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Luteolin inhibits Epstein-Barr virus lytic reactivation by repressing the promoter activities of immediate-early genes

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ABSTRACT

The lytic reactivation of Epstein-Barr virus (EBV) has been reported to be strongly associated with several human diseases, including nasopharyngeal carcinoma (NPC). Inhibition of the EBV lytic cycle has been shown to be of great benefit in the treatment of EBV-associated diseases. The administration of dietary compounds is safer and more convenient than other approaches to preventing EBV reactivation. We screened several dietary compounds for their ability to inhibit EBV reactivation in NPC cells. Among them, the flavonoid luteolin showed significant inhibition of EBV reactivation. Luteolin inhibited protein expression from EBV lytic genes in EBV-positive epithelial and B cell lines. It also reduced the numbers of EBV-reactivating cells detected by immunofluorescence analysis and reduced the production of virion. Furthermore, luteolin reduced the activities of the promoters of the immediate-early genes Zta (Zp) and Rta (Rp) and also inhibited Sp1-luc activity, suggesting that disruption of Sp1 binding is involved in the inhibitory mechanism. CHIP analysis revealed that luteolin suppressed the activities of Zp and Rp by deregulating Sp1 binding. Taken together, luteolin inhibits EBV reactivation by repressing the promoter activities of Zp and Rp, suggesting luteolin is a potential dietary compound for prevention of virus infection.

1. Introduction

Epstein-Barr virus (EBV) is a member of the γ-herpesviridae and is known to infect more than 95% of the human population. After infection, EBV becomes latent. Lytic replication can be activated in latently infected cells by chemical induction, e.g. treatment with 12-O-tetradecanoyl-phorbol-1,3-acetate (TPA) and sodium butyrate (SB), cross-linking of immunoglobulin, and cytokine stimulation. Upon reactivation, EBV expresses sequentially immediate early (IE), early (E) and late (L) proteins and eventually releases the mature virions (Rickinson and Kieff, 2001). The immediate-early proteins Zta and Rta play key roles in initiation of the lytic cycle and, ectopic expression of either of these proteins is sufficient to trigger the lytic cascade (Fixman et al., 1992). In other words, blocking their expression of Zp or Rp or inhibiting the activities of their promoters can inhibit lytic reactivation of EBV (Chang et al., 2004; Feederle et al., 2000).

Primary infection usually does not cause serious illnesses but results in latent EBV resting in circulating B cells (Maeda et al., 2009). However, EBV infection has been shown to play a causative role, not only in infectious mononucleosis (IM), hairy leukoplakia, and post-transplant lymphoproliferative disorder (PTLD) (Zhong, 2012), but also to have relevance to autoimmune and cardiovascular diseases (Binkley et al., 2013). It is highly associated with several human malignancies, e.g. Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). Furthermore, accumulating evidence indicates that inhibition of the EBV lytic stage may be beneficial for prevention or have therapeutic value. Acyclovir (ACV) has been used for the treatment of IM patients (Ernberg and Andersson, 1986), and treatment with anti-EBV compounds can

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Abbreviations

EBV	Epstein-Barr virus
NPC	nasopharyngeal carcinoma
BL	Burkitt's lymphoma
IM	infectious mononucleosis
PTLD	post-transplant lymphoproliferative disorder
TPA	12-O-tetradecanoylphorbol-13-acetate
SB	sodium butyrate
EGCG	epigallocatechin gallate
SFN	sulforaphane
CC_{50}	cytotoxicity concentration
Zp	Zta promoter
Rp	Rta promoter
ZRE	Zta response element
RRE	Rta response element
ACV	acyclovir
GCV	ganciclovir
IgG	immunoglobulin G

cause regression of OHL (Kessler et al., 1988; Resnick et al., 1988; Triantos et al., 1997). Administration of ACV or gancyclovir (GCV) can prevent the development of PTLD or cause it to regress (Funch et al., 2005; McDiarmid et al., 1998). Moreover, because EBV lytic reactivation is considered to be highly associated with several human malignancies, blocking the EBV lytic cycle could repress reactivation-induced genomic instability or tumorigenesis in a mouse model (Fang et al., 2009; Hong et al., 2005a, 2005b). Hence, for this purpose, administration of compounds that inhibit the EBV lytic cycle is a more promising approach to clinical treatment than more complex methods, such as transfection of Zta siRNA (Chang et al., 2003) or antisense RNA (Liu et al., 1997). Several types of compounds have been documented: (i) Nucleoside analogs, e.g. acyclovir and ganciclovir, are able to inhibit EBV lytic replication and are used extensively for antiviral therapy (Ernberg and Andersson, 1986; Lin et al., 1986). (ii) Multifunctional drugs, e.g. maribavir, has been found to block EBV transcription and replication (Wang et al., 2009). (iii) In addition to chemicals, dietary ingredients such as retinoic acid, epigallocatechin gallate (EGCG), curcumin and sulforaphane (SFN) also have been suggested to have the potential to inhibit the EBV lytic cycle (Chang et al., 2003; Hergenhahn et al., 2002; Sista et al., 1993; Wu et al., 2013). Regarding clinical application, dietary compounds are more attractive for inhibition of the EBV lytic cycle because of their low cost and convenience. To achieve this objective, our laboratory screened several dietary compounds to identify those that are able to inhibit EBV lytic cycle. After screening, we were surprised to find that luteolin, a flavonid, has the ability to inhibit EBV lytic reactivation effectively.

Luteolin (3,4,5,7-tetrahydroxyflavone), a natural flavonoid, is abundant in various medicinal herbs, fruits and vegetables, e.g. broccoli, onion, parsley, green peppers, citrus, celery and chamomile (Lin et al., 2008b; Miean and Mohamed, 2001). Luteolin has many beneficial properties, including antioxidant, anti-inflammatory, anti-cancer, anti-diabetic and cardio-protective effects (Miean and Mohamed, 2001). It is also well known to have good effects in anti-angiogenesis, anti-metastasis, anti-inflammation and estrogenic regulation and can regulate many signal pathways (Lopez-Lazaro, 2009; Seelinger et al., 2008a). Based on these reports, luteolin is considered to have potential clinical value for cancer prevention and therapies (Seelinger et al., 2008b). However, although a few studies have been published, the questions whether luteolin has effective anti-viral activity and through what mechanism are less well elucidated.

In this study, we demonstrate that luteolin has an inhibitory effect on EBV lytic reactivation through a distinct pathway. It inhibits the initiation of the EBV lytic cycle by repressing the activities of viral promoters by disrupting Sp1 binding. These findings give a new insight into the antiviral effect of luteolin and may provide an alternative choice for the therapy of EBV-related malignancies.

2. Materials and methods

2.1. Compounds and antibodies

Luteolin, and the EBV induction agents (TPA and SB) were purchased from Sigma-Aldrich Co. Antibodies used in this study include anti-Sp1 (clones PEP2 and 1C6, Santa Cruz), normal rabbit IgG (Santa Cruz), anti-EBV Rta 467 (unpublished), anti-BMRF1 (EAD) 88A9 (Tsai et al., 1991), anti-EBV Zta 4F10, anti-DNase 311H (Tsai et al., 1997) and anti-β-actin (Sigma-Aldrich Co.).

2.2. Cell lines

NA is an EBV-positive cell line obtained by co-cultivation of rAkata cells with TW01 cells, a human NPC cell line from Taiwan (Lin et al., 1990). HA is another EBV-positive cell line obtained by co-cultivation of rAkata cells with HONE1 cells, a human NPC cell line from China (Glaser et al., 1989). NA and HA were both selected by G418 (Sigma-Aldrich Co) treatment (Chang et al., 1999). All of the NPC cell lines and their derivatives were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum (FCS). Raji (Epstein et al., 1966), P3HR1 (Hinuma et al., 1967) and Akata (Takada et al., 1991) are EBV-positive Burkitt's lymphoma cell lines and were maintained in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% FCS.

2.3. Cytotoxicity assay

The cytotoxicity of luteolin to each cell line was determined by WST-1 assay (Invitrogen) according to manufacturer's instructions. Briefly, NA and HA (1×10^4 cells/well) cells were seeded in 96 well plates for 24 h and 48 h. Each cell line was incubated with various concentrations of luteolin (0, 1, 5, 10, 20, 50 and 100 μM) for 24 or 48 h and the cytotoxicity analyzed by WST-1 assay. The fluorescence of each sample was measured with a microplate reader (Infinite M200, Tecan). The half maximum of cytotoxicity concentration (CC_{50}) was defined as the concentration of luteolin which killed 50% of the cell populations. The mean and standard deviation were calculated from at least three independent experiments.

2.4. EBV induction and luteolin administration

Two EBV-positive NPC cell lines, NA and HA, and three EBV-positive Burkitt's lymphoma cell lines, Raji, P3HR1 and Akata were seeded for 24 h before carrying out the experiments. To detect inhibition of reactivation, the cells were pre-treated with various concentrations of luteolin for 1 h, and TPA (40 ng/ml) and SB (3 mM) were added to co-treat the cells (NA, HA, Raji and P3HR1) for EBV induction. After 24 or 48 h incubation, the cells and their extracts were collected for further studies. For EBV induction of Akata cells, 0.8% (vol/vol) goat anti-human immunoglobulin G (IgG) was added to activate EBV. To determine whether luteolin can reactivate EBV in Burkitt's lymphoma cell lines, luteolin was added to the cells for 48 h. Cells and lysates were collected for further analyses.