes are expressed, the virus is replicated by a virally encoded DNA polymerase and infectious viral particles are released. Without Zta or Rta, the virus cannot complete its lytic cycle (Feederle *et al.,* 2000).

The EBV reactivation from latency and persistent replication contribute to viral spread and disease pathogenesis. The current treatment strategy for EBV is to eradicate replication and infection of EBV *in vivo* using anti-virus drugs such as acyclovir and ganciclovir (Gershburg and Pagano, 2005). However, because of their side effects, low antiviral potency and long treatment period, the actual effects of these treatments are neither ideal nor adequate. Therefore, today, there is an urgent need to search for alternative therapies that may have a similar degree of efficacy. Chinese herbal medicine has been used for diseases for thousands of years in China, and their efficiency has been confirmed by modern biological technology. The identification of compounds from medicinal plants may provide an opportunity to develop a new

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Abbreviations: APS = Astragalus polysaccharide; EBV = Epstein-Barr virus; SB = sorbol butyrate; TPA = 12-O-tetradecanoylphorbol-13-acetate

class of antivirus agents. APS is one of the main efficacious compounds of radix Astragali (*Astragalus membranaceus*), which has been used in medicine for centuries in China and is believed to exhibit immune-stimulatory, anti-viral, anti-oxidation and anti-tumor effects (Dang *et al.,* 2004; Yuan *et al.,* 2008).

The aim of this study was to investigate the effects of APS on the expression of immediate early EBV proteins during lytic cycle in Raji cells.

Materials and Methods

Astragalus polysaccharide. APS was purchased from Shanxi Undersun Biomedtech Co. Ltd., China.

Cells. EBV-positive Raji cells were grown in RPMI 1640 medium (Gibco) containing 10% FBS. After culturing for 24 hr, the cells were treated with APS. After incubating for 2 hr, Raji cells were treated with 50 nmol/l TPA and 3 mmol/l SB (Sigma-Aldrich) to induce the EBV lytic cycle.

Immunofluorescence assay. Raji cells were plated on poly-L-lysine-coated overslips and fixed with 3.7% paraformaldehyde for 20 min at 4°C. The cells were washed twice times with PBS and permeabilized with 0.5% Triton X-100 for 10 min at RT. Cells were rinsed twice with PBS, and non-specific binding was reduced by blocking with 1% BSA for 30 min. After washing twice, the cells were incubated with anti-Zta (dilution 1:50, Santa Cruz), anti-Rta (1:100, Argene) and anti-EA-D (1:200, ABI) antibodies for 1 hr, and then with FITC-conjugated IgG antibody (Zhongshan Golden Bridge) for 30 min at RT in the dark. After staining with DAPI (Sigma), the percentage of EA-D-positive cells and the expression of Zta, Rta, EA-D was examined under a fluorescence microscope (Leica).

Western blot analysis. Approximately 2×10^6 Raji cells were harvested at indicated time points. The cells were washed twice by ice cold PBS, and the whole-cell lysates were prepared using radioimmunoprecipitation assay lysis buffer (Applygen Technologies, China) according to the manufacturer's protocol. Protein concentration of the cell lysates was determined by BCA Protein Assay Kit (Pierce). Equal amounts of proteins (50 μg) were subjected to SDS-PAGE and then electroblotted to PVDF membrane (Millipore). The blots were blocked with 5% skimmed milk and then incubated with the antibodies against Zta, Rta and EA-D, followed by washing and incubating with HRP-conjugated secondary antibodies (Zhongshan Golden Bridge, China). Equal protein loading was verified by detection of GAPDH with the specific antibody (Kangchen, China). Bands were visualized by the ECL system (Amersham Pharmacia Biotech).

Flow cytometry. Raji cells were incubated with antibodies as described for immunofluorescence assay, resuspended in 1% paraformaldehyde and analyzed with a FACS Calibur flow cytometer (BD Biosciences).

MTT assay. Viability of Raji cells was assessed using MTT dye reduction assay (Sigma). Cells were seeded in a 96-well plate at a density of 1×10^4 cells/well, cultured for 24 hr, and then treated with different concentrations (0, 7.5, 15, 30, and 60 μg/ml) of APS. At the end of the treatment, MTT was added and the cells were incubated for another 4 hrs. DMSO was added to each well after removal of the supernatant. After shaking the plate for 10 min, cell viability was assessed by measuring the absorbance at A_{490} using an Enzyme-labeling instrument (Multiskan Mk3); all measurements were performed three times.

Statistical analysis. Data representing the means of values from three independent experiments are presented as means + error of means. The statistical significance of differences was tested by Student´s t-test using the Statgraphics Plus software (Statistical Graphics Corp.). A difference with $P \leq 0.05$ was considered significant.

Results

The cytotoxicity effect of APS on proliferation of Raji cells

In order to investigate whether APS treatments affected cell viability, the toxicity of APS to Raji cells was determined using the MTT method. The APS at concentrations of 15 μg/ml and 30 μg/ml killed only 10% and 15% of Raji cells after 48 hr of culturing (Fig. 1). The APS at the concentration of 60 μg/ml killed almost 55%. These results demonstrated that APS did not influence the cell viability at concentrations under 30 μg/ml.

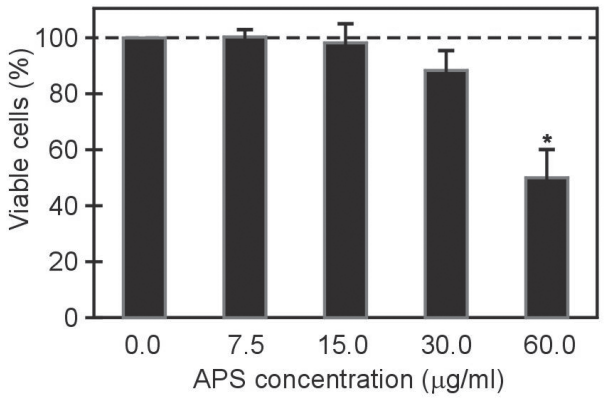

Fig. 1

The cytotoxicity of APS

MTT assay was used to detect the viability of Raji cells treated with APS after lytic induction. The data are presented as means ± SD from three independent experiments. *P <0.05 represents a significant difference from the values of the control group.

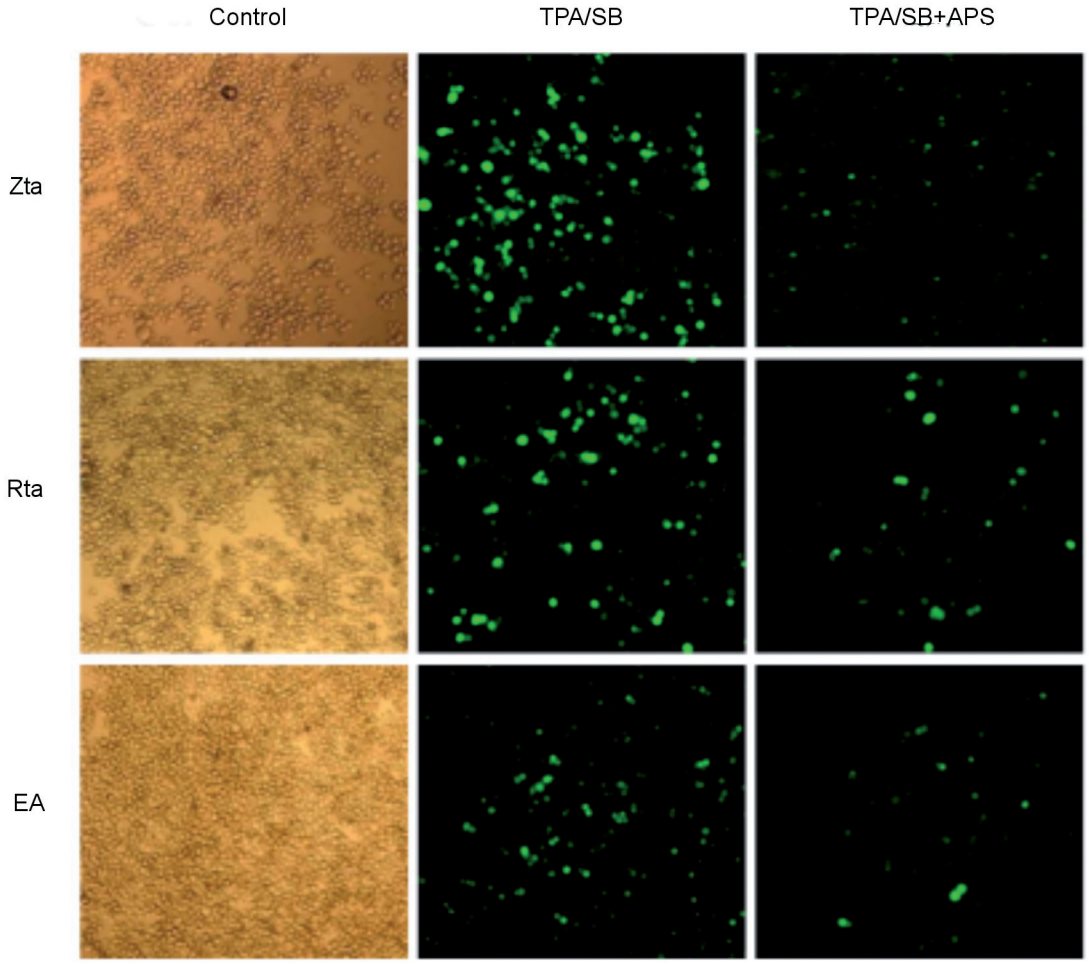

Fig. 2

Immunofluorescence analysis of APS effects on the expression of immediate early EBV proteins
The effects of 30 μg/ml APS on Zta, Rta and EA-D were examined 48 hr after addition of TPA/SB and APS to Raji cells.

Effects of APS on the expression of immediate early EBV proteins

The EBV-positive cell line Raji, treated with TPA/SB for 48 hr to induce the EBV lytic cycle, were incubated in the absence or presence of 30 μg/ml APS before induction. As shown in Fig. 2, Zta, Rta and EA-D were expressed 48 hr after TPA/SB treatment. The treatment with 30 μg/ml APS prior to lytic induction significantly inhibited the expression of these three poteins.

Protein profile of lytic EBV infection of Raji cells is shown in Fig. 3. Raji cells were treated with TPA/SB for 48 hrs to induce the EBV lytic cycle. As expected, Zta, Rta, and EA-D level strongly increased during incubation with EBV lytic cycle inducers. However, the expression was inhibited in the cells induced in the presence of the APS. APS down-regulated Zta, Rta, and EA-D expression. Also, APS was found to down-regulate their expression in a dose-dependent manner. These results indicated that APS inhibits the expression of EBV lytic proteins.

The expression of lytic proteins was further confirmed by flow cytometry analysis. The percentage of Raji cells that expressed Zta, Rta and EA-D was 57.2%, 70.8%, and 55.1%, respectively, following TPA/SB treatment (Fig. 4). Notably, after adding 30 μg/ml APS before lytic induction, the percentage of Zta, Rta and EA-D-positive cells was dramatically decreased to 29.4%, 40.6%, and 14.3% (Fig. 4), respectively. We noted that APS treatment resulted in a significant disappearance of the positive cells, possibly implying that viral lytic proteins were blocked by APS.

Discussion

EBV is one of the most successful viruses, which ubiquitously infect humans and persist for the lifetime (Bornkamm and Hammerschmidt, 2001). Primary EBV infection is usually asymptomatic in childhood however it may induce acute infectious mononucleosis (Cohen, 2000). An earlier study demonstrated that the lytic reactivation of EBV in B lymphocytes may be an important step toward the oncogenesis of epithelial cells, including nasopharyngeal carcinoma (Feng *et al.,* 2000). The viral lytic reactivation, which produces infectious virions, is likely to be important for periodic expansion of the pool of virus-infected cells within the host and for horizontal transmission of the virus (Hopwood *et al.,* 2002). For the treatment of herpesvirus infection, it is recommended to use herpesvirus-specific drugs, including acyclovir and ganciclovir. Both agents in this class of compounds are activated by phosphorylation by virus-encoded thymidine kinase. The resulting nucleoside triphosphate analog inhibits viral DNA replication by termination of DNA chain during elongation and by direct

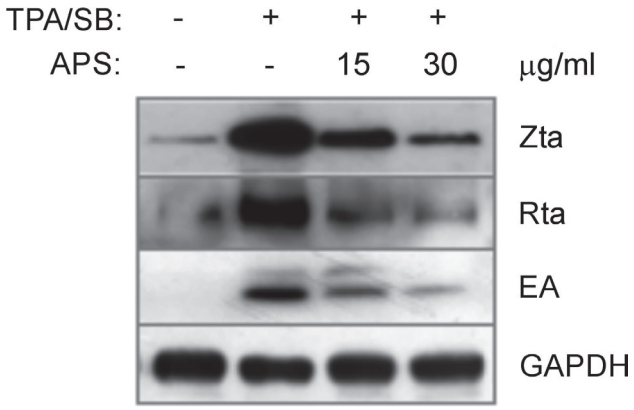

Fig. 3

Western blot analysis of APS effects on the expression of immediate early EBV proteins
The effects of 15 μg/ml and 30 μg/ml APS on Zta, Rta and EA-D were examined 48 hrs after addition of TPA/SB and APS to Raji cells.

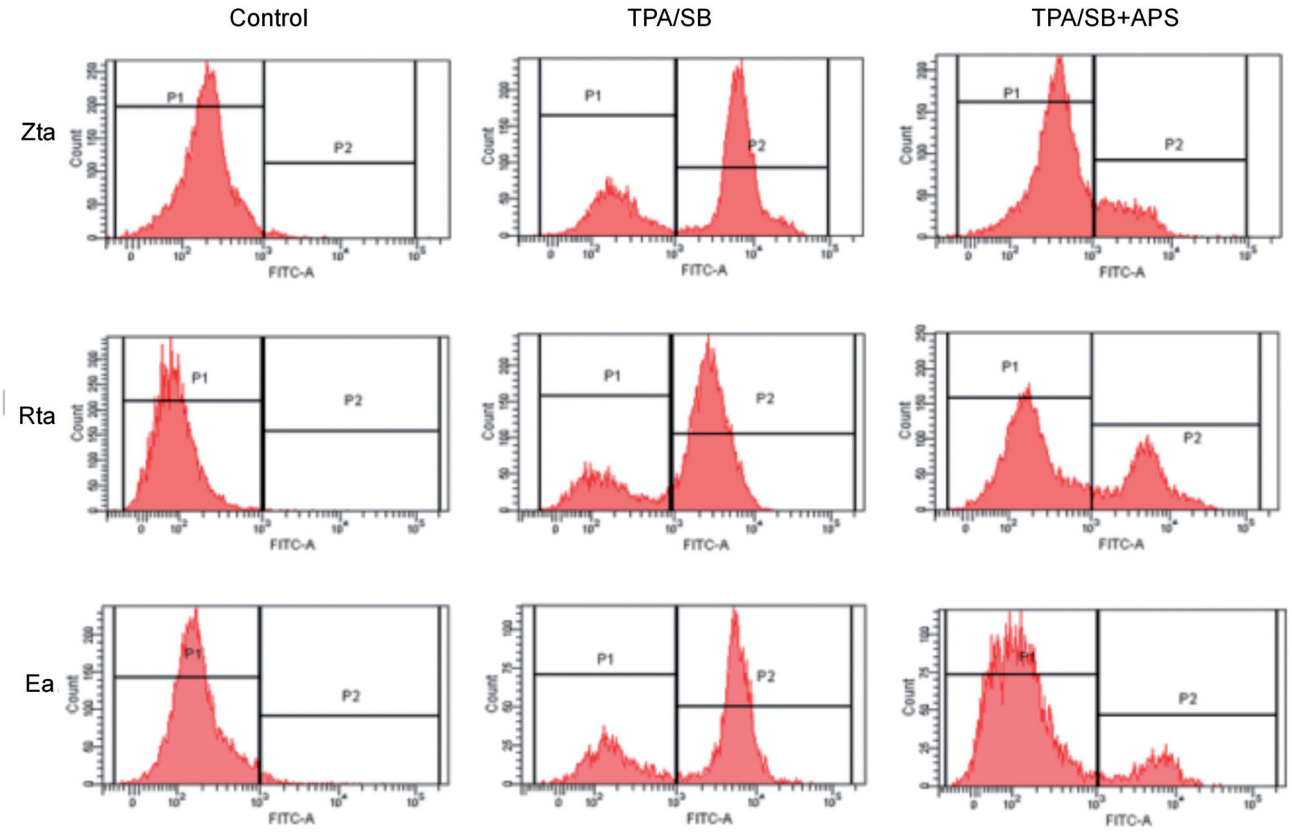

Fig. 4

Flow cytometry analysis of APS effects on the expression of immediate early EBV proteins
The effects of 30 μg/ml APS on Zta, Rta and EA-D were examined 48 hr after addition of TPA/SB and APS to Raji cells.

inhibition of herpesvirus DNA polymerase (Gershburg and Pagano, 2005). However, because of their side effects, low antiviral potency, and long treatment period, the actual effects of these treatments are neither ideal nor adequate. Therefore, development of an effective strategy to inhibit the lytic cycle may be valuable in reducing the disease risk.

Traditional Chinese medicine and herbal medicine in particular have been used in the treatment of virus for thousands of years. APS has been used in medicine for centuries in China and is believed to exhibit anti-viral effects (Dang *et al.,* 2004; Yuan *et al.,* 2008). No detailed investigation of the mechanism of this antiviral activity has yet been published. In this study we have found that APS inhibits the EBV lytic cycle in three cell lines. When Raji, B95-8 and Daudi cells were treated with TPA/SB, a large increase in the number of cells expressing the replicative antigens was observed. Concomitant treatment with APS resulted in a reduction of the number of cells supporting viral replication, indicating that APS blocked the EBV replicative cycle.

Zta and Rta play a critical role in the life cycle of EBV. They are the first proteins expressed in EBV reactivation. The expression of Zta and Rta switches EBV infection from the latent to lytic state, leading to the replication and dissemination of EBV (Hicks *et al.,* 2001). It was of interest to compare the level of their expression in induced and APS-treated induced cells. The results revealed that APS markedly reduced the expression of the EBV immediate-early proteins, Zta, Rta and EA-D using different experimental approaches. Overall, our results indicate that TPA/SB lead to a significant induction of EBV replication and indeed APS inhibits such induction. Furthermore, our results also revealed that APS has low cytotoxicity toward Raji cells when concentration below 30 μg/ml is used. Thus, it is potentially useful in preventing the lytic development of EBV and other related herpesviruses.

Acknowledgements. The authors thank Lu Qian, Institute of Basic Medical Sciences, Beijing for helpful discussions.

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