Determination of the temporal pattern and importance of BALF1 expression in Epstein-Barr viral infection

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7.340 Research Proposal

Introduction

Apoptosis, or programmed cell death, is a vital process that helps maintain homeostasis in normal multicellular organisms. This process eliminates cells that have been produced in excess, developed improperly, or sustained DNA damage (4). The process can be induced by many different signals, but the final cell death pathway is conserved even between organisms as diverse as humans and nematodes (4). In contrast to necrotic cell death, apoptosis is a carefully controlled process that does not result in an inflammatory response. An apoptotic cell undergoes cytoskeletal disruption, activation of cytoplasmic proteases, cell shrinkage, degradation of nuclear DNA, and membrane blebbing and is eventually engulfed by phagocytic cells (4). One important regulator of the apoptotic pathway is BCL-2, a protein that inhibits apoptosis by preventing the activation of the caspases that are the direct effectors of cell death. BCL-2 is frequently expressed in cancer cells and is associated with a poor prognosis; inhibition of BCL-2 in such cells can often induce apoptosis (4).

Apoptosis frequently occurs in virally-infected cells, disrupting the viral life cycle and depriving the viral particles of their host. However, a number of effective pathogens like African swine fever virus, adenoviruses, and gammaherpesviruses such as Epstein-Barr virus have developed the ability to inhibit apoptosis in their host cells (3, 4). Many such viruses express genes that are highly homologous to bcl-2, known as the v-bcl-2 families (3). Along with other mechanisms for evading the host immune response, this inhibition of apoptosis allows the viruses to establish persistent infections.

Epstein-Barr virus, or EBV, is a ubiquitous pathogen that infects the majority of the world's adult population. While the infection frequently produces no clinical symptoms, primary infection in adolescents or adults can cause mononucleosis, and long-term EBV infections have

been associated with cancers such as Burkitt lymphoma and Hodgkin's disease (1). Recently research has revealed two v-bcl-2 genes in the EBV genome, BHRF1 and BALF1. BHRF1 suppresses apoptosis, is expressed primarily during lytic infection, and is not necessary for lymphocyte transformation; it appears to function primarily to prolong the lifespan of cells undergoing viral replication (3). BALF1 has also been shown to suppress apoptosis and to associate with two cellular pro-apoptotic proteins, Bax and Bak (3). The timing of BALF1 expression during infection and the relative importance of BALF1 and BHRF1 are unknown.

Specific Aims

1. We will determine whether BHRF1 and BALF1, the EBV homologs of *bcl-2*, are expressed at different times during viral infection. The temporal pattern of expression for each gene will be analyzed by both RT-PCR and immunofluorescence assays.

2. We will determine whether BALF1 is necessary to prevent apoptosis in EBV-infected cells. Cells will be infected with either wild-type EBV or a BALF1-knockout strain, and the degree of apoptosis in each case examined by flow cytometric analysis of subdiploid nuclei.

Experiments and Expected Results

To ascertain the temporal patterns of expression for BHRF1 and BALF1 during EBV infection, Akata cells (B-cell lymphoma line) will be infected with wild-type EBV and levels of gene expression analyzed by RT-PCR and immunofluorescence. Expression will be assayed at a variety of timepoints ranging from 0 to 120 hours post-infection; this series of assays should be broad enough to cover the initiation and termination of expression of the two genes, but may be extended if necessary to give a more complete picture of the temporal expression patterns. For

RT-PCR analysis of transcriptional expression, we will isolate total RNA from wtEBV-infected cells at each timepoint using the RNeasy Mini Kit (Qiagen). RT-PCR's will be performed using the SuperScript RT-PCR System (Life Technologies) and primers for BHRF1, BALF1, and GAPDH (or another constitutively expressed gene) as a control. The RT-PCR products for each timepoint will then be analyzed by gel electrophoresis. To analyze the viral protein (translational) expression patterns by immunofluorescence, uninfected and wtEBV-infected cells will be treated with fluorescence-conjugated antibodies to BALF1 and BHRF1 at a range of timepoints after infection. The anti-BALF1 antibody will be conjugated to fluorescein, and the anti-BHRF1 antibody to rhodamine, so that the presence of the two viral proteins can be distinguished when treated cells are examined by immunofluorescence microscopy.

We hypothesize that BHRF1 and BALF1 will be expressed at different times during EBV infection. Since both genes are both *bcl-2* homologs and function to inhibit apoptosis, it would be less advantageous for the virus to express them at the same time. We therefore expect to see non-coinciding (but possibly overlapping) temporal patterns of expression for the two genes (Figure 1). BHRF1 is known to be expressed during lytic infection; during this time, we expect to see BHRF1 bands on the RT-PCR gel and rhodamine fluorescence under the microscope. BALF1 expression is likely to occur either before or after BHRF1 expression (or both); this is when we expect to see BALF1 bands on the gel and fluorescein fluorescence. If BALF1 and BHRF1 are expressed at the same time, we expect to see timepoints at which both RT-PCR bands are present and both red and green fluorescence are visible in infected cells. A final possibility is that expression (for example, of BALF1) is regulated post-transcriptionally; if this is the case, we expect to see the BALF1 band from the RT-PCR but not the corresponding green fluorescence under the microscope.

To determine whether BALF1 is necessary for the prevention of apoptosis in EBV-infected cells, we will assay the degree of apoptosis in cells infected with either wild-type EBV or BALF1-knockout EBV. The knockout strain will be constructed by treating wtEBV with appropriate restriction enzymes to remove the region containing the BALF1 gene. The restriction fragments can be separated by gel electrophoresis, and the non-BALF1-containing fragments can be purified and ligated together (Figure 2). The reconstructed virus could then be sequenced to insure that BALF1 has been disrupted, and that the rest of the genome is intact. Akata cells will then be infected with either wtEBV or the BALF1 knockout strain (b-EBV). Uninfected Akata cells will serve as negative controls. In two separate trials, each of the three cell groups will be exposed either to anti-Fas/IFNγ (control: IFN alone) or to camptothecin (control: DMSO alone); the treatments both induce apoptosis, but by different pathways. The degree of apoptosis for each trial will be assayed by flow cytometric analysis for subdiploid nuclei. The flow cytometric analysis will count the number of cells that have less DNA than normal cells; it is therefore a measure of DNA fragmentation, one indicator of apoptosis.

For both the anti-Fas and camptothecin experiments, we expect to see a high degree of apoptosis in uninfected cells, somewhat less in b-EBV-infected cells, and much less in wtEBV-infected cells (Figure 3). Although BALF1 is suspected to be important in preventing apoptosis during EBV infection, EBV has other genes that also appear to interfere with the apoptotic machinery, such as BHRF1 and LMP-1. It is therefore unlikely that the BALF1 knockout will be as susceptible to apoptosis as uninfected cells. If the b-EBV-infected cells were as susceptible as uninfected cells, that could indicate that BALF1 is very important for preventing apoptosis; perhaps it is a common downstream target for several pathways that interfere with the process. Alternatively, it could indicate that BALF1 is the only viral apoptotic inhibitor that functions

during the time apoptosis was induced; perhaps BHRF1 and LMP-1 were not being expressed when the cells were exposed to the apoptosis-inducing factors. On the other hand, if the b-EBV-infected cells were as resistant to apoptosis as the wtEBV-infected cells, that would indicate that BALF1 was not an important inhibitor of apoptosis at the time the cells were exposed to apoptosis-inducing factors. Data from the previous experiments concerning the temporal expression patterns could suggest when the BALF1 knockout strain would be most resistant to apoptosis; when BALF1 protein expression is high, we would expect the knockout to be sensitive to apoptosis. When BALF1 expression is low, apoptosis-resistance is more likely due to other factors such as BHRF1, so the BALF1 knockout would not be more sensitive to apoptosis at these times.

Discussion and Conclusions

We have proposed a series of experiments to further elucidate the function of BALF1, a *bcl-2* homolog, in inhibiting apoptosis during EBV infection. First, we will determine the temporal expression patterns of BALF1 and BHRF1 by (1) examining mRNA expression by RT-PCR and (2) examining protein expression by immunofluorescence microscopy. Since these genes both appear to disrupt the apoptotic pathway, we hypothesize that they will be expressed at different times during the viral life cycle. Next, we will investigate the importance of BALF1 in preventing apoptosis by comparing levels of apoptosis in cells infected with wild-type EBV or BALF1-knockout EBV. In two different experiments, infected cells will be treated with anti-Fas/IFNγ or with camptothecin to induce apoptosis. We hypothesize that the b-EBV-infected cells will be more resistant to apoptosis than uninfected cells, but less resistant than wtEBV-infected cells, because EBV expresses other genes that also interfere with the apoptotic pathway.

Future experiments might further investigate the importance of BALF1 in resisting apoptosis. The importance of this particular gene could vary as a function of time after infection with EBV. For example, if our analysis of temporal expression patterns shows that BALF1 is expressed immediately after EBV infection and BHRF1 expression begins six hours later, then BALF1 will probably be instrumental in preventing apoptosis in recently-infected cells but not after longer infection. Exposing wtEBV-infected cells and b-EBV-infected cells to apoptosis-inducing factors at various timepoints after infection and assaying for apoptosis could be informative. Futhermore, BHRF1-knockout strains of EBV could be constructed and subjected to the same analysis to determine its relative importance in preventing apoptosis. Finally, a double-knockout strain lacking both BALF1 and BHRF1 could be analyzed. Investigation of apoptosis resistance in several EBV mutant strains at different times after infection would help elucidate the relative importance of various possible genes and their "windows" of functionality.

EBV is a ubiquitous pathogen that persistently infects most of the world's adults. If the primary infection occurs late in life, it can cause mononucleosis; long-term infections have been associated with many types of cancer, such as Burkitt lymphoma and Hodgkin's disease (1). EBV's success as a pathogen is partly due to its ability to inhibit apoptosis in the cells it infects, and this inhibition is partly dependent on the functions of BALF1 and BHRF1. Many other viruses, such as gammaherpesviruses, adenoviruses, and African swine fever virus have similar capabilities (3, 4). A better understanding of the processes that allow these viruses to inhibit apoptosis and establish persistent infections may allow us to generate vaccines containing attenuated viruses that cannot establish latent infections (4).

References

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Figures

Figure 1: Sample expected results for RT-PCR analysis of BHRF1 and BALF1 expression. Rectangles represent bands of ethidium bromide staining after gel electrophoresis. RT-PCR will be performed using primers for BALF1, BHRF1, and GAPDH at various times after infection of Akata cells with wild-type Epstein-Barr virus.

<u>**RT-PCR**</u>   <u>**Hours Post-Infection**</u>

	0 hours	2 hours	8 hours	16 hours	36 hours
BALF1		▇	▇	▇	
BHRF1			▇	▇	▇
GAPDH	▇	▇	▇	▇	▇
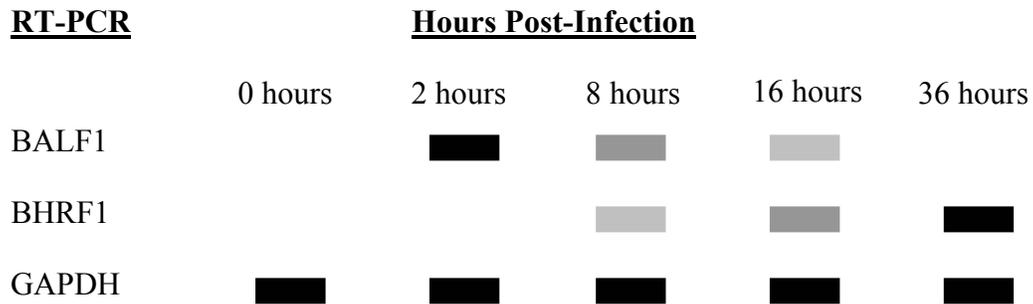
Figure 2: Diagram of BALF1 knockout strain of EBV.
A BALF1-knockout strain of EBV will be constructed by digesting the wild-type viral genome with appropriate restriction enzymes and ligating the non-BALF1-containing fragments back together.

Restriction Sites

wild-type EBV genome

BALF1

Fragment A   0.7 kb   Fragment B

Restriction Digest and Ligation

Ligation Site

BALF1-KO EBV genome

Fragment A   Fragment B
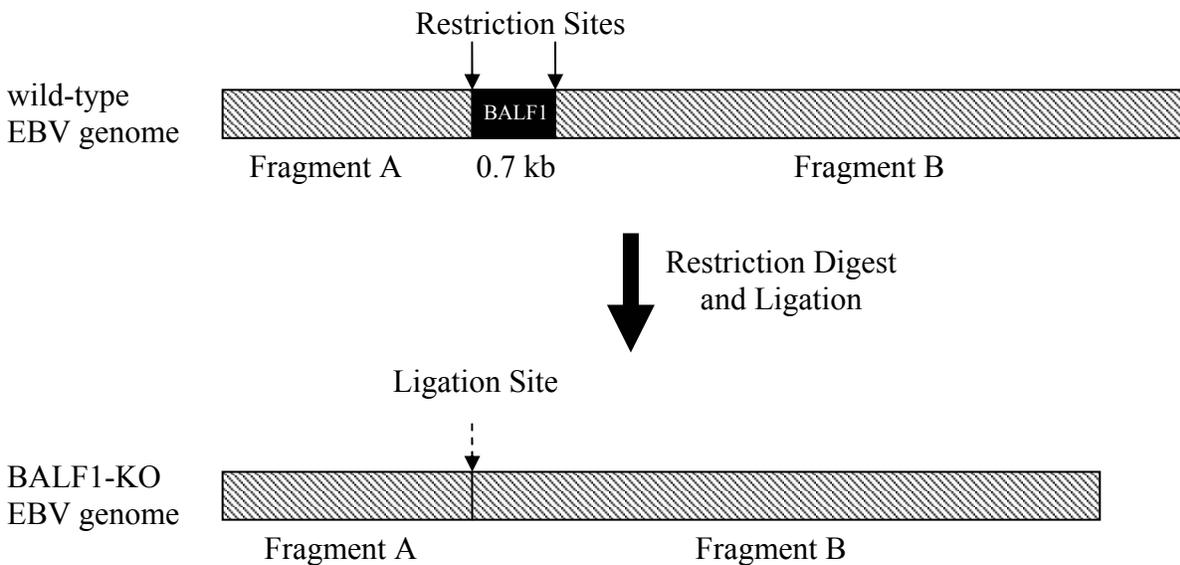
Figure 3: Sample expected results for apoptosis resistance assay.

Three types of cells (uninfected, wild-type EBV-infected, and BALF1-knockout EBV-infected) will be treated with camptothecin to induce apoptosis. The percentage apoptotic nuclei will be determined by flow cytometric analysis for subdiploid nuclei. Similar results are expected when cells are treated with anti-Fas/IFNγ instead of camptothecin.