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Epstein-Barr virus-encoded LMP1 promotes cisplatin-induced caspase activation through JNK and NF-κB signaling pathways

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Abstract

Our previous studies have shown that Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) potentiates chemotherapeutic agent-induced apoptosis in human cell lines of epithelial origin: cervical carcinoma-derived HeLa cells and nasopharyngeal carcinoma-derived TW03 cells. LMP1 acted upstream of caspase-dependent mitochondrial perturbation, and the effect was mapped to the C-terminal signaling domain of LMP1, designated CTAR2. CTAR2 is known to engage the c-Jun N-terminal kinase (JNK) and NF-κB pathways, and we show here that SP600125, a selective JNK inhibitor, suppresses LMP1 potentiation of cisplatin-induced mitochondrial damage and caspase activation in HeLa cells. Moreover, the potentiation of cisplatin-triggered caspase activation was blocked by Bay11-7082, a potent inhibitor of NF-κB. Similar results were obtained when a dominant negative form of IκB, a specific repressor of NF-κB, was co-expressed with LMP1. The current data support the notion that LMP1 modifies stress-induced apoptosis in epithelial cells through molecular interactions downstream of its C-terminal signaling domain.

Keywords: Apoptosis; Epstein-Barr virus; Caspase; c-Jun-N-terminal kinase; Latent membrane protein 1; Nuclear factor-κB

Epstein-Barr virus (EBV) encodes a number of proteins with transforming ability. Among these proteins, latent membrane protein 1 (LMP1) is essential for EBV-mediated transformation of resting primary human B cells into indefinitely proliferating lymphoblastoid cell lines [1]. EBV is associated with human lymphoid tumors, such as Burkitt's lymphoma (BL) arising in immunocompetent individuals, and lymphoblastoid cell-like lymphomas in immunocompromised patients. Importantly, early studies showed that LMP1 confers apoptosis resistance in B cells through transcriptional regulation of the anti-apoptotic protein Bcl-2 [2]. Recent studies have yielded a more complex picture insofar as LMP1 was shown to upregulate expression of multiple genes with opposing activities in B cell proliferation and apoptosis [3].

EBV infection is also tightly associated with the undifferentiated form of nasopharyngeal carcinoma (NPC), a human tumor of epithelial origin. Thirty to sixty percent of the EBV-associated NPCs are also positive for LMP1 [4,5]. LMP1 inhibits terminal differentiation of human epithelial cells [6]. LMP1 also affects apoptosis in epithelial cells, but the outcome appears to vary in a context-dependent manner [7–10]. Moreover, in several NPC cell lines, LMP1 was shown to repress DNA repair and enhance sensitivity to DNA-damaging agents, as assayed by micronucleus formation [11]. The latter findings support an additional role for LMP1 in the pathogenesis of NPC, insofar as disruption of DNA repair by LMP1 may contribute to genomic instability of epithelial cells.

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The cellular effects of LMP1 are mostly attributed to the two functional domains in its cytoplasmic region: a membrane proximal domain, termed the C-terminal activation region 1 (CTAR1), and a membrane distal domain, CTAR2 [12]. Activation of NF-κB is the hallmark of the biological activities of LMP1 [13], and CTAR1 has been shown to contribute to 30% of the NF-κB activation and to the initiation of cell proliferation exerted by LMP1 in some cell types [14]. CTAR2, in turn, contributes to 70% of the NF-κB activation as well as to the activation of c-Jun-N-terminal kinase (JNK) [15].

Our previous studies have shown that LMP1 regulates apoptosis in the epithelial cell line HeLa in a stimulus-dependent manner [9]. Hence, expression of LMP1 diminished tumor necrosis factor (TNF)-induced apoptosis, but enhanced apoptosis triggered by other forms of cellular stress, such as etoposide. We also observed that the regulation of cellular stress-induced apoptosis by LMP1 occurred upstream of caspase-dependent mitochondrial perturbation, and this effect was mapped to CTAR2 [10]. The latter observations suggested that molecular interactions involving the membrane distal functional domain of LMP1 may be responsible for the regulation of stress-induced apoptosis. However, the underlying signaling pathways or factors involved have remained elusive. In the present study, we investigated the involvement of NF-κB and JNK in the regulation of stress-induced apoptosis in human cell lines of epithelial origin.

Materials and methods

Cell lines and plasmids. HeLa cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). Raji, an EBV-positive BL-derived cell line [16], was from the Department of Microbiology, Tumor, and Cell Biology at Karolinska Institutet. HeLa cells were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μl/ml streptomycin, 2 mg/ml geneticin, 500 μg/ml hygromycin B, and 1 μg/ml tetracycline (Stratagene, La Jolla, CA, USA). Raji cells were cultivated in RPM1-1640 medium (Gibco) supplemented with 10% FCS and antibiotics. The κB.ConA.LUC reporter plasmid contains three NF-κB enhancer element upstream of a conalbumin promoter driving a luciferase gene [17], and the β-galactosidase-encoding plasmid pCMV βGal was obtained from Dr. Martin Rowe (University of Wales, Cardiff, UK). Dual JNK reporter plasmids pFA+pFR from Strategene were kindly provided by Dr. Aris Eliopoulos (University of Birmingham, Birmingham, UK). Transient transfection of a plasmid expressing a dominant negative (DN) inhibitor of NF-κB (IκB) [termed Gly-Ala repeat (GAr) IκB] (the generous gift of Dr. Anatoly Sharipo, Karolinska Institutet) [18] was performed using Lipofectamine 2000 (Invitrogen, Paisley, Scotland) according to instructions from the manufacturer.

Transfection procedures. Transfections with tetracycline transactivator (tTA) expressor plasmid and responder plasmid with inserts of LMP1 cDNA were done as previously described [9]. Cells with tetracycline-regulated LMP1 expression were selected by subcloning. For reporter assays, monolayer cells were plated in 6-well tissue culture plates, and incubated overnight at 37 °C. Cells were then co-incubated with 4 μg/ml lipofectin (Invitrogen) mixed with 300 ng κB. ConA.LUC reporter plasmid or 50 ng pFA+pFR and 300 ng pCMV βGal plasmids. The introduced genes were expressed for 24 h during which time the HeLa cells were cultured in the presence or absence of tetracycline. Cells were then harvested in cell culture lysis reagent (CCLR) (Promega, Madison, WI, USA). Transfection efficiency was normalized by assessment of β-galactosidase activity, as described previously [12].

Western blotting. To monitor the tetracycline-regulated expression of LMP1 in HeLa cells, the same cell lysate mentioned above was mixed with an equal volume of sampling buffer (65 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol with protease inhibitors) and heated by boiling at 95 °C, whereupon total proteins were separated by 7.5% polyacrylamide gel electrophoresis (PAGE). After electroblotting, the membranes were probed using the anti-LMP1 antibody S12 [10] or the anti-β-actin antibody AC-15 (SIGMA, St. Louis, MO, USA). Horseradish peroxidase (HRP)-linked anti-mouse goat immunoglobulins (Bio-Rad, Hercules, CA, USA) were used as secondary antibodies and membranes were developed with ECL reagents, as recommended by the manufacturer (Amersham, Uppsala, Sweden).

Apoptosis induction. Apoptosis of HeLa cells was triggered by the addition of 5 μg/ml of cisplatin (Sigma). For pharmacologic inhibition of JNK and NF-κB, cells were pre-incubated with 10 μM SP600125 (Calbiochem, Nottingham, UK) or Bay11-7082 (Calbiochem), respectively, while control cells were cultivated in the presence of solvent alone (DMSO). Effectiveness of apoptosis induction/inhibition was routinely monitored by the assessment of detached and floating cells in the culture medium (data not shown). The apoptosis assays described below were performed on floating cells pooled with the remaining, adherent cells detached by trypsinization.

Mitochondrial membrane potential. The drop of mitochondrial membrane potential (MMP) upon apoptosis induction was determined as previously reported [10], using the cationic fluorescent dye tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes, Leiden, The Netherlands). Data were acquired using a FACScan (Becton–Dickinson, San Jose, CA, USA), operating with the CellQuest software (Becton–Dickinson), and results are depicted as the percentage of cells with depolarized mitochondria.

Caspase activation. Caspase-2 and caspase-3 activities were measured as reported previously [10]. Briefly, cell lysates and peptide substrates (VDVAD-AMC or DEVD-AMC) (Calbiochem) were combined in a standard reaction buffer [19] and added to a 96-well plate. Cleavage of the fluorogenic substrates was monitored by AMC liberation in a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden). Fluorescence units were converted to pmol of AMC using a standard curve generated from free AMC. Data were analyzed by linear regression and are displayed as pmol AMC release per min.

Results

To evaluate the role of JNK activation for LMP1 modulation of apoptosis, HeLa cells were transiently transfected with JNK reporter plasmids (Fig. 1A). LMP1 induction of JNK was observed, and the addition of SP600125 [20] decreased JNK activation, as expected (Fig. 1B). Cells were then pre-treated with SP600125 before administration of cisplatin to trigger apoptosis. As seen in Fig. 1C, the drop of MMP was increased in LMP1-expressing cells, in line with our previous studies [10]. Importantly, SP600125 abrogated the dissipation of MMP in LMP1-expressing cells, but not in the LMP1-negative cells. Furthermore, induction of caspase-2 (Fig. 1D) and caspase-3 (Fig. 1E) activity upon exposure of the LMP1-expressing cells to cisplatin was blocked by the JNK inhibitor. Together, these data suggest that stress (cisplatin)-induced apoptosis in LMP1-expressing HeLa cells is JNK-dependent.

We also asked whether NF-κB-dependent pathways were involved in stress-induced apoptosis in our model