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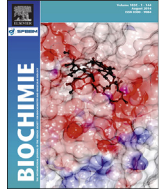

Research paper

Sodium arsenite induces apoptosis and Epstein–Barr virus reactivation in lymphoblastoid cells

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ABSTRACT

Epstein–Barr virus (EBV) is associated with several malignancies, including carcinomas, such as nasopharyngeal carcinoma, and lymphomas, such as Burkitt's lymphoma and Hodgkin's lymphoma. The Latent Membrane Protein 1 (LMP1) is the major oncogene protein of EBV as its expression is responsible for the induction of cell transformation, immortalization and proliferation. Arsenic trioxide was shown to induce a cytotoxic effect on nasopharyngeal cancer cells associated with LMP1 down-regulation. However, the effect of arsenic on EBV-associated lymphoproliferative malignancies has been less studied.

We investigated the effect of two different arsenical compounds, arsenic trioxide (As_2O_3) and sodium arsenite ($NaAsO_2$) on the induction of cell death in P3HR1 cells, an Epstein–Barr virus-positive Burkitt lymphoma derived cell line. Both compounds inhibited cell growth and induced cell death. By flow-cytometry and Western blot analysis, we provide evidence that $NaAsO_2$ induced caspase-dependent apoptosis whereas As_2O_3 triggered autophagic cell death. Furthermore, we show that $NaAsO_2$ treatment led to a dramatic decrease of the expression level of LMP1 and the cellular protein PML. Importantly, this down-regulation was associated with a reactivation of EBV lytic cycle through the induction of immediate-early proteins Zta and Rta. These results are in agreement with a model in which LMP1 maintains EBV in a latent state by stabilizing PML expression. Altogether, our results suggest that $NaAsO_2$ would represent a better therapeutic candidate than As_2O_3 in EBV-induced B lymphoma for its capacity to promote viral reactivation.

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1. Introduction

Epstein–Barr virus (EBV) is a human virus member of the *Herpesviridae* family and is the causative agent of infectious mononucleosis. EBV is also associated with a variety of cancers, including Burkitt's lymphoma, Hodgkin's lymphoma and nasopharyngeal carcinoma [1]. An important part of EBV oncogenicity lies in its ability to infect and induce proliferation of B lymphocytes, where it usually remains latent. However, in a small percentage of cells, latent EBV can become reactivated and undergo lytic replication. EBV can establish three distinct latency types, each having its own distinct pattern of gene expression: Type I latency, which is found in Burkitt's lymphoma, type II latency, which is exemplified in nasopharyngeal carcinoma and Hodgkin's disease and type III latency, which is typical of early phases of EBV lymphoproliferative syndromes. Most EBV-infected cell lines such as lymphoblastoid cell lines (LCLs) are in type III latency and express six nuclear antigens (EBNAs), three latent membrane proteins (LMP1, LMP2A and LMP2B) and the non-coding EBER-1 and -2 RNAs.

LMP1 is the major transforming protein of EBV and is a *bona fide* oncogene as it has the ability to transform rodent fibroblasts [2–4]. Its expression is required for EBV-mediated immortalization of B-cells to proliferating LCLs [4,5]. Furthermore, LMP1 maintains EBV in latency by preventing its reactivation through different mechanisms [6,7].

LMP1 acts as a constitutively active ligand-independent receptor, and triggers multiple cell signaling events that induce cell survival and growth. LMP1 was shown to up-regulate interferon stimulated gene products (ISGs), thus conferring viral resistance

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[8,9]. Ectopic expression of LMP1 in human lung adenocarcinoma and nasopharyngeal carcinoma cells increases promyelocytic leukemia (PML) expression and the intensity of PML nuclear bodies (NBs) observed by immunofluorescence [10]. In contrast, down-regulation of PML expression or its arsenic trioxide- (As_2O_3)-induced degradation is associated with an inhibition of LMP1 expression, EBV reactivation and cell death in nasopharyngeal carcinoma cells [10,11], thus suggesting that maintenance of viral latency by LMP1 was dependent of PML expression and PML NB integrity [10]. Since As_2O_3 treatment can restore cell susceptibility to ganciclovir, it was proposed as a potential therapeutic agent for EBV-induced cancers and co-treatment of As_2O_3 and ganciclovir was found to reduce tumor volume in a murine xenograft model of nasopharyngeal carcinoma [12].

Altogether, these observations strongly suggest that PML and LMP1 expression are tightly connected to maintain EBV latency and regulate its reactivation, *via* a mechanism that has not yet been elucidated.

PML is an ISG product implicated in both intrinsic and innate immunity [13]. PML is expressed in the cytoplasm, in the nucleoplasm and in the nuclear matrix-associated NBs. PML NBs are multiprotein structures present in cell nuclei of almost all human tissues and PML is essential for their formation [14]. The involvement of PML and PML NBs in cell development, apoptosis, senescence, cell signaling and antiviral defense underline the multiple functions of PML due to its ability to interact with various partners [15–19].

PML protein is fused to the retinoic acid receptor alpha in patients suffering from acute promyelocytic leukemia (APL). Treatment of APL patients with As_2O_3 reverses the disease phenotype by a process involving the proteasome-dependent degradation of the fusion protein *via* its PML moiety and induction of both apoptosis and differentiation of leukemic blasts [20]. Since As_2O_3 is a therapeutic agent and has potent cytotoxic and antitumor activities *in vitro* and *in vivo*, numerous studies have been conducted to elucidate molecular mechanisms responsible for its cytotoxicity in normal and cancer cells. Among the various forms of cell death, three main morphologies have been described: apoptosis, necrosis and autophagy [21]. Apoptosis is a process of programmed cell death that involves the coordinated action of proteases and nucleases. Morphological characteristics of apoptosis include DNA fragmentation, plasma membrane blebbing, cell shrinkage, chromatin condensation and cellular decomposition into membrane-bound apoptotic bodies. Necrosis, or necrotic cell death refers to a type of cell death that provokes a gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent leakage of cell constituents into the extracellular space. Autophagy is a programmed homeostatic response to diverse types of cellular stress that disposes of long-lived proteins, organelles, and invading microbes within double-membrane vesicles called autophagosomes. Unlike apoptosis or necrosis, autophagy can also play a cell-adaptive role for survival or repair under stress conditions. There are many interconnections between autophagy, necrosis and apoptosis, as these different processes can either cooperate or inhibit each other [22–24].

As_2O_3 was shown to cause cells an oxidative stress through the generation of reactive oxygen species (ROS), resulting in the induction of caspase-dependent apoptosis (reviewed in Refs. [25–27]). However, caspase-independent death pathways have also been reported to be activated by this agent in myeloma cells and may mediate pro-apoptotic signals [28]. In addition, it was reported that As_2O_3 could also induce autophagy in certain cellular models [29–31].

In this report, we investigated the effect of two arsenical compounds on an EBV positive LCL named P3HR1. These compounds, As_2O_3 and sodium arsenite ($NaAsO_2$), are the two main forms of trivalent arsenic among inorganic arsenicals [32]. Their molecular structure is presented in Fig. 1A. First, we show that both As_2O_3 and $NaAsO_2$ trigger cell death through two distinct mechanisms. While $NaAsO_2$ induces caspase-dependent apoptosis, As_2O_3 promotes autophagy. Furthermore, we show that, unlike As_2O_3, $NaAsO_2$ induces a dramatic decrease of LMP1 and PML protein levels that is correlated with a reactivation of EBV lytic protein expression. These observations strongly reinforce the hypothesis that LMP1 maintains EBV in latency through an up-regulation of PML expression and a stabilization of PML NBs. Finally, our results suggest that $NaAsO_2$ would be a better therapeutic agent than As_2O_3 in the case of EBV-associated lymphoma.

2. Materials and methods

2.1. Chemicals and antibodies

As_2O_3 and $NaAsO_2$ were purchased from Sigma–Aldrich. Rabbit polyclonal anti-PML (H238, sc-5621), rabbit polyclonal anti-Beclin-1 (H-300, sc-11427), mouse monoclonal anti-caspase 8 (8CSP03, sc-56070) and mouse monoclonal anti-Bcl-2 (100, sc-509) antibodies were from Santa-Cruz Biotechnology. Rabbit polyclonal anti-caspase 3 antibodies were from Cell Signaling Technology, rabbit anti-LC3B and HRP-conjugate monoclonal anti-actin antibodies were from Sigma–Aldrich, Rabbit polyclonal anti-Sp100 antibodies were from Abcam (ab43151) and mouse monoclonal anti-Epstein–Barr Virus LMP1 (Clone CS.1–4) antibody was from Dako.

2.2. Cell culture

P3HR1 (ATCC® HTB-62™) cells are an EBV-positive Burkitt lymphoma derived cell line. Ramos (RA 1) (ATCC® CRL-1596™) cells are an EBV-negative B-lymphoblastoid cell line derived from a Burkitt lymphoma. Both cell lines were grown at 37 °C in RPMI-1640 medium (Gibco, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 25 mM Hepes and 1 mM sodium pyruvate. The cells were maintained in culture between 3×10^5 and 2×10^6 cells/ml.

2.3. Cell viability assay

P3HR1 cells were incubated with increasing concentrations of As_2O_3 or $NaAsO_2$ for 24, 48 or 72 h. At the indicated time, cells were re-suspended and the proportion of living cells was estimated by trypan blue exclusion assay.

2.4. Clonogenicity test

Double-layer soft agar clonogenic assay procedure was used. The underlayer containing complete culture medium supplemented with agar (Sigma) at 0.5% (w/v) serves as feeder layer. The second soft agar layer is then plated and contains 5000 cells/ml in complete culture medium supplemented with agar at 0.3% (w/v) and 1 or 5 μM of As_2O_3 or $NaAsO_2$. The plates were placed at room temperature for 10 min and incubated under standard condition for 10 days. Each experimentation condition was done in quadruplet. The colonies containing 50 cells or more were counted using inverted microscope (×40) for each well. The Plating Efficiency (PE) represents (Number of formed colony/Number of plated cells) × 100. The Surviving Fraction (SF) was calculated as following: (PE of treated cells/PE of control cells) × 100.

2.5. Western blot analysis

Cells were lysed in hot Laemmli sample buffer and boiled for 5 min. Protein extracts were loaded on a 10% (LMP1, PML, Sp100

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