EBV Infection of Human B Lymphocytes Leads to Down-Regulation of Bim Expression: Relationship to Resistance to Apoptosis

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EBV infects a large proportion of the human population worldwide and is one of the major viruses with human B lymphocyte tropism. It can immortalize human B lymphocytes and controls their resistance to apoptosis. EBV infection is associated with several lymphomas, including Burkitt’s lymphoma. In this report we show that EBV infection leads to the post-transcriptional down-regulation of expression of the proapoptotic protein Bim. This process involves the phosphorylation of BimEL by the constitutive EBV-activated kinase ERK1/2, followed by its degradation through the proteasome pathway. We also show that ectopic expression of BimEL in EBV-positive Burkitt’s lymphoma cells can enhance the sensitivity of these cells to serum deprivation-dependent apoptosis. Thus, EBV-mediated resistance to growth factor deprivation in human B lymphocytes is dependent on BimEL expression. Our data suggest that this regulatory pathway is an important contributor to the oncogenic potential of EBV.

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EBV belongs to the herpes virus family and infects >90% of the human population worldwide. It shows strong tropism for human B lymphocytes and weaker tropism for T cells and epithelial cells. EBV causes infectious mononucleosis, which is usually harmless. However, it can also be associated with the development of malignant lymphomas, including Burkitt’s lymphoma, Hodgkin’s lymphoma, post-transplant plantomas, some T cell lymphomas, and nasopharyngeal carcinomas (for review, see Refs. 1 and 2). In vitro infection of human B lymphocytes leads to the immortalization of these cells. Immortalization is characterized by rapid proliferation and resistance to various apoptotic stimuli, including growth factor deprivation (1, 2). The molecular basis of EBV-mediated resistance to apoptosis is still not totally understood, although such resistance appears to be associated with the expression of some viral proteins, such as latent membrane protein 2A (3) and the Bcl-2-related BamH1 rightward reading frame (BHRF1) (4).

Murine studies have shown that the BH3-only protein Bim, a member of the Bcl-2 family, plays a central role in the control of lymphocyte homeostasis. Indeed, T and B cells accumulate in Bim-deficient animals, and these animals have high levels of serum Ig and develop autoimmune diseases (5). In addition, Bim regulates the apoptosis of T cells in response to various cytotoxic stimuli, including cytokine withdrawal-induced apoptosis (5), and is essential for the elimination of autoreactive murine T cells (6) and B cells (7). Moreover, the loss of Bim favors the development of murine acute leukemia, and B cells from Bim-deficient Eμ-MyC mice can survive in the absence of cytokines in culture (8). Several groups, including ours (9, 10), have recently demonstrated that Bim is also involved in the regulation of human B lymphocyte apoptosis. This led us to investigate the role of Bim during the resistance to apoptosis promoted by EBV infection in human B lymphocytes.

Materials and Methods

Reagents

The murine DA44 Ab (anti-human IgM, IgG1) was obtained from hybridoma cell lines (American Type Culture Collection) and was purified from ascitic fluids on protein A-Sepharose columns (Pharmacia Biotech). Recombinant human TRAIL was obtained from R&D Systems. MG262, U0126, and PD98059 were obtained from Calbiochem.

Cells

Normal B cells were isolated from human tonsils. Single-cell suspensions were depleted of T cells by two cycles of rosetting with 2-aminoethylisothiouronium-treated SRBC and were depleted of monocytes by adhesion to plastic. B cells were then separated into density fractions on discontinuous Percoll gradients (Pharmacia Biotech). The Burkitt’s lymphoma BL41 cell line was provided by Drs. A. Calender and G. Lenoir (Centre International de Recherche sur le Cancer, Lyon, France). The B95.8 and Ramos, Raji, P3HR1, and Jiyoye Burkitt’s cell lines were obtained from the European Collection of Cell Cultures. EBV-positive cells were generated from these cell lines or B cells by in vitro infection with the EBV-encoded nuclear Ag-2 (EBNA-2)-deficient P3HR1 strain (BL41/P3HR1) or the wild-type B95.8 strain (BL41/B95.8 and lymphoblastoid cells) as previously described (11). Resting tonsillar B cells were infected in vitro with B98.8 supernatant and incubated for 5 wk (with the medium changed three times per week) until the appearance of growing, lymphoblastoid, EBNA-1-positive cells. We then analyzed Bim levels in uninfected and EBV-infected cells from the same donor (Fig. 1D). All cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 1-glutamine (2 mM),

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3 C.C. and B.M. contributed equally to this paper.

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5 Abbreviation used in this paper: BHRF, BamH1 rightward reading frame; EBNA-2, EBV-encoded nuclear Ag-2.
RT-PCR, real-time quantitative PCR, and transfection

Total cellular RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. For real-time quantitative PCR, RNA was treated with RNase-free DNase I (Qiagen).

RT-PCR

The first-strand cDNA was generated by RT of 5 μg of total RNA using oligo(dT) nucleotides. PCR was performed with the primers 5′-TGATG TAAAGTTCTGACTGTTG-3′ and 5′-ACGTAAGATAGCTGTTAGAT-3′ for Bim, 5′-TTAGAGACGTTTCTTGAGC –3′ and 5′-CATTTCCCAG GTCCGTGATCCT-3′ for EBNA-1, and 5′-GTTGAAAGTGCGAGTGCAA CGGA-3′ and 5′-GAGGGAGTCTGGCTCCTGAGAA-3′ for GAPDH. PCR products were separated by electrophoresis in 1–2% agarose gels and were visualized by ethidium bromide staining.

Real-time PCR

We diluted the cDNAs 1/10 for use as templates for real-time PCR analysis. PCR was conducted on triplicate cDNA samples, using the Quantitect SYBR Green PCR kit (Qiagen) and sequence-specific primers for human BimEL (forward, 5′-ATCCCCGCTTTTCATCTTTA-3′; reverse, 5′-AGGACTTGGGGTTTGTTTTG-3′).

We normalized BimEL expression in each sample with respect to mRNA for the housekeeping gene β-actin, the levels of which were determined using the following primers: forward, 5′-AGTGTCGTTACAC CTGTCCTTT-3′; and reverse, 5′-CACCCTTACCGCTTACGTT-3′. All data collected were subjected to efficiency correction (e) for each amplification:

\[ e = 10^{-1/slope} \]

The normalized ratio of calibrators (equimolar pools for each sample) and of each sample was determined as follows:

\[ \log_{10} \frac{C_{\text{samples}}}{C_{\text{calibrator}}} = \log_{10} \left( \frac{C_{\text{calibrator, BimEL}}}{C_{\text{calibrator, β-actin}}} \right) \left( \frac{C_{\text{β-actin}}}{C_{\text{β-actin, BimEL}}} \right) \]

Transfection
cDNAs corresponding to the BimEL, -L, and -S isoforms were isolated and inserted into a pcDNA6b His-Myc plasmid (Invitrogen Life Technologies). Ramos-AW cells were transfected with a pcDNA6 vector (Invitrogen Life Technologies) containing the BimEL cDNA by electroporation at 0.24 mV and 960 μF using a Bio-Rad apparatus. All constructs were checked by sequencing. Stable transfectants were selected by incubating the cells with 30 μg/ml blasticidin (Invitrogen Life Technologies) for ~3 wk. Stable clonal transfectants were isolated from blasticidin-resistant cells using the limiting dilution technique. BimEL levels in the various clones were analyzed by Western blotting with the anti-c-Myc 9E10 mAb (Sigma-Aldrich).

Western blotting

Cells were lysed in 20 mM Tris (pH 7.4) and 0.5% SDS in the presence of 10 U of recombinant TRAIL (200 ng/ml) or 48 h in complete medium in the presence of anti-μ mAb (DA44; 10 μg/ml) or recombinant TRAIL (200 ng/ml).

Detection of apoptotic cells

Cells were cultured for 96 h in culture medium with no FCS or for 48 h in complete medium in the presence of anti-μ mAb (DA44; 10 μg/ml) or recombinant TRAIL (200 ng/ml).

Analysis of dot-blot light scatter profiles.

Cells (10⁶) were washed in PBS and resuspended in 1 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodide in 1 ml of 50% ethanol) for 30 min. Aliquots of the supernatants were used for protein determination (microBCA protein assay; Pierce). Cells lysates were subjected to SAGE-PAGE, and the proteins were then electrophoretically transferred onto nitrocellulose filters. The filters were probed with anti-Bim (Stressgen and Santa-Cruz Biotechnology); anti-Bad (Stressgen and Sigma-Aldrich); anti-Bcl-2 (Stressgen and Santa-Cruz Biotechnology); and anti-Puma (Stressgen and Sigma-Aldrich). Abs binding was detected by incubation with sheep anti-mouse or anti-rabbit IgG HRP-conjugated Abs and chemiluminescence (West-pico or West femto; Pierce). Images were captured using a DDC camera (LAS-1000; Fuji).

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flow cytometric analysis of the propidium iodide fluorescence of individual nuclei using a FACScan flow cytometer (BD Biosciences). Debris was excluded from analysis by raising the forward scatter threshold. The DNA content of the intact nuclei was registered on a logarithmic scale. Apoptotic cells were identified on the basis of their nuclei having hypodiploid DNA, emitting fluorescence in channels 10–200. These cells were counted, and their number was expressed as a percentage of the total population.

Results

We studied the interaction between Bim and EBV by analyzing Bim production in various B cell populations, including EBV-negative normal tonsillar B cells, EBV-positive lymphoblastoid cells (Fig. 1A), and various EBV-negative and EBV-positive Burkitt’s lymphoma cell lines (Fig. 1B). Our initial results suggested variable, but significant, levels of Bim expression in EBV-negative tonsillar cells and Burkitt’s lymphoma cell lines (Fig. 1) and in resting peripheral B cells (data not shown). The most abundant isoform of Bim observed was BimEL (Fig. 1, lanes 1, 4, 5, 8, and 10). In contrast, Bim was expressed in only very small amounts in all EBV-positive B cells analyzed (lymphoblastoid cells (Fig. 1, lanes 2, 3, 6, and 7) and EBV-positive Burkitt’s cell lines (Fig. 1, lanes 9, 11, and 12)). No change in the level of Bim expression over time was observed in these established cell lines (data not shown). We investigated whether the lower level of BimEL in EBV-positive cells was due to the presence of the EBV genome by studying two EBV-negative Burkitt’s lymphoma cell lines (BL41 and Ramos) before and after in vitro infection with different strains of EBV, including BL41/P3HR1, BL41/B95.8, and Ramos-AW (Fig. 1C) and normal B cells before and after the establishment of lymphoblastoid cells resulting from in vitro infection with B98.8 supernatants (Fig. 1D). EBV infection led to a drastic decrease in the amount of BimEL in all these cells (Fig. 1, C and D), even after infection with EBNA-2-deficient P3HR1 supernatants (BL41/P3HR1 cells in Fig. 1C). In contrast, EBV infection did not decrease the levels of other BH3-only proteins, including Bid, Bad, and Puma. In addition, EBV infection did not modify Bax expression in these cells, whereas it moderately increased the expression of Bcl-xL and Bcl-2 (see BL41/B95.8 vs BL41). No such effect of EBV infection was observed in BL41/P3HR1 cells, suggesting that the expression of these proteins may be more sensitive to EBNA2. Our data are consistent, at least in the case of Bcl-2, with previously reported observations (13).

Because Bim expression can be regulated at either the transcriptional (14–17) or post-transcriptional (9, 18–23) level, we investigated a possible mode of action by which EBV infection might regulate BimEL expression (Fig. 1C). Because we and others have previously reported observations (13).

Because constitutive ERK1/2 activation is associated with EBV infection (25, 26), we next checked the presence of active, phosphorylated ERK1/2 in Burkitt’s lymphoma cells positive or negative for EBV. Constitutive activation of ERK2 was observed in BL41/B95.8 and Ramos-AW cells (EBV positive), but not in the EBV-negative cell lines, BL41 and Ramos (Fig. 2B). We then checked whether the activation of ERK was correlated with the low level of BimEL observed in EBV-positive cells. We found that treatment of EBV-positive cells (Ramos-AW (Fig. 2C) or BL41/B95.8 (Fig. 2D)) with the specific inhibitor of the ERK pathway, PD98059, led to a significant increase in the level of BimEL observed after 24 or 48 h of treatment. Similar results were obtained with another inhibitor of the ERK1/2 pathway, UO126, whereas the SB203580 inhibitor specific for p38 had no effect (data not shown). Accordingly, the levels of mRNAs corresponding to BimEL and to the shorter isoforms, L and S (27, 28), were similar in EBV-negative (Fig. 3A, lane 1; Ramos) and EBV-positive cells (Fig. 3A, lane 2; Ramos-AW). Real-time PCR also confirmed that the lower levels of Bim proteins observed in EBV-positive Ramos-AW cells (Fig. 3B, lane 2) than in EBV-negative Ramos cells (lane 1) were not due to transcriptional regulation. In the course of this study we also observed that EBV-positive Burkitt’s Raji cells expressed significantly more Bim protein than Ramos-AW (Fig. 3B) or BL41/B95.8 (data not shown); they also produced more Bim mRNA (Fig. 3B). Addition of the inhibitor PD98059 significantly increased the expression of BimEL protein levels in Raji cells (Fig. 3C), consistent with our observation that post-transcriptional mechanisms (based on ERK-mediated phosphorylation and proteasome degradation) controlled the levels of BimEL protein in EBV-infected human B cells.
We assessed the biological relevance of this down-regulation of BimEL by producing it ectopically in EBV-positive Ramos-AW cells. All the isolated clones produced moderately, but consistently, larger amounts of BimEL protein (BimEL/a in Fig. 4A) than their EBV-negative counterpart, Ramos (BimEL/b in Fig. 4A). The moderate levels of BimEL (no >20% of the total amount of BimEL protein in uninfected Ramos control cells, as suggested by Western blot scans) appear to be due to threshold levels of production that are tolerated by host cells without leading to cell death. Indeed, the overproduction of BimEL in transient transfection assays (data not shown) (27) resulted in significant levels of cell death. We analyzed the influence of BimEL in EBV−/Bim− (Ramos), EBV+/Bim− (Ramos-AW), and EBV+/Bim+ (clones A37 and A53) cells in response to three different stimuli that can promote apoptosis in Ramos cells, including growth factor deprivation (−FCS), BCR-mediated activation with soluble anti-μ Ab (anti-μ), and activation with recombinant TRAIL (Fig. 4B). EBV infection rendered Ramos cells (Ramos-AW) resistant to apoptosis after serum deprivation and treatment with anti-μ Ab, whereas these cells remained sensitive to TRAIL-induced apoptosis. BimEL production (clones A37 and A53) did not restore sensitivity to anti-μ Ab-induced apoptosis (Fig. 4E) even if the time of exposure to various concentrations of anti-μ Ab was modified (data not shown). In contrast, the production of BimEL in Ramos-AW cells restored the sensitivity of these cells to apoptosis triggered by serum deprivation (Fig. 4D), which was maximal after 72–96 h of culture (Fig. 4C), in a dose-dependent manner (Fig. 4D). The apoptotic cell death observed in these conditions was associated with chromatin modifications, because the number of apoptotic events determined by dot-blot, light-scatter profile analysis was correlated with the results of the analysis of sub-G₀ nuclei (Fig. 4E). These data suggest that EBV-mediated resistance to growth factor deprivation is directly dependent on BimEL.

Discussion
EBV infection leads to the immortalization of B lymphocytes, which is associated with an increase in the resistance of transformed cells to various apoptotic stimuli, including growth factor deprivation and BCR-mediated apoptosis. It was previously reported that the resistance to apoptosis promoted by EBV infection was associated with the expression of a viral homologue of Bcl-2, such as BHRF1, and the up-regulation of antiapoptotic members of the Bcl-2 family, including Bcl-x₁ and Bcl-2 (4, 13). Indeed, we also observed that EBV infection could lead to higher Bcl-2 levels in certain Burkitt’s lymphoma cell lines (BL41), but not in others (Ramos). These data suggest that the up-regulation of Bcl-2 and Bcl-x₁ may depend on the cell line analyzed, rather than being generally promoted by EBV infection, at least in Burkitt’s lymphomas. Studies in mice have highlighted the key role of another protein of the Bcl-2 family, the BH3-only Bim, in the control of B lymphocyte homeostasis. Our data show that EBV infection may be associated with the preferential down-regulation of Bim production in various types of human B lymphocytes, including normal and lymphoma cells (Burkitt’s). These cells spontaneously produce significant amounts of the BimEL isoform, the precise location of which has not yet been precisely determined, but the inactivation of which is not dependent on its association with tubulin, as observed in epithelial cells (10, 18, 29). EBV infection led to a drastic drop in the levels of BimEL in EBV-negative cells, whereas the levels of other BH3-only proteins, including Bid, Bad, and Puma, were not modified. Interestingly, it has been reported that Chlamydia infection significantly alters the level of Bad, Puma, and Bim (24), suggesting that different infectious agents may have different modes of action (Chlamydia) (24). Chlamydia regulated the patterns of these different BH3-only proteins, including Bim, through a more general BH3-dependent regulatory pathway, whereas the EBV-mediated regulation of BimEL was based on post-transcriptional mechanisms.

The BimEL and L isoforms are phosphorylated by various kinases, including the MAPK serine/threonine kinases JNK and ERK (9, 10, 15, 19, 20, 22, 23, 30). Phosphorylation by these kinases may control either the level of sequestration of Bim by tubulin, as shown for JNK (20), or the proteasome-dependent degradation of the BimEL isoform by ERK (9, 10, 22). Indeed, it has been reported that among the various possible residue targets for ERK, serine 69, which is phosphorylated only in the BimEL isoform, is essential for the regulation of BimEL during its degradation by the proteasome pathway. Our data are consistent with these observations and show that the control of Bim production by EBV infection is directly related to the ability of EBV to promote the constitutive activation of ERK. Therefore, the specific inhibition of ERK activation and of the proteasome pathway may restore significant levels of BimEL in EBV-positive cells (Fig. 5). Similar control of BimEL expression was observed in the EBV-negative BL41 and Ramos cells after extensive BCR cross-linking, which also induced prolonged activation of ERK (data not shown), and therefore seems to be a general pathway for BimEL regulation in human B lymphocytes.

In addition to the direct regulation of B cell homeostasis, our data suggest that Bim is involved in some modification of sensitivity to apoptosis promoted by EBV infection. EBV infection rendered Ramos cells (Ramos-AW) resistant to apoptosis after serum deprivation and treatment with soluble anti-μ Ab. The ectopic production of BimEL had different effects for these two apoptotic

![FIGURE 3. Down-regulation of BimEL expression by EBV infection is a post-transcriptional event. A. The mRNAs specific for the various Bim isoforms were determined by real-time quantitative PCR and compared with those in the EBV-positive Ramos-AW and Raji cells. The mRNA levels in Ramos, Ramos-AW, and Raji cells were normalized to the mRNA levels of β-actin. Bim protein expression in these cells was analyzed by Western blotting, using an anti-Bim Ab (b). C, Raji cells were cultured for 24 h with or without the specific MKK1 inhibitor PD98059 for 24 h. Cell extracts (30 μg) were subjected to SDS-PAGE, and BimEL was detected by immunoblotting with an anti-Bim Ab.](http://www.jimmunol.org/content/177/6/2971/F3)

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stimuli. Whereas BimEL significantly decreased the resistance of these cells to growth factor deprivation, the production of BimEL in EBV Ramos-AW Burkitt’s cells did not restore their sensitivity to anti-
/H9262 Ab-induced apoptosis. There may be several possible reasons for this. There may be too little Bim in these cells to respond to this apoptotic stimulus. Alternatively, the apoptotic pathways mobilized by soluble anti-
/H9262 Ab in human Burkitt’s lymphoma cells may be independent of Bim (10) and may therefore differ from the pathway observed in murine B cells (7). Finally, the resistance to soluble anti-
/H9262 Ab-induced apoptosis conferred by EBV infection may involve other pathways dependent on the presence of viral proteins, such as latent membrane protein 2A (26) or the Bcl-2-related protein BHRF1 (4). Interestingly, Bim has been reported to inhibit the prosurvival function of Bcl-2, but not that of BHRF1 (27). In contrast, we found that the production of BimEL in Ramos-AW cells restored the sensitivity of these cells to apoptosis triggered by serum deprivation. This suggests that EBV-mediated resistance to growth factor deprivation is, at least in Burkitt’s cells, dependent on BimEL. Bim has been reported to play a key role in controlling growth factor deprivation-dependent apoptosis in hemopoietic and nonhemopoietic cells (14–16, 21, 31, 32). However, most of these reports found that Bim expression was controlled at the transcriptional level through the activation of forkhead transcription factors, although one study recently reported that Bim phosphorylation plays a role in this process (33). In our experimental conditions, the resistance to serum deprivation-induced apoptosis conferred by EBV infection was independent of the transcriptional machinery and relied on the control of BimEL protein levels by the proteasome. This pattern is probably important for EBV, because this virus may be oncogenic in its acute phase, and this biological outcome may take advantage of this regulatory pathway. In contrast, proapoptotic viruses, such as HIV, regulate Bim activation by a different pathway, based on the disruption of tubulin by TAT proteins, leading to Bim activation (34).

![FIGURE 4. EBV-induced resistance to growth factor deprivation is mediated by BimEL.](image)

A. Whole-cell extracts (30 µg) from Ramos, Ramos-AW, or Bim-transfected Ramos-AW (clones A37 and A53) cells were subjected to SDS-PAGE. Myc-tagged BimEL was detected by immunoblotting with an anti-Myc Ab or an anti-Bim Ab (a). Endogenous BimEL was detected with the anti-Bim Ab (b). B, Ramos, Ramos-AW, A37, and A53 were incubated with small amounts of FCS (0.1%) for 96 h or with soluble anti-
/H9262 Ab (10 µg/ml) or recombinant TRAIL (200 ng/ml) for 48 h. The responses of A37 and A53 cells differed significantly from that of Ramos-AW cells (p < 0.001, by Student’s t test). Apoptotic cells were identified by flow cytometry (shrunken cells with high side scatter and low forward scatter). C, Ramos, Ramos-AW, A37, and A53 cells were incubated with small amounts of FCS (0.1%) for various periods of time. D, Ramos-AW, A37, and A53 cells were incubated with various amounts of FCS for 96 h. SD from triplicate analyses are shown. E, Apoptosis analysis of A37 cells cultured for 96 h in the presence of 10% FCS (a and b) or 0.1% FCS (c and d). Cells were analyzed by flow cytometry. Apoptotic cells, identified as having high side scatter (SSC) and low forward scatter (FSC) properties, were counted, and their numbers were expressed as a percentage of total population (a and c). Cell nuclei were stained with propidium iodide, and the hypodiploid DNA peak corresponding to apoptotic nuclei was quantified (b and d). The percentage of apoptotic cells is shown for each panel.
In conclusion, our data show that EBV infection leads to the post-transcriptional inhibition of Bim expression, leading to its inactivation. This process involves constitutive phosphorylation of Bim by ERK 1/2, followed by its degradation through the proteasome pathway. This regulatory pathway directly controls the sensitivity of B cells to serum starvation and may therefore contribute to the oncogenic potential of EBV.

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