Induction of Lytic Epstein-Barr Virus (EBV) Infection in EBV-associated Malignancies Using Adenovirus Vectors in Vitro and in Vivo

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ABSTRACT

The consistent presence of EBV genomes in certain tumor types (in particular, AIDS-related central nervous system lymphomas and nasopharyngeal carcinomas) may allow novel, EBV-based targeting strategies. Tumors contain the latent (transforming) form of EBV infection. However, expression of either of the EBV immediate-early proteins, BZLF1 and BRLF1, is sufficient to induce lytic EBV infection, resulting in death of the host cell. We have constructed replication-deficient adenovirus vectors expressing the BZLF1 or BRLF1 immediate-early genes and examined their utility for killing latently infected lymphoma cells in vitro and in vivo. We show that both the BZLF1 and BRLF1 vectors efficiently induce lytic EBV infection in Jijoye cells (an EBV-positive Burkitt lymphoma cell line). Furthermore, lytic EBV infection converts the antiviral drug, ganciclovir (GCV), into a toxic (phosphorylated) form, which inhibits cellular as well as viral DNA polymerase. When Jijoye cells are infected with the BZLF1 or BRLF1 adenovirus vectors in the presence of GCV, viral reactivation is induced, but virus replication is inhibited (thus preventing the release of infectious EBV particles); yet cells are still efficiently killed. Finally, we demonstrate that the BZLF1 and BRLF1 adenovirus vectors induce lytic EBV infection when they are directly inoculated into Jijoye cell tumors grown in severe combined immunodeficiency mice. These results suggest that induction of lytic EBV infection in tumors, in combination with GCV, may be an effective strategy for treating EBV-associated malignancies.

INTRODUCTION

EBV is a human herpesvirus that infects >90% of humans (1). It causes infectious mononucleosis and persists throughout life after the primary infection, being contained by the immune system of the host (1). The EBV genome has been found in a number of different tumor types, including Hodgkin’s disease, B-cell lymphomas, nasopharyngeal carcinoma, gastric carcinomas, and leiomysarcomas (1–9). EBV-associated B-cell lymphomas are a particular problem in immunocompromised hosts. In AIDS patients, essentially all primary CNS1 lymphomas contain the EBV genome (6), as do many of the peripheral lymphomas (8, 9). Although CNS lymphomas rarely metastasize, they have a very poor prognosis and often respond minimally to conventional therapy.

Given the frequent presence of the EBV genome in certain tumor types, we are developing therapeutic strategies that specifically target EBV-containing cells for destruction. One such strategy is to convert EBV infection in tumor cells from a latent into a cytoytic form. In infected tumor cells, EBV normally exists in one of three latency types (1). EBV-transforming proteins are expressed during latency types II and III, whereas induction of lytic infection results in host cell killing (1, 10, 11). The switch from latent to lytic EBV infection can be induced by expression of either of the EBV IE proteins, BZLF1 and BRLF1. BZLF1 and BRLF1 are transcriptional activators that activate expression of EBV early genes (12–18). BZLF1 expression is sufficient to trigger induction of lytic infection in both B cells and epithelial cells (12–14, 19–22), and BRLF1 expression can induce lytic EBV infection efficiently in epithelial cells (22). Thus, delivery of either the BZLF1 or BRLF1 proteins into latently EBV-infected tumors using gene delivery methods would be expected to result in specific killing of tumor cells.

A potential problem with this approach is that lytic infection of tumor cells may result in the release of infectious viral particles. However, it is possible that the combination of lytic EBV infection with the antiviral drug, GCV, could abort viral replication and prevent release of infectious EBV, while still allowing EBV-specific killing. Phosphorylation of GCV into its triphosphate form is initiated by the early (or lytic cycle) proteins of several herpesviruses, including the HSV-TK protein and the CMV UL97 protein (23, 24). Phosphorylated GCV is cytoxic, and it inhibits not only virally encoded DNA polymerases but also the cellular DNA polymerase, resulting in death of the host cell (25–27). Delivery of the HSV-TK gene into tumor cells, in combination with GCV, is, thus, being investigated as a potential method for treating cancer (25–27). Although the EBV genome contains homologues to both the HSV-TK and CMV UL97 genes, it is uncertain if either (or both) of these lytic EBV proteins can phosphorylate GCV (28, 29). However, GCV is effectively phosphorylated in lytically EBV-infected cells (30) and lytic EBV replication is inhibited by GCV (31). Thus, we hypothesized that the combination of lytic EBV infection and GCV would result in cellular death, while preventing the release of infectious EBV.

In this study, we have used adenovirus vectors expressing the BZLF1 or BRLF1 proteins to induce lytic EBV infection in a Burkitt lymphoma cell line in vitro as well as in vivo. We demonstrate that GCV is phosphorylated into its active form in lytically EBV-infected Burkitt lymphoma cells. The BZLF1 and BRLF1 adenovirus vectors can, thus, efficiently kill EBV-positive Burkitt cells, without concomitant release of infectious virus, when combined with GCV. The induction of lytic EBV infection, combined with GCV administration, may be a promising therapeutic approach for specifically killing EBV-positive tumors.

MATERIALS AND METHODS

Human Cell Lines. Jijoye is an EBV-positive Burkitt’s lymphoma cell line. The CB95 cell line is an EBV-transformed lymphoblastoid B-cell line. CB95-TK cells were stably transduced with a retroviral vector expressing the HSV-1 TK gene, as described previously (32). The EBV-positive epithelial cell line, NPC-KT, is derived from the fusion of a human adenoidal epithelial cell line and a primary EBV-positive nasopharyngeal carcinoma (33). Primary human fibroblasts were established from neonatal human foreskin. Saos-2 is a human osteosarcoma line. B-cell lines were maintained in RPMI 1640 with 10% FBS and penicillin/streptomycin, and NPC-KT cells were maintained in DMEM with 10% FBS and penicillin/streptomycin. Saos-2 cells were grown in McCoy’s 5A medium with 15% FBS and penicillin/streptomycin. Primary human fibroblasts were grown in MEM with 10% FBS and nonessential amino acids.

Construction of Adenovirus Vectors. The EBV IE genes BZLF1 and BRLF1 and the control lacZ gene were initially cloned under the control of the IE promotor.
CMV IE promoter into a shuttle vector containing a loxP site, the left adenovirus terminal repeat, and a packaging signal (Fig. 1 and Ref. 34). The BZLF1 shuttle vector contained the BZLF1 cDNA (a gift from Paul Farrell, Ludwig Institute for Cancer Research, St. Mary’s Hospital, London, United Kingdom), and the BRLF1 shuttle vector contains the genomic EBV BgIII-HindIII fragment from position 105,413 to 103,080 (35). The shuttle vectors were then inserted through cre-loxP-mediated recombination into an adenovirus type 5 derivative, which is missing the E1 and E3 genes and packaging sequences. This parent adenovirus is a derivative of Ad d1309 (Stephen Hardy, Cell Genesis; Ref. 34) and can only be packaged after recombination with the shuttle vector. Virus stock was tiered on the 293 cell line and purified by double cesium chloride gradient, followed by dialysis.

Adenovirus Infection. Monolayer cells were plated 1 day prior to adenovirus infection and then overlaid with the appropriate medium containing 2% FBS and adenovirus virions. B cells were concentrated at 1 × 10^6 per 100 μl in RPMI 1640 with 2% FBS before adding adenovirus virions. After 3 h of incubation at 37°C, fresh medium containing 10% FBS was added to the cells. β-Galactosidase Staining. For in vitro staining, cells were fixed in 0.5% glutaraldehyde and then stained for β-galactosidase expression, as suggested by the manufacturer (Promega). Tumor tissue pieces were fixed in 2% paraformaldehyde-0.2% glutaraldehyde and then stained for β-galactosidase staining.

Immunoblot Assays. Twenty to 50 μg of total cellular protein were loaded onto a 10% denaturing polyacrylamide gel, and immunoblot analyses were then performed as described previously (36). A 1:40 dilution of the monoclonal antibody 9240 (Capricorn, Scarborough, ME) was used to detect induction of the EBV lytic early protein, BMRF1. Proteins were visualized using a chemiluminescence kit from Amersham.

EBV Termini Assays. DNA was isolated from Jijoye cells 3 days after adenovirus infection, cut with BamHI, run on a 0.8% agarose gel, and blotted onto a Hybond nylon membrane. The filter was hybridized with a 32P-labeled riboprobe spanning the EBV termini (1.9-kb XhoI fragment; a gift from Nancy Raab-Traub; Ref. 37).

FACS Analysis. Cells were fixed in 60% ice-cold acetone and washed in PBS with 1% BSA. The following dilutions of antibodies in PBS with 1% BSA were used: monoclonal antibody 9240 (Capricorn) to detect BMRF1, 1:25; monoclonal antibody BZ.1 (Dako, Carpinteria, CA) to detect BZLF1, 1:20; monoclonal antibody 8C12 (Argene, Inc., North Massapequa, NY) to detect BRLF1, 1:25; and monoclonal antibody B0172 (Virotech International, Inc. Rockville, MD) to detect viral capsid antigen, 1:20. FITC-conjugated anti-mouse IgG (GAM-FITC, 1:100; Sigma Chemical Co., St. Louis, MO) was used as a secondary antibody. FACS was performed on a Becton-Dickinson apparatus. Annexin V binding was detected using the ApoDETECT Annexin V-FITC kit (Zymed, South San Francisco, CA) following the manufacturer’s instructions.

Intracellular Nucleotide (GCV) Anabolism Assays. GCV was synthesized at Wellcome Research Laboratories (Research Triangle Park, NC). A total of 2 × 10^6 Jijoye or NPC-KT cells were infected with a MOI of 50 of the BZLF1 or lacZ adenovirus vectors or were mock-infected. The cells were pulse-labeled with 27 μM [8-3H]GCV (Moravek Biochemicals, Brea, CA) at various time points postinfection in the appropriate medium. After incubation at 37°C for either 6 or 18 h, the pulse media were removed, and the cells were rinsed with ice-cold PBS. Following extraction with ice-cold 0.5 M perchloric acid, the cellular extracts were clarified by centrifugation (770 × g, 10 min). GCV anabolites were quantitated by passage through a cation exchange column, as described previously (38). Column effluent was counted using a Packard 2500TR liquid scintillation counter.

Ganciclovir Viability Assays. Cells were infected with adenovirus particles as described above. After 3 h of virus incubation, 10 μM ganciclovir was added to the appropriate medium containing 10% FBS. Cells were maintained under optimal growth conditions (logarithmic phase) with and without the drug. The number of live cells was determined by trypan blue exclusion.

Animal Experiments. Four- to 5-week-old SCID mice (NIH) were injected s.c. with 5 × 10^7 Jijoye cells into both flanks. After 10–14 days, tumors developed and were injected with 2 × 10^7 adenovirus particles containing BZLF1, BRLF1, or lacZ. Two days later, the mice were sacrificed, and the
tumors were removed. Tumor tissue was frozen in methanol/dry ice and used for protein extraction and immunoblot analysis or β-galactosidase staining.

RESULTS

The BZLF1 and BRLF1 Adenovirus Vectors Disrupt Latency in EBV-positive B Cells and Epithelial Cells. In transfection experiments, we have previously shown that BZLF1 disrupts latency in EBV-positive epithelial cells and B cells, whereas BRLF1 disrupts latency in an epithelial cell-specific manner (22). To determine the effect of the BZLF1 and BRLF1 adenovirus vectors, we infected the nasopharyngeal carcinoma cell line NPC-KT and the Burkitt lymphoma line Jijoye with the adenovirus vectors (using a MOI of 50) and examined the expression of the early lytic cycle EBV protein BMRF1 2 days later. In the absence of adenovirus infection, both NPC-KT cells and Jijoye cells are primarily latently EBV infected and, thus, do not express BMRF1. As shown in Fig. 2A, infection with the lacZ adenovirus vector did not activate lytic EBV infection in either cell type. In contrast, infection with either the BZLF1 or BRLF1 adenovirus vectors induced BMRF1 expression in both NPC-KT cells and Jijoye cells. Thus, in the context of an adenovirus vector, BRLF1 expression (like BZLF1) disrupts EBV latency in both B cells and epithelial cells.

Lytic EBV infection follows a set pattern of gene expression: IE genes are expressed first, then early viral genes are expressed, and finally, late genes are expressed. To determine whether infection with the BZLF1 and BRLF1 adenoviruses results in fully lytic infection, we performed FACS analysis to quantitate early and late lytic EBV gene expression. As shown in Fig. 2B, when Jijoye cells were infected with the adenovirus vectors at an MOI of 50, essentially all of the cells expressing the BZLF1 or BRLF1 IE proteins also expressed the early BMRF1 protein. In addition, within 2 days after infection, a late EBV protein, viral capsid antigen, was also clearly induced by the BZLF1 adenovirus vector using an MOI of 50 and by both the BZLF1 and BRLF1 adenoviruses using an MOI of 150. Thus, infection of Jijoye cells with either the BZLF1 or BRLF1 adenovirus vectors results in fully lytic infection and, hence, would be expected to result in release of infectious EBV.

Lytic EBV Replication Induces GCV Phosphorylation. We next investigated whether induction of lytic EBV replication in Jijoye and NPC-KT cells using the BZLF1 and BRLF1 adenovirus vectors results in phosphorylation of GCV. NPC-KT and Jijoye cells were either mock-infected or infected with the BZLF1 or lacZ adenovirus vectors, and 2 days later, they were incubated with 3H-labeled GCV. The amount of mono-, di-, and triphosphorylated GCV was determined as described in “Materials and Methods” and compared with levels obtained in a lymphoblastoid cell line (CB95-TK) stably transduced with the HSV-1-TK gene. As shown in Fig. 3, when ~50% of NPC-KT and Jijoye cells were infected by the BZLF1 adenovirus vector, there was a 4–5-fold increase in GCV phosphorylation in comparison to lacZ-infected cells (correlating to an 8–10-fold increase if 100% of cells were lytically infected). Furthermore, the amount of GCV phosphorylation induced by lytic EBV infection was comparable to that observed in lymphoblastoid cells stably transduced with the HSV-1 TK gene, which we have previously shown to...
be extremely susceptible to GCV-induced cell killing (32). Therefore, the level of GCV phosphorylation induced by the BZLF1 and BRLF1 adenovirus vectors in EBV-positive tumor cells should be sufficient to promote GCV-mediated cell death.

**GCV Inhibits Lytic EBV Replication.** We next determined whether GCV inhibits lytic EBV replication in cells infected with the BZLF1 and BRLF1 adenovirus vectors. Lytic replication was quantitated by Southern blot analysis using the termini assay (37). As shown in Fig. 4, Jijoye cells infected with the lacZ adenovirus contain only the latent (episomal) form of the EBV genome. Infection with the BZLF1 adenovirus vector efficiently induces the lytic (linear) form of the EBV genome in Jijoye cells (Fig. 4A). The BZLF1 adenovirus vector induces early lytic EBV protein expression in Jijoye cells in the presence or absence of acyclovir or GCV (data not shown). However, treatment with either acyclovir or GCV completely abolishes the ability of BZLF1 to form linear EBV genomes, which result from lytic viral replication and are required for productive EBV infection. Similar results were obtained using the BRLF1 adenovirus in Jijoye cells (Fig. 4B), although BRLF1 was less efficient than BZLF1 in inducing fully lytic replication. EBV cannot be packaged into infectious mature virions unless it is lytically replicated (1). Therefore, concomitant GCV administration should prevent the release of infectious EBV particles when tumor cells are treated with the BZLF1 or BRLF1 adenovirus vectors.

**The BZLF1 and BRLF1 Adenovirus Vectors Kill Jijoye Cells in the Presence or Absence of GCV.** The above results predict that the BZLF1 and BRLF1 adenovirus vectors will induce cell killing in Jijoye cells in the presence or absence of GCV, although the mechanism of cell death is presumably different. To confirm this, we infected Jijoye cells with the BZLF1, BRLF1, or lacZ adenovirus vector and examined cell viability in the presence and absence of GCV. As shown in Fig. 5, in the absence of lytic EBV infection, GCV has little effect on the viability of Jijoye cells because latent EBV infection does not phosphorylate GCV. Infection of Jijoye cells with either the BZLF1 or BRLF1 adenovirus vectors (MOI of 50) induced dramatic cell killing (97–99%) in the presence or absence of GCV. Interestingly, this amount of cell killing was significantly greater than the percentage of infected cells (~35%) as
determined by FACS analysis using BZLF1- and BRLF1-specific antibodies). Thus, the induction of lytic EBV infection in tumor cells results in cell death, in the presence or absence of GCV.

**Effect of the BZLF1 and BRLF1 Adenovirus Vectors in EBV-negative Cells.** Although it has been reported previously that BZLF1 expression is nontoxic in EBV-negative cells (39, 40), BZLF1, in fact, induces a cell cycle block in these cells (39). The effect of BRLF1 expression in EBV-negative cells is unknown. Unfortunately, we were unable to efficiently infect a variety of EBV-negative B-cell lines with adenovirus vectors (data not shown). Therefore, to examine the effect of BZLF1 and BRLF1 adenovirus vectors in EBV-negative cells, we infected two different cell types, normal human fibroblasts, and the osteosarcoma cell line, Saos-2, with the lacZ, BZLF1, and BRLF1 adenovirus vectors and quantitated cell numbers 3 days later. These two cell lines were chosen because they represent different extremes in terms of tumor suppressor dysregulation; Saos-2 cells are deleted in both p53 and Rb, whereas normal human fibroblasts are not. As shown in Fig. 6A, infection of Saos-2 cells with either the BZLF1 or BRLF1 adenovirus vectors had relatively little effect upon cell growth in comparison to the lacZ control vector. However, infection of primary human fibroblasts with either the BZLF1 or BRLF1 adenovirus vectors resulted in significant growth inhibition.

Because a BZLF1-induced cell cycle block could cause growth inhibition in EBV-negative cells without killing them, we examined the level of annexin V binding in primary fibroblasts infected with the lacZ or BRLF1 adenoviruses. Annexin V binds to cells expressing phosphatidylserine on the outer membrane, which is a sensitive marker for cells undergoing early apoptosis or necrosis (41). As shown in Fig. 6B, BZLF1 adenovirus infection of normal human fibroblasts does not significantly increase the binding of FITC-labeled annexin V. Therefore, BZLF1 expression inhibits growth of primary human fibroblasts (due to a cell cycle block) but does not kill the cells. In contrast, BRLF1 induced apoptosis in a variety of cell types.4 Thus, BZLF1-induced cell killing appears to be more EBV-dependent than BRLF1-induced killing, suggesting that BZLF1 may be preferable for treatment of tumors in vivo.

**Disruption of EBV Latency in Vivo.** To determine whether the BZLF1 and BRLF1 adenovirus vectors can disrupt viral latency in vivo, SCID mice were injected s.c. with Jijoye cells, resulting in the formation of palpable tumors 2 weeks later. The Jijoye tumors were grown s.c. in SCID mice and directly inoculated with the lacZ-, BZLF1-, or the BRLF1-expressing adenovirus vectors. Two days later, the mice were sacrificed and tumor extracts were examined for expression of the lytic EBV protein, BMRF1, using immunoblot analysis.

The consistent presence of EBV in certain tumor types may open novel, EBV-based targeting strategies. Here, we have investigated the feasibility of converting EBV latency into lytic EBV infection in Burkitt lymphoma cells. Using adenovirus vectors, we demonstrated that lytic infection can be induced successfully in vitro and in vivo with either of the two EBV IE proteins. We have also demonstrated that lytic EBV infection phosphorylates GCV, converting it into a cytotoxic agent that kills the host cell while simultaneously preventing completion of the viral replicative cycle and release of infectious EBV particles. Therefore, disrupting EBV latency by gene delivery methods, with concomitant GCV administration, may be an effective method for treating EBV-associated tumors.

Because we were previously unable to show that transfected BRLF1 protein disrupts EBV latency in B cells (22), we were surprised to discover that the BRLF1 adenovirus vector efficiently induces lytic infection in Jijoye cells. The BRLF1 adenovirus vector likewise disrupts viral latency in another Burkitt cell line, Akata.5 Because another group has recently reported that BRLF1 expression (delivered by transfection) in B cells disrupts EBV latency (42), the ability of the BRLF1 adenovirus vector to induce lytic infection in Burkitt lines is probably due to a greatly increased level of BRLF1 expression from the adenovirus vector versus our plasmid vectors rather than a requirement for an adenovirus-encoded “helper” effect. In any event, our finding that the BRLF1 adenovirus vector disrupts EBV latency in both B cells and epithelial cells indicates that either BZLF1 or BRLF1 gene delivery vectors can be considered for the induction of lytic infection of EBV-associated B-cell lymphomas in vivo.

Although both BZLF1 and BRLF1 disrupt viral latency, they are significantly different in other respects. For example, BZLF1 blocks the cell cycle (39), whereas BRLF1 activates cell cycle progression.4 There may also be differences in the in vivo toxicity of BZLF1 versus BRLF1 in EBV-negative cells. Therefore, it will be important to compare the efficacy and toxicity of BZLF1 versus BRLF1 adenoviruses in vivo to determine which is the better vector for disrupting tumor latency. Should the expression of the BZLF1 or BRLF1 proteins prove to be excessively toxic to EBV-negative cells in vivo, these proteins could be placed under the control of EBV-specific promoter elements (such as the EBNA-1-dependent oriP enhancer or the EBNA-2-responsive BamHI C promoter; Refs. 43 and 44) to make this system even more EBV dependent.

Previous investigators have delivered the HSV-TK gene to promote GCV-induced killing of tumor cells. Here, we have devised a novel strategy in which we do not deliver a virally encoded TK gene but instead induce the EBV genome within tumor cells to express proteins that phosphorylate GCV. This strategy has the advantage of being completely EBV specific, because the expression of BZLF1 or BRLF1 in EBV-negative cells obviously cannot induce virally encoded proteins. Furthermore, because phosphorylated GCV has been shown to produce bystander killing in certain cell types (25–27), the BZLF1/GCV (or BRLF1/GCV) combination could potentially kill more cells than delivery of the IE proteins alone. Although we have been unable to observe bystander killing with the HSV-TK/GCV combination in EBV-positive B-cell lines in vitro, bystander killing occurred with the HSV-TK/GCV combination in EBV-positive lymphomas in vivo (32). Thus, the use of GCV with EBV IE gene delivery vectors will not only prevent release of infectious EBV but may actually improve the efficiency of tumor cell killing in vivo. EBV contains homologues to both the HSV-TK gene and the CMV

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5 E.M. Westphal and S. C. Kenney, unpublished observations.
REFERENCES


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