Università degli Studi di Roma "La Sapienza"

"Effects of Resveratrol on Epstein-Barr Virus (EBV) - infected B lymphocytes"

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1. Introduction

1.1 Resveratrol

Antioxidant compounds present in dietary items have gained interest because of their beneficial effects on human health. Resveratrol (3,40,5-trihydroxy-trans-stilbene, RV), a polyphenolic phytoalexin found in the skin of red grapes and a variety of other fruits, functions in plants to protect against fungal infections, injury, ultraviolet (UV) radiation (Shakibaei et al., 2009). It was first isolated from the roots of white hellebore *(Veratrum grandiflorum O. Loes)* in 1940 (Takaoka M.J. 1940), and later, in 1963, from the roots of *Polygonum cuspidatum*, a plant used in traditional Chinese and Japanese medicine. Initially characterized as a phytoalexin, resveratrol attracted little interest until 1992, when it was proposed to explain some of the cardioprotective effects of red wine. A variety of studies have reported that resveratrol has antioxidant, anti-inflammatory, cardioprotective, antibacterial, and antiviral activities (Bhat et al., 2001; Yu et al., 2012). In addition, resveratrol was found to inhibit proliferation and induce apoptosis in several human tumor cells (Joe AK et al., 2002; Aggarwal BB et al., 2004).

1.1.1 Antioxidant activity

Resveratrol prevents oxidative stress by attenuating hydrogen peroxide-induced cytotoxicity and intracellular accumulation of reactive oxygen species (ROS) at a concentration of 1–100 μmol/l

(Jang and Surh, 2001; Liu et al., 2003; Chen et al., 2004; Leiro et al., 2004; Yu et al., 2009). Recent studies have demonstrated that this polyphenol can also reduce aging- and exercise-induced oxidative damage and mitochondrial dysfunction (Ryan et al., 2010; Jackson et al., 2011; Sun et al., 2011). Its antioxidant activities can be explained because of its action on different mechanisms. First, resveratrol has been reported to be a strong inhibitor of nicotinamide-adenine dinucleotide phosphate (NADPH)- and adenosine 5'-diphosphate (ADP)-Fe^{+}-lipid peroxidation as well as UV light-induced lipid peroxidation, and an efficient scavenger of radicals (Miura et al., 2000). Second, it is able to activate the sirtuin class of NAD^{+}-dependent histone deacetylases (Chen et al., 2009; Yu et al., 2009). Sirtuins regulate many intracellular pathways via the activation of transcription factors and enzymes responsive to nutrient availability. SIRT1, the major sirtuin activated by resveratrol, mediates the beneficial effects on longevity and health (Brooks and Gu, 2009).

1.1.2 Anti-inflammatory activity

There is considerable evidence demonstrating the anti-inflammatory properties of resveratrol, including inhibition of ROS production in neutrophils, monocytes and macrophages (Martinez and Moreno, 2000) and release of various cytokines from macrophages and lymphocytes (Feng et al., 2002). The important roles of cyclooxygenase (COX)-2 in various tumors and inflammatory diseases have been demonstrated (Kong et al., 2002). COX-2

is strongly induced in activated monocytes and macrophages. Subbaramaiah et al. (1998) indicated that resveratrol suppressed the synthesis of PGE2 by inhibiting COX-2 enzyme activity. Recent studies demonstrated that resveratrol significantly attenuated COX-2 expression and inhibited inflammatory cytokines such as TNF-a, IL-6, and IL-8 through a decrease of intracellular $Ca2^{+}$ levels and extracellular-signal-regulated protein kinases (ERK 1/2), as well as through inactivation of the transcription factor NF-kB (Csaki et al., 2009; Kang et al., 2009). In particular, treatment with resveratrol not only suppressed NFkB- regulated gene products involved in inflammation but also inhibited apoptosis by up-regulating Bcl-2, Bcl-xL, and TNF-α receptor-associated factor 1, and preventing the activation of caspase-3 (Csaki et al., 2009). Most agents that activate NF-kB also activate another transcription factor AP-1 (Karin et al., 1997; Pervaiz, 2003). Resveratrol has been shown to inhibit TNF-induced activation of AP-1 by c-Jun N-terminal protein kinase (JNK) and the upstream kinase MEK (mitogen-activated protein kinase kinase or MAPKK) activities (Manna et al., 2000).

1.1.3 Inhibition of platelet aggregation

Regular consumption of red wine is often credited as the explanation for the 'French Paradox' (Renaud S. & Gueguen R. 1998; Renaud S. & de Lorgeril M. 1992), a term coined to describe that the French enjoy a low risk of cardiovascular disease despite a diet that is high in saturated fat (Richard J.L. 1997). Although regular and moderate consumption of any alcoholic beverage seems to be beneficial to

cardiovascular health, epidemiological studies indicate that red wine confers significant additional benefits (Gronbaek M. et al., 1995; Bohm M. et al., 2004).

Platelet aggregation is one of the first steps in the formation of a blood clot that can occlude a coronary or cerebral artery, resulting in myocardial infarction or stroke, respectively. Moreover, inappropriate platelet activation is another major contributor in the process of atherosclerosis. Platelets adhesion has also been linked to the synthesis of eicosanoids from arachidonic acid (AA) (Fre´mont, 2000). Crescente et al. (2009) found that resveratrol inhibited AA-induced platelet aggregation. The mechanisms underlying this effect may be associated with the inhibition of PKC activation and protein tyrosine phosphorylation.

1.1.4 Effects on neurological conditions

Experimental and epidemiological studies gave evidences that resveratrol can exert protective activity against a number of neurodegenerative disorders (e.g., Alzheimer's and Parkinson's diseases, multiple sclerosis, amyotrophic lateral sclerosis). This polyphenol could exert beneficial effects on cells not only through its antioxidant potential but also through the modulation of the synthesis/ degradation of the amyloid β peptide. It has been demonstrated that moderate consumption of wine is associated with a lower incidence of Alzheimer's disease (AD) (Wang et al., 2006). Marambaud et al. (2005) showed that resveratrol (20–40 μM) could markedly reduce the levels of secreted and intracellular

amyloid-β peptides by promoting their proteosomal degradation. Resveratrol may also be useful in dopaminergic neurodegenerative disorders such as Parkinson's disease (PD) by repressing the excitatory neurotransmitter toxicity associated with glutamate. In this respect, experimental data have shown that pretreatment of SH-SY5Y human neuroblastoma cells with resveratrol resulted in an inhibition of dopamine cytotoxicity (Lee et al., 2007).

1.1.5 Anticancer activity

In various types of cancers resveratrol has been reported to inhibit all three phases of tumor development: initiation, promotion, and progression (Table I). The induction of apoptosis is a key mechanism for most anti-tumor therapies. Resveratrol exerts strong anti-proliferative activity in many cultured cancer cell lines by arresting cell cycle progression and by inducing apoptosis (Bai et al., 2010; Gatouillat et al., 2010; Jiang et al., 2010; Kweon et al., 2010; Vanamala et al., 2010; Wang et al., 2010). The pro-apoptotic protein nuclear factor p53 plays a key role in protecting a cell from tumorigenesis, blocking the cell cycle or initiating apoptosis if the cell is damaged. Resveratrol initiates p53-dependent apoptosis in human cancer cells, including prostate cancer, colon cancer, and melanoma (Gatouillat et al., 2010; Kai et al., 2010; Vanamala et al., 2010). Resveratrol also inhibits cell proliferation and contributes to the apoptotic cell death by suppressing the expression of cyclin D1 that causes a G1/S arrest in the cell cycle progression (Bai et al., 2010; Gatouillat et al., 2010; Wang et al., 2010).

Table 1.

Effects of resveratrol on Various Human Cancers in 2010. (Wei Yu et al., 2012)

Modes	Cell lines used	Cellular effect
Prostate cancer	PCa cells	Apoptosis
	LNCaP	Apoptosis, Cell cycle
Medulloblastoma	UW228-3	Intracellular bioavailability
Leukemia	K562	Cell cycle arrest, Cell proliferation
	AML-2	Apoptosis, Cell cycle arrest
	K562	Apoptosis
	CML cells	Autophagy
Breast cancer	MCF-7	DNA strand breaks
Colon cancer	HT-29, SW480	Apoptosis, Cell proliferation
Bladder cancer	T24	Apoptosis, Cell cycle arrest
Glioma cells	U251	Cell proliferation, Apoptosis
Melanoma	B16/DOX	Cell cycle arrest, Apoptosis
Endometrial cancer	HEC1B	Apoptosis
Hepatocarcinoma	HepG2, Hep3B	Anti-invasive activity

1.1.6 Antimicrobial activity

Plant polyphenols have been known to possess potential antibacterial, antifungal and antiviral activities. Rodriguez-Vaquero et al. (2007) have shown that grape wine inhibited microbial growth, especially of *Escherichia coli*, in concentration-dependent manner. Moreover, alcohol-free extracts of red and white wine exhibited antimicrobial activity to some pathogens such as *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (Papadopoulou C. et al.; 2005). Other studies reported that phenolic compounds inhibited some food-borne species such as *Salmonella typhimurium* (Sivarooban T. et al., 2008) and *Listeria monocytogenes* (Luther M. et al., 2007).

On the other side, the antiviral activity of resveratrol 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).

1.2 Molecular targets of resveratrol

The mechanisms by which resveratrol exerts its beneficial effects across species and disease models is not yet clear. Experiments in vitro have shown promising effects, and have led to the identification of multiple targets for this compound.

1.2.1 Effects on cell cycle regulators

The anti-proliferative and pro-apoptotic effects of resveratrol have been extensively studied *in vitro* and confirmed in several *in vivo* models of neoplastic growth. Resveratrol, at micromolar concentrations, has been shown to modulate the major cell cycle regulators and arrest cancer cells proliferation at the G1/S phase of the cell cycle. The anti-proliferative activity of resveratrol involves the induction of p21WAF1 and p27KIP1 and downregulation of cyclins D1/D2/E, Cdks 2/4/6, and hyperphosphorylated pRb. In other cell types, resveratrol has been reported to arrest the cell cycle at the S-phase as well as at the G2/M-phase, by inhibiting Cdk7 and p34Cdc2 kinases (reviewed in Athar M et al 2009). Resveratrol upregulates the p53 tumor suppressor protein, stimulates its post-translational modification (Zhang S et al., 2004), induces the expression of p53-responsive genes (p21WAF1, p300/CBP, APAF1,

and Bak) and causes Bcl2 down-regulation (Narayanan BA et al., 2003). In addition, p53-independent induction of p21WAF1 and subsequent cell cycle arrest in cells lacking wild-type p53 protein has been documented. Taken together, the anti-proliferative activity of resveratrol involves the differential regulation of multiple cell cycle targets depending on both concentration and cell type.

1.2.2 Effects on apoptosis-related proteins

Apoptotic cell death depends on the modulation of genes related to apoptotic and survival pathway. Caspase activation is regulated by the inhibitor of apoptosis proteins (IAPs) and survivin. IAPs bind to caspases and antagonize their activity. p53 induces a number of mitochondria-related apoptotic genes, such as Bax, Noxa, PUMA, and BID, and represses the anti-apoptotic Bcl2 and cIAPs. Resveratrol, through p53 activation, induces the expression of these pro-apoptotic proteins, and inhibits the expression of anti-apoptotic Bcl2, Bcl-XL, and Mcl-1, directly affecting the mitochondrial death pathway. Furthermore, resveratrol was shown to potentiate the apoptotic effects of bortezomib and thalidomide through the suppression of NF-κB and STAT3 in human multiple myeloma cells. In particular, it was observed the down-regulation of anti-apoptotic genes included cyclin D1, cIAP-2, XIAP, survivin, Bcl-2, Bcl-xL, Bfl-1/A1, TRAF2 and up-regulation of Bax/caspase-3-associated apoptosis (Bhardwaj A et al., 2007).

1.2.3 Inhibition of PI3K/AKT and MAPK pathway

Resveratrol has also been reported to interfere with the phosphatidylinositol- 3 kinase (PI3K)/ AKT and mitogen activated protein kinase (MAPK) pathway, two key survival cascades. Resveratrol derivatives were found to inhibit cell transformation by blocking EGF-induced activation of PI3K/AKT in mouse JB6 epidermal cells (Kolesnick R., 2002). Moreover, the ability of the polyphenol to control tumor growth by inhibiting MAPK signaling pathway was demonstrated in cervical carcinoma cells exposed to UV irradiation (Scarlatti F et al., 2003). However, the antitumor activity of resveratrol has also been shown to require MAPK-induced p53 activation for subsequent induction of apoptosis (Trincheri NF et al., 2007; Chambers AF et al., 1997). In particular, extracellular signal-regulated kinase(ERK), c-Jun N-terminal kinase (JNK) and p38 kinase were found to mediate resveratrol-induced activation of p53 in JB6 mouse epidermal cells. The functional impact of resveratrol on MAPK signaling may seems to depend, at least in part, on the concentrations of the polyphenol used in different settings. In this respect, low concentrations of resveratrol (from 1 pM to 10 μM) were reported to induce phosphorylation of ERK1/2 in human neuroblastoma cells, whereas higher concentrations (50–100 μM) negatively interfered with phosphorylation of MAP kinases (Bernhard EJ et al., 1994).

1.2.4 Modulation of Transcription Factor NF-κB

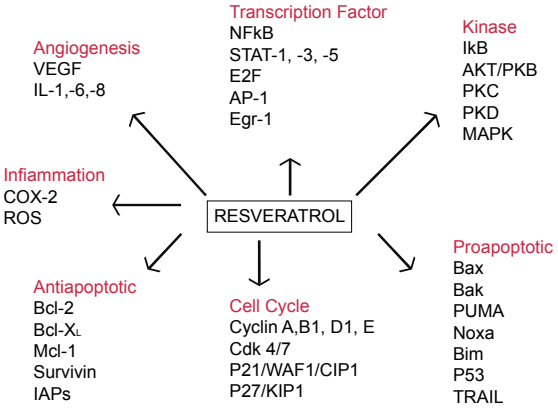
The transcription factor nuclear factor-kappa B (NF-κB) is implicated in a number of cellular processes, including immune and stress responses, inflammation, apoptosis, and regulation of cell growth. Deregulated NF-κB activity has been implicated in various stages of tumorigenesis and linked to a variety of cancers. NF-κB is composed of homo- and heterodimeric complexes, consisting of linked to a variety p50, p65/RelA, c-Rel, p52, and RelB. In its inactive state, NF- κB is sequestered in the cytoplasm, via non-covalent interactions with the family of inhibitor proteins, IκBs. External stimuli such as mitogens, cytokines, UV, ionizing radiation, and bacterial toxins, cause the IkB kinase (IKK) mediated-phosphorylation of IkBα and its subsequent degradation. Dissociation of IκBα from NF-κB allows nuclear translocation of the activated free NF-κB dimer, where it binds to the specific sequence in the promoter of target genes and induces their expression. Resveratrol can suppress IKK phosphorylation and can block the degradation of IκBα, thereby inhibiting the activation of NF-κB in TPA-stimulated mouse skin (Kundu JK et al., 2006). Resveratrol also blocked NF-kB activation induced by PMA, LPS, H_2O_2, okadaic acid, and ceramide (Manna et al., 2000). In addition, the polyphenol may down-regulate the expression of NF-κB-regulated genes, including interleukin-6, Bcl-2, Bcl-xL, XIAP, c-IAP, vascular endothelial growth factor (VEGF), and matrix

metalloproteinase-9 (MMP-9) (Sun C et al., 2006).

AP-1

The transcription factor AP-1 is composed by a number of different dimeric combinations of the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) families and Jun dimerization partners (JDP1 and JDP2) and activating transcription factor (ATF2, LRF1/ATF3 and B-ATF) subfamilies. AP-1 plays important roles in the proliferation of tumor cells, and induces COX-2, urokinase-type plasminogen activator, Fos, MMP-9, cyclin D1, and VEGF (Lee KW, Lee HJ 2006). Down-regulation of c-Jun and suppressed AP-1 activity by resveratrol involve the inhibition of mitogen-activated protein kinase (MEK)1 /extracellular signal-regulated kinase ERK1/2 signaling (Kim AL et al., 2006).

Figure 1.
Mechanisms of action of resveratrol. (Shankar S et al., 2007)



1.3 Resveratrol and viruses

1.3.1 Antiviral activity of resveratrol against members of the Herpesviridae family

The effects of resveratrol on the *Herpesviridae* family , in particular on HSV-1 (herpes simplex virus 1) and HSV- 2, are among the most widely studied. The first report, published in 1999, showed that addition of resveratrol in the first 6 h of HSV-1 infection blocked viral replication (Docherty J.J. et al., 1999). This inhibition didn't affect either the virus capsid directly or its attachment to the plasma membrane, since pre-incubation of the virus or the cells with the drug had no effect on viral replication, suggesting that resveratrol acted after entry of the virus into the cell. Additional studies demonstrated the protective effect of resveratrol *in vivo* against HSV-1 and HSV-2 infection by using a hairlessmouse model of cutaneous HSV infection and a mouse model of intravaginal infection (Docherty J.J. et al., 2005, 2004). Other members of the *Herpesviridae* family have been shown to be susceptible to resveratrol treatment. The replication of VZV (Varicella Zoster virus) and HCMV (human cytomegalovirus), was inhibited by resveratrol in a dose-dependent manner (Docherty, J.J. et al., 2006; Evers, D.L. et al., 2004). As observed for HSV-1, resveratrol affected the immediate-early phase of VZV replication by blocking the expression of essential viral proteins and it reduced early and late gene expression of HCMV. However, in contrast with what described for HSV, resveratrol could inhibit the early phases of the HCMV entry in the cell and the

early cellular signal transduction pathways.

Another member of the family, EBV (Epstein–Barr virus), has been shown to be affected by resveratrol. In an *in vitro* assay, treatment of a Burkitt's lymphoma cell line with the polyphenol largely inhibited EBV early antigen induction (Kapadia, G.J et al., 2002). Finally, Dyson O.F. et al. (2012) demonstrated that resveratrol, by lowering ERK1/2 activity and Egr-1 expression in KSHV-infected cells, suppressed viral reactivation from latency.

1.3.2 Activity of resveratrol on retroviruses

Several reports have analyzed the effects of resveratrol on HIV-1 infection. They propose a possible therapeutic effect of resveratrol on HIV infection, suggesting a different mechanisms of action for the compound. The ability of the drug to induce the lytic cycle of HIV-1 was evaluated by Krishnan and Zeichner (2004). This approach consists in combining a drug such as resveratrol, capable of inducing the lytic cycle of the virus from the latently infected cells, with an antiviral drug in order to eliminate the viral reservoirs in the organism. For this purpose, they treated latently HIV-infected ACH-2 cells with resveratrol and assessed the production of the viral lytic antigen p24. Resveratrol was shown to induce an increase of approx. 4-fold in the levels of p24. This effect was attributed to the activation of EGR-1 (early growth response gene product 1), a protein involved in cell-cycle regulation and differentiation. Another approach by Heredia and co-workers consists in evaluating the synergistic effect of resveratrol on the inhibition of HIV-1

replication when administrated together with known inhibitors of the viral cycle (Heredia, A. et al., 2000; Wang, L.X. et al., 2004). In this case, resveratrol , arresting the cell cycle in S-phase would allow a longer exposure of the virus to nucleoside analogue inhibitors. Resveratrol alone had only a marginal effect on inhibition of viral replication on activated infected-PBMCs (peripheral blood mononuclear cells). However, the drug strongly increased the antiviral activity of various nucleoside analogues. Although the activity of resveratrol on the replication of other retroviruses has not yet been reported, resveratrol treatment inhibits the growth of HTLV-1 (human T-cell lymphotrophic virus-1) infected cell lines (Hayashibara, T. et al., 2002), suggesting a possible antiviral activity of the chemical compound also on HTLV-1.

1.3.3 Antiviral activity of resveratrol on other viruses

Another virus whose replication was shown to be severely inhibited by resveratrol is the murine polyomavirus. Resveratrol inhibited polyomavirus replication in a dose-dependent manner by blocking the synthesis of viral DNA in an *in vitro* system (Berardi, V. et al., 2009). A strong antiviral activity of resveratrol has been also demonstrated against influenza virus *in vitro* and *in vivo* (Palamara, A.T et al., 2005). As observed for HSV-1 and polyomavirus, resveratrol treatment did not cause virus particle inactivation or inhibition of viral adsorption but induced a decrease in the translation of late viral proteins. Recently, Zang et al. (2011) provided evidence that resveratrol was a potent suppressor of

Respiratory Syncytial Virus (RSV) infection, inflammation, and the airway hyperresponsiveness (AHR) response associated with RSV infection. However, resveratrol significantly enhanced Hepatitis C virus (HCV) replication making it unsuitable for an antioxidant therapy in chronic hepatitis C (Nakamura M et al., 2010).

1.4 Epstein Barr Virus (EBV)

EBV, known as the causative agent of infectious mononucleosis, is a member of the Herpesviridae family. Like other herpesviruses, EBV is an enveloped virus that contains a DNA core surrounded by an icosahedral nucleocapsid and a tegument. Family members include herpes simplex I and II and varicella-zoster virus (alphavirus subfamily), cytomegalovirus and human herpesvirus 6 and 7 (betaherpesvirus subfamily), and human herpesvirus 8 and EBV (gammaherpesvirus subfamily; Roizman, B 1990). Human tumors have been attributed to both human herpesvirus 8 (Kaposi's sarcoma, primary effusion lymphoma, and Castleman's disease) and to EBV. Up to date, tumors related to EBV infection include Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's (HD) and non-Hodgkin's lymphomas, gastric cancer, breast cancer, leiomyosarcomas arising in immunocompromised individuals, central nervous system lymphomas associated with HIV, post-transplant lymphoproliferative disorders (PTLDs). The B-lymphotropic nature of EBV is evidenced by the ability of the virus to immortalize normal resting B lymphocytes *in vitro*, converting them into permanently growing lymphoblastoid cell lines (Nilsson, K 1992).

Two subtypes of EBV are known to infect humans: EBV-1 and EBV-2. They differ in the organization of the genes that code for the EBV nuclear antigens (EBNA-2, EBNA- 3a, EBNA-3b, and EBNA-3c; Sample, J. et al., 1990). EBV-2 transforms B cells less efficiently

than EBV-1 *in vitro*, and the viability of EBV-2 lymphoblastoid cell lines is less than that of EBV-1 lines (Buisson, M. et al., 1994). The differences in transforming efficiency of the EBV subtypes may relate to divergence in the EBNA-2 sequences (Cohen J et al., 1989, Rickinson A. et al., 1987).

EBV-1 and EBV-2 differ in geographic distributions. EBV-1 is observed more frequently in most populations. However, EBV-2 is nearly as prevalent as EBV-1 in New Guinea, as well as in equatorial Africa (Young, L. S et al., 1987; Sixby, J. W. 1989). Endemic Burkitt's lymphoma and holoendemic malaria are common in equatorial Africa, and it has been shown that almost half of all African Burkitt's lymphoma tumors carry EBV-2. In contrast, 85% of nasopharyngeal carcinomas in Taiwan contain EBV-1 (Shu, C. H. et al., 1999). Immunocompromised patients also more commonly harbor both subtypes of EBV (Borisch, B. et al., 1992). Taken together with the attenuated transforming ability of EBV-2, these data suggest that it may be necessary a preexisting immunosuppressed condition (HIV or malaria) for EBV-2 B-lymphocytic infection and transformation (Buisson, M. et al., 1994).

1.4.1 Molecular Biology of EBV

EBV is an herpesvirus with a 184-kbp long, double-stranded DNA genome that encodes about 100 proteins (Kieff E., and Rickinson A. B 2001). The viral genome carries two 0.5-kb terminal repeats at either end and internal repeat sequences, resulting into short

and long unique sequence domains that have most of the coding capacity (Cheung, A., and Kieff, E 1982). Similar to other herpesviruses, EBV has a toroid-shaped protein core containing the DNA, a nucleocapsid with 162 capsomeres, a protein tegument between the nucleocapsid and envelope, and an outer envelope with external glycoprotein spikes (Rickinson, A. B., and Kieff E 2001). After infection, the EBV DNA becomes a circular episome with a characteristic number of terminal repeats, depending on the number of those in the parental genome, with variation introduced during viral replication. If the infection is permissive for latent infection but not replication, future generations will have EBV episomes with the same number of terminal repeats. Therefore, the number of terminal repeats in a group of latently infected cells can be useful in eludicating if these cells arose from a single cancer-infected progenitor or from multiple progenitors (Baumforth, K. R. N et al., 1999).

1.4.2 EBV Infection

Although herpesviruses are ubiquitous in nature, humans are the only natural host for EBV. It is now known that EBV infects > 90% of the world's adult population. Upon infection, the individual remains a lifelong carrier of the virus (Henle G. et al., 1979). EBV is transmitted from host to host via saliva. Primary infection begins at the oropharyngeal epithelium. B lymphocytes are infected when they are in proximity to these cells. During acute infection, EBV expresses proteins that induce cell proliferation in

B lymphocytes. An EBV-specific CTL response occurs in healthy people and probably is responsible for the decrement in infected B cells from levels as high as 10% in acute EBV infection to 1 in 10^6 cells with convalescence. In a primary EBV infection, three classes of antibodies (-IgG, -IgM, and -IgA) are produced against EBV viral capsid antigen, two (-IgG and -IgA) are produced in response to early antigen D, and one (-IgG) is produced in response to early antigen R (Linde A. 1996). During a latent infection, EBNA-3A, EBNA-3B, and EBNA-3C all elicit specific CTL responses, the dominant latency response to EBV proteins (Tan, L. C. et al., 1999; Murray, R. J et al., 1992; Khanna, R. et al., 1992). EBV infection of B cells begins with the attachment of the gp 350/220 viral membrane glycoprotein to the CD21 molecule on these lymphocytes (Nemerow G. et al., 1985). CD21 becomes cross-linked, triggering an initial signal able to prepare the cell for EBV infection. EBV binding to CD21 immediately activates tyrosine kinase *lck* and mobilizes calcium (Cheung, R., and Dosch, H 1991; Gordon, J. et al., 1986). This event is followed by an increment in mRNA synthesis, blast transformation, homotypic cell adhesion, surface CD23 expression (a characteristic marker for activated B cells), and interleukin (IL)-6 production (Tanner J. et al., 1987, 1996; Alfieri C. et al., 1991). The virion is then uncoated and the viral DNA is transported into the nucleus where it immediately circularizes. Circularization and W promoter expression promote an ordered cascade of events that leads to the expression of all of the EBNA proteins and the two latent membrane proteins (LMPs; Allday M. et al., 1989). The EBV

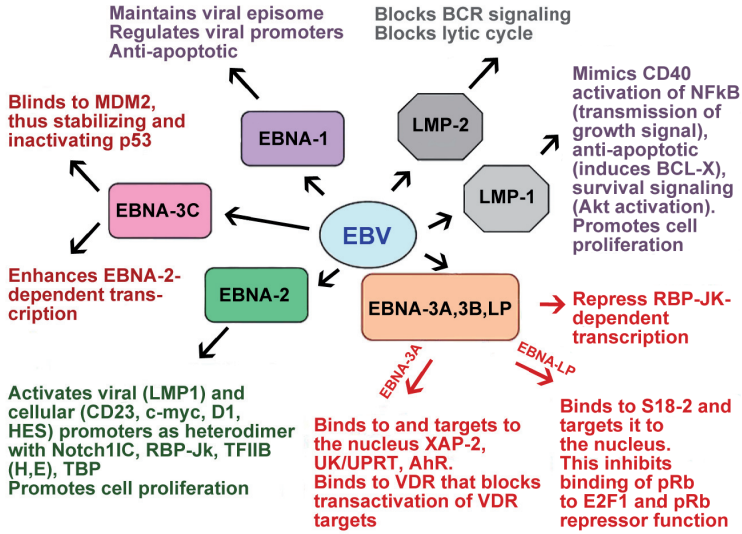
nuclear antigen leader protein (EBNA-LP) and EBNA-2 proteins are the first proteins to be detected upon EBV infection (Hennessy, K., and Kieff, E 1985). EBNA products are involved in transcriptional control and participate in the activation of the expression of the viral LMP-encoding genes (LMP-1 and LMP-2) and several cellular genes. The combined action of these viral and cellular proteins stimulates cellular S-phase 24–48 h after infection (Rowe, D 1989). The viral genome is generally episomal and present in low numbers in the host cell's nucleus. Immunosuppressive states favor spontaneous replication of the viral episome in circulating B cells, as observed in acute infectious mononucleosis. Loss of the EBV-specific CTL may permit the development of lymphoma. Besides its well-known tropism for B cells, EBV also infects epithelial cells, T cells, and cells of the macrocytic, granulocytic, and natural killer lineages. These cells may be infected by different mechanism from the CD21-mediated internalization typical in B cells.

1.4.3 EBV latent products

EBV encodes a number of products that interact with or exhibit homology to a wide variety of antiapoptotic molecules, cytokines, and signal transducers, thus promoting EBV infection, immortalization, and transformation. During the latent phase, EBV expresses a limited set of viral gene products, consisting of six nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA leader protein (EBNA-LP)), three latent membrane proteins (LMP1, LMP2A, and LMP2B) and two small

non-polyadenylated (and therefore non-coding) RNAs (EBER 1 and 2). The function of EBERs transcripts is not clear but they are consistently expressed in all forms of latent EBV infection.

Figure 2.
Some of the functions of the EBV-encoded proteins expressed in latent infection of B-lymphocytes. (Klein G. et al., 2010)



1.4.4 Patterns of EBV Gene Expression

All EBV-associated cancers involve the viral latent cycle. Three types of latent gene expression have been described and characterize a heterogenous group of malignancies (Table 2). During latency I, EBNA-1 and the EBERs are expressed. Latency I is generally associated with the EBV-related malignancy Burkitt's lymphoma (Sbih-Lammali, F.et al., 1996). Latency II has been associated

with Hodgkin's disease, T-cell non-Hodgkin's lymphoma, and nasopharyngeal carcinoma (Cesarman, E., Mesri, E. A. 1999). EBV gene expression in latency II is usually limited to EBNA-1, the EBERs, LMP-1, and LMP-2A and LMP-2B (Liebowitz, D., and Kieff, E. 1993). The final pattern of gene expression (latency III) occurs mainly in immunocompromized individuals afflicted by post-transplant lymphoproliferative disorders (PTLDs), AIDS-related proliferative disorders, and in lymphoblastoid cell lines (LCLs) (Niedobitek, G. et al., 1997). Latency III usually involves the expression of all EBNAs, EBERs, and LMPs (Liebowitz, D., and Kieff, E. 1993).

Table 2.
EBV latency pattern and associated malignancies. (Matthew P. Thompson and Razelle Kurzrock. 2004)

Latency type	Viral genes expressed	Associated malignancies
Latency I	EBNA-1 EBERs	Burkitt 's lymphoma
Latency II	EBNA-1 EBERs LMP-1 LMP-2	Hodgkin 's disease Nasopharyngeal carcinoma Peripheral T/NK lymphoma
Latency III	All EBNAs EBERs LMP-1 LMP-2	AIDS-associated lymphomas Posttransplant lymphoproliferative disord Lymphoblastoid cell lines

1.4.5 Oncogenic Features of EBV

To be oncogenic, EBV must maintain its viral genome in the cell, establishing latent infection in B lymphocytes. The EBV genome is maintained in these cells, either as a multicopy circular

episome or by integrating the viral DNA into the cellular genome. In normal individuals, cytotoxic T-cell responses against latent viral proteins prevent the expansion of these activated B cells. Through normal differentiation of these cells, EBV arrives in the resting B-cell memory compartment. In these lymphocytes EBV expresses only EBNA-1 and escapes immune surveillance because cytotoxic responses against this viral latent protein are rare. Intermittently, these cells may enter the lytic cycle with subsequent lysis/death of infected cells, releasing virions to infect more cells. In a state of immunosuppression, latently infected cells in the peripheral blood or persistently infected cells on the oropharynx increase in number. Through diverse virally expressed genes that stimulate multiple intersecting cellular transduction pathways, involved in control of proliferation, EBV can become oncogenic. LMP1 is the main latent viral product with oncogenic features.

EBNA-1

EBNA-1 is a sequence-specific DNA binding phosphoprotein that is required for the replication and maintenance of the EBV genome (Middleton, T., and Sugden, B. 1994). EBNA-1 binds to the origin of latent plasmid replication *(oriP)*, which is composed of two distinct EBNA-1 binding elements (Ambinder, R. et al., 1990). These are the family of repeats (FRs) and the dyad symmetry (DS). The family of repeats and the dyad symmetry binding elements both contain multiple 18-bp EBNA-1 binding

sites (Wysokenski, D., and Yates, J 1989). The family of repeats element contains 20 binding sites, whereas the dyad symmetry element only contains 4 EBNA-1 binding sites. Following EBNA1 binding to the plasmid origin of replication, EBV uses host enzymes to mediate all remaining steps in replication. EBV episomes are present at low copy numbers and replicate only once per cell cycle. Their maintenance at a stable copy number in dividing cells also requires a mechanism to ensure segregation during cell division. The mitotic segregation or partitioning of the EBV episomes depends on EBNA1 and the oriP FR elements. EBNA1 functions by tethering the EBV episomes to the cellular chromosomes in mitosis and the pairing of EBV episomes on sister chromatids ensures their equal distribution to the daughter cells (Delecluse HJ. Et al., 1993).

LMP-1

LMP-1 is involved in transformation by acting as a constitutively active receptor (CD40), thus miming the cellular growth signal that normally results from the binding of CD40 ligand (Zimber-Strobl, U et al., 1996; Gires, O. et al., 1997). LMP-1 has been most directly linked to oncogenesis because of its ability to recruit a number of cellular genes. It also inhibits apoptosis by elevating levels of Bcl-2 (Zimber-Strobl, U et al., 1996). LMP-1 is an integral membrane protein with six hydrophobic membrane-spanning segments and a COOH-terminal cytoplasmic tail, representing the effector (Farrell, P. J 1998). LMP-1 aggregates in patches on the plasma

membrane (Clausse, B. et al., 1997) and mutational analyses have demonstrated that the N-terminal and the trans-membrane segments of LMP-1 are responsible for membrane aggregation and that this aggregation is essential for immortalization (Moorthy, R., and Thorley-Lawson, D 1993). LMP-1 simulates CD40 by associating with the same tumor necrosis factor receptor-associated factors (TRAFs; Eliopoulos, A. et al., 1996). The COOH-terminal domain of LMP-1 interacts with TRAFs and with tumor necrosis factor receptor-associated death domain protein through different regions known as transformation effector sites (Thompson MP. et al., 2003; Izumi, K., and Kieff, E. 1997). At least four signaling pathways, namely NF-kB, c-Jun N-terminal kinase s (JNKs), p38 MAPK, and Janus kinase (JAK)/signal transducers and activators of transcription (STATs) are implicated in the function of LMP-1 (Huen DS. et al., 1995; Eliopoulos AG. et al., 1998, 1999; Gires O.et al., 1999). The activating cascades associated with LMP-1 lead to the enhanced expression of B-cell adhesion molecules (LFA1, CD54, and CD58), enhanced expression of B-cell activation markers (CD23, CD39, CD40, CD44, and HLA class II), and morphological changes such as cellular clumping (Zimber-Strobl U. et al., 1996, Izumi, K., and Kieff, E. 1997, Martin J et al., 1993; Wang, D. et al., 1988;Wang, F.et al., 1990).

1.4.6 EBV-associated Cancers

EBV, the first human tumor virus discovered, has been implicated in the development of a wide range of cancers (Table 3).

Table 3.
Characterization of EBV-associated malignancies. (Matthew P. Thompson and Razelle Kurzrock. 2004)

Malignancy	Subtype	EBV gene expression pattern
Burkitt's lymphoma	Endemic Nonendemic	Latency I
Hodgkin's disease	MC LD NS LP	Latency II
Non-Hodgkin's lymphoma	Nasal T/NK Angioimmunoblastic Lymphadenopathy	Latency II Latency II
Nasopharyngeal carcinoma	Anaplastic	Latency II
Breast Cancer	Medullary carcinoma Adenocarcinoma	Not clear
Gastric Cancer	Lymphoepithelioma-like Adenocarcinoma	Controversial novel LMP1 (-) Latency III
PTLDs		Latency III
AIDS-associated lymphomas	IP-CNS Other	Latency III
Leiomyosarcomas in immunosuppressed individuals	Leiomyosarcomas varies	Unclear

Burkitt's Lymphoma

In 1958, Denis Burkitt (Burkitt, D. P 1958) described a common cancer in children of specific regions of Africa. Burkitt believed a virus might be responsible for the cancer, given the climatic and geographic distribution of the cases. EBV was first identified in 1964 when Anthony Epstein's group observed virus-like particles by electron microscopy in a cell line that had been established from a Burkitt's lymphoma biopsy (Epstein, M. A. et al., 1964). Later, it was found that sera from patients with the Burkitt's lymphoma, had much higher EBV antibody titers than controls without the lymphoma. The subsequent detection of EBV DNA in Burkitt's

lymphoma and the experimental production of lymphomas in animal models established EBV as the first virus clearly implicated in the development of a human tumor (Epstein, M. A. et al., 1964). Burkitt's lymphoma is a particularly aggressive lymphoma, characterized by a chromosomal translocation between chromosome 8 and either chromosomes 14, 2, or 22 (Gulley, M. L 2001; Manilov, G et al., 1986; Dalla-Favera, R. et al., 1982; Taub, R. et al., 1982). Because of this translocation, the oncogene *c-myc* (chromosome 8) is juxtaposed to the immunoglobulin heavy-chain (chromosome 14) or light-chain genes (chromosomes 2 or 22), resulting in the deregulation of its own expression. The relationship between EBV, Burkitt's lymphoma, and the *c-myc* translocation is complicated by the existence of two types of Burkitt's lymphoma: endemic (EBV positive) and non endemic (generally EBV-negative). They differ in the breakpoints within the genes involved and presumably in the mechanism mediating juxtaposition (Baumforth, K. R. N et al., 1999). Endemic Burkitt's lymphoma occurs primarily in equatorial Africa and Papua New Guinea, with EBV being present in > 90% of cases (Lenoir, G. et al., 1984). The role of EBV in Burkitt's lymphomas is strongly supported by observations of the Akata Burkitt's lymphoma cell line. Akata subcultures that have lost EBV have decreased growth and have not induced tumors in mice (Shimizu N. et al., 1994). Reinfection of the Akata cells with EBV reestablishes the malignant phenotype (Komano, J. et al., 1998). Non endemic Burkitt's lymphoma is found in the West and its incidence has increased dramatically because of its high prevalence

in AIDS patients. Only 15–30% of non endemic Burkitt's lymphoma cases are associated with EBV in the United States (Subar, M. et al., 1998), however, the percentage is 85% in Brazil. As with malaria in endemic Africa, coinfection is thought to increase the oncogenic potential of the B cell (Araujo, I. et al., 1996).

EBV-associated Lymphomas in Immunocompromised Individuals

Several distinct classes of EBV-associated lymphoproliferative disorders exist in immunocompromised individuals. Among them, there are lymphomas associated with immunosuppressive drugs given to transplant recipients, and AIDS-related lymphoproliferative disorders. The most common gene expression pattern in these disorders is latency III. Mostly, EBV-associated lymphomas in the immunocompromised patients are aggressive and difficult to treat.

Posttransplant Lymphoproliferative Disorders (PTLDs)

These heterogeneous lymphoproliferative disorders occur in the setting of therapeutic immunosuppression after organ transplantation (Cesarman, E., Mesri, E. A. 1999). This condition, leading to primary EBV infection or reactivation of latent EBV infection, is followed by polyclonal expansion of B-cell populations with a selective growth advantage. These cells are susceptible to genetic changes and other molecular aberrations that drive malignant growth. The incidence of post-transplant lymphoproliferative disorders ranges

from 0.5 to 30%, depending on the organ being transplanted, the EBV status of the transplant recipient and donor, and the therapies used to obtain immunosuppression (Knowles, D. M 1998). A variety of distinct post-transplant lymphoproliferative disorders have been described, including plasmacytic hyperplasia, polymorphic lymphoproliferative disorder, malignant non-Hodgkin's lymphoma, and multiple myeloma (Knowles, D. M 1998). Most posttransplant-lymphoproliferative disorders are B-cell neoplasms. PTLDs arising in bone marrow transplant recipients are generally of donor origin, whereas those in solid organ recipients are usually of recipient origin. The diagnosis and monitoring of patients affected by these diseases (Gulley, M. L 2001), revealed correlation between PTLDs and EBV viral load measured by quantitative PCR of the peripheral blood.

AIDS-Related Lymphoproliferative Disorders

AIDS-related lymphoproliferative disorders arise in the presence of HIV-associated immunosuppression that permits the unchecked proliferation of EBV-infected lymphocytes. These disorders include both central nervous system and systemic lymphomas. AIDS-related central nervous system lymphomas are derived from germinal center B cells and (Cesarman, E., Mesri, E. A. 1999) include immunoblastic and large lymphomas. The AIDS-related systemic lymphomas include diffuse large cell lymphomas, immunoblastic lymphomas, Burkitt's lymphomas, and small, Burkitt's-like lymphomas. EBV positivity for these lymphomas

ranges from 30 to > 90% (Cesarman, E., Mesri, E. A. 1999, Davi, F. et al., 1998; Shibata, D. et al., 1993).

1.4.7 Treatment

Despite the growing understanding of the role of EBV in the pathogenesis of disease, the control of EBV remains unsatisfactory and novel therapeutic approaches are explored.

There are several antiviral compounds that have entered the clinical setting and have some anti-EBV activity. However, it has been difficult to demonstrate reproducible antitumor effects. The majority of these drugs are broad-spectrum anti-herpesvirus and anticytomegalovirus agents that vary in their effectiveness against EBV. They include ganciclovir, famcyclovir, acyclovir, valaciclovir (a prodrug of acyclovir), foscarnet, and cidofovir. Moreover, a variety of non conventional compounds may have antiviral effects. For instance, flavanones (amorilin and lupinifolin) from plant extracts block EBV early antigen activation in vitro (Itoigawa, M. et al., 2002). Additionally, flavonoid derivatives synthesized from morin and quercetin and several herbal remedies may have varying degrees of anti-EBV activity (Iwase, Y. Et al., 2001, Kapadia, G. et al., 2002). The drawback of antivirals is that they have no effect on the latent EBV infection .

Adoptive immunotherapy, using EBV-specific CTLs, may overcome this problem (Gottschalk, S. et al., 2002). CTLs may be isolated from the recipient's own lymphocytes, expanded in

vitro, and infused back into the patient. This approach has been proven effective in the treatment of PTLD (Heslop et al., 2010), but unsuccessful in patients with NPC or HD.

Many studies have been directed to develop an EBV vaccine that would be used both for preventive as well as therapeutic purposes. Vaccination with recombinant gp350 viral glycoprotein or CTL epitope-based peptides have been successful in generating viral immunity in animal models (Jackman WT et al., 1999) and clinical vaccine trials in healthy individuals demonstrated the appearance of neutralizing anti-EBV antibodies in vaccinated individuals (Moutschen M et al., 2007). Another therapeutic approach is based on antibodies directed against B-cell antigens, such as CD20 to obtain the depletion of EBV-infected B-cells. The anti-CD20 monoclonal antibody designated Rituximab has been efficiently used in the treatment of a variety of CD20-expressing lymphomas as well as of EBV-related lymphoproliferative disorders and PTLDs. However, rituximab treatment is unsuitable for immunosuppressed patient since normal B cells are also destroyed increasing the risk of concomitant infections (Lee MY et al., 2007).

In the last years, attempts have been made to use antiviral compounds in conjunction with drugs that can induce lytic replication through epigenetic modifications with valproic acid or SAHA (Vorinostat), both histone deacetylase inhibitors. Azydothymidine (AZT), bortezomib, gemcitabine, and doxorubicin can also induce lytic replication and expression of viral thymidine kinases, so their use in combination with other treatment modalities has also been

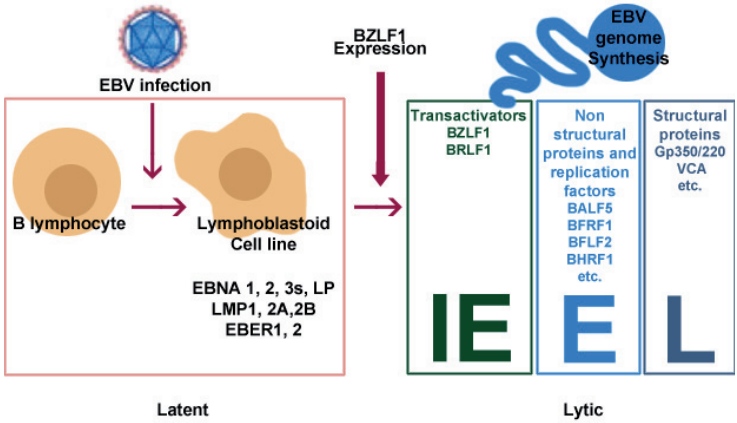
proposed as a form of oncolytic therapy. These approaches have shown results in experimental systems, but results from clinical trials have not been published.

1.4.8 EBV and lytic cycle

The latency to lytic switch has implications for the pathogenesis of EBV-associated malignant and non malignant diseases. While latency may be the predominant life cycle form in virus-associated cancers, the virus must replicate in order to be transmitted between cells and among individuals. This lytic phase of infection is clinically associated with infectious mononucleosis and oral hairy leukoplakia (Rickinson and Kieff, 2007). The cascade of events in the lytic phase of the EBV life cycle is divided into three phases of regulated gene expression: immediate–early (IEA), early (EA) and late (LA) antigens. Although the latency to lytic cycle switch can be manipulated in cell culture, by the addition of one or more chemical-inducing agents, little is understood about the physiologic event *in vivo*. The stimulus may be external, for example ralterations of activating or inhibitory signals, or may be internal, reflecting the metabolic state of the cell and its position in the cell cycle.

Figure 3.

Switch from latent to lytic cycle.



Virally encoded lytic cycle activator genes (IEA)

ZEBRA, encoded by the viral BZLF1 gene, is a member of the basic zipper (bZIP) group of transcriptional activators like mammalian cellular members c-Fos and c-Jun. ZEBRA binds to canonical AP-1 sites as well as ZEBRA response element (ZRE) in promoters of viral and cellular genes and activates their transcriptions. ZEBRA is essential to activate lytic cycle genes (Feederle et al., 2000), in particular the expression of another transcription factor, Rta, encoded by the viral BRLF1 gene (Kolman et al., 1996). ZEBRA and Rta then act in synergy to activate transcription of early lytic cycle genes many of them required for lytic viral DNA replication (Quinlivan et al., 1993; Ragoczy and Miller, 1999). However, Rta seems to be able of activating a distinct set of viral genes independent of ZEBRA (Chen et al., 2005; Ragoczy and Miller, 1999). ZEBRA

represses the action of Rta on some promoters in a temporally controlled manner (El-Guindy and Miller, 2004; Ragoczy and Miller, 1999). ZEBRA is involved in viral lytic DNA replication by binding to the origin of lytic cycle replication (oriLyt) and by interacting with and recruiting viral proteins that are essential for lytic replication (Baumann et al., 1999; Fixman et al., 1992; Gao et al., 1998; Lieberman et al., 1990; Schepers et al., 1993, 1996).

During latency the BZLF1 and BRLF1 genes are probably repressed by epigenetic mechanisms such as chromatization and DNA methylation of the BZLF1 promoter, Zp, and BRLF1 promoter, Rp, as well as occupancy of these promoters by specific repressors. Following application of an inducing stimulus, cellular activators ,such as Sp1, c/EBPα, AP-1, and CREB, likely bind to Zp and Rp. These cellular factors lead to low-level expression of ZEBRA and Rta, which then amplify the signal by autostimulating and cross-stimulating each other's expression. Rta autostimulates Rp by an indirect mechanism that involves interaction with other proteins such as those of the Sp1 family (Ragoczy and Miller, 2001). Rta may influence the activity of cellular transcription factors by protein–protein interactions and by activating signaling cascades that lead to posttranslational modification of the cellular transcription factors (Adamson et al., 2000).

ZEBRA and Rta synergistically activate expression of many downstream viral target genes, resulting in synthesis of early antigen (EA) and late antigens (LA), with the final production of the progeny virus.

Functions of the main early antigens (EA)

BALF5

After induction of viral productive replication, the EBV genome is amplified 100-1,000-fold by viral replication machinery composed of BALF5 DNA polymerase, BMRF1 polymerase processivity factor, BALF2 single-stranded DNA-binding protein, and BBLF4-BSLF1-BBLF2/3 helicase-primase complex in replication compartments in the nucleus (Daikoku, T et al., 2005; Fixman, E. D. et al., 1995). BALF5 possesses intrinsic DNA polymerase and 3'-to-5' exonuclease activities (Tsurumi, T et al., 1994) and forms a complex with the BMRF1 polymerase accessory protein increasing its processivity (Tsurumi, T 1993).

BFRF1 and BFLF2

One common feature shared by several viruses during the envelopment process to exit from the nucleus is the modification of the nuclear envelope structure. The nuclear envelope is composed of the outer and inner nuclear membranes and filamentous proteins called lamins, attached to the nuclear membrane by integral membrane proteins and collectively forming the nuclear lamina. For herpesviruses, dismantling of the nuclear lamina during HSV-1 infection has been reported by Scott and O'Hare (Scott, E. S., and P. O'Hare 2001), and a similar effect has been attributed to

protein kinase C (PKC)-mediated phosphorylation of the nuclear lamina induced by the BFRF1 and BFLF2 homologs during mouse cytomegalovirus infection (Muranyi, W et al., 2002). Gonnella R et al. (2005) reported the association of the BFRF1-BFLF2 complex with lamin B at the nuclear membrane, showing the possible involvement of these proteins in affecting nuclear lamina structure and function. In addition, an interesting observation is represented by the profound nuclear alterations induced by expression of BFRF1 and even more by BFRF1/BFLF2 coexpression, thus suggesting the involvement of the two viral products.

Viral homologues of anti-apoptotic members of the BCL-2 family

Herpesviruses are known to encode homologues of cellular anti-apoptotic BCL-2 proteins, which protect the infected host cell from apoptosis during viral synthesis (Boya et al., 2004). EBV even encodes two viral BCL-2 proteins, BHRF1 and BALF1, highly expressed within the first 24 h post infection, hardly detectable in latently infected cells and again maximally expressed during the lytic cycle. Genetic analyses demonstrated that primary resting human B cells infected with a Bcl-2-negative EBV did not enter the cell cycle and died of immediate apoptosis. Moreover, the inactivation of these genes disabled EBV's capacity to transform the primary B cells but did not negatively affect virus de novo synthesis in established, latently infected cells (Altmann and Hammerschmidt, 2005). The expression of the two viral BCL-2

proteins were previously thought to be induced by BZLF1, during EBV's lytic phase. However, the early expression of viral Bcl-2 genes in the early phase of infection (Altmann and Hammerschmidt, 2005) suggests that their expression may be also not regulated by BZLF1 activation.

1.4.9 Lytic program reactivation in vitro

In latently infected cells, the induction of the lytic phase of EBV's life cycle can be achieved by different compounds *in vitro*. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is able to trigger the reactivation of EBV through PKC signaling pathway (Baumann M. et al., 1998), while transforming growth factor beta 1 (TGF-beta1) stimulates MAPK cascade (Fahmi H. et al., 2000). Finally, histone deacetylase (HDAC) inhibitors promote the switch from latent to lytic phase in PKC independent manner, leading to the derepression of silenced promoters and subsequent changes in gene expression (Jenkins P.J. et al., 2000). All these agents activate expression of the viral immediate-early protein BZLF1.

Of the different ways to reactivate the lytic program in vitro, treatment with anti-Ig antibodies seems to be more physiologically relevant since it mimics B-cell antigen-receptor (BCR) signaling (Iwakiri and Takada, 2004; Takada, 1984; Tovey et al., 1978). Upon antigen binding, BCR aggregates and activates protein tyrosine kinases Lyn, a member of Src family of tyrosine kinases, and Syk. Simultaneously, antigen binding causes activation of PI3K (through

BCR co-receptor CD19), which, along with Lyn and Syk, is involved in activation of Bruton's tyrosine kinase (Btk). Once Lyn, Syk, and Btk are activated, the signal is further propagated through activation of phospholipase Cc2 (PLCc2). PLCc2 hydrolyzes phosphatidylinositol diphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 generation causes the mobilization of Ca_2+ ions required for the activation of several transcription factors (such as NF-kB), whereas DAG activates conventional protein kinase C (PKC) isotypes which regulate MAPK family. Following activation, these kinases phosphorylate different sets of transcription factors, which then regulate expression of certain genes. Other similar 'downstream' effectors are Akt and Ca_2+-calmodulin. A number of studies demonstrated that activation of PI3K, PKC, MAPK, and NF-kB pathways is crucial for activation of EBV lytic program (Daibata et al., 1990; Darr et al., 2001; Davies et al., 1991; Gao et al., 2001; Iwakiri and Takada, 2004; Lazdins et al., 1987; Mori and Sairenji, 2006; Oussaief et al., 2011; Satoh et al., 1999); however, other reports suggested that inhibition of PI3K or NF-kB pathways facilitates reactivation of lytic program of several gammaherpesviruses (Brown et al., 2003; Liu et al., 2008; Peng et al., 2010).

Superinfection of latently infected cells with EBV defective substrains or ectopic expression of viral immediate early genes are also methods used to promote reactivation *in vitro* (Takada K. e Ono Y., 1989; Adamson A.L. e Kenney S.C., 1998).

2. Aim of the project

Resveratrol is a potent chemopreventive agent with in vivo and in vitro proved efficacy against a broad spectrum of malignant cells and among them, it has been reported that can inhibit the growth of malignant B-cell lines in a dose- and time-dependent manner (Gupta SC et l., 2010, 2011).

As described above, EBV infects B cells and transforms them by subverting multiple cellular pathways that control proliferation and apoptosis. However, the virus, during the latent phase of infection established in most of EBV-related malignancies, differentially expresses its antigens that may vary from one (latency I), to nine (latency III). Recently it was reported that different Burkitt's lymphomas express different combinations of EBV antiapoptotic genes indicating that the virus contributes survival functions of these tumors in multiple ways. We hypotesized that EBV-related tumors that vary for the pattern of viral antigens expressed might show distinctive responsiveness to the antiproliferative activity of resveratrol.

One goal of this project was therefore to assess whether resveratrol would be able to inhibit the growth of malignant B cells in which EBV infection might confer additional survival potential, also depending on the different programs of latent gene expression adopted by the virus.

To this end, in the same cellular background, I have investigated the contribution that different patterns of EBV gene expression give

to resveratrol-induced susceptibility to apoptosis and studied the molecular mechanisms underneath. In particular, the role played by the viral oncogene LMP1 and strategies to down-regulate its expression, have been examined.

EBV productive cycle allows horizontal spread of the virus and favors B cell tumors development. Moreover, large amounts of virus are produced in infectious mononucleosis (IM) and hairy leucoplakia (HLP) of the tongue, in AIDS patients.

Therefore, the second goal of this project was to evaluate the activity of resveratrol against EBV replication. For this purpose, I have examined the effects of the polyphenol in two Burkitt's lymphoma cell lines where is possible to activate EBV lytic cycle by treating the cells with different inducing agents. In particular, in cross-linked Akata cells, a system for EBV activation that most likely mimics the mechanism of viral reactivation in vivo, I evaluated the effects of resveratrol on EBV lytic genes expression and viral particles production. Drug targets that might affect EBV replicative activity, have been studied.

3. Materials and Methods

3.1 Reagents

Resveratrol (Sigma), prepared a 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.

3.2 Cell culture and treatment with resveratrol

Human Burkitt's lymphoma (BL)-derived cell lines, either positive or negative for EBV, and human B lymphoblastoid cell lines were used. EBV-positive Raji and EBV(-) Ramos cells were purchased from American Type Culture Collection (LGC Promochem). Akata with the EBV genome (Takada K 1984;Takada K, Ono Y. 1989), the isogenic EBV(-) Akata subline 2A8 (Shimizu N, et al., 1994), and Jijoye M13 cells (Minarovits J. et al., 1991) were obtained from Dr. P. Trivedi (Sapienza University, Rome, Italy). Lymphoblastoids LCL1087 and LCL1260 were gifts from Dr. R. Dolcetti [Cancer Bioimmunotherapy Unit, Aviano (PN), Italy]. All cell lines were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) and antibiotics in a 5% CO2 atmosphere and maintained at a cell density of 3.5 x 10^5/mL. Cells, at a density of 3 x 10^5 cells/mL, were incubated in RPMI-1640/10% FCS with different concentrations of resveratrol (Sigma) from a stock solution of 50

mg/mL dissolved in ethanol and kept at 20°C protected from light. Control cells were treated with the diluent. At different times, viable cells excluding Trypan blue dye were counted in a Burker chamber. The percentage of proliferation was determined as proliferation of treated cells x 100/proliferation of untreated cells.

3.3 EBV lytic cycle induction

EBV-positive Raji cells, characterized by a latency III pattern and an EBV abortive lytic cycle and Akata cells showing a latency I phenotype, were used. To induce EBV lytic cycle, Raji cells (1.5 x 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 EA 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.

3.4 Cytofluorimetric analysis

Cell-cycle studies were conducted by fluorescence-activated cell-sorting (FACS) analysis after DNA staining with propidium iodide (PI). Cell samples were washed with phosphate buffer saline (PBS) and centrifuged for 5 min at 300 × g. The cell pellet was fixed for 1 h at 4°C with 70% ethanol, washed with PBS, and treated for 1 hour with 100 μg/ml of PI and 100 μg/ml RNase. DNA content was assessed by using an Epics Coulter XL flowcytometer. Apoptosis was evaluated by Annexin V-FITC apoptosis detection kit (Sigma), which measures Annexin V binding to phosphatidylserine in conjunction with propidium iodide (PI) staining, according to the accompanying procedure.

3.5 Western blot analysis

Western blot analysis on whole-cell lysates was conducted. Cells were collected and washed with PBS before being lysed in Laemmli buffer. To detect phosphorylated proteins, cells were resuspended in lysis buffer (0,1% NaN_3, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 0.5 μM aprotinin, 4 μM leupeptin, 2 mM PMSF, 10 mM NaF, 10 mM iodoacetamide, 1 mM Na_3VO_4, 1% Triton-X 100 in PBS) and kept for 30 min on ice. Where needed, nuclei were isolated (Foisy S et al., 1997) and lysed in Laemmli buffer. 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 blots were probed with the following primary antibodies: cyclin A (1:1,000), cyclin E (1:1,000), cdk1 (1:1,000), p27 (1:50), Mcl-1 (1:200; all by Santa Cruz Biotechnology); cdk2 (1:1000; Biomol); PARP (1:1,000; Alexis Biochemicals, Enzo Life Sciences); active caspase 3 (1:1000; AbCam); XIAP and IAP1 (1:500; R&D System); FLIP (1:500; Upstate Biotechnology); phospho-p38 (catalogue no. 9211), total p38, phospho-ERK1/2 (catalogue no. 9101), total ERK1/2 (1:1000; Cell Signaling); LMP1 (1:7500; Pharmingen); 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 (BioRad) and signals were visualized by ECL detection kit (Amersham).

3.6 Electrophoretic Mobility Shift Assay (EMSA)

To determine NF-kB and AP1 activation, an electrophoretic mobility shift assay (EMSA) was carried out. Whole cell extracts were obtained after lysis in a high salt extraction buffer (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X100, 0.5% NP40, 10% glycerol, 2 mM DTT, 2 μM aprotinin, 2 μM leupeptin, 1 mM Na_3VO_4, 2 μM pepstatin, 1 mM

PMSF). 20 μg were incubated with 30 fmol of a DIG-labeled (DIG oligonucleotide 3' end-labeling kit, Roche Applied Science) NF–kB (Zabel et al., 1991) and AP-1 (Saatian et al., 2006) DNA probe, in a binding buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 10% glycerol) containing 1 μg BSA, 0.5 μg poly d(I-C), for 20 min at room temperature. Complexes were resolved by nondenaturing 4% polyacrylamide gel electrophoresis, transferred to nylon membrane and detected by chemiluminescence (DIG luminescent detection kit, Roche Applied Science). The specificity of binding was also verified by competition with the unlabeled oligonucleotides. For supershift assay, cell extracts were incubated with antibodies against either p50 or p65 of NF-kB (Santa Cruz Biotechnology) for 30 minutes at room temperature and then analyzed by EMSA. Quantitative evaluation of NF-kB complex formation was carried out by ImageJ Freeshare Software (http://rsbweb.nih.gov/ij).

3.7 Fluorescence microscopy

Cells incubated with resveratrol or with diluent for 24 hours were washed with PBS, spotted on slides, and fixed with methanol for 5 minutes at - 20°C. Fixed cells were incubated with p65 NF-kB subunit antibodies (1:500; Santa Cruz Biotechnology) for 1 hour at 37°C, rinsed in PBS, and then incubated with fluorescein isothiocyanate– conjugated secondary antibodies for 30 minutes at room temperature. Subsequently, cells were rinsed and nuclei stained with 4',6-diamidino-2-phenylindole (DAPI; 0.2 μg/mL) for 15 minutes. The coverslips were mounted with 0.1% (w/v)

p-phenylenediamine in 10% (v/v) PBS, 90% (v/v) glycerol, pH 8 (18), and the specimens observed by immunofluorescence microscopy using a LeicaDM4000 fluorescence microscope equipped with an FX 340 digital camera.

3.8 LMP1 silencing and overexpression

To obtain LMP1 downregulation, we used a human H1 RNA polymerase III promoter–based short hairpin RNA (shRNA) vector, pSilencer 3.1-H1 hygro (Ambion), to express siRNA that targets the EBV LMP1 in Raji cells. The sense- and antisense-strand oligonucleotides for LMP1 shRNA were 5'-gatccGGA ATTTGCACGGACAGGCttcaagagaGCCTGTCCGTGCAAA TTCCtttttttggaaa-3' and 5'-agcttttccaaaaaaaaGGAATTTGCA CGGACAGGCtctcttgaaGCCTGTCCGTGCAAATTCCg- 3'. A pSilencer hygro vector expressing a hairpin siRNA with limited homology to any known sequences in the human genome was used as a negative control (control siRNA). The constructs were prepared by ligating the annealed oligonucleotides into the BamH1 and HindIII of pSilencer and the resulting vectors analyzed by DNA sequencing. A total of 1.5 x 10^7 cells were electroporated with 30 μg of siRNA expression vectors at 975 μF, 260 V, using a Gene Pulser II system (Bio-Rad). To select clones, electroporated cells were grown in medium containing 100 μg/mL hygromycin (Sigma). After 3 weeks, resistant clones were grown up and tested for LMP1 protein expression. Transient expression of siLMP1 in LCLs was obtained by electroporation with the specific siRNA and

the control plasmid according to the conditions used for Raji cells. To enforce transient LMP1 expression, 1.5 x 10^7 EBV (-) Akata 2A8 cells were electroporated (960 µF, 230 V) with 10 µg of LMP1 expression vector pSG5-LMP1 (a kind gift of Dr Martin Rowe, Birmingham University Medical School, United Kingdom) or of the corresponding control vector pSG5.

3.9 Semiquantitative RT-PCR assay

Total RNA was extracted from 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) 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'cagcgattctggctgttgtggttt; 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'ttcgacagtcagccgcatcttctt; GAPDH-R, 5'gcccaatacgaccaaatccgttga. Amplification products were resolved on 1.5% agarose gels and stained by GelRed (Biotium).

3.10 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 300xg 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. A reporter dye 6-carboxyfluorescein (FAM)-labeled probe for the viral EBNA1 gene 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.

3.11 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 x 10^5cells), incubated for the last 15 min with 40 μM 5,6-carbossy-2',7'-diclorohydro fluoresceine diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) were collected at 30, 60 and 120 min. ROS production was analyzed by measuring the oxidized fluorescent derivative DCF with a flow-cytometer (excitation: 488 nm, emission: 530 nm).

The levels of ROS detected in H_2O_2 treated cells have been used as positive controls.

3.12 Statistical analysis

Statistical analysis was carried out using the ANOVA followed by post hoc tests (Bonferroni/Dunn). The level of significance was set at $P < 0.05$.

4. Results

4.1 Resveratrol inhibits proliferation of EBV-positive BL cells

As we aimed to determine whether EBV gene expression played a role on the susceptibility of BL cells to resveratrol, we tested 2 EBV(-) BL cell lines (2A8 and Ramos) and 3 EBV(+) BL cell lines, the latter expressing different patterns of EBV latency genes. In particular, Raji cells show a latency III phenotype; Jijoye M13, a subline of Jijoye cells classified as group II like (Minarovits J. et al. 1991), express EBNA1 and LMP2A, whereas Akata cells display a latency I program. The cell lines, exposed to resveratrol concentrations increasing from 20 to 300 μmol/L for various periods of time, showed different degrees of sensitivity to the polyphenol. Figure 4 shows that the growth of each cell line was inhibited by resveratrol in a concentration- and time-dependent manner. The concentrations reducing cell proliferation by 50% at 48 hours were of about 300 μmol/L for Raji, 130 μmol/L for Jijoye M13 cells, and 40 μmol/L for Akata and EBV(-) BL cells. The values determined for latency III and latency II cells were significantly different between each other and with respect to latency I and EBV (-) cells ($P < 0.05$). Altogether, resveratrol cytotoxicity for EBV(-) and Akata cells was approximately 3- and 7-fold greater than that observed for Jijoye and Raji cells, respectively.

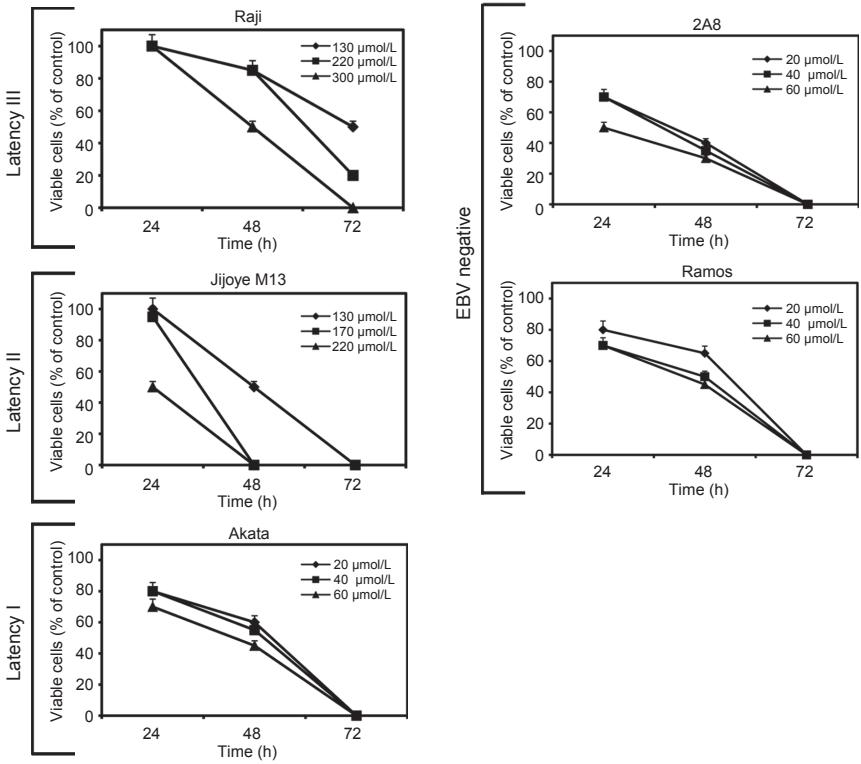


Figure 4.

Resveratrol-induced inhibition of BL cell proliferation. Cells were incubated with resveratrol at the concentrations indicated. After 24, 48, and 72 hours, cell counts were assessed by Trypan blue staining.

Percentage of proliferation was determined as proliferation of treated cells x 100/ proliferation of control cells treated with the diluent. Each point represents the mean ± SD of 3 similar experiments.

4.2 Resveratrol induces cell-cycle arrest and apoptosis of EBV-infected BL cells

To investigate the antiproliferative effect of resveratrol on BL cells, we analyzed the cell-cycle distribution after incubation with the polyphenol. To compare the different cell lines, all the following experiments were carried out by exposing each cell line to the resveratrol concentration reducing cell proliferation by 50% at 48 hours, as previously determined. Cells were harvested at 12 and 24 hours, and the DNA content was determined by PI staining and flowcytometric analysis. The bar graph in Figure 5A shows that 12-hour treatment with resveratrol increased significantly ($P < 0.01$) the percentage of cells in the G1 phase with a reduction of cells in S and G2–M phases. After 24-hour incubation, the G1 population further increased in Raji and Jijoye M13 cells whereas decreased in Akata cells, 2A8, and Ramos cells. In the latter (latency I and EBV(-) Burkitt's lymphoma), the proportion of cells in the sub G0–G1 phase, representing apoptotic events, augmented after 24-hour incubation (data not shown), suggesting a relation between cell-cycle arrest and apoptosis. Therefore, we evaluated apoptosis in cells incubated with resveratrol for 12 and 24 hours, after staining with Annexin V and PI. Annexin V binding to phosphatidylserine, on the outer side of the cell membrane, characterizes the cells entering apoptosis, whereas cells double stained by Annexin V and PI represent the fraction of late apoptotic/ necrotic cells. As it appears from Figure 5B, the ratio between Annexin V and Annexin

V/PI-positive cells appeared to be dramatically different among the cell lines. In fact, after 24-hour exposure to resveratrol, the percentages of early apoptotic cells in Raji and Jijoye were about 30% and 55% whereas the fractions of late apoptotic/ necrotic cells were about 8% and 12%, respectively. In addition, the percentage of Annexin V–positive cells in Jijoye M13 was significantly higher than that measured in Raji ($P < 0.0001$). Conversely, in Akata cells as well as in 2A8 and Ramos cells, at both time points, the percentages of cells double stained by Annexin V and PI were larger than those of cells stained only by Annexin V. In particular, in EBV(-) 2A8 and Ramos, Annexin V/PI-positive cells reached about 30% whereas single stained cells at 24 hours were about 12% and 22%, respectively. In Akata, the fraction of double-positive cells was lower than those measured for EBV(-) cell lines but still higher than that of cells stained only by Annexin V. Taken together, these data seemed to indicate that EBV(-) BL cells and latency I Akata were more prone to resveratrol-induced apoptosis than latency II and latency III cell lines. Western blot analysis of PARP processing confirmed that resveratrol differentially induced apoptosis in BL cells. As illustrated in Figure 5C, after 12-hour incubation, the 85-kDa cleavage product of PARP appeared in latency I Akata and EBV(-) cell lines and further increased after 24 hours. In contrast, PARP apoptotic form was detectable in Jijoye M13 cells after 24-hour exposure to resveratrol and in Raji cells only after 48 hours (data not shown), indicating that latency III BL cells were more resistant to resveratrol induced apoptosis than the other cell lines.

To investigate the apoptotic pathway, we analyzed the activation of effector caspase 3 by measuring the levels of the 17-kDa cleaved form. Data reported in Figure 5C showed higher levels of the biologically active caspase 3 along with increased amounts of the processed PARP, thus indicating that resveratrol induces apoptosis by the caspase-dependent pathway.

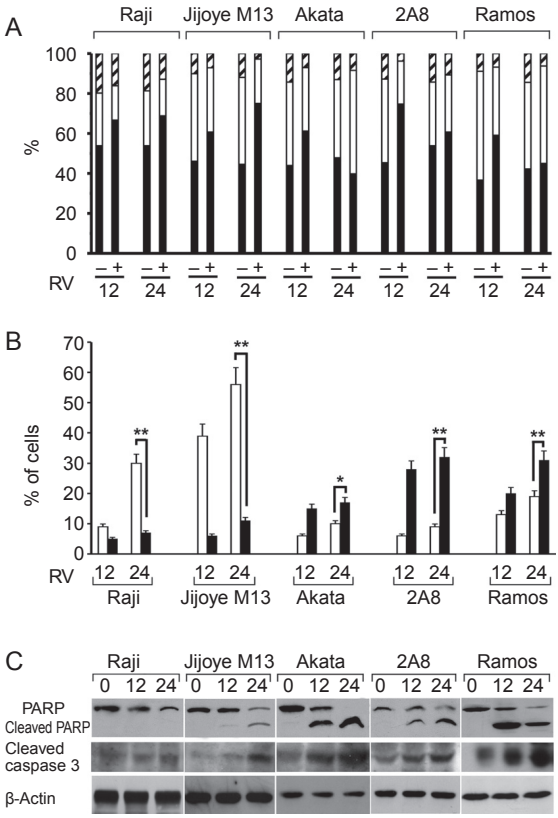


Figure 5.

Resveratrol (RV) induces cell-cycle arrest in G1 phase and apoptosis of BL cells. Each cell line was cultured with the resveratrol concentration reducing proliferation by 50% at 48 hours or with the diluent for 12 and 24 hours. A, fixed cells were stained with PI and analyzed by flow cytometry. The percentages of cells in G1 (black bars), S (white bars) or G2–M phase (hatched bars) are shown. B, evaluation of apoptosis by FACS analysis after Annexin V and PI double staining. Annexin V–positive cells (empty columns) and Annexin V/PI-stained cells (black columns), mean values ± SD. *, $P < 0.01$ or **, $P < 0.001$ vs. Annexin V/PI. C, detection of PARP cleavage and active caspase 3. At the indicated times, cell lysates were prepared and analyzed by Western blots. β-Actin was used as the internal marker. The data are representative of those obtained in at least 3 independent experiments with similar results.

4.3 Modulation of cell-cycle and apoptosis-associated proteins by resveratrol

To clarify the molecular mechanism of resveratrol-induced cell-cycle arrest in G1 phase and the subsequent apoptosis, we examined by Western blot analysis the expression of several cell-cycle and apoptosis-related proteins (Fig. 6). Exposure to resveratrol increased the expression of the cdk inhibitor p27 and that of cyclin E in all cell lines; in contrast, cyclin A, cdk1, and cdk2 were downregulated in Raji and Jijoye M13 cells but did not vary significantly in Akata and in the EBV(-) cell lines (Fig. 6A). Because S-phase cdk1/2 and cyclin A are essential for DNA synthesis, their decrement along with the increment of cdk inhibitor p27 indicate that resveratrol blocks specific cell-cycle–associated activity involved in the progression from G1 to S-phase. Moreover, the levels of the c-myc oncogene were strongly downregulated in Raji and Jijoye M13 cells and a significant decrement was detected in Akata as well as in 2A8 and Ramos cells. The levels of expression of the antiapoptotic proteins IAP1, FLIP, and XIAP appeared to diminish in all cell lines, whereas Mcl-1 levels were reduced by 80% in Raji and 40% in Jijoye M13 but only slightly in the other cell lines (Fig. 6B). Conversely, the Bcl-2 protein, whose gene is deleted in Ramos, was overexpressed in the other cell lines; furthermore, the tumor suppressor p53 gene, deleted in Akata, was upregulated in Raji, Jijoye M13, and Ramos cells (Fig. 7).

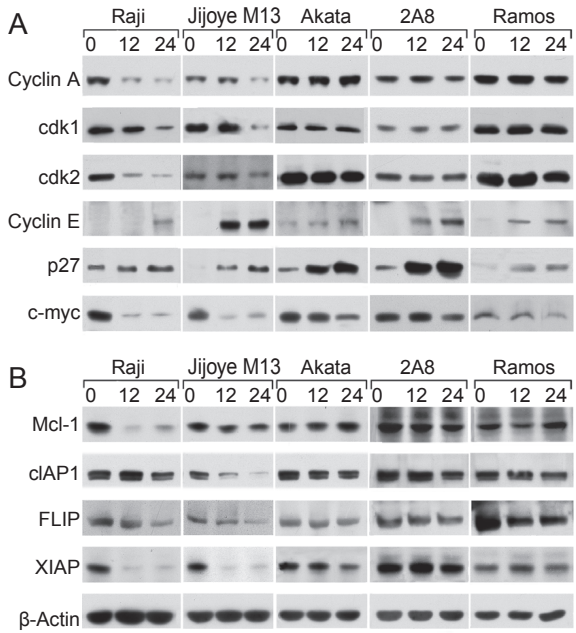


Figure 6.

Western blot analysis of cell cycle (A) and apoptosis (B) regulatory proteins following resveratrol treatment of BL cells. At the indicated times (hours), cell lysates were analyzed by 12% SDS-PAGE followed by immunoblot analysis. Specific signals were visualized and quantified as described in Materials and Methods. A representative result of 3 independent experiments is shown. β-Actin was used as the internal marker.

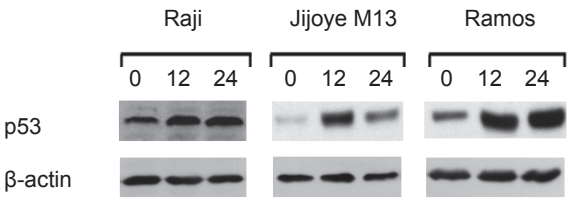


Figure 7.
Western blot analysis of p53 protein following resveratrol treatment of BL cells. At the indicated times (hours), cell lysates were analyzed by SDS-PAGE followed by immunoblot analysis. Specific signals were visualized and quantified as described in Methods. A representative result of three independent experiments is shown. β-actin was used as the internal marker.

4.4 Resveratrol activates p38 and inhibits ERK1/2 MAPK pathways

It has been reported that p38 MAPK and ERK signaling pathways may be important in determining cell proliferation or apoptosis. To investigate whether resveratrol treatment of BL cell lines affected MAPK pathways, we incubated EBV(+) and EBV(-) BL cells with the polyphenol for different periods of time and analyzed the cell lysates by Western blotting with phospho-specific antibodies (Fig. 8). For each cell line, a positive control for MAPK activation pathway was obtained by exposing the cells to 12-O-tetradecanoylphorbol-l3-acetate (TPA) for 1 hour. Activation of p38 in Akata and Ramos cells was detected 3 hours after the addition of resveratrol, as the signal identifying phospho-p38 elevated to about 4- and 5-fold, respectively, with respect to that at time 0. A similar strong activation of p38 MAPK was observed for Raji and Jijoye M13 after 6 hours of

incubation with resveratrol. In contrast, the signals corresponding to phospho-ERK1/2, highly represented in untreated cells, rapidly disappeared following the addition of resveratrol, indicating that this pathway was dramatically inhibited by exposure of the cells to the polyphenol. Unexpectedly, both MAPK signaling pathways did not appear to be modulated by resveratrol in 2A8 cells, although they were both activated by TPA treatment. In addition, the analysis of the phosphorylation pathways of c-jun-NH2-kinase and protein kinase C did not reveal significant variations in all BL cell lines exposed to resveratrol (data not shown).

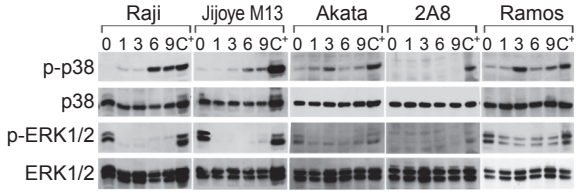


Figure 8.
Western blot analysis of phosphorylation of p38 and ERK1/2 MAPK in resveratrol-treated BL cells. At the indicated times (hours), cell lysates were prepared and the phosphorylation pattern of p38 and ERK was evaluated by Western blot analysis with the specific antibodies for each protein and its phosphorylated (p-) form; C^+, TPA-treated cells as the positive control. Specific signals were visualized and quantified as described in Materials and Methods.

4.5 Resveratrol inhibition of NF-kB activity in BL cells

In BL tumor cells, c-myc overexpression is linked to chromosomal translocation in one of the Ig gene loci; it has been shown that NF-kB activity is required for c-myc expression and constitutive NF-kB DNA-binding activity in different types of B-cell malignancies, including Burkitt's lymphoma, has been reported. To investigate the effect ofresveratrol on NF-kB activity in BL cells, EBV(+) and EBV (-) cell lines were treated with resveratrol or control diluent for up to 9 hours. After different periods of incubation, whole-cell extracts were used to measure NF-kB activity by EMSA. Figure 9A shows that Raji and Akata cells displayed high constitutive NF-kB DNA-binding activity. Resveratrol caused a marked inhibition of NF-kB activity in Akata cells already after 1 hour of treatment. In Jijoye M13, 2A8, and Ramos cells, after a rapid and transient increment, NF-kB activity decreased under control levels. In contrast, exposure of Raji cells to resveratrol did not significantly alter NF-kB DNA-binding activity during the first 9 hours of treatment. To confirm that the retarded band visualized by EMSA was indeed NF-kB, cell extracts were incubated with antibodies to either p50 or p65 and then analyzed by EMSA. Antibodies to either subunit of NF-kB shifted the band to higher molecular weight, suggesting that the active complex consisted of p50 and p65 subunits (Fig. 10). The effect of resveratrol on NF-kB activity seemed to be specific for this transcription factor because EMSA carried out to measure AP1 activity in parallel samples did not reveal a significant decre-

ment in EBV-positive cell lines and variable levels were detected in EBV(-) cells (Fig. 11). NF-kB–binding activity decreased, however, in all cell lines treated for prolonged time with resveratrol (data not shown). Immunofluorescence studies and Western blot analysis conducted on Raji cells exposed to the polyphenol for 24 hours showed a dramatic decrement of the p65 subunit (Fig. 9B and C). In particular, the fluorescent signal decorating the nucleus and the cytoplasm of untreated cells in bright patches appeared weak and mainly localized to the cytoplasm in resveratrol-treated cells.

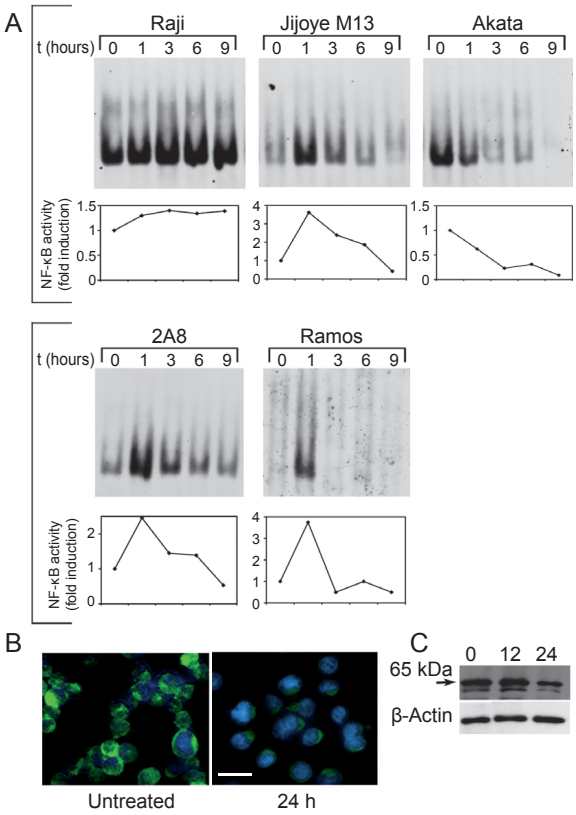


Figure 9.

NF-kB activity in resveratrol-treated BL cells. A, at the indicated times (hours), whole-cell extracts were incubated with a consensus NF-kB–binding site oligonucleotide to evaluate NF-kB activity by EMSA (see Materials and Methods). The signals, quantified by densitometry, are expressed as fold induction of time 0. The data shown are representative of 3 independent experiments with similar results. B, Raji cells untreated or treated with resveratrol for the times indicated were analyzed with RelA/p65 antibodies by indirect immunofluorescence; DNA was visualized with DAPI, and the merge images are displayed; scale bar, 20 μm. C, 50 μg of nuclear proteins from Raji cells untreated (0) or treated with resveratrol for 12 and 24 hours were subjected to SDS-PAGE and Western blot analysis with RelA/p65 antibodies as described (see Materials and Methods).

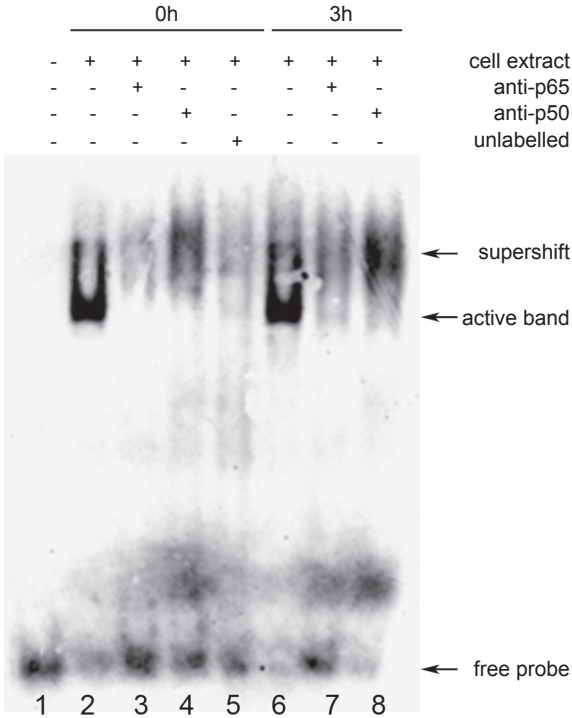


Figure 10.

Supershift assay . The cell extracts from control cells and cells treated for three hours with RV, were incubated with anti-p65 or anti-p50 antibodies and analyzed by EMSA. Lane 1, without cell extract; Lane 2 and 6, kB probe alone; Lane 3 and 7, with p65 antibody; Lane 4 and 8 with p50 antibody; Lane 5, unlabeled probe.

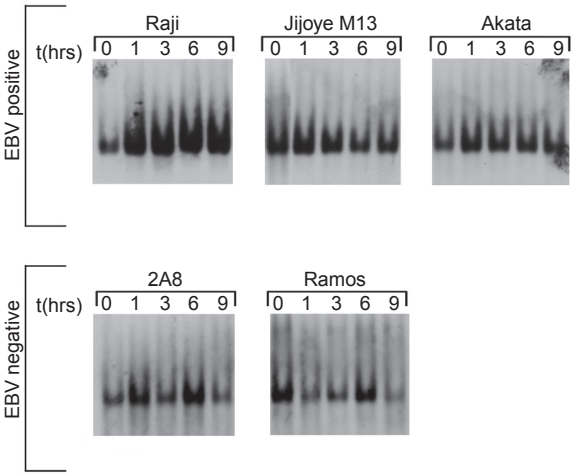


Figure 11.

AP1 activity in resveratrol-treated BL cells. (a) At the indicated times (hours), whole-cell extracts were incubated with a consensus AP1 binding site oligonucleotide to evaluate AP1 activity by EMSA (see Methods).

4.6 LMP1 expression modulates the sensitivity of BL cells to resveratrol

Because Raji cells seemed to be more protected from resveratrol-mediated apoptosis, we looked for EBV genes expressed in the latency III program that would confer additional survival advantage against the effects of the polyphenol. LMP1 is the principal EBV oncogene with the potential to antagonize apoptosis as well as to promote cellular survival and proliferation (Li HP et al.2003; Dirmeier U et al. 2005; Soni V et al.2007). We therefore set to investigate the effects of resveratrol after silencing LMP1 expression in Raji cells. Raji 5E, a clone selected after transfection of Raji cells with a construct expressing LMP1 siRNA, and Raji control

transfected with a control plasmid were exposed to resveratrol or to the diluent for 24 hours and viable cells determined by Trypan blue staining. Figure 12A shows that in Raji 5E, cell proliferation was reduced by about 40% as compared with that measured in the control. This result clearly indicated that inhibition of LMP1 expression in clone 5E, as confirmed by Western blot analysis (Fig. 12B), dramatically increased the sensitivity of latency III Raji cells to resveratrol. The analysis of NF-kB DNA-binding activity by EMSA in Raji control and Raji 5E revealed in the latter a strong reduction of the band corresponding to the DNA–protein complex, indicating that inhibition of LMP1 expression in Raji 5E resulted in a lower stimulation of the transcription factor (Fig. 12C). To further investigate links between LMP1 expression and sensitivity of BL cells to resveratrol, we transfected EBV (-) 2A8 cells with the LMP1 plasmid or the control vector; 24 hours later, cells were exposed to either resveratrol or the diluent and viable cells determined by Trypan blue staining after a further 24-hour period. Figure 12D shows that 24 hours after transfection, LMP1 plasmid had efficiently promoted the expression of the protein. As expected, cell proliferation of EBV(-) 2A8 cells transfected with the control plasmid was strongly reduced by exposure to resveratrol as compared with control cells treated with the diluent. In contrast, proliferation of 2A8 cells expressing the viral oncogene and treated with resveratrol was similar to that observed for the control cells (Fig. 12E). These results clearly indicate that LMP1 expression increases the resistance of BL cells to the polyphenol.

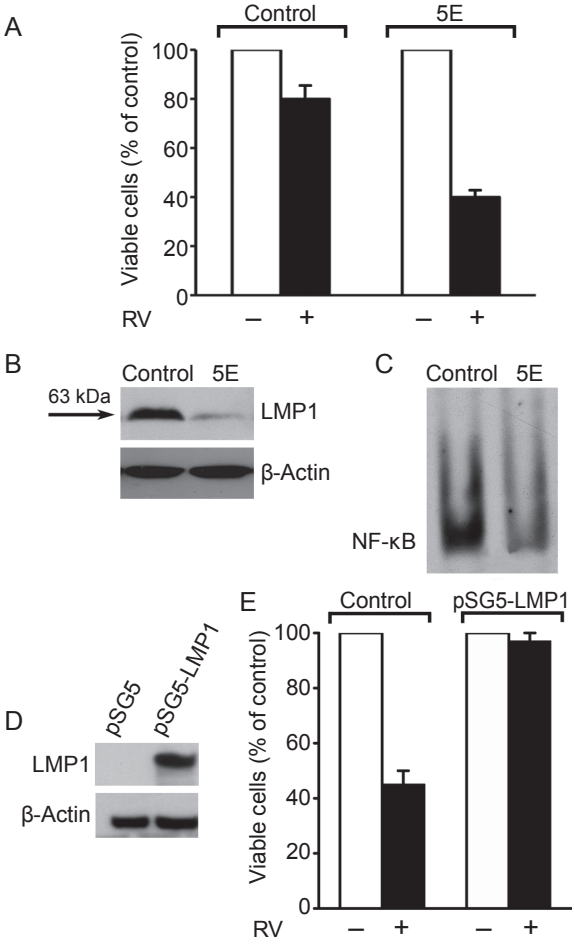

A
Control
5E
Viable cells (% of control)
100
80
60
40
20
0
RV
–
+
–
+
B
Control
5E
63 kDa
LMP1
β-Actin
C
Control
5E
NF-κB
D
pSG5
pSG5-LMP1
LMP1
β-Actin
E
Control
pSG5-LMP1
Viable cells (% of control)
100
80
60
40
20
0
RV
–
+
–
+

Figure 12.
Influence of LMP1 expression on Burkitt's lymphoma sensitivity to resveratrol (RV). A, Raji cells expressing control siRNA (control) or siRNAs targeting LMP1 (5E) were treated with resveratrol (black columns) or with diluent (empty columns) for 24 hours and cell viability was measured by Trypan blue staining; values are means ± SD of 3 different experiments and are presented as the percentage of control; B, LMP1 protein levels in Raji cells expressing control siRNA (control) or siRNAs targeting LMP1 (5E) were assessed by Western blot analysis; β-actin was used as a loading control. C, NF-kB DNA-binding activity in Raji control and Raji 5E was detected by EMSA (see Materials and Methods).
D, 2A8 cells were electroporated with either pSG5 or pSG5-LMP1 plasmid and 24 hours later analyzed by Western blotting for LMP1 expression. E, 2A8 cells treated as in (D) were exposed to resveratrol (black columns) or the diluent (empty columns) for 24 hours and cell viability was measured by Trypan blue staining; values are means ± SD of 3 different experiments and are presented as the percentage of control.

4.7 LMP1 downregulation increases the antiproliferative effect of resveratrol on LCLs

To validate the cooperative effects of LMP1 suppression and resveratrol treatment in inhibiting cell proliferation of EBV-infected latency III cells, we exposed to the polyphenol 2 LCLs silenced for LMP1. To this end, cells were first electroporated with LMP1 siRNA or control plasmid and 24 hours later treated with or without resveratrol for the next 24 hours at concentration previously found to effectively inhibit LCLs proliferation (Fig. 13). Cell counts were determined by Trypan blue exclusion assay, and apoptosis levels were assessed by detection of PARP cleavage on Western blots. Figure 14A shows that electroporation of LCL1260 with control plasmid (siRNA C) did not affect LMP1 expression;

in contrast, transfection of the cells with LMP1siRNA decreased LMP1 levels by about 30% to 40% at 24 hours and by about 80% at 48 hours. Following 24-hour exposure of the cells to 130 or 220 μmol/L resveratrol, proliferation of LCLs with control siRNA was inhibited by 45% and 55%, respectively (Fig. 14B). However, LMP1 downregulation by siRNA dramatically reduced the viability of the cells treated with the polyphenol and strongly induced apoptosis as revealed by the high levels of PARP cleavage product (Fig. 14C). Similar results were obtained with LCL1087 (data not shown). Altogether, our data indicated that LCLs expressing lower levels of LMP1 were more sensitive to resveratrol induced apoptosis.

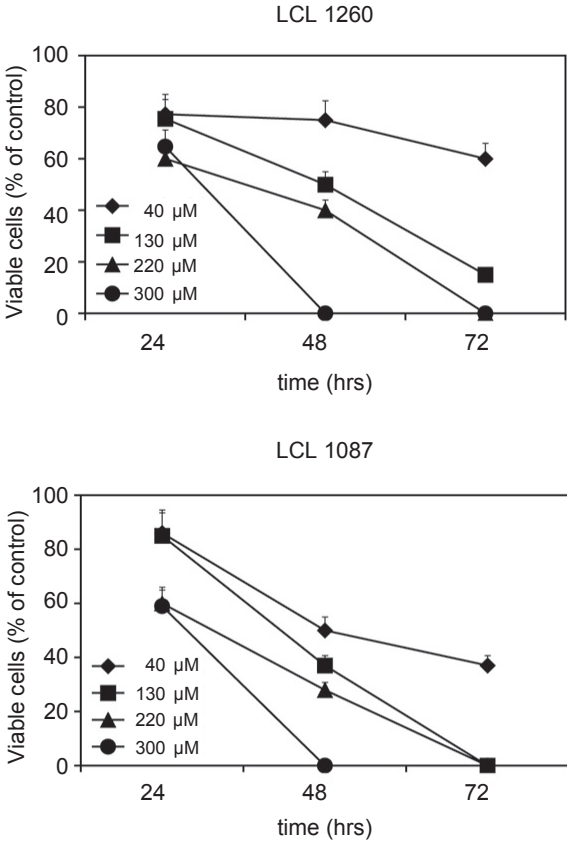


Figure 13.

Resveratrol-induced inhibition of LCLs proliferation. Cells were incubated with RV at the concentrations indicated. After 24, 48 and 72 hours, cell counts were assessed by trypan blue staining and the values expressed as percentage of control cells treated with the diluent. Each point represents the mean ± SD of three similar experiments.

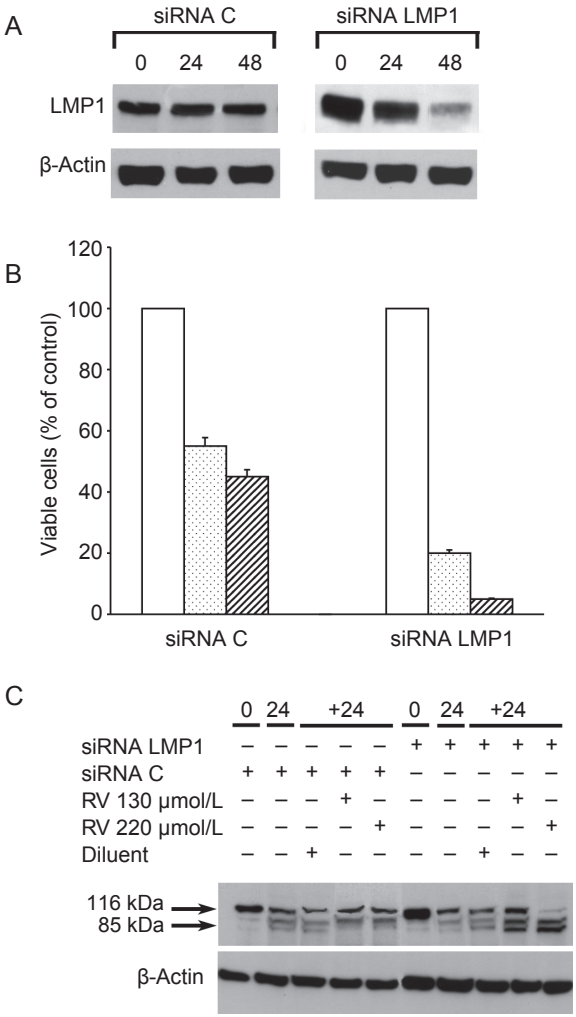

A
siRNA C
siRNA LMP1
0 24 48
0 24 48
LMP1
β-Actin
B
Viable cells (% of control)
120
100
80
60
40
20
0
siRNA C
siRNA LMP1
C
0 24 +24 0 24 +24
siRNA LMP1 – – – – – + + + + +
siRNA C + + + + + – – – – –
RV 130 μmol/L – – – + – – – – + –
RV 220 μmol/L – – – – + – – – – +
Diluent – – + – – – – + – –
116 kDa
85 kDa
β-Actin

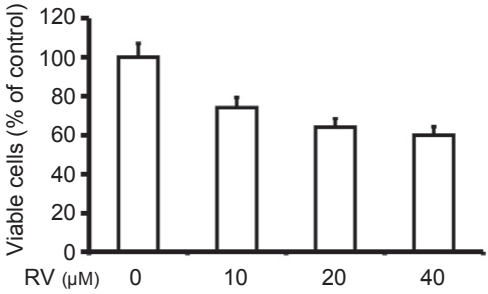
Figure 14.
Downregulation of LMP1 expression increases resveratrol (RV)- induced cell growth inhibition and apoptosis of LCLs. A, cells were electroporated with control siRNA (siRNA C) or siRNAs targeting LMP1 (siRNA LMP1) and, at the time indicated, LMP1 protein levels analyzed by Western blotting; B, LCLs treated as in (A) and incubated for 24 hours were exposed to 130 μmol/L (pointed columns), 220 μmol/L (dashed columns) resveratrol, or to diluent (empty columns) for 24 hours. Cell counts were determined by Trypan blue staining, and the values expressed as the percentage of control cells treated with the diluent. Each value represents the mean ± SD of 3 similar experiments. C, samples of cells treated as in (B) were collected after electroporation (0), 24 hours later (24), and following exposure for 24 hours to either resveratrol or the diluent (+24). A total of 20 μg of cell lysates was resolved on 8% to 16% acrylamide gel and analyzed by Western blotting with PARP antibodies.
Data shown are representative of 3 independent experiments.

4.8 Evaluation of the cytotoxicity of resveratrol on Akata cells upon EBV lytic cycle induction

The concentrations of the polyphenol causing a 50% reduction in the proliferation of Raji and Akata cells at 48 h, equal to 300 and 40 μM, respectively, were chosen as the highest values of a range used to examine the effects of RV on EBV replicative cycle in the two cell lines. EBV lytic cycle was induced in Raji cells by electroporation of the plasmid expressing the immediate early gene BZLF1 and in Akata cells by IgG cross-linking. In the latter cellular system for EBV replication, we evaluated a putative cytotoxic effect of RV by Trypan blue exclusion assay and by cytofluorimetric determination of apoptotic/necrotic cells after FITC-Annexin V and propidium iodide (PI) staining. The data reported in Figure 15 show that cell counts, determined after 24 h incubation of Akata cells with 40

μM RV, were reduced by about 40% with respect to control cells incubated with IgG in the absence of the compound, while only a 10–20% decrement was measured after exposure of the cells to RV concentrations of 10 and 20 μM respectively. In agreement with these data, in the presence of 40 μM RV, the percentage of the cell population stained by Annexin V was about 30% higher than that measured in the absence of the polyphenol, while an increment of 10–20% was detected with RV concentrations of 10 and 20 μM (Fig. 15B). Interestingly, the percentages of necrotic cells (stained with PI), and those of late apoptotic cells (Annexin V/PI-positive) detected after 24 h incubation, were substantially low and not significantly affected by the treatment with RV. These results clearly show that at the concentrations used for our experiments, RV inhibits the proliferation of Akata cells without increasing cell death.

A



B

	AnnV(%)	PI(%)	AnnV+PI(%)
RV 0 μM	27	1,7	0,9
RV 10 μM	34	1,5	1,1
RV 20 μM	47	1,2	2
RV 40 μM	58	1	3

Figure 15.

Antiproliferative effect of RV on Akata cells upon EBV lytic cycle induction.

IgG cross-linked Akata cells were treated with RV at the concentrations indicated for 24 h. (A) cell counts were assessed by Trypan blue staining. The values represent the mean ±SD of three similar experiments. (B) Samples of cells treated as in (A) were subjected to Annexin V (AnnV) and PI staining and analyzed by flowcytometry as described in the Methods. The table reports the results of a representative experiment as percentages of apoptotic cells (AnnV-positive), necrotic cells (PI) and late apoptotic cells (AnnV and PI positive).

4.9 Down-regulation of EBV lytic antigens expression by resveratrol

To examine the effects of RV on EBV lytic antigens expression, Raji cells were treated with concentrations of the polyphenol varying from 50 to 300 μM for 24 h, while Akata cells with concentrations varying from 10 to 40 μM for six hours. Cell lysates were analyzed by Western blot with antibodies specific for the two EBV immediate early (IE) antigens BZLF1 and BRLF1 and for the early (E) proteins, BFRF1, BFLF2 and the viral DNA polymerase BALF5. Fig. 16 shows that 24 h after electroporation of Raji cells with the BZLF1 plasmid, EBV immediate early and early antigens were efficiently expressed. In contrast, viral lytic antigens were not expressed in Raji cells electroporated with the CMV vector (data not shown).When 300 μM RV was added to the cell culture, BZLF1 and BRLF1 signals, as well as those representing BFRF1, BFLF2 and BALF5 antigens, were barely detectable. Moreover, a dose-dependent effect on the expression of EBV early antigens and BRLF1 was observed with lower concentrations of the polyphenol. In contrast, concentrations of RV lower than 300 μM increased BZLF1 levels suggesting a stimulatory effect on the expression of the BZLF1 plasmid. The results obtained in Akata cells show that EBV IE and E genes were highly expressed after 6 h incubation with IgG. The addition of RV to the cell cultures inhibited the expression of all EBV lytic antigens in a concentration-dependent manner so that at 40 μM, the reduction of protein levels, measured

by densitometric evaluation of the specific signals, was close to 90%. Because RV similarly inhibited EBV lytic antigens expression in Raji and Akata cells, the effects of the polyphenol appeared to be independent of the method used to trigger viral reactivation. Therefore, further experiments have been performed on Akata cells that allow full completion of EBV lytic cycle.

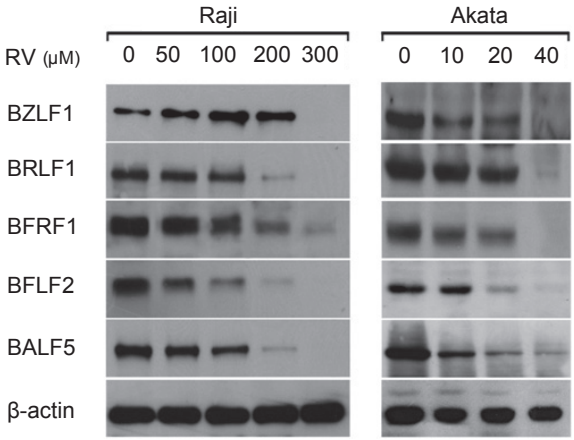


Figure 16.
Expression of EBV lytic antigens in Raji and Akata RV-treated cells. Raji and Akata cells treated as described in the Methods to induce EBV lytic cycle, were incubated in the absence or in the presence of RV at the indicated concentrations for 24 and 6 h, respectively. Cell lysates (40 μg) were resolved by SDS–PAGE, blotted on membrane and probed with antibodies for EBV antigens. Specific signals were visualized by ECL. β-Actin was used as the internal marker. The data are representative of those obtained in at least 3 independent experiments with similar results.

4.10 Modulation of EBV lytic gene expression occurs at the post-transcriptional level

To gain insights into the regulation of EBV lytic gene expression by RV, we measured mRNA levels of the viral IE genes in untreated and RV-treated cells by semi-quantitative RT-PCR. As shown in Fig. 17A, even at the highest concentration, RV did not significantly affect the transcription of BZLF1 and BRLF1 genes. Similar results were obtained when the levels of EBV early genes BHRF1 and BALF5 were evaluated (data not shown). Therefore, the down-regulation exerted by the polyphenol on EBV lytic gene expression appeared to occur at the post-transcriptional level. To investigate whether inhibition of EBV mRNAs translation could account for the decrement of the viral lytic antigens observed after RV administration, EBV lytic cycle was induced in the presence of either the protein synthesis inhibitor cycloheximide (CHX) or RV, each added at time zero (together with the IgG), 2 or 4 h later. The levels of EBV lytic antigens were measured byWestern blot of cells harvested at 8 h. As shown in Fig. 17B, inclusion of CHX or RV for the 8 h duration of the experiment strongly inhibited the synthesis of BZLF1, BRLF1 and BALF5 induced by IgG (lanes 0–8). The addition of the two compounds 2 and 4 h after EBV induction, completely abolished their inhibitory effect on EBV lytic antigens expression. In fact, similar levels of the viral products were detected upon addition of either CHX or RV at 2 or 4 h after EBV induction. All together these results suggest that

down-regulation of EBV lytic antigens expression observed in the cells simultaneously exposed to IgG and RV, might involve a block in the synthesis of protein factors required in the early phases of EBV productive cycle. We also measured the abundance of BZLF1 mRNA by RT-PCR in the samples harvested at 8 h. Fig. 17C shows that the mRNA levels were similar in the cells treated with either RV or CHX, implying that the expression of BZLF1 mRNA was independent of new protein synthesis.

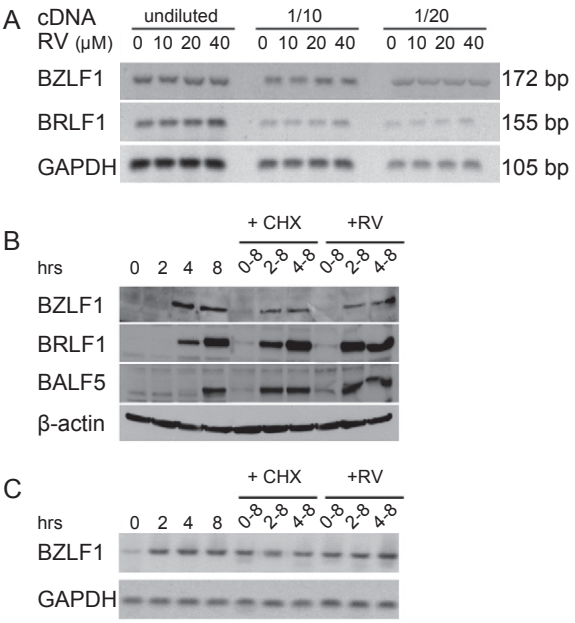


Figure 17.

EBV lytic genes expression in RV-treated cells. (A) EBV lytic cycle was induced in Akata cells in the absence or in the presence of 10, 20 or 40 μM RV for 6 h. Total RNA was purified and subjected to reverse transcription as described in the Methods. Undiluted and diluted cDNA were analyzed by PCR with the specific primers for BZLF1 and BRLF1 genes. GAPDH amplification was used to verify equal amounts of total RNA. (B) Akata cells treated with IgG were harvested at 0, 2, 4, and 8 h. CHX or 40 μM RV were added with the inducing agent (lane 0–8) or at 2 h (lane 2–8) and at 4 h (lane 4–8) after EBV lytic activation. Cell lysates were prepared from all treated cells 8 h after starting the experiment. Expression of EBV antigens was analyzed by immunoblotting. β-actin was used as loading control. (C) Total RNA was extracted from Akata cells treated as in (B) and RT-PCR was performed as described in the Methods. Representative results of 3 independent experiments are shown.

4.11 Inhibition of viral particles production by resveratrol

The strong down-regulation of EBV lytic gene expression observed after exposure of the cells to RV led us to investigate the effects of the polyphenol on EBV replication. To this end, EBV copies were measured in the medium of Akata cells exposed for 24 h to IgG with or without different RV concentrations. The results, illustrated in Fig. 18A, show that EBV particles production was modulated by RV in a dose-dependent manner. In the presence of 10 μM RV, EBV copies released in the medium were about 65% of those measured in the absence of the polyphenol. A progressive decrement of EBV particles was observed when the induction of the EBV lytic cycle was carried out in the presence of either 20 or 40 μM RV as the EBV copies measured under these conditions were about 45 and 30% respectively, of those released in the absence of the compound.

4.12 Resveratrol inhibition of EBV lytic cycle is associated with reduced ROS generation

It has been well established that many pharmacological actions of RV are due to its anti-inflammatory and anti-oxidant properties. To clarify whether RV down-regulation of EBV lytic cycle was associated with a decrement of ROS, the cellular redox state was analyzed in RV-treated or untreated Akata cells after EBV activation. After 30, 60 and 120 min, cell samples were subjected to flow-cytometric analysis to quantify ROS levels by the ROS-sensitive fluorescent product DCF. We found that ROS markedly decreased in the cells exposed to RV during the induction of EBV lytic cycle (Fig. 18B). After 30 min, ROS levels measured in RV-treated cells were about 45% of those measured in the absence of the polyphenol. In addition, the values obtained after 60 and 120 min of incubation, were about 55 to 40% and 60 to 42% of the control, respectively, depending on RV concentrations and the time elapsed from EBV lytic cycle activation.

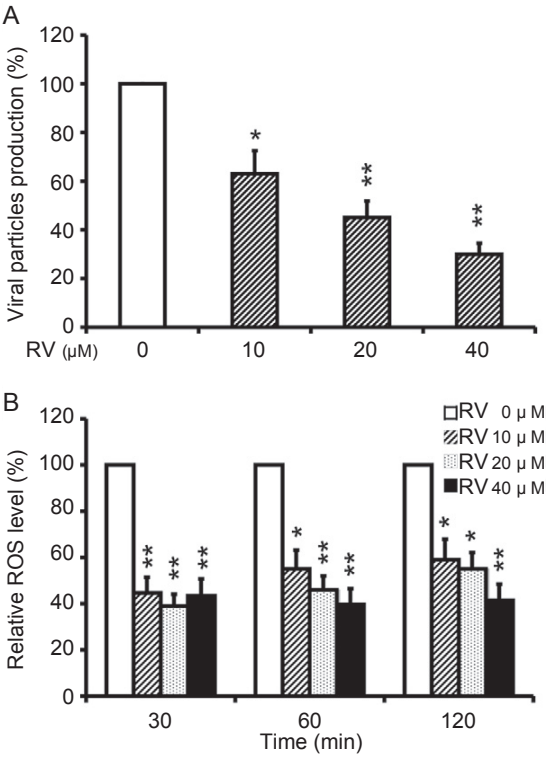


Figure 18.

(A) Inhibition of viral particles release from RV-treated cells. Akata cells were treated for 24 h with IgG in the absence or in the presence of RV at the concentrations reported. The culturing medium was collected and EBV DNA measured by real-time PCR, as described in the Methods. The values in the bargraph are means ± SD of 3 different experiments and are presented as the percentage of control (in the absence of RV).*P < 0,001 or **P < 0,0001. (B) Inhibition of ROS production in RV-treated cells. Akata cells were exposed to IgG in the absence or in the presence of RV at 10, 20 or 40 μM. At the indicated times, samples were analyzed to determine ROS levels as described in the Methods; values in the bar-graph are means ± SD of 3 different experiments and are presented as the percentage of control (in the absence of RV).*P < 0.05 or **P < 0.01.

4.13 Effects of resveratrol on EBV-mediated transcription factors activation

Since RV has been reported to inhibit the activity of several transcription factors, we measured NF–kB and AP-1 DNA binding capacity after activating EBV in Akata cells exposed or not to RV. As shown in Figure 19, within three hours following induction of EBV lytic cycle,NF–kB DNA-binding activity rapidly increased reaching a three fold higher level than that measured at time zero; there after it declined and by 6 h returned to the levels measured before EBV reactivation. Addition of RV during EBV induction rapidly inhibited the initial phase of NF–kB activation. In fact, the signals corresponding to the DNA–protein complexes were similarly high in untreated and RV-treated cells after one hour of treatment but had declined to base levels already after three hours exposure of the cells to the polyphenol. Similar results were obtained when cells extracts were assessed for AP-1 DNA binding activity, clearly indicating that RV treatment inhibited the boosted activity of transcription factors induced by EBV lytic switch. To assess whether the decrement of transcription factors activities could be ascribed to decreased amounts of the constituting subunits, the levels of p65 and p50 NF–kB components were measured by Western blot analysis in the cell extracts used for EMSA. The data illustrated in Figure 20 show that treatment of Akata cells with IgG in the absence or in the presence of 40 μM RV did not alter the levels of NF–kB protein components, indicating that the polyphenol mainly affected the binding activity of the transcription factor.

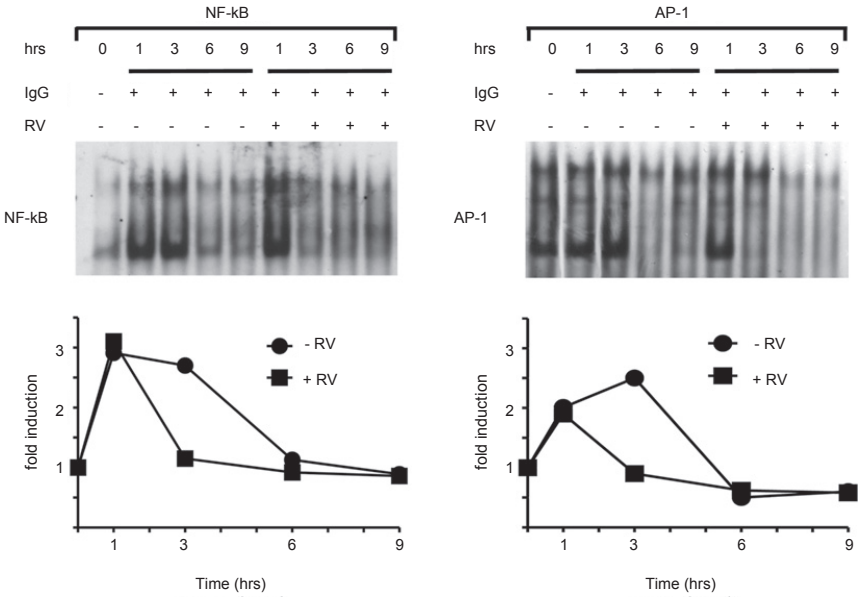


Figure 19.

NF–kB and AP-1 activities in RV-treated cells. Akata cells were exposed to IgG and 40 μM RV. At the times indicated, whole-cell extracts were obtained and equal amounts of proteins incubated with the NF–kB or AP-1 probe. Transcription factors activities were evaluated by EMSA (see Materials and Methods). The signals, quantified by densitometry, are expressed as fold induction of time 0. The data shown are representative of 3 independent experiments with similar results.

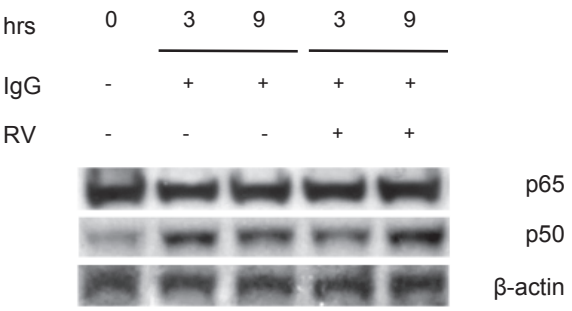


Figure 20.
Evaluation of NF–kB subunits expression in RV-treated cells. 30 µg of cell lysates from Akata cells untreated (0) or IgG cross-linked and treated or not with 40 µM resveratrol for 3 and 9 hours, were subjected to SDS-PAGE and Western blot analysis with RelA/p65 and p50 antibodies as described (see Materials and Methods). β-Actin was used as the internal marker. The data are representative of those obtained in at least 3 independent experiments with similar results.

5. Discussion

Three main patterns of differential EBV expression have been described in infected cells in lymphoproliferative disorders, as Latency I, II, or III. The prototypic malignancy with Latency I is Burkitt lymphoma, while Latency II is seen in Hodgkin lymphoma and Latency III in AIDS-related diffuse large B cell lymphoma (immunoblastic subtype) and post-transplantation lymphoproliferative disorders.

In terms of how to use this knowledge to develop new antivirals, it is evident that by inhibiting EBNA1, the only gene expressed in all phenotypes, it could be possible to diminish the EBV effects in the largest number of viral lymphomas. Moreover, because EBNA 1 protein is also involved in episome maintenance, it might be conceivable to eliminate the viral genomes from the lymphoma cells . However, although RNA interference to EBNA1 used in a Burkitt lymphoma line, decreased cell proliferation (Hong M et al. 2006), dependency of infected tumor cells on EBNA1 is not very clear.

In fact, some Burkitt lymphomas were found to express a viral protein called BHRF1, which is a viral homolog of BCL2, an important antiapoptotic protein (Kelly GL et al., 2009). Moreover, one endemic Burkitt's lymphoma case was found to be unusually heterogeneous and in early-passage cultures yielded clones that, besides c-myc translocation, differed in EBV status showing 3 forms of restricted latency correlated with specific degree of

protection from apoptosis (Kelly GL et al., 2002, 2005, 2006). These findings indicate that multiple viral genes can block apoptosis and different Burkitt's lymphomas express different combinations of these antiapoptotic genes that contribute survival functions of these tumors in multiple ways. Therefore, inhibition of these proteins or of the signaling pathways they activate, could be useful to treat the lymphomas with type II and III latency where these viral products are expressed. These include Hodgkin's lymphoma, post-transplant lymphoproliferative disorders, and a subset of AIDS related lymphomas.

A great variety of naturally occurring compounds have been shown to inhibit, delay, or reverse cellular events associated with carcinogenesis by acting on multiple targets and therefore representing a promising alternative or support to conventional therapies for the management of cancer (Benner SE, Hong WK 1993). Among them, resveratrol is a dietary chemopreventive phytochemical that has gained considerable attention for its remarkable inhibitory effects on different stages of carcinogenesis (Jiang H et al., 1997).

The experiments carried out in 3 EBV-positive BL lines that express just EBNA1 as in Akata, a latency II-like pattern with the exception of LMP1 (EBNA1^{+}/LMP2A^{+}) as in Jijoye M13, and the latency III phenotype as in Raji, respectively, have allowed us to evaluate the antiproliferative effect of resveratrol on cells in which EBV infection was associated with a specific degree of protection from apoptosis. We found that resveratrol causes cell-cycle arrest and apoptosis of

EBV-infected BL cells independently of the latency program the virus established in the host cells. However, the efficacy of the polyphenol seems to be inversely related to the restriction pattern of viral gene expression. Thus, latency I infection in Akata cells confers a small but significant protective effect whereas latency II-like infection in JijoyeM13 and latency III in Raji cells, further broadening EBV latent genes expression pattern, progressively diminish the sensitivity of BL cells to resveratrol-induced apoptosis. The different susceptibility of BL cells to resveratrol is reflected in the variable concentrations of the polyphenol necessary to reduce proliferation by 50%. Our results show that the growth inhibitory potential of resveratrol is mainly due to the induction of cell-cycle arrest and apoptosis because a significant fraction of the cell population accumulated in the G1 phase of the cell cycle after exposure to the polyphenol. Cytofluorimetric evaluation of Annexin V and Annexin V/PI-positive cells revealed that G1 block triggered a high percentage of EBV(-) and latency I BL cells to rapidly proceed toward late apoptosis/necrosis, whereas the proportion of early apoptotic cells further increased in latency II-like and latency III BL cells during 24-hour treatment with the polyphenol. Resveratrol-induced cell-cycle arrest in G1 seemed to be associated with the modulation of cell-cycle regulatory proteins involved in the G1–S transition because a decreased expression of cyclin A, cdk1, and cdk2 as well as an up-regulation of cyclin E and the cdk inhibitor p27 were detected.

It has been shown that c-myc down-regulation is a critical molecular

event of resveratrol-mediated antiproliferative activity, closely associated with growth suppression, cell cycle arrest, and apoptosis (Zhang W et al., 2007; . Paul S et al., 2009). Our data indicate that resveratrol suppresses c-myc expression in all BL cell lines, regardless of EBV status. Because c-myc is the master transcription factor responsible for proliferation of BL cells, EBV (-) and latency I BL cell lines might be very sensitive to c-myc repression and cell-cycle arrest and readily die by apoptosis or necrosis rather than sustaining a prolonged arrest of proliferation. Moreover, the higher protection of Raji and Jijoye M13 cells from apoptosis, despite the striking and rapid decrement of c-myc levels, seems most likely due to the expression profile of EBV genes in these cell lines. With respect to Jijoye M13, protection from apoptosis has been previously reported in LMP2A-expressing cell lines (Fukuda M et al., 2005; Mancao C and Hammerschmidt W 2007). PARP cleavage and caspase 3 activation detected in all BL cells suggest that resveratrol may induce cell death, at least in part, through caspase-dependent pathway. We have found that resveratrol-induced apoptosis occurs in conjunction with down-regulation of the antiapoptotic proteins cIAP1, FLIP, and XIAP. Because these proteins inhibit caspase activity (Deveraux QL et al., 1998; Hideshima T et al., 2003; Mitsiades N et al., 2002), it is conceivable that resveratrol might induce apoptosis by down-regulating their expression. Our data concerning the analysis of cell cycle and apoptosis related factors in resveratrol-treated BL cells also showed that, overall, the variations of protein levels were much more pronounced in Raji and Jijoye

M13 cells than in Akata or EBV(-) cell lines. A likely explanation for the lower reactivity of the latter cell lines can be found in the rapid and massive cell death that follows resveratrol-induced block of cell-cycle progression.

A great deal of experimental evidence has been provided for p38 MAPK involvement in apoptosis triggered by a variety of agents, as well as in the regulation of cell cycle G1–S and G2–M checkpoints in response to cellular stress and DNA damage (Xia Z et al., 1995; Thornton TM, Rincon M 2009). Although p38 MAPK is generally believed to be a kinase that mediates cell death, ERK1/2 MAPK pathways have been shown to promote survival and cell growth (Meloche S, Pouyssegur J 2007). Our findings indicating inhibition of ERK1/2 activity and the concomitant activation of p38 signaling cascade confirm the opposite effects these kinases play in determining cell survival/death and may account for the observed antiproliferative activity of resveratrol on BL cells. It has been proposed that ERK1/2 kinases regulate G1 phase progression through various mechanisms including cyclin D1 induction, enhanced c-myc protein stability, p27 down-regulation, and decreased expression of antiproliferative genes (Meloche S, Pouyssegur J 2007; Yamamoto T et al., 2006). Although cyclin D1 protein levels were barely detectable in resveratrol-treated BL cells, our results showing c-myc suppression and the increment of p27 protein levels suggest the involvement of ERK1/2 pathway in mediating resveratrol-induced cell-cycle arrest.

NF-kB activation has been linked to several aspects of oncogenesis,

including the control of cell migration, cellcycle progression, and differentiation, as well as apoptosis (Karin M 2006). In general, NF-kB exerts an antiapoptotic activity by switching on genes that hamper the effects of proapoptotic stimuli, thereby suppressing cell death pathways. Genes whose activity is positively regulated by NF-kB include members of the Bcl-2 family, TRAF1/2, cIAP1/2, cFLIP, and XIAP (Karin M et al., 2002). BL cells often present aberrant NF-kB regulation and express constitutive high levels of NF-kB activity (Ni H et al., 2011, Piva R et al., 2005). Our data, indicating that resveratrol markedly inhibits NF-kB activity in EBV(-) 2A8 and Ramos, latency I (Akata), and latency II-like (Jijoye M13) BL cells, are in agreement with what previously reported on the inhibitory effect of the polyphenol on the activity of the transcription factor (Gupta SC et al., 2010) although, in latency III Raji cells expressing the EBV oncogene LMP1, suppression of NF-kB activity was observed only after prolonged treatment with the polyphenol.

Among the BL cell lines examined in this study, only Raji expresses the EBV oncogene LMP1 because the Jijoye subline M13, deleted for EBNA2, lacks the viral latent membrane protein. LMP1, as the critical activator of NF-kB (Cahir McFarland ED et al., 1999, Wu L et al., 2006), is responsible for the dramatic differences in the growth pattern and phenotype of cells driven into proliferation by either c-myc or the EBV latency III program (Dirmeier U et al., 2005). We have found that LMP1 down-regulation decreases the protection of Raji cells to resveratrol-induced apoptosis and

conversely that LMP1 overexpression protects EBV(-) 2A8 cells from resveratrol treatment. Because of the LMP1 stimulatory effect on NF-kB, it is conceivable that LMP1 increases the resistance of latency III BL cells to resveratrol by transcription factor activation. All together, for what concerns EBV latent infection of B lymphocytes, our data show that in BL cells, induction of apoptosis mediated by resveratrol is clearly different between EBV(-) and EBV-infected cells that sustain a type I, II, or III latency program. These results suggest that the assessment of the different viral latency programs might have great impact for the treatment and sub-classification of Burkitt's lymphoma and other EBV-related malignancies.

In vivo, the activation of the lytic cycle, in a relatively small number of infected B cells leads to the production of viral particles that spread EBV infection. Lytic activation is also observed in acute infectious mononucleosis as well as in post-transplant lymphomas and lymphoproliferative diseases that occur in immunocompromized patients.

In these studies we found that resveratrol is able to inhibit EBV lytic cycle in Burkitt's lymphoma cells when induced either by the ectopic expression of the immediate early gene BZLF1 as in Raji cells, or by activation of the B-cell receptor signaling pathway as in Akata cells. In the latter that support the complete replicative cycle, we showed that RV treatment reduced EBV particles production in a concentration dependent manner, without significantly affecting cell viability. The data reported here, indicate that upon EBV lytic

cycle induction, RV antiproliferative effects during productive infection are even more pronounced since, in the two cell lines, the concentrations able to reduce by 50% the proliferation of EBV latently-infected cells at 48 h, caused similar decrements 24 h after induction of EBV lytic cycle. However, despite the increment of early apoptic (AnnV-positive) cells with increasing RV concentrations, the percentages of late apoptotic and necrotic Akata cells did not represent any relevant value, indicating that cell viability was not affected even by the highest concentration of the drug. Moreover, because EBV induction blocks the cell cycle at G1/S (Wu et al., 2004), the effects of RV on EBV lytic cycle have to be considered independent of the arrest of cellular proliferation. We demonstrate that down-regulation of EBV genes expression by RV occurs without variation of the corresponding mRNA levels, indicating that the polyphenol acts at a post-transcriptional level. We also show that in the absence of significant alterations in the amounts of the transcripts, the expression of EBV lytic products is largely suppressed when lytic cycle is carried out in the presence of either RV or CHX, and that the inhibitory effect of the two compounds is lost when they are added to the cells 2 or 4 h after IgG stimulation. This result, while strongly suggesting a similar mechanism for the two compounds, indicates that both exert their effects only in the very early phases of EBV induction. Because, at least CHX, is known to block protein synthesis, it was surprising to find that cells exposed to the drugs either two or four hours after EBV induction, showed levels of EBV lytic antigens higher than

those detected in the control cells, collected at two and four hours after addition of IgG. A likely explanation for this result could rely on a decreased availability and/or activity of the two drugs within the 8 h period of incubation, as previously reported for CHX (Martin et al., 1990). Recently, an effect of RV on protein synthesis through the modulation of mTOR-dependent and independent pathways has also been reported in hepatic cells (Villa-Cuesta et al., 2011). Further investigations will be required to assess the involvement of mTOR pathways in EBV lytic cycle suppression by RV. Our data are apparently in contrast with those obtained by Yiu et al. who showed that in P3HR1 cells transfected with BRLF1 or BZLF1 plasmids and treated with RV and sodium butyrate, the activity of the two promoters was inhibited by the polyphenol (Yiu et al., 2010). However, because sodium butyrate activates EBV gene expression by favoring histone acetylation and RV has been reported to stimulate the deacetylase activity of SIRT1 (Borra et al., 2005), the polyphenol might annihilate the effect of sodium butyrate on EBV immediate early antigens promoters. In this respect, we consider the activation of EBV lytic cycle by IgG crosslinking in a cellular context that allows the coordinated expression pattern of EBV lytic genes, a more suitable model to study the possible targets/mechanisms of RV antiviral activity in vivo.

Resveratrol has proven to exert potent antiviral activity against various families of DNA and RNA viruses by altering molecular pathways within cells affecting the viral life cycle (Campagna and Rivas, 2010). ROS production is considered as one of the host

defense reaction against microbes. We and others have reported an increment of ROS levels following EBV lytic cycle activation (Gargouri et al., 2011; Matusali et al., 2009). Moreover, it has been shown that oxidative stress contributes to the reactivation of the EBV lytic cycle through the induction of BZLF1 expression (Lassoued et al., 2010). Here we demonstrate that RV caused a strong reduction of ROS levels generated upon EBV activation. The anti-oxidative effect of the polyphenol was slightly reduced with time when concentrations lower than 40 μM were used, these results suggesting that increased concentration of RV can prolong the inhibitory effect on the burst of ROS levels generated during EBV lytic cycle activation.

Following B-cell receptor cross-linking, activated signaling pathways lead cell-encoded transcription factors to bind to the promoter region of EBV immediate early gene BZLF1 and initiate the lytic replication process (Amon and Farrell, 2005). We report here that RV-mediated EBV lytic cycle inhibition involves the rapid suppression of NF–kB and AP-1 activation. In a similar cellular context, RV was found to inhibit KSHV reactivation by lowering the level of Egr-1, a transcription factor mediating the expression of RTA, the analog of BZLF1 (Dyson et al., 2012). The impairment in the activities of NF–kB and AP-1 might similarly affect both upstream, as well as downstream events of BZLF1 expression. However, our data showing that RV does not alter BZLF1 transcription, suggest a prominent inhibitory effect on the trans-activating function of the viral product.

NF–kB and AP-1 are known to be redox-sensitive transcription factors, positively regulated by ROS levels during stress response, inflammation and cellular proliferation (Angel and Karin, 1991; Lin and Karin, 2003). Numerous anti-oxidative and anti-inflammatory phytochemicals have been used to alter abnormal cellular signaling mediated via NF–kB or AP-1 and prevent tumor promotion or progression (Ralhan et al., 2009; Shu et al., 2010). RV is demonstrated to modulate the expression and/or activity of transcription factors involved in critical pathways of carcinogenesis (Manna et al., 2000; Whitlock and Baek, 2012) and to inhibit COX2-induced AP-1 DNA binding activity in TPA-treated murine skin (Kundu et al., 2006). In addition, RV was found to block NF–kB activation in HCMV-infected cells (Evers et al., 2004), HSV-1, HSV-2 (Faith et al., 2006) and VZV (Docherty et al., 2006). Therefore, it is conceivable that the RV-mediated inhibition of the EBV lytic cycle occurs, at least in part, through the down-regulation of critical transcription factors involved in the initial phases of EBV reactivation.

6. Conclusions

Taken together, our data indicate that in Burkitt's lymphoma cells, Resveratrol is able to suppress the proliferation of latently infected cells and inhibit EBV productive infection upon activation of the lytic cycle. In particular, concerning the latent infection, we showed that silencing of the LMP1 viral oncogene dramatically increased the antitumor potential of resveratrol in latency III BL cells and LCLs, thus enlightening a previously unknown aspect of the chemotherapeutic potential of resveratrol, that is its use in combination with the delivery of siRNA for silencing latent EBV genes whose expression characterize the latency pattern of the tumor.

Concerning the lytic cycle, RV treatment caused down-regulation of EBV lytic genes at the post-transcriptional level by affecting multiple cellular targets. The molecular mechanisms involved include: (i) inhibition of protein synthesis; (ii) a strong decrement of ROS levels and (iii) rapid suppression of NF–kB and AP-1 activities, boosted by EBV lytic cycle reactivation.

Several studies are currently evaluating whether the resveratrol can prevent or even cure lifestyle diseases, such as cancer, diabetes and cardiovascular diseases. In these respects, while promising results have been presented at the Resveratrol 2012 International Conference and trials in humans are under way, clinical studies meant to assess the antiviral effects of the polyphenol are still lacking. The results obtained by this research project might provide

the experimental basis for further investigating in a mouse model the antiviral activity of this natural product against EBV infection.

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