Published in final edited form as:

Semin Cancer Biol. 2014 June ; 0: 60–68. doi:10.1016/j.semcancer.2014.01.002.

Regulation of the latent-lytic switch in Epstein–Barr virus

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Abstract

Epstein–Barr virus (EBV) infection contributes to the development of several different types of human malignancy, including Burkitt lymphoma, Hodgkin lymphoma, and nasopharyngeal carcinoma. As a herpesvirus, EBV can establish latent or lytic infection in cells. EBV-positive tumors are composed almost exclusively of cells with latent EBV infection. Strategies for inducing the lytic form of EBV infection in tumor cells are being investigated as a potential therapy for EBV-positive tumors. In this article, we review how cellular and viral proteins regulate the latent-lytic EBV switch in infected B cells and epithelial cells, and discuss how harnessing lytic viral reactivation might be used therapeutically.

Keywords

Ataxia-telangiectasia mutated (ATM); Hypoxia; Genome methylation; Lytic induction therapy; TGF- β

1. Introduction

Epstein–Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis. It is also associated with the development of certain malignancies, including African Burkitt lymphomas (BL), B-cell lymphomas of immunocompromised patients, nasopharyngeal carcinomas (NPC), Hodgkin's disease, and, occasionally, with T-cell lymphomas and gastric cancers [1]. Like all herpesviruses, EBV can infect cells in either latent or lytic forms [1,2]. Latent infection occurs in memory B cells, allowing the virus to evade the host immune response and to persist indefinitely within humans [1,2]. Regardless of cell type, all EBV-associated malignancies largely consist of latently infected cells in which EBV-encoded

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Conflict of interest

None declared.

transforming proteins and non-coding RNAs are expressed. The presence of a limited number of lytically infected cells may enhance tumor growth through release of growth factors and immunosuppressive cytokines [3–5].

Lytic EBV infection is essential for production of infectious viral particles, enabling virus transmission from cell to cell and host to host [1,2]. Lytic infection occurs in differentiated oropharyngeal epithelial cells [6,7], and tonsillar plasma cells [8]. *In vitro* studies indicate that B-cell receptor (BCR) stimulation [9], hypoxia [10], and transforming growth factor-beta (TGF- β) [11–13] can also induce lytic replication under some circumstances. EBV's ability to remain latent in memory B cells, yet lytically reactivate under appropriate circumstances, likely explains its near universality in humans. Furthermore, by inducing lytic reactivation in EBV-positive tumors, one could potentially selectively kill EBV-positive malignant cells.

Here, we highlight some recent findings relating to how cellular and viral factors promote or inhibit EBV reactivation and discuss how “lytic induction therapy” might be used to treat patients with EBV-positive tumors. We refer readers to prior review articles for coverage of the older literature on these and related topics [2,14–22].

2. EBV lytic reactivation from latent infection

2.1. Overview

In latently infected cells, the double-stranded DNA genome of EBV is maintained as a nuclear episome replicated once per cell cycle by the host DNA polymerase. It is usually highly methylated, existing in a repressive chromatin structure. Following reactivation, the lytic genes of EBV are expressed in a temporally regulated manner. The first ones transcribed are the viral immediate-early (IE) lytic genes, *BZLF1* and *BRLF1* (Fig. 1A). They encode the transcription factors, Z (aka Z, ZTA, ZEBRA) and R (aka R, RTA), respectively. Neither *BZLF1* nor *BRLF1* is expressed in latently infected cells due to silencing by multiple cellular transcriptional repressors. The promoters of these genes (Zp and Rp, respectively) are initially activated by cellular transcription factors (Fig. 1B and C). Subsequently, the Z and R proteins activate both their own and one another's promoters to greatly amplify their lytic-inducing effects. They then cooperatively activate the promoters of early (E) lytic genes that encode the viral replication proteins. Following viral genome replication, the late (L) viral genes are expressed. The latter encode structural proteins required for viral genome encapsidation into infectious virion particles.

2.2. Z-mediated lytic reactivation

In most EBV-positive cell lines, synthesis of Z protein is sufficient to induce the switch from a latent to lytic form of viral infection. Z, a member of the bZip family, binds as a homodimer to AP-1-like motifs known as Z-responsive elements (ZREs). Z protein contains an amino-terminal transactivator domain, a DNA-binding domain homologous to the basic DNA-binding domains of c-Jun and c-fos, and a carboxy-terminal bZip homodimerization domain [23]. Z interacts directly with histone acetylating complexes such as CBP and p300 and the general transcription factors TFIID and TFIIA. During viral reactivation (in cells in which the EBV genome is highly methylated), Z initially activates transcription from Rp. Z

and R then activate transcription from multiple early lytic viral promoters which often contain binding sites for both [25]. They are both required for expression of many, but not all, of the early-lytic genes within the context of the intact viral genome [24].

Z also plays an important role in lytic EBV DNA replication, binding directly to a series of essential ZRE sites located within the lytic origin of replication, *oriLyt*. The role(s) Z plays in mediating lytic EBV DNA replication are distinct from its transcriptional functions [26], with direct interactions between Z and core viral replication proteins likely promoting formation of replication complexes.

2.3. R-mediated lytic reactivation

R can also induce the switch from latent to lytic infection in some EBV-positive cell lines, particularly epithelial cells. The closely related Kaposi's sarcoma herpesvirus (KSHV) exclusively uses its R homolog (ORF50; RTA), rather than a Z-like protein (K8), to disrupt viral latency. R contains an amino-terminal DNA-binding domain, a homodimerization domain, and a carboxy-terminal transcriptional activation domain. It binds directly to GC-rich motifs known as R-responsive elements (RREs) (consensus 5'-GNCCN₉GGNG-3') located within the promoters of early lytic genes, functioning as a powerful enhancer when bound to these sites [27]. R directly interacts with both the general transcription factors TBP and TFIIB and the histone acetylases CBP and p300. Data from transient transfection reporter assays indicate that R activates both its own promoter and Zp by indirect mechanisms involving protein interactions with the Sp1, MCAF1 and Oct-1 transcription factors, and induction of cellular kinases that activate the c-Jun and ATF-2 transcription factors [28–32]. However, given the powerful enhancer activity of R-bound RREs, we speculate that a primary mechanism by which R activates Rp/Zp transcription in the context of the intact viral genome may well be through direct binding to RREs located potentially thousands of base pairs away from transcription initiation sites (and, thus, not present in the reporter constructs used in the transient transfection assays) [27].

At least two EBV-encoded proteins differentially regulate the ability of R to disrupt viral latency. The early-lytic viral protein Na (encoded by BRRF1) activates phosphorylation of c-Jun and cooperates with R to induce transcription from Zp in the context of the intact viral genome [33,34]. In contrast, the early-lytic viral protein LF2 directly interacts with R, sequestering it in an inactive form in the cytoplasm [35]. Presumably, the opposing effects of BRRF1 and LF2 help to fine-tune the transcriptional effects of R during the various stages of EBV lytic replication.

3. Factors contributing to maintenance of EBV latency

3.1. Overview

The ability of EBV to switch from a latent to lytic form of infection in host cells is initially determined by the availability of cellular transcription factors that repress *versus* stimulate transcription from Zp and Rp. However, activation of these IE promoters *per se* is not sufficient to induce viral reactivation. Rather, EBV encodes multiple, redundant mechanisms to ensure it remains latent in B cells, yet can lytically reactivate when B cells differentiate into plasma cells. As detailed below, Z and R activation of viral gene expression is also

strongly influenced by the viral genome's methylation state and the presence or absence of B- *versus* plasma-cell-specific proteins that inhibit or promote, respectively, viral reactivation through effects on Z and R functional activities. Although much less is currently known about how EBV is regulated in epithelial cells, it is likely to be strongly influenced by the differentiation state in this cell type as well.

3.2. Negative regulation of EBV IE promoters

Silencing of transcription from Zp by multiple cellular factors (including YY1, E2-2, MEF-2D, and the ZEBs) plays a critical role in establishment and maintenance of viral latency in B cells. The binding sites of these factors on the Z promoter are shown in Fig. 1B. MEF2D binds the ZIA, ZIB, and ZID elements of Zp, repressing *BZLF1* gene expression during latency by attracting type II histone deacetylating complexes (HDACs) to the promoter [36,37]. These ZI motifs also function as positive regulators of Zp transcription when MEF2D switches to an activator in the presence of lytic inducers discussed below.

The ZV and ZV' elements surrounding the Zp transcription initiation site are also strong silencers of transcription. In this case, they synergistically bind the two zinc-finger regions present in the E-box-binding proteins ZEB1 (aka TEF8) and ZEB2 (aka SIP1) [38–41]. Expression of the ZEBs is strongly negatively regulated by members of the cellular 200 family of micro RNAs (miRs) *via* a double negative feedback loop. Thus, EBV-positive cell lines in which the infection is highly latent usually contain high levels of ZEB1 and/or ZEB2 and very little of the 200b and 429 miRNAs. The converse is true in cell lines such as AGS in which the EBV infection is highly lytic [42–44]. As expected, addition of these miRNAs to latently infected cells induces lytic reactivation [43,44]. Other cellular and EBV-encoded miRNAs also contribute to maintenance of EBV latency [19–21,45,46]. ZEB1 and ZEB2 are also both Smad-interacting proteins (SIPs), with ZEB1 shown to function as a component of activator complexes in some cell lines [47]. Thus, as with MEF2D, they may switch from repressor to activator of Zp transcription under some conditions such as presence of TGF- β 1 (Fig. 2B).

ZIV elements are bound by YY1, HI elements by the E box-binding protein E2-2 [48], and the ZIIR element by an as-yet-unidentified protein [49]. The latter element can synergistically contribute to strong silencing of transcription from Zp. For example, an EBV genome containing mutations in both ZEB-binding ZV elements and the ZIIR element exhibits a high level of constitutive lytic-gene expression *in vitro* and *in vivo* and is highly defective in transforming primary blood B cells *in vitro* [41,50]. Nevertheless, this super-lytic mutant still induces B-cell lymphomas in mice with a humanized immune system due to inability to achieve fully lytic infection in this model [50].

The factors silencing *BRLF1* gene expression during latency in B cells have been much less well studied. Cellular transcription factors known to bind to and regulate Rp are shown in Fig. 1C. YY1 [51] and, possibly, ZEB1/2 [38] have both been reported to decrease Rp activity. The other transcription factors identified as binding to this promoter activate transcription through either direct (*e.g.*, Z, Sp1, EGR-1, NF1) or indirect (*e.g.*, BLIMP-1) [52] mechanisms.

Silencing of transcription from Zp and Rp is also essential for EBV latency in epithelial cells, yet only beginning to be understood. We recently discovered that Np63, a spliced variant of the p53 family member p63, plays a key role in inhibiting EBV reactivation [53]. Lacking the amino-terminal transcriptional activator domain of p63, Np63 is highly expressed in undifferentiated basal epithelial cells, with its expression lost during differentiation. It is also highly expressed in NPCs [54]. Knock-down of Np63 expression in EBV-infected telomerase-immortalized normal oral keratinocytes (NOK) cells [25] induces lytic EBV gene expression, whereas its over-expression in EBV-infected AGS cells suppresses lytic gene expression. Cyclin D was recently shown to do likewise in EBV-infected nasopharyngeal epithelial cells [55]. The mechanisms by which Np63, cyclin D, and other yet-to-be-identified factors silence IE gene expression in latently infected epithelial cells remain to be determined.

3.3. Negative regulation of Z and R functional activities

Achieving long-term latency in B cells is an essential component of EBV's life cycle, and multiple mechanisms are employed by EBV to promote latent infection in B lymphocytes. For example, we recently reported that infection of B cells in a humanized mouse model system with a "super-lytic" EBV mutant (in which the ZV, ZV', and ZIIR elements in Zp are all mutated) leads to high-level Z protein expression in many of the infected cells, yet few of them also express early lytic proteins, and almost none express late viral proteins [50]. Thus, lytic replication can be inhibited in B cells even in the presence of high-level Z expression.

How might this occur? As shown in Fig. 2A, the ability of EBV to switch from the latent to lytic form of infection in B cells is tightly associated with the differentiation state of the cell. For example, two B-cell-specific factors, PAX5 and Oct-2, promote viral latency by directly interacting with Z protein, thereby inhibiting Z's ability to bind ZREs within lytic EBV promoters [56,57]. Importantly, BCR stimulation and plasma cell differentiation lead to loss of expression of these factors (Fig. 2A). Likewise, reactivation of EBV in lymphoblastoid cell lines is negatively correlated with EBF1 expression, another factor lost during plasma cell differentiation [58]. Thus, EBV reactivation likely occurs, in part, by enhancement of Z transcriptional activity as a direct consequence of loss of Oct-2 and PAX5. We recently discovered that in the absence of R expression, Ikaros (another cellular transcription factor abundantly present in B cells), contributes to maintenance of EBV latency, in part, by increasing Oct-2 expression [59]; interestingly, once R is expressed, Ikaros promotes lytic gene expression and directly associates with R [59]. The transcriptional activity of Z is also suppressed, in part, by SUMOylation at Z lysine 12 [60]. In addition, insufficient phosphorylation of Z serine 173, which is required for Z-mediated lytic replication and late gene expression [26], may also present a barrier to fully lytic infection under some circumstances.

4. Factors contributing to EBV reactivation

4.1. Positive regulation of the EBV IE promoters

Expression of the *BZLF1* and *BRLF1* genes can be activated in B cells by a variety of physiological stimuli and chemical agents, including BCR engagement, TGF- β , hypoxia,

DNA damage, and chemical agents (Fig. 2). The ZI and ZII elements of Zp (Fig. 1B) are both critical for EBV reactivation by B-cell receptor engagement as well as numerous other lytic-inducing stimuli, including phorbol esters, calcium ionophores, and chemotherapeutic agents. MEF2D bound to the ZII element can be converted from a negative to positive regulator of Zp by BCR engagement which induces dephosphorylation of MEF2D [61]. BCR engagement also leads to changes in the activities of multiple other factors regulating Zp [62], including phosphorylation of type II HDACs (resulting in their relocation to the cytoplasm) [63]. The ZII element resembles a CREB-responsive element (CRE), binding CREB, CCAAT/enhancer binding proteins, ATF-1, AP-1, ATF-2/c-Jun heterodimer, and the spliced variant of XBP-1 [64,65]. Interestingly, most of these transcription factors become activated *via* phosphorylation, and many of the lytic-inducing stimuli do so *via* activating cellular kinases, including JNK, MAPK/p38, ERK, PKC, PKD, and/or PI3K/AKT. Conversely, inhibitors of these kinases reduce the effectiveness of several lytic-inducing stimuli [13,28,29,62,66]. NF- κ B activation is also required for induction *via* BCR engagement and TGF- β , likely through effects on MAPKs [62,67]. Once expressed, Z can bind to and activate its own promoter (Fig. 1B).

Induction of EGR-1 expression also likely plays a central role in mediating EBV reactivation in response to a variety of lytic-inducing agents. EGR-1 levels are enhanced by BCR engagement [68], phorbol esters [69], the viral LMP2A protein (a BCR mimic) [70], DNA damaging agents [71], and, even, direct binding of Z to the EGR1 promoter [72,73]. BCR engagement likely induces EGR1 through activation of MAPKs which phosphorylate the cellular transcription factor ELK1, which then drives *EGR1* gene expression. EGR1 activates Rp through two EGR1 binding motifs [69] [72] (Fig. 1C). Although the mechanism(s) by which EGR1 activates Zp are not yet well defined, one pathway likely involves activating expression of hypoxia inducible factor (HIF)-1 α (Fig. 2D). Consistent with EGR-1's central role, knock-down of EGR-1 inhibits lytic-gene expression in EBV-positive 293 cells, and the Rp response to chemotherapy requires its EGR-1 binding sites [74]. Rp also contains several Sp1 sites, required for constitutive promoter activity and efficient autoactivation by the R protein, as well as multiple ZREs (Fig. 1C).

TGF- β , a cytokine secreted by T cells, can induce EBV reactivation *via* activating *BZLF1* gene expression in some type I BL cell lines [12,13,67]. This effect is mediated in large part *via* binding of TGF- β 1 to the TGF- β type II receptor (T β RII), thereby triggering heterodimerization and transphosphorylation of the TGF- β type I receptor (T β RI) which then phosphorylates Smad2 and Smad3, which then complex with Smad4 (Fig. 2B). This activated Smad complex then translocates to the nucleus where it directly binds a series of Smad-binding elements (SBEs) located throughout Zp [13] (Fig. 1B). Zp may also be activated in part by the formation of ZEB/Smads/co-activator complexes bound at the transcription initiation site. However, given ERK inhibitors can inhibit EBV reactivation by TGF- β [67], factors that bind the ZII element of Zp likely contribute to activation *via* non-canonical TGF- β signaling pathways as well.

Hypoxic growth conditions can also induce lytic-gene expression in some EBV-infected cell lines *via* transcriptional effects on Zp [10]. Under normal oxygen levels, alpha subunits of HIFs are hydroxylated at conserved proline residues by HIF prolyl hydroxylases, allowing

their recognition and ubiquitination by the VHL E3 ubiquitin ligase, which labels them for rapid degradation by the proteasome (Fig. 2C). However, these hydroxylases require iron and oxygen to function. In the absence of either one, the HIFs accumulate to high levels, binding to hypoxia-responsive elements (HREs) in complexes with co-activators. We recently identified the HRE located within Zp through which HIFs and iron chelating drugs can induce EBV reactivation [75] (Fig. 1B). HIF-1 α /HIF β heterodimers directly bind this HRE, inducing lytic-gene expression in some p53-positive epithelial and B cell lines latently infected with EBV, but not cells infected with HRE mutants of EBV. Drugs that induce accumulation of HIF-1 α in cells also induce EBV reactivation *via* this HRE.

Finally, the cellular kinase, ataxia-telangiectasia mutated (ATM), which is induced by both DNA-damage and reactive oxygen species (ROS), is required for EBV reactivation by multiple different types of stimuli, including BCR engagement, TGF- β , H₂O₂, and HDAC inhibitors (HDACi) [76] (Fig. 2D). Most of the various stimuli that reactivate EBV share the ability to induce either DNA damage and/or ROS. ATM likely contributes to EBV reactivation through multiple different signaling pathways (Fig. 2D). ATM phosphorylates and activates p53. p53 enhances Zp activity, not only by directly interacting with Sp1 protein bound to Zp ZI motifs (Fig. 1B), but also potentially through its ability to increase *EGR1* gene expression [34,75,77,78]. However, since ATM is required for efficient viral reactivation even in cells with mutant p53 [76], additional ATM targets such as KAP1, TIP60, and H2AX likely also contribute to EBV reactivation.

4.2. Activation by plasma and epithelial cell differentiation

EBV reactivation in humans is probably largely triggered by BCR stimulation and plasma cell differentiation [8,58]. Two cellular proteins that play critical roles during plasma cell differentiation of B cells are X-box binding protein-1 (XBP-1) and B lymphocyte-induced maturation protein-1 (BLIMP-1; aka PRDM1) (Fig. 2A). XBP-1 is a member of the CREB/ATF family. Its spliced variant, XBP-1s, is synthesized both during plasma cell differentiation and following ER stress. XBP-1s directly binds the Zp ZII element (Fig. 1B), activating transcription from Zp in reporter assays [64,79]; it can also activate transcription from Rp [80]. XBP-1s expression is sufficient to reactivate EBV in some latently infected cell lines. BLIMP-1, a zinc-finger DNA-binding protein which usually functions as a repressor, induces reactivation in some EBV-infected cell lines and activates transcription from both Zp and Rp in reporter assays [52,81,82]. The mechanisms by which it does so are not yet known. By affecting expression of both IE lytic genes, XBP-1s and BLIMP-1 amplify their lytic-inducing effects and likely expand the range of specific cell types in which they can reactivate EBV.

Understanding regulation of the EBV latent-lytic switch in epithelial cells has been hampered by lack of non-transformed epithelial cell lines that can be latently infected with EBV. EBV does not efficiently infect normal undifferentiated epithelial cells [83]. Studies performed on tissue obtained from patients with oral hairy leukoplakia (*i.e.*, EBV-infected lesions in the epithelium along the side of the tongue) suggested that EBV infection of epithelial cells normally occurs in the more differentiated cell layers, resulting in lytic infection [6]. In contrast, EBV-positive NPCs are largely composed of undifferentiated

epithelial cells latently infected with EBV [1], suggesting that undifferentiated epithelial cells are capable of supporting latent EBV infection.

Recently, we showed that undifferentiated telomerase-immortalized normal oral keratinocytes can be latently infected long-term with the Akata strain of EBV [25]. As with B-cell differentiation, we found using the “rafting” technique that lytic EBV proteins are expressed in the more differentiated cell layers of EBV-infected NOK cells while the undifferentiated basal layers remain latently infected [53]. Likewise, EBV super-infected squamous carcinoma cells that can be partially differentiated in response to methylcellulose suspension also express lytic EBV proteins along with markers of cellular differentiation, an effect that may be mediated through activated forms of ATF-1 and/or CREB binding to the ZII element in Zp [84,85] (Fig. 1B). Interestingly, BLIMP-1, which is required for terminal differentiation of epithelial cells and is expressed only in the more differentiated layers of epithelium, may play a role in activating transcription from Zp and Rp during epithelial as well as B-cell differentiation [81].

5. Role of genome methylation

Methylation of cytosines provides yet another level at which the EBV latent-lytic switch is regulated (Fig. 3). ZREs in the EBV genome often contain CpG motifs which become methylated in latently infected cells. While such methylated bases inhibit most transcription factors from activating gene expression, the Z protein actually has a preference for the methylated forms of many viral and cellular promoters [25,73,86–90]. Z binding to methylated ZREs requires serine residue 186 located within its DNA-binding domain [87,91]; the equivalent position in c-Jun and c-fos contains an alanine residue. Conversion of these alanines to serines enables these latter proteins to also activate methylated Rp [91]. Z’s unique ability to activate methylated promoters helps to ensure rapid EBV reactivation following Z synthesis, despite the EBV genome being methylated in most latently infected cells (Fig. 3).

In contrast, viral genome methylation inhibits R activation of most early lytic EBV promoters [25] by decreasing R’s ability to induce histone acetylation of chromatin while not affecting R’s DNA binding (Fig. 3). Importantly, while lytic EBV promoters are methylated in latently infected B cells, they remain hypomethylated in NOK-Akata cells. This finding may explain why R, but not Z, can reactivate EBV in NOK-Akata cells, while the converse is true for most EBV-infected B-cell lines [92]. Thus, cellular factors which activate Zp are presumably essential for inducing lytic reactivation in cells containing methylated latent viral genomes, while cellular factors that activate Rp are more important for inducing lytic-gene expression in cells with unmethylated viral genomes (Fig. 3). Nevertheless, maximal lytic-gene expression and genome replication requires both Z and R regardless of the methylation status of the viral genome [25,91]. This synergy is achieved, in part, by Z and R activating each other’s promoters. It likely also involves post-transcriptional mechanisms [25,91].

6. Lytic induction therapy for treatment of EBV-positive tumors

6.1. Laboratory studies

Whereas very few normal cells in EBV-infected humans contain EBV, essentially all cells in EBV-positive tumors do. This fact suggests that EBV reactivation into lytic-gene expression in these malignancies may provide a method to promote EBV-dependent tumor cell killing (Fig. 4). This approach, called “lytic-induction therapy” [15–17], requires identification of drugs and other treatments that induce viral reactivation in EBV-positive tumor cells without causing unacceptable toxicity to normal cells. Agents that can do so in a majority of EBV-positive cells have yet to be identified. Thus, current lytic-induction strategies take advantage of two EBV-encoded kinases: a thymidine kinase encoded by the early lytic *BXLF1* gene; and a protein kinase (PK) encoded by the early lytic *BGLF4* gene. These kinases can convert the nucleoside analogs 2'-fluoro-2'-deoxy-1- β -D-arabinofuransyl-5-iodo-uracil (FIAU) and ganciclovir (GCV), respectively, into cytotoxic drugs that both kill the EBV-positive tumor cells and prevent release of infectious virus from them [93,94]. Since the phosphorylated form of GCV can also be transferred to nearby tumor cells through gap junctions, activation of ganciclovir phosphorylation in even a small percentage of tumor cells results in “bystander killing” of a much greater percentage of them. In another approach, ^{131}I -labeled FIAU successfully inhibited growth of EBV-positive BL cells in a xenograft model, despite only 10% of the tumor cells exhibiting lytic infection, due to selective retention of the phosphorylated FIAU by the lytically infected cells along with radiation-induced killing of nearby cells [94].

Drugs that can induce EBV reactivation in tumor xenograft models and/or *in vitro* at relatively non-toxic doses include HDAC inhibitors, bortezomib (a proteasome inhibitor), and a variety of chemotherapy agents [74,94–96]. Low-dose γ -irradiation also does so [95,97]. These agents induce ATM activation, consistent with ATM and its downstream target, p53, playing key roles in promoting EBV reactivation (Fig. 2D). HDAC inhibitors are particularly attractive agents for lytic-induction therapy given they reactivate EBV through several mechanisms including: (a) increasing acetylation of viral chromatin [98]; (b) activating ATM and p53 [99]; and (c) decreasing expression of Oct-2 and PAX5 [56,57]. Nevertheless, they need to be used in combination with other lytic-inducing agents to achieve efficient reactivation *in vitro*. Unexpectedly, some HDAC inhibitors (*e.g.*, voronistat) are more effective than others (*e.g.*, valproic acid) for inducing EBV lytic-gene expression despite their inducing similar levels of acetylated viral chromatin [100]. Likely, “off target” effects (*e.g.*, inducing ATM, inhibiting Oct-2 or PAX5 synthesis) explain this finding.

6.2. Clinical studies

Few patients with EBV-positive cancers have been treated with lytic-induction regimens to date. Nevertheless, results are promising. The largest published study enrolled 15 patients with EBV-positive lymphomas refractory to conventional therapy [101]. They were treated with a combination of arginine butyrate (an HDACi) and ganciclovir. Four had complete and six had partial remissions. This finding indicates HDAC inhibitors may be more effective at inducing EBV reactivation in humans than cells cultured *in vitro*. However,

whether the observed therapeutic effect was due to lytic induction is unclear since HDAC inhibitors can also inhibit growth of EBV-negative lymphomas [102] and EBV lytic-gene expression was not measured.

Recently, three patients with end-stage EBV-positive NPCs refractory to conventional therapy were treated with a combination of gemcitabine (a nucleoside analog used in chemotherapy), valproic acid, and ganciclovir [103]. Gemcitabine plus valproic acid had previously been shown to synergistically induce reactivation in EBV-positive NPC cells *in vitro* [96]. This treatment regimen led to stabilization of disease in all three patients. Again, whether lytic induction was responsible for the outcomes is unclear. Nevertheless, based upon these promising results, a larger-scale clinical trial has been initiated to determine the efficacy of this regimen for EBV-positive NPCs.

Studies directly comparing the efficacy of routine chemotherapy alone *versus* chemotherapy plus ganciclovir for the treatment of a variety of EBV-positive lymphomas in humans have yet to be initiated. However, data obtained with animal models indicate that addition of ganciclovir to routine chemotherapy may improve outcomes in human patients with such lymphomas provided: (a) the chemotherapeutic agents induce lytic gene expression in the EBV-positive tumor cells; and (b) the drug combination is not overly toxic [74]. Also, a study examining tumor biopsies obtained before *versus* after routine treatment of pediatric Burkitt's lymphoma patients with Cytosin (i.e., cyclophosphamide) indicated Cytosin may, indeed, induce lytic EBV infection in tumor cells [104]. Thus, a study comparing the efficacy of Cytosin alone *versus* Cytosin plus ganciclovir for treating such patients may be warranted.

7. Conclusions

Over the past few years, significant progress has been made toward identifying the cellular signaling pathways, transcription factors, and viral proteins that together determine whether EBV infection remains latent or becomes lytic. In both epithelial and B cells, the differentiation state plays a major role, with the factors involved in this process better known now. Recent studies illustrate how EBV can also use cellular stress signals associated with DNA damage and hypoxia to reactivate. Enhanced understanding of the multiple mechanisms regulating EBV reactivation will, hopefully, lead to the development of minimally toxic, highly specific, and potent agents for inducing lytic EBV infection in tumors. In the interim, the use of HDAC inhibitors and/or DNA damaging agents (e.g., chemotherapy) in combination with ganciclovir appears to be a promising approach for treating some patients with EBV-positive tumors.

Acknowledgments

This research was supported by grants R01-CA58853, R01-CA66519, R01-CA174462, P01-CA22443, and P30-CA14520 from the US National Institutes of Health.

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Fig. 1. Schematics (not drawn to accurate scale) showing (A) the locations of the *BZLF1* and *BRLF1* genes within the context of the EBV genome, and (B and C) factors known to play roles in regulating transcription from the promoters of these genes, Zp and Rp, respectively. *Cis*-acting elements are indicated by gray rectangles, with their corresponding *trans*-acting factors shown directly above or below them. ZRE, Z-responsive element; SBE, Smads-responsive element; and HRE, hypoxia-responsive element.

Fig. 2.

Some cellular signaling pathways that can lead to EBV reactivation. Shown here are highly simplified schematics indicating some of the key players involved in inducing transcriptional activation of Zp and/or Rp by (A) differentiation of B cells into plasma cells, (B) TGF-β1, (C) hypoxia, and (D) a variety of agents that induce DNA damage or reactive oxygen species (ROS). See text for details. P, phosphorylation.

Fig. 3.

Schematic indicating role of EBV genome methylation in determining whether EBV reactivation is initially induced by Z or R protein in B cells *versus* telomerase-immortalized normal oral keratinocytes, respectively. Once either Z or R protein is synthesized in a cell type-dependent manner, it induces synthesis of the other immediate-early protein, with the two proteins then inducing the cascade of events that leads to production of infectious virus particles. See text for details.

Fig. 4.

Strategy of lytic-induction therapy for treating patients with EBV-positive tumors using a combination of EBV lytic-inducing agents and ganciclovir. Lytic EBV reactivation in a portion of the tumor cells by one or more inducing agents leads to synthesis of the EBV-encoded thymidine and protein kinases (TK and PK, respectively). The PK then converts GCV to an active form by phosphorylation. Phosphorylated GCV inhibits both cellular and viral DNA replication, leading to cell death. It also spreads to nearby cells, killing these cells as well. Cells lacking phosphorylated GCV continue to replicate their DNA normally. See text for details.