Antiviral Research 96 (2012) 196–202

Contents lists available at SciVerse ScienceDirect

Antiviral Research

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

Resveratrol inhibits Epstein Barr Virus lytic cycle in Burkitt's lymphoma cells by affecting multiple molecular targets

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ARTICLE INFO

Article history:
Received 27 July 2012
Revised 4 September 2012
Accepted 5 September 2012
Available online 15 September 2012

Keywords:
Epstein Barr Virus
Resveratrol
Lytic infection
ROS
Protein synthesis
Transcription factors

ABSTRACT

Resveratrol (RV), a polyphenolic natural product present in many plants and fruits, exhibits anti-inflammatory, cardio-protective and anti-proliferative properties. Moreover, RV affects a wide variety of viruses including members of the Herpesviridae family, retroviruses, influenza A virus and polyomavirus by altering cellular pathways that affect viral replication itself. Epstein Barr Virus (EBV), the causative agent of infectious mononucleosis, is associated with different proliferative diseases in which it establishes a latent and/or a lytic infection. In this study, we examined the antiviral activity of RV against the EBV replicative cycle and investigated the molecular targets possibly involved. In a cellular context that allows *in vitro* EBV activation and lytic cycle progression through mechanisms closely resembling those that *in vivo* initiate and enable productive infection, we found that RV inhibited EBV lytic genes expression and the production of viral particles in a dose-dependent manner. We demonstrated that RV inhibited protein synthesis, decreased reactive oxygen species (ROS) levels, and suppressed the EBV-induced activation of the redox-sensitive transcription factors NF–kB and AP-1.

Further insights into the signaling pathways and molecular targets modulated by RV may provide the basis for exploiting the antiviral activity of this natural product on EBV replication.

1. Introduction

The vast majority of people carry latent Epstein Barr Virus (EBV) infection for their lifetimes without any symptoms, but the clinical feature of infectious mononucleosis (IM) may arise in adolescents and young adults. The primary cellular targets are resting B lymphocytes that are induced to proliferate by the virus. In an immune-competent host, the virus-induced proliferation is limited by a strong T cell response which allows spontaneous resolution of EBV primary infection. However, the virus maintained in a pool of latently infected memory B cells, may be reactivated in the immune-deficient host (Rickinson and Kieff, 2007). Immunodeficiency-related B cell lymphoma, including post transplant lymphoproliferative disorders (PTLD), are directly caused by EBV. In AIDS patients EBV causes hairy leucoplakia (HLP) of the tongue whose lesions produce large amount of virus (Greenspan et al., 1985). Moreover, EBV is associated with a variety of tumors including Burkitt's lymphoma (BL), Hodgkin's lymphoma, T-cell lymphoma and nasopharyngeal carcinoma (Kutok and Wang, 2006).

Except for IM and HLP, all the other EBV diseases are malignancies characterized by latent infection. However, also in the latter, the EBV productive cycle allows horizontal spread of the virus and favors B cell tumors development by promoting lytically-infected B cell secretion of several growth factors and cytokines (Cayrol and Flemington, 1995; Hong et al., 2005; Hsu et al., 2008; Jones et al., 2007; Mahot et al., 2003; Miyazaki et al., 1993). Moreover, recent studies carried out in a mouse model, found that EBV lymphoma formation is less frequent in animals infected with a lytic replication-defective virus than the control virus, thus supporting an important role for lytic EBV infection in the development of B cell lymphoma (Ma et al., 2011).

Treatments of EBV infection typically include (alone or in combination) antivirals, radiotherapy, chemotherapy, CD20 antibodies and adoptive T-cell therapy (De Paoli, 2010; Villegas et al., 2010). Generally, the use of antiviral compounds is limited by toxic side effects, poor oral bioavailability and the risk for the emergence of drug-resistent virus strains.

Resveratrol (trans-3,4′,5 trihydroxy–stilbene, RV), a polyphenolic phytoalexin produced by a variety of plants, has gained

Abbreviations: RV, resveratrol; EBV, Epstein Barr Virus; IE, immediate early; E, early; NF–kB, nuclear factor kappa-light-chain-enhancer of activated B cells; AP-1, activator protein 1.

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http://dx.doi.org/10.1016/j.antiviral.2012.09.003

considerable attention as a cancer chemopreventive agent and to control fungal, bacterial and viral infections (Bhat et al., 2001; Yu et al., 2012). We have recently shown that RV is able to induce apoptosis of EBV infected Burkitt's lymphoma cells with an efficacy inversely related to the restriction pattern of viral latent gene expression (De Leo et al., 2011). Moreover, the antiviral activity of RV has also been reported for several members of the Herpesviridae family (Docherty et al., 2005, 2006; Evers et al., 2004; Faith et al., 2006; Yiu et al., 2010), HIV (Zhang et al., 2009), influenza A virus (Palamara et al., 2005) and polyomavirus (Berardi et al., 2009).

In this study, we have examined the effects of RV on EBV replication in two Burkitt's lymphoma cell lines that allow EBV lytic cycle induction through different treatments. We show that, independently of the method used to trigger EBV activation, RV strongly inhibits lytic cycle initiation. Moreover, in cross-linked Akata cells, a system for EBV induction that most likely mimics the mechanism of viral reactivation *in vivo*, we demonstrate that RV inhibited EBV lytic genes expression and viral particles production in a dose-dependent manner. We provide evidences that the down-regulation of EBV gene expression occurs at the post-transcriptional level, involving the inhibition of protein synthesis, the reduction of reactive oxygen species (ROS) and the suppression of redox sensitive NF–kB and AP-1 activities stimulated by EBV lytic cycle activation.

2. Materials and methods

2.1. Reagents

Resveratrol (Sigma), prepared as 50 mg/ml stock solution in ethanol and kept at −20 °C protected from light, was diluted to final concentrations in RPMI 1640 medium.

Cycloheximide (CHX) purchased from Sigma, was dissolved in DMSO at 100 mg/ml and used at 50 μg/ml.

2.2. Cell lines, EBV lytic cycle induction and resveratrol treatment

EBV-positive Raji cells, characterized by a latency III pattern and an EBV abortive lytic cycle and Akata cells showing a latency I phenotype, are Burkitt's lymphoma (BL)-derived cell lines. Cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and antibiotics, in a 5% CO_2 atmosphere and maintained at a cell density of 3.5×10^5 /ml.

To induce EBV lytic cycle, Raji cells (1.5×10^7) were electroporated with 10 μg of pCMVgenZ (a kind gift of Dr. G. Miller, Yale University School of Medicine, USA) using a Bio-Rad Gene Pulser (0.26 kV and 960 μF) and thereafter diluted to $5x10^5$ /ml. Control Raji cells were electroporated with 10 μg of the CMV vector. To activate the virus in Akata cell line, cells were diluted to 10^6 /ml and treated with 10 μg/ml of goat anti-human IgG (Sigma, St. Louis, MO, USA). The efficiency of EBV lytic cycle induction was evaluated, in both cell lines, by counting positive cells after immunofluorescence staining with FITC-labeled antibodies specific for EBV early antigens (Matusali et al., 2009).

Statistical analysis of the cells expressing BZLF1 revealed that in Raji as well as in Akata cells, EBV lytic cycle was activated in about 30% of the cell population, regardless of the method used to initiate the process.

2.3. Cell counts and flowcytometry

Following a 24 h period of incubation with IgG and RV at the concentrations of 10, 20 and 40 μM, Akata cells were counted in a Burker chamber by Trypan blue exclusion assay. The percentage of proliferation was determined as proliferation of treated cells × 100/proliferation of untreated cells. Apoptosis was evaluated by Annexin V-FITC apoptosis detection kit (BD Pharmingen) which measures Annexin V binding to phosphatidylserine in conjunction with propidium iodide (PI) staining, according to the accompanying procedure.

2.4. Immunoblots

Raji and Akata cells, treated as described, were incubated in the absence or in the presence of either resveratrol or CHX, collected at different times and analyzed by Western blot, as previously described (Matusali et al., 2009). Equal amounts of proteins, as determined by RC-DC Protein Assay (Bio-Rad, Hercules, CA, USA), were resolved by 4–20% Bio-Rad TGX gels and electroblotted to an Amersham Hybond PVDF membrane (GE Healthcare, Milan, Italy). The following primary antibodies were used: BZLF1(1:100 Argene); BRLF1 (1:2000 Argene), BFRF1(1: 1000) and BFLF2 (1: 10), kindly provided by Dr. A. Farina, Dept. of Experimental Medicine, Univ. of Rome "Sapienza", Italy; BALF5 (1: 40) was a kind gift of Dr. F. Graesser, GSF-Forschungszentrum, Munich, Germany; NF–kb RelA/p65 (1: 1000) and NF–kBp50 (1:3000) were purchased from Millipore; β-actin (1:1500 Sigma). Membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) and signals were visualized by ECL detection kit (Amersham).

2.5. Semiquantitative RT-PCR assay

Total RNA was extracted from 10^6 cells by NucleoSpin RNAII columns and treated with DNase I (Macherey–Nagel, Duren, Germany). A total of 3 μg of the RNA was subjected to MMLV reverse transcriptase (Invitrogen) with 2.5 μM oligo$(dT)_{23}$ primers (Sigma) in a 50 μl reaction mixture according to the manufacturers' protocols. 3 μl of RT products were analyzed undiluted or 1/10 and 1/20 diluted, by conventional PCR. As controls, PCR of RNA samples not subjected to RT and of RT products in the absence of primers, were used. Amplifications (25 cycles) were carried out with the following primers: BZLF1-F, 5′agaagcacctcaacctggagacaa; BZLF1-R, 5′cagc gattctggctgttgtggttt; BRLF1-F, 5′tcactacacaaacagacgcagcca; BRLF1-R, 5′aatctccacactcccggctgtaaa; BALF5-F, 5′cggaagccctctggacttc; BALF5-R, 5′ccctgtttatccgatggaatg; BHRF1-F, 5′gtcaaggtttcgtctgtgtg; BHRF1-R, 5′ttctcttgctgctagctcca; and GAPDH-F, 5′ttcgacagtcagccg-catcttctt; GAPDH-R, 5′gcccaatacgaccaaatccgttga. Amplification products were resolved on 1.5% agarose gels and stained by GelRed (Biotium).

2.6. Real-time PCR

Akata cells were treated for 24 h with IgG in the absence or in the presence of RV at 10, 20 and 40 μM. Cells were collected by centrifugation for 5 min at 300×*g* and culture supernatant used to determine EBV DNA copies by real-time PCR. The assay was performed essentially as described (Gaeta et al., 2009). Briefly, extracted DNA was analyzed by a commercially available kit (Nanogen Advanced Diagnostics S.r.l.) based on TaqMan technology.A5′ reporter dye 6-carboxyfluorescein (FAM)-labeled probe for the viral EBNA1gene was used for PCR reactions. Dilutions of plasmid carrying the specific viral gene were used to construct the reference curve and quantify viral DNA in the samples. PCR reactions were carried out with ABI PRISM 7000 (Applied Biosystem, USA) according to the manufacturer's protocol. Computer software quantified the viral DNA load referred to the sample extracted volume.

2.7. Production of reactive oxygen species (ROS)

Akata cells were treated with anti-human IgG in the absence or in the presence of 10, 20 or 40 μM RV. Aliquots (2.5×10^5cells),