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BZLF1 Controlled by Family Repeat Domain Induces Lytic Cytotoxicity in Epstein-Barr Virus-positive Tumor Cells

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Abstract. *Background: BZLF1, an EBV (Epstein-Barr virus) immediate early gene, is required for EBV lytic replication that causes the death of host cells. EBNA1, the product of EBV latent gene, binds to the family repeats (FR) of the origin of replication (Orip) regulating EBV replication. Materials and Methods: A vector pFR-Z (BZLF1 controlled by FR domain of EBV) was constructed and transfected into EBV-positive 5-8F and-negative HNE3 nasopharyngeal carcinoma cells and BZLF1-induced cytotoxicity was tested. Results: EBNA1 expression was detected in 5-8F but not HNE3 cells and, in agreement, pFR controlled luciferase expression was activated in 5-8F cells but inhibited in HNE3 cells. Gardella gel assay demonstrated that pFR-Z effectively induced EBV lytic replication in 5-8F but not HNE3 cells. The lytic cytotoxicity was confirmed by a diminished cell survival and the induction of lytic proteins EA-D and gp125. The cytotoxicity was also strikingly enhanced by addition of GCV (gancyclovir) that kill cells with lytic form EBV. Conclusion: pFR-Z is a specific gene therapy vehicle for EBV-positive carcinomas.*

EBV (Epstein-Barr virus) is a human herpes virus that infects 90% of human beings. Although infection of EBV is primarily in lymphocytes and epithelial cells (1, 2), increasing number of tumor types are found to be EBV-positive (3-9). Like all other herpes viruses, EBV induces both lytic and latent forms of infection. The lytic form of infection requires transmission of virus from cell to cell, which results in the death of host cells (1, 2). Almost all the EBV-positive tumor cells contain the latent form of EBV infection. It is thus reasonable to believe that gene modifications should be approached to kill EBV-positive tumor cells if the latent form of EBV can be switched into the lytic form (10-16). Lytic replication requires the coordinated expression of two viral immediate-early proteins, BZLF1 (also known as Zta, ZEBRA and EB1) and Rta (BRLF1) (17). Most interestingly, BZLF1 is expressed in the lytic but not the latent form and BZLF1 transcriptionally activates all of the EBV genes required for the lytic replication (18). Therefore, induction of lytic viral infection by delivering either the BZLF1 or BRLF1 gene may promise a gene therapy target specific and efficient for inhibition of EBV-positive carcinomas. The anticancer potential using the herpes simplex virus TK gene has been combined with the antiviral GCV (Gancyclovir) and phosphorylated GCV inhibits the cellular DNA polymerase as well as the viral polymerase (19, 20). Interestingly, lytic EBV infection activates the phosphorylation of GCV that induces a significant cytotoxicity in tumor cells, although it may suppress viral replication (10, 11). Thus, the anticancer potential of lytic form of EBV infection may be enhanced when BZLF1 expression is combined with such antiviral agents.

OriP, a 1.8-kb region of the EBV genome identified based on its ability to support the maintenance of EBV genome in the host cells, is activated by EBV-encoded EBNA-1 (21, 22). OriP depends on two cis-acting components, the DS and the FR (23). The DS, named for a dyad symmetry within it, is a cluster of four EBNA-1 binding sites that functions as applicators in the presence of EBNA-1 (24-26). On the

Abbreviations: EBV, Epstein-Barr virus; FR, family repeats; GCV, gancyclovir; LMP1, latent membrane protein 1.

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plasmids supported by oriP, replication appears to initiate at or near the DS (27). The FR, named for a family of repeats, contains 20 EBNA-1 binding sites within 20 copies of a 30-bp sequence. Combination of EBNA-1 with the FR region also functions to keep the plasmids in mitotic cells (28), apparently by allowing them to be tethered by EBNA-1 to condensed human chromosomes during mitosis (29). In this study, to test if BZLF1 gene can be used as a specific tumor gene target, we designed an expression vector containing the transcriptional cassette of the oriP-FR elements along with a CMV promoter cloned in juxtaposition with either reporter (Luciferase) or BZLF1 gene. We demonstrated that expression of BZLF1 induced in EBV-positive 5-8F but not negative HNE3 lines of human NPC cells causing a significant cytotoxicity. In addition, a synergistic cytotoxicity was induced when BZLF1-transfected cells are treated with GCV.

Materials and Methods

Construction of plasmids. To construct the pLuc, a 1689-bp Hind III – XbaI fragment containing the Luciferase gene was excised from plasmid pGL3-Basic (Clontech. Inc.). After PCR tailing, it was constructed into T easy vector (Clontech. Inc.) and then cut by EcoRI and subcloned into pcDNA3.1 (Invitrogen Inc). The FR sequence was amplified by PCR with EBV genome using the following primer: 5'-GAA GAT CTC GGC ATA TGC AAA GGA TAG C-3', 5'-GAA GAT CTA TTC ACG AGG TCG CTG AGA G-3', cut by BglII and then subcloned into pcDNA3.1 BglII site. BZLF1 gene was obtained from p294: Zp-552wt plasmids (a gift from Dr. Paul. J. Farrell, ref. 30) by cutting with BamHI and XhoI. After PCR tailing, it was constructed into T easy vector (Clontech. Inc.) and cut with EcoRI. The 1.1kb BZLF1 fragment was cloned into pLuc fragment by replacing the Luc gene and the new plasmid was named pZ. The pFR- Z was constructed by inserting the FR sequence into pZ as mentioned above.

Cell culture and transfection. The negative NPC HNE3 and EBV-positive NPC 5-8F cell lines derived from NPC tissue were maintained in RPMI 1640 supplemented with 10% FBS. B95.8 cell line, an EBV-positive marmoset B cell line, was obtained from ATCC (No. CCL-1612) and maintained in 1640 supplemented with 15% FBS. Raji cell lines, an EBNA-positive lymphoblast-like cell line established from a Burkitt's lymphoma, was purchased from ATCC (No. CCL-86) and maintained in RPMI 1640 supplemented with 10% FBS. HeLa cell line (ATCC No. CCL-2) was maintained in RPMI 1640 supplemented with 10% FBS. To detect the clonogenic survival, 10^7 cells were transfected for 24 h with 20 μg plasmids and cells were plated at a density of 10^5 cells/ml in 24-well plates in the medium with or without GCV (Sigma Chemical Co.). Clonogenicity was scored 2 weeks after the treatment.

PCR amplification of EBNA1. PCR amplification of EBNA1 was performed to determine EBV status in cells. The primers used were 5'-CCCAGTAGTCAGTCATCATC -3' and 5'-AGGAGAGCTCTTAAACCTTC -3'; the length of target product was 297 bp. Amplifications were performed in 50 μl reaction mixtures containing 10mmol/L Tris-HCl (pH 9.0), 2.5mmol/L $MgCl_2$, 50 mmol/L KCl, 25pmol of each primer, 1 mmol/L each dNTP, 1ml of DNA solution of each cell line (100ng) and 2 U of Taq DNA polymerase. After predenaturing, the fragment was enhanced by 30 cycles and one-tenth of each product was electrophoresed and visualized by ultraviolet radiation.

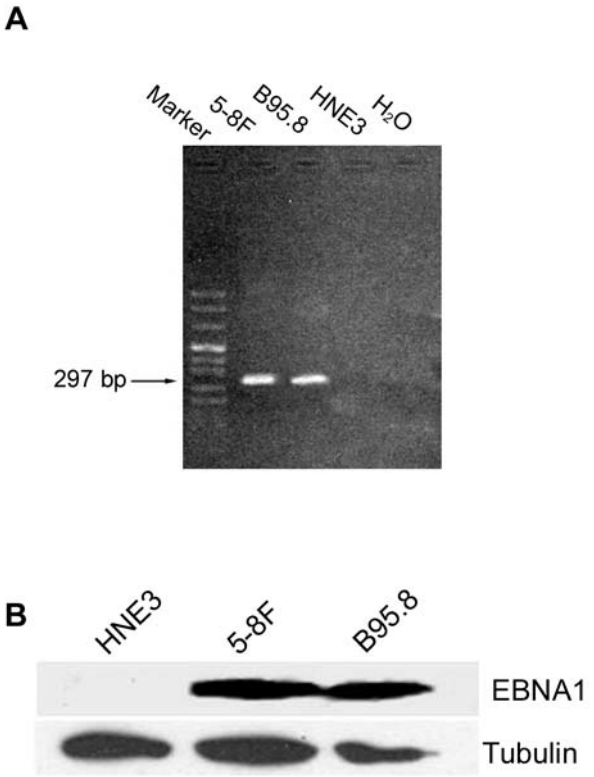

Figure 1. *EBNA-1 expression was detected in EBV-positive 5-8F but not EBV-negative NPC cell lines. A: PCR amplification of EBNA1 in HNE3, 5-8F and B95.8 (used as positive control) cell lines. B: Western blot analysis of EBNA1 expression in HNE3, 5-8F and B95.8 cell lines. The same blot was reprobed with a tubulin-specific antibody (bottom panel) to control for protein loading. The data were representative of three repeated experiments.*

Western blot analysis of EBV encoded proteins. Cells were seeded in 60mm plates for 24 h and transfected for 3 h with plasmids p3.1, pZ, or pFR-Z by Superfectamine (Qiagen). The transfected cells were cultured with complete medium for 48 h and cell extracts were prepared with lysis buffer (0.1 mol/L Tris-HCl, 0.1% SDS, 10 mmol/L EDTA and 2 mmol/L DTT) and protein concentrations were determined using the BCA protein assay (Pierce). Immunoblotting was carried out with modification as described previously (31). Briefly, samples containing equal amounts of protein (100mg) were loaded onto 5% -12% SDS-PAGE gels, electrophoresed for 5 h at 135 V and transferred onto nitrocellulose membranes. The membranes were incubated with blocking buffer containing 5% low-fat milk for 1 h and probed with antibodies of BZLF1 (dilution 1:100, Dako), EA-D (dilution 1:100, Chemicon), gp125 (dilution 1:100, Chemicon) and EBNA1 (dilution 1:100, Chemicon). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody. The specific complexes were detected using chemiluminescence (Pierce).

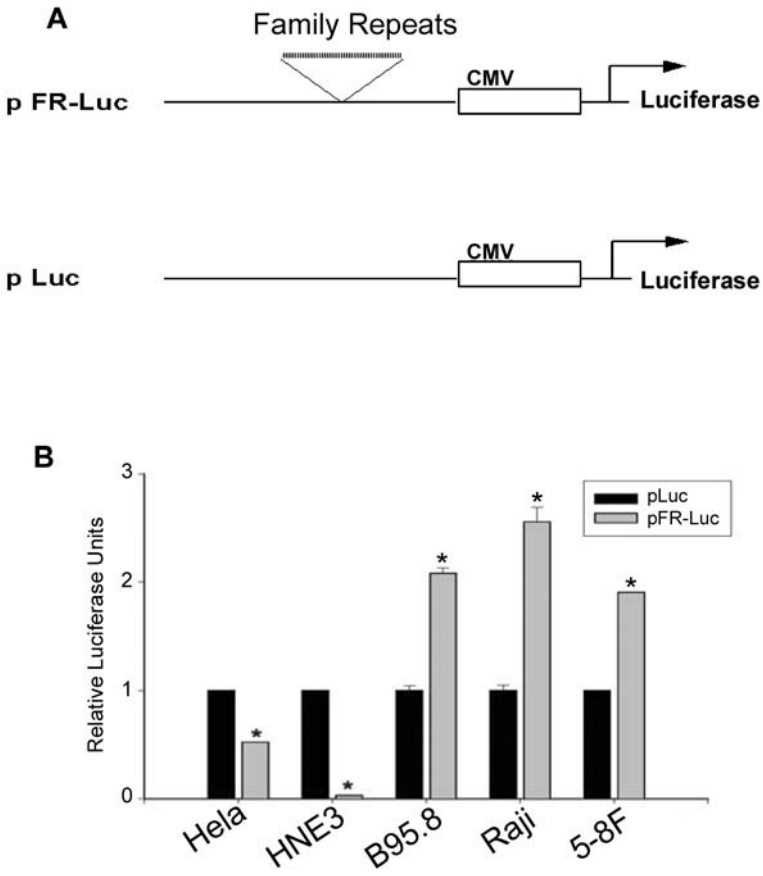

Figure 2. *Luciferase assay of EBV-negative and EBV-positive cell lines transfected by control pLuc and pFR-Luc vectors. A: Scheme of pLuc and pFR-Luc reporters. B: The indicated cell lines were transfected with pLuc or pFR-Luc plasmids with Superfectamine as described in Materials and Methods. EBV-negative cell lines are HeLa and HNE3 and EBV-positive are B95.8, Raji and 5-8F. pFR-Luc data was normalized to the mean of control pLuc and data are presented as relative luciferase activity. All the results were adjusted by β-gal plasmid transfection efficiency. Error bars represent the standard error (N=3, *p<0.05).*

Quantitative evaluation of pLuc and pFR- Luc expression. To evaluate transgene expression from pLuc or pFR-Luc in HNE3 and 5-8F cells, luciferase activities were measured using the Luciferase assay system kit (Promega). The HeLa cell line was also included as a negative control and Raji and B95.8 cell lines were used as positive controls. Cells were seeded in 12-well culture plates and transfected by pLuc and pFR-Luc as well as β-gal control plasmids with Superfectamine (Qiagen). Cultures were incubated in growth medium for 36 h. Cell lysates were analyzed for luciferase activities using the luciferase assay kit and a Luminometer (Bioscan luni-scint v3.2a) according to the manufacturer's protocols. The lysates were also analyzed for β-gal (β-galactosidase enzyme assay system, Promega Inc.).

Gardella gel analysis. Gardella gels were prepared as previously described (32). Cells were loaded (0.5 to 1 x 10^6 per well) and gels were run at 4˚C with 1.5V/cm for 3 h, followed by 5v/cm for 18 h. After electrophoresis, gels were stained with ethidium bromide and photographed. The upper section containing the sodium dodecyl sulfate and proteinase K was removed and DNA was transferred onto nylon membrane used as a Southern blot. The membrane was then hybridized with radioactive-labeled PCR products of 307bp BZLF1 gene produced by PCR with primers: 5'-CTGCAGCAGTTGCTT AAACTTG-3', and 5'-GGACAACAGCTAGCAGACATTG-3'. The following condition was used: 50 μl reaction mixtures containing 10 mmol/L Tris-HCl (pH 9.0), 2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 25 pmol of each primer, 1 mmol/L each $dAT^{32}P$, dCTP, dGTP and dTTP; 1 μl of DNA solution of p294:Zp-552wt (50ng DNA) and 2 U of Taq DNA polymerase. The mixture was predenatured and the fragments were enhanced by 35 cycles.

Effect of pZ, pFR-Z on cell viability. Cells were transfected with plasmids p3.1, pZ, pFR-Z for 3 h in 96-well plates with 180 μl medium, followed by incubation for 24 h with 0.2 ml of 10% FBS medium. Cell viability was assessed using the MTT assay after GCV was added. For MTT assay, MTT (Sigma Chemical Co.) was dissolved in PBS (5mg/ml) and 10 μl of the stock solution was added to each well and incubated at 37˚C for 4 h. After pouring the medium, 100 μl DMSO was added to each well and mixed thoroughly to dissolve the blue MTT organza crystals. The plates were subsequently read on a Bio-Rad 3350 microplate reader at a wavelength of 570 nm. For clonogenic survival, cells were transfected with the above plasmids and 5 x 10^3 cells/well of HNE3 and 5-8F cells were seeded in 6-well plates with or without GCV treatments. After 14 days incubation, clones were fixed with methanol for 20 min, stained for 15 min with 0.4% crystal violet in water, washed with water and the number of clones with more than 50 cells was calculated.

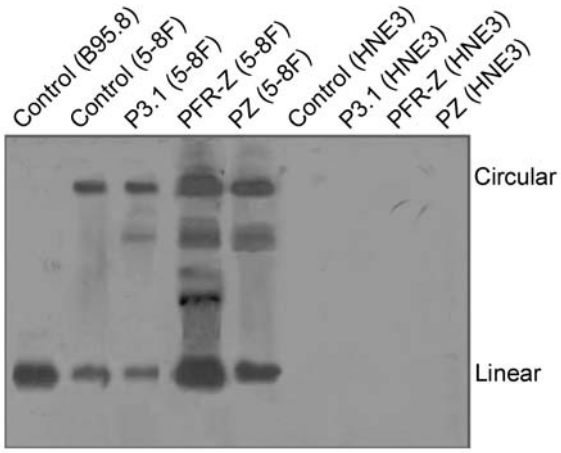

Figure 3. *Gardella gel analysis of EBV DNA in EBV-positive 5-8F and EBV-negative HNE3 cell lines transfected with pZ and pFR-Z. p3.1, pZ, or pFR-Z transfected 5-8F and HNE3 cells, as well as uninfected parental cells, were analyzed by Gardella gel electrophoresis under the conditions described in Materials and Methods. B95.8 cell supernatant was added as a linear EBV control. Episomal and linear EBV DNA is indicated as circular and linear (representative of three experiments).*

Statistics. Values were expressed as x ± s, and assessed by one-way ANOVA and Student's *t*-test. When $p<0.05$, the results were considered to be statistically significant.

Results

EBNA1 was expressed in NPC 5-8F cells. We used PCR to detect EBNA1, one of the EBV latent genes, which is expressed in all EBV latent phases in nasopharyngeal carcinoma cell line 5-8F (EBV-positive) and HNE3 (EBV-negative). Western blot with anti-EBNA1 monoclonal antibody (Chemicon) was used to detect EBNA1 protein levels in these cells. Figure 1 shows that both the PCR and Western blot results were negative for HNE3 cells whereas EBNA1 gene expression and proteins were detected in 5-8F cells, which is comparable to the level of EBV-positive control B95.8. Therefore, 5-8F and HNE3 were considered to be EBV-positive and -negative NPC cell lines and studied further.

FR sequence functioned as a gene expression enhancer in EBV-positive NPC 5-8F cells. FR sequence was reported to function as an enhancer in EBV-positive cells and as a repressor in EBV-negative cells (33). To determine whether FR functions in our system, luciferase reporters pLuc and pFR-Luc were transfected into HNE3, 5-8F, HeLa (negative control), Raji and B95.8 (positive controls) shown in Figure 2. Luciferase activity of pFR-Luc was about 33-fold lower than that induced by pLuc in EBV-negative HNE3 cells. In the negative control HeLa cells, the luciferase activity produced by pFR-Luc transfection was 1.89-fold lower than the control vector pLuc, suggesting that it is probably true that FR acts as a gene repressor in EBV-negative cells. In contrast, the EBV-positive 5-8F cells showed a 2.5-fold increase of pFR-Luc transfection compared to the control vector pLuc. In support, similar activation was induced in EBV-positive controls, B95.8 and Raji cells. These results suggest that FR sequence functions as a gene expression enhancer in EBV-positive tumor cells.

Gardella gel analysis of EBV in plasmids-treated 5-8F cells. We examined the configuration of viral DNA using Gardella gel analysis that is able to detect episomal (circular) and linear (resulting from lytic replication) EBV DNA according to their mobility in the gel. The results were shown in Figure 3. We used B95.8 cell supernatant as lytic replication controls, because the supernatant contains EBV viron, the genome in it is linear. 5-8F cells showed a limited lytic replication. However, when transfected with pFR-Z (FR and CMV promoter driving BZLF1) or pZ (CMV promoter driving BZLF1), all the 5-8F cells showed both episomal and linear EBV DNA, demonstrating a mixed state of latent and lytic infection. PZ and pFR-Z-transfected 5-8F cells showed more linear EBV DNA than controls of non-transfection and control vectors p3.1 (pcDNA3.1; Invitrogen). In addition, transfection of pFR-Z in 5-8F cells showed more linear EBV DNA than pZ transfection. These results thus strongly suggest that EBV lytic replication took place in pZ and pFR-Z-transfected 5-8F cells with more lytic replication by transfection with pFR-Z plasmid.

EBV encoded lytic protein expression in 5-8F cells. To determine whether pFR-Z-induced cell killing was caused by EBV lytic replication, we further analyzed three EBV proteins expressed in immediate early, early and late phase of EBV replication, namely BZLF1, EA-D and gp125. EA-D, an early product of EBV functions with DNA polymerase gp125, a glycoprotein, contributes to EBV capsid. Detection of all these viral proteins will provide direct evidence suggesting that EBV lytic replication takes place and causes the cell killing. Our results shown in Figure 4A and 4B demonstrate that, compared to the basal levels, 5-8F cells transfected with pFR- Z showed a significant increase in all three proteins. In contrast, the EBV-negative HNE3 cells, although pFR-Z and pZ-induced BZLF1 expression, showed no EA-D and gp-125 protein expression. Therefore, this FR-controlled BZLF1 expression system appears to be specifically activated in EBV-positive 5-8F NPC cells.

Cytotoxic effects of pZ, pFR-Z and GCV on 5-8F cells. To evaluate the cytotoxic effects of pZ, pFR-Z and GCV, 5-8F and HNE3 cells were seeded in 96-well plates and

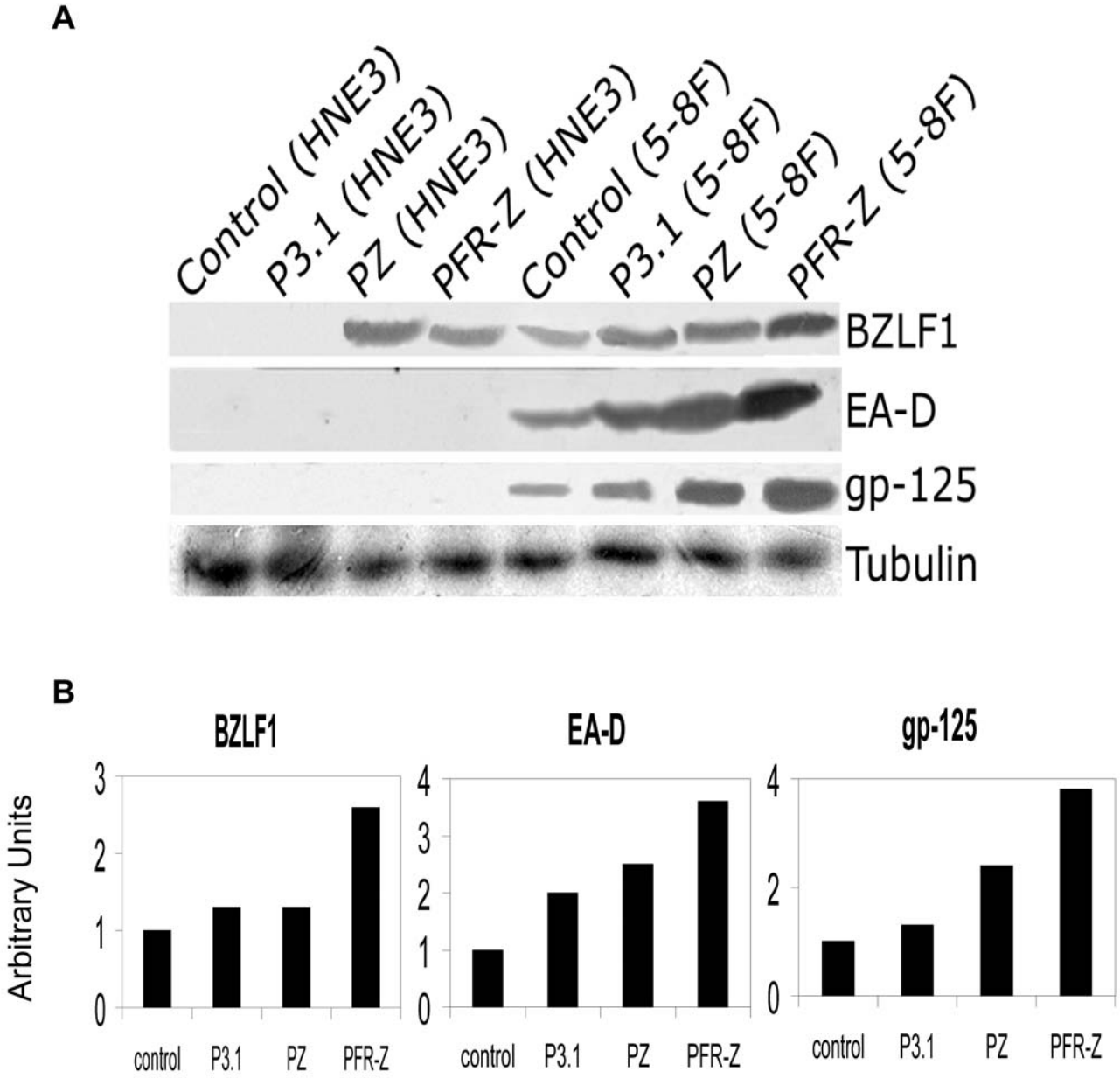

Figure 4. *pFR-Z induced lytic EBV replication in EBV-positive 5-8F cells. A: EBV-positive 5-8F and EBV-negative HNE3 cells were transfected with pFR-Z and pZ plasmids. Non-transfected as well as control vector p3.1-transfected 5-8F and HNE3 cells were included in the analysis. Western blotting analysis was performed 48 h after transfection using the BZLF1, EA-D and gp125 antibodies to detect expression of these lytic viral proteins. The same blot was then reprobed with a tubulin-specific antibody (bottom panel) to control protein loading. The data were representative of three repeated experiments. B: Densitometry quantitation of protein levels in 5-8F cells as the ratio of target protein:Tubulin.*

transfected with the plasmids of pZ, pFR- Z or p3.1 with or without treatment of GCV (100mg/ml). GCV treatment alone was used as a control. Cell viability detected by MTT assay (Figure 5A) shows a 28% decrease in pZ-transfected 5-8F cells and a 32 % decrease in pFR-Z transfection, suggesting a potential cytotoxicity induced by BZLF1 expression. In addition, when GCV was added, cell viability was further decreased by 82% and 76%, respectively, in the transfectants of pFR-Z and pZ. However, the p3.1 transfection and GCV treatment alone showed little cytotoxic effect. In EBV-negative HNE3 cells, no cytotoxic effect was detected by transfection of pFR-Z (data not

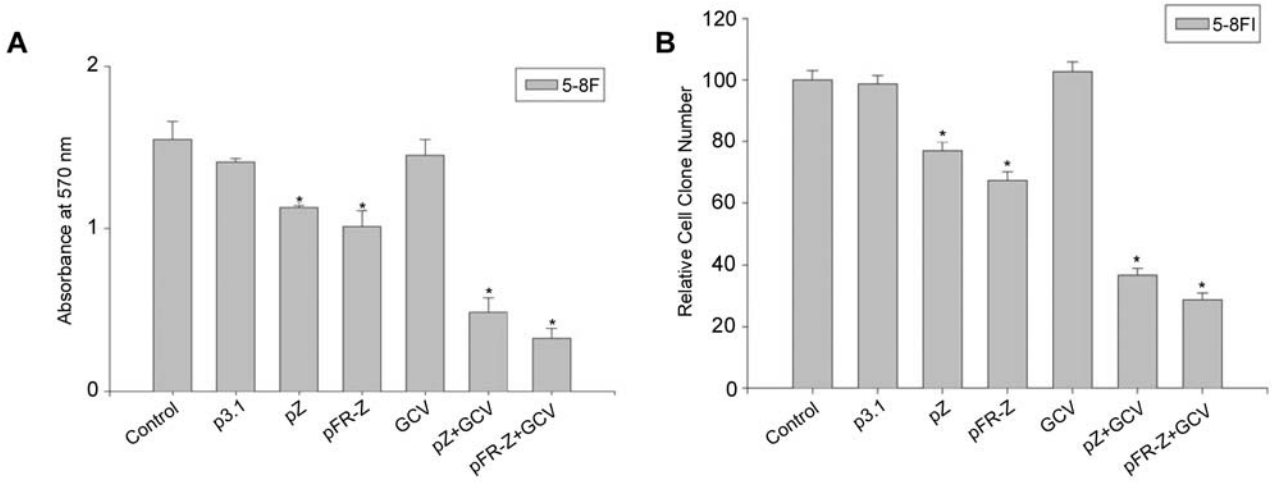

Figure 5. *Cytotoxicity of pFR-Z, pZ and GCV. A: MTT viability assay was performed on p3.1, pZ, pFR-Z-transfected 5-8F cells with or without GCV. Cell viability was determined with MTT assay and measured by the absorbance at 570 nm (N=3, *p<0.05). B: Clonogenic survival was determined and the percentage of viable cells (in comparison with untreated cells) was calculated by counting the clone number of treated and control cells. Transfection efficiency was about 28% in all the experiments (N=3, *p<0.05).*

shown). The cytotoxic effects were also accessed by clonogenic survival. Figure 5B demonstrates that clonogenic survival was decreased by 23.1% and 32.7% in 5-8F cells transfected, respectively, with pZ and pFR-Z. GCV further increased the cell killing by 63.2 % and 71.2%, respectively for pZ and pFR-Z transfectants. Control vector and GCV treatment alone did not induce any effect on clonogenic survival. No obvious cytotoxicity was detected in EBV-negative HNE3 cells (data not shown).

Discussion

The consistent presence of the EBV genome in certain tumor types suggests a novel therapeutic strategy for the treatment of EBV-positive carcinomas. Based on the idea of converting the latent (transforming) form of EBV infection into the lytic (cytotoxic) form that induces lytic cytotoxicity, we report here for the first time that FR directing BZLF1 expression causes cell death in EBV-positive NPC cells.

BZLF1 is an EBV immediate early protein that binds to the EBV gene promoter Zta response element (ZRE) and induces the gene expression for virus replication. Some strategy has been developed to use BZLF1 to treat EBV-positive carcinomas. CMV-BZLF1 is shown to efficiently induce EBV lytic replication and cause cell death in EBV-positive carcinoma (10, 11, 13-16). However, a major limitation of these approaches is related to their potential transformation effects by infection of BZLF1 and the possible side-effect of promoting metastasis (34). In the present report, we used FR in control of BZLF1 that specifically enhanced BZLF1 expression in EBV-positive 5-8F cells and, in contrast, lowered the expression of BZLF1 in EBV-negative HNE3 cells. These results, therefore, suggest a novel gene therapy approach by targeting BZLF1 in EBV-positive carcinomas.

EBV exists in many EBV-associated carcinoma cells in a state of latent infection. EBV-encoded EBNA1 is essential for the maintenance of the EBV genome in its episomal state. Binding of EBNA1 to FR sequences in the EBV genome enhances the transcriptional activity of downstream genes. This property of EBV infection has been extensively studied for therapeutic applications by Judde *et al.* (33). They put the FR sequence upstream of the TK promoter, which resulted in a 5 to 38-fold increase in luciferase activity over that programmed by the TK promoter alone in the EBV-containing cell lines. Interestingly, in all the EBV-negative cell lines tested, FR repressed the TK promoter by 12 to 28-fold. In the present report, we found a limited induction of luciferase activity induced in EBV-positive 5-8F cells ($\sim$2-fold; Figure 2). However, a 30-fold decrease in FR-mediated gene transcription was induced in EBV-negative HNE3 cells (Figure 2), suggesting a lesser side-effect in EBV-negative normal tissues if applied as a gene therapy vector.

We also found that all the three EBV proteins expressed in immediate early, early and late phase of EBV replication, namely BZLF1, EA-D and gp125, showed a basal expression in EBV-positive 5-8F cells (Figure 4). Significant increases of such virus proteins were induced by pFR-Z

transfection, indicating that FR efficiently activates BZLF1-mediated transcription. Gardella gel further demonstrates that transfection of pFR-Z induced EBV-positive cell death through the lytic replication of EBV. The transfection efficiency of both pZ and pFR-Z vectors was about 28% (data not shown), which is in agreement with the decreased cell viability detected by MTT and clonogenic assay in 5-8F cells (about 30%; Figure 5). Furthermore, cytotoxicity was increased by 80% when GCV was added, indicating a synergistic cytotoxicity with anti-virus agent. Since GCV functions only in the lytic form of EBV infection, it suggests a lytic virus replication in pFR-Z transfectants. Although the specific EBV proteins that phosphorylate GCV remain uncertain, it is likely that either the EBV TK protein (35,36) or the EBV homologue (BGLF4) or cytomegalovirus protein UL97 (37) phosphorylates GCV during lytic EBV infection. It should be mentioned that, in addition to expressing BZLF1, the current system may be further developed to express other molecules specifically in EBV-containing cells. Such molecules include cytokines or antigenic epitopes capable of making the tumor cells more susceptible to immunological surveillance. These should be studied in the future.

In conclusion, this study used a gene therapy delivery system of EBV FR domain to control EBV IE gene BZLF1 to test its anticancer potential in EBV-positive carcinoma cells. Two EBV-specific elements were applied in this system, *i.e.*, FR sequence that induced transgene expression in EBNA1-expressing cells and BZLF1 that induces cell death *via* lytic replication of EBV in EBV-positive carcinoma cells. Therefore, this specific tumor vector represents a double specific strategy for the treatment of EBV-positive carcinomas.

Acknowledgements

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