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Epstein-Barr Virus BHRF1 Protein Protects Intestine 407 Epithelial Cells from Apoptosis Induced by Tumor Necrosis Factor Alpha and Anti-Fas Antibody

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Tumor necrosis factor (TNF) and cytotoxic T lymphocytes, which utilize Fas to induce apoptosis in target cells, are known to play a critical role in the host defense against viral infection. In this study, the Epstein-Barr virus BHRF1 protein was stably expressed in intestine 407 cells which were susceptible to cell death mediated through both the TNF receptor and Fas. WST-1 conversion assays and acridine orange staining showed that vector-transfected control cells were killed by TNF-α or anti-Fas antibody in a dose-dependent manner, whereas BHRF1-expressing cells were resistant to apoptosis induced by these mediators. DNA fragmentation, a characteristic of apoptosis induced by TNF-α and the anti-Fas antibody, was suppressed in BHRF1-expressing cells. These results indicate that the BHRF1 protein protects cells from apoptosis mediated by the TNF receptor and Fas. The role of BHRF1 as an inhibitor of cytokine-induced apoptosis during the Epstein-Barr virus lytic cycle in vivo is discussed.

The Epstein-Barr virus (EBV) BHRF1 protein, which is encoded in the EBV *Bam*HI fragment H rightward open reading frame 1 (1, 17), shows partial sequence homology to Bcl-2, a potential human oncogene activated in human follicular lymphomas (2). Although abundant expression of the BHRF1 mRNA and protein appears early after lytic induction (16), the role of the BHRF1 protein in the viral life cycle remains to be determined. Several studies with the recombinant virus have shown that the BHRF1 protein is not required in vitro for viral replication or virus-induced cell transformation (12, 13). However, the BHRF1 gene is present in all natural virus isolates examined to date, suggesting that its protein product plays an important role in the viral life cycle in vivo. Recently, several reports have shown that the BHRF1 protein can suppress apoptosis induced by exogenous stimuli, including DNA-damaging agents, heterologous viral infection, serum depletion, and the calcium ionophor ionomycin (6, 19).

The tumor necrosis factor (TNF) receptor and Fas (APO-1) play important roles in physiological forms of cell death. These mediators have been shown to induce cell death either by activation their respective ligands or by cross-linking with agonist antibody (8, 15, 20, 21, 23). Programmed cell death triggered by these receptors is especially relevant to the viral life cycle, since both receptors have been implicated in viral clearance. TNF alpha (TNF-α) itself is a potent antiviral agent (22). Cytotoxic T cells, which comprise a major component of the antiviral defense system, utilize Fas to induce apoptosis in target cells (5, 9, 10, 18). To investigate whether the BHRF1 protein can modulate cytokine-induced apoptosis, we expressed the gene for BHRF1 in the intestine 407 cell line, which is susceptible to cell death mediated through both the TNF receptor and Fas.

The BHRF1-expressing vector pRcCMV-BHRF1 was constructed by B. Tarodi et al. (19) and contains the cytomegalovirus (CMV) IE promoter in front of the BHRF1-encoding sequence (residues 1 to 191) fused in frame with the nine-amino-acid epitope of the influenza virus hemoagglutinin (HA) gene (4). Intestine 407 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 6% (vol/vol) new born calf serum (CS). Detached cells were suspended in phosphate-buffered saline–0.3 M mannitol (1:1) at a density of 5×10^6 cells per ml. Plasmids were introduced into the cells by electroporation by using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) at 100 V/cm and 25 μF. Cells were seeded onto culture plates in Eagle's MEM containing 6% CS. The medium was replaced 48 h later by fresh MEM containing 6% CS and was supplemented with 1 mg of G418 per ml.

To determine expression of the BHRF1 protein, cells were solubilized in gel sample buffer (50 mM Tris buffer [pH 6.8], 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue), sonicated for 20 s, and then boiled for 5 min. Samples containing 0.5×10^6 cells in 20 μl were separated by discontinuous gel electrophoresis employing a 5% acrylamide stacking gel and a 13% acrylamide resolving gel and were then blotted onto polyvinylidene difluoride membranes. Excess protein-binding sites on the blotted membranes were blocked by incubation for 2 h in 29 mM Tris-buffered saline (TBS; pH 7.5) containing 5% dried skim milk (TBS-milk). The membranes were then incubated at 4°C overnight with the primary antibodies diluted in TBS-milk. The immunoblotted membranes were washed in TBS containing 0.1% Tween 20 before incubation for 2 h with the peroxidase-conjugated secondary antibodies in TBS-milk. After extensive washings in TBS-Tween 20, the filters were incubated in an ECL detection reagent (Amersham) and exposed to RP X-Omat film (Kodak). Monoclonal antibodies against HA (12 CA5) were obtained from Boehringer Mannheim.

To assay cytotoxicity, cells were plated in 96-well tissue culture plates at a density of 4×10^3 per well. Twenty-four hours later, the media were replaced with media containing either cycloheximide (CHX), TNF-α plus CHX, or anti-Fas antibody plus CHX. Each condition was repeated in quadruplicate. After 16 h of incubation, viability was determined by highly water-soluble formazan production from sulfonated tetrazolium salt (WST-1) assay (7). The absorbance at 450 nm (A_{450}) was determined for each well. After subtraction of the value of the

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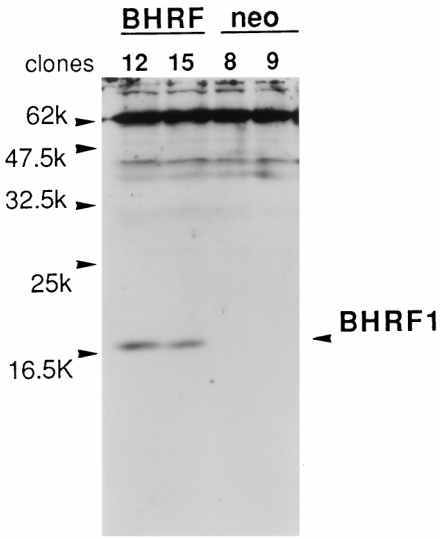

FIG. 1. Expression of the BHRF1 protein in subclones of BHRF1-transfected intestine 407 cells. Protein extracts from BHRF1 transfectants (BHRF-12 and BHRF-15) and control clones (neo-8 and neo-9) were immunoblotted and probed with monoclonal antibodies specific for HA.

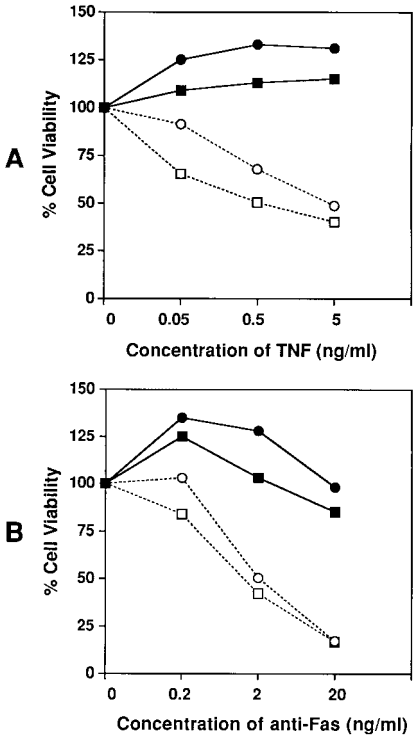

FIG. 2. Effects of TNF-α and the anti-Fas antibody on viability of BHRF1 and neo transfectants. BHRF1 transfectants (BHRF-12 and BHRF-15) and control clones (neo-5 and neo-8) were treated with increasing concentrations of TNF-α (A) or the anti-Fas antibody (B) for 16 h in the presence of 2-μg/ml CHX. Cell viability was assessed by the WST-1 assay. ■, BHRF-12; ●, BHRF-15; □, neo-5; ○, neo-8.

background absorbance from each measurement, the viability of each clone in response to treatment with TNF-α plus CHX or anti-Fas plus CHX was calculated by the following equation: (A_{450} TNF-α plus CHX or anti-Fas plus CHX)/(A_{450} CHX). Apoptotic cells were characterized by acridine orange staining and internucleosomal DNA fragmentation. The cells were stained with 2 μg of acridine orange per ml and were viewed immediately under a fluorescence microscope. The staining clearly distinguishes between viable cells and cells showing the condensed chromatine staining characteristic of apoptosis (14). DNA laddering was analyzed as described before (11).

Both TNF-α and the anti-Fas antibody can induce apoptosis in subcloned intestine 407 cells in the presence of CHX. To determine the effect of the EBV-encoded BHRF1 protein on TNF-α- and anti-Fas antibody-mediated apoptosis, intestine 407 epithelial cells were transfected with pRcCMV-BHRF1 and control vector pRcCMV. G418-resistant clones were isolated and were used for BHRF1 expression by immunoblotting. As shown in Fig. 1, a 17-kDa (approximate) BHRF1 protein was identified in BHRF-12 and -15 clones by immunoblotting with murine monoclonal antibodies against HA.

To determine whether the BHRF1 protein can block apoptosis induced by TNF-α or anti-Fas antibody, two BHRF1-expressing clones, BHRF-12 and BHRF-15, and three control clones, neo-5, neo-8, and neo-9, were employed. Cells were treated with various concentrations of TNF-α or anti-Fas antibody for 16 h in the presence of 2 μg of CHX per ml. Cell viability was determined by measuring the ratio of WST-1 conversion between treated (TNF-α plus CHX or anti-Fas antibody plus CHX) and control (CHX alone) cells. As shown in Fig. 2, the viability of control clones was reduced dose dependently by treatment with TNF-α or the anti-Fas antibody. In contrast, the viability of BHRF1-expressing clones determined by the WST-1 assay was slightly enhanced by TNF-α or the anti-Fas antibody. It is unlikely that this enhanced cell viability seen in the presence of TNF-α and the anti-Fas antibody is caused by an increase in cell numbers, because CHX was present throughout the assay. In fact, cell numbers determined by trypan blue exclusion were approximately the same between untreated cells and cells treated with TNF-α or anti-Fas antibody, and cell viability was greater than 95% (data not shown). Since WST-1 assaying is based on determination of dehydrogenase activities (7), lower concentrations of TNF-α or anti-Fas may slightly enhance the enzyme activities.

To determine whether cytotoxicity determined by WST-1 assay is due to apoptosis, BHRF-12 and neo-8 clones were treated with TNF-α and anti-Fas antibody for 16 h in the presence of CHX and were stained with acridine orange. As shown in Fig. 3, greater than 95% of BHRF-12 clone cells were viable after treatment with 5-ng/ml TNF-α or 100-ng/ml anti-Fas antibody. On the contrary, apoptotic cells were increased in number in a dose-dependent manner after treatment of neo-8 cells with TNF-α or the anti-Fas antibody.

In order to clarify the effect of the BHRF1 protein on DNA ladder formation (a characteristic of apoptosis induced by TNF-α or anti-Fas antibody), agarose gel electrophoresis of low-molecular-weight DNA fragments from BHRF-12 and neo-8 was performed. Cells were treated with TNF-α or anti-Fas antibody at the indicated concentrations for 6 h in the presence of 2-μg/ml CHX followed by cellular DNA isolation. As shown in Fig. 4, the formation of DNA laddering was observed in the neo-8 clone treated with TNF-α or the anti-Fas antibody. In contrast, much less fragmentation was seen in the BHRF-12 clone (Fig. 4). Similar results were obtained with other BHRF1-expressing clones. These results indicate that the BHRF1 protein can effectively protect intestine 407 cells from apoptotic cell death induced by TNF-α and the anti-Fas antibody.

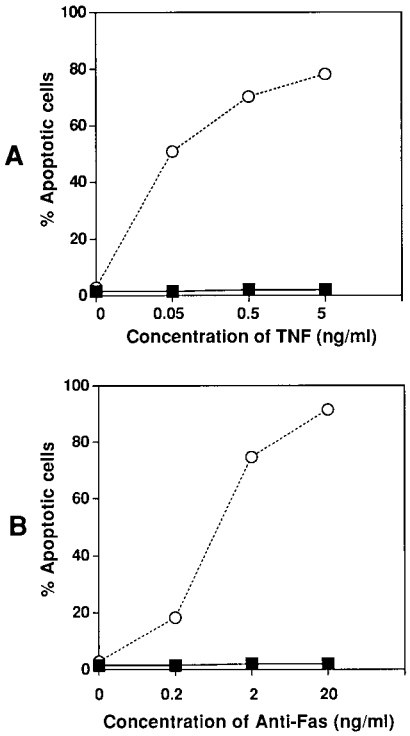

FIG. 3. Effects of BHRF1 on apoptosis mediated by TNF-α and the anti-Fas antibody. BHRF-12 and neo-8 clones were treated with increasing concentrations of TNF-α (A) or the anti-Fas antibody (B) for 16 h in the presence of 2-μg/ml CHX. Percentages of apoptotic cells were assayed by acridine orange staining. ■, BHRF-12; ○, neo-8.

TNF-α and the anti-Fas antibody induce apoptotic cell death via interaction with their respective receptors. The levels of two TNF receptors, TNFR1 and TNFR2, and Fas in the BHRF-expressing clones were found to be similar to those for the control clones, as determined by flow cytometric analysis (data not shown). Furthermore, there were no apparent differences in expression of apoptosis-related cellular genes such as Bcl-2, Bax, and c-Myc between BHRF1-expressing and control clones (data not shown). Therefore, it is apparent that BHRF1 itself blocks cell death triggered by the different receptors, TNF receptors and Fas.

Recent reports have shown that BHRF1 protects different cell types from various external stimuli, such as DNA-damaging agents, heterologous viral infection, serum depletion, or the calcium ionophore ionomycin (6, 19). The findings presented here demonstrate an important new function of the BHRF1 protein, namely, the inhibition of cytokine-induced apoptosis. TNF-α and the Fas ligand play important roles in the immune system (5, 9, 10, 18, 22). A major mechanism of viral clearance is immune system-mediated apoptosis. The BHRF1 protein is expressed early in the EBV replication cycle. Therefore, it can be speculated that BHRF1 protects infected cells from attack of TNF-α or cytotoxic T cells through an anti-apoptotic mechanism, thus allowing effective viral replication to occur in vivo. Recently, it has been reported that BHRF1 expression delays the terminal differentiation of epithelial cells through the prevention of apoptosis, resulting in the promotion of productive EBV infection (3). In epithelial cells, the BHRF1 protein may play an important role in EBV replication via its protection of infected cells from the host defense system, as well as from the final terminal differentiation.

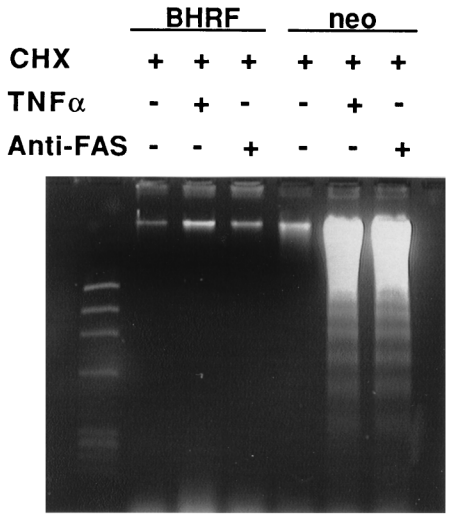

FIG. 4. Gel electrophoresis of low-molecular-weight DNA fragments extracted from control and BHRF1 transfectants treated with TNF-α or the anti-Fas antibody. BHRF-12 and neo-8 clones were treated with 5-ng/ml TNF-α or 15-ng/ml anti-Fas antibody for 6 h in the presence of 2-μg/ml CHX. The first lane shows the DNA size markers (*Hae*III-digested ϕX174 DNA).

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