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Effects of ER stress on unfolded protein responses, cell survival, and viral replication in primary effusion lymphoma

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ABSTRACT

Primary effusion lymphoma (PEL), a subtype of non-Hodgkin's B-lymphoma, is an aggressive neoplasm caused by Kaposi's sarcoma-associated herpesvirus (KSHV) in immunosuppressed patients. Endoplasmic reticulum (ER) stress induces activation of the unfolded protein response (UPR), which induces expression of ER chaperones, which in turn decrease ER stress, leading to ER homeostasis. The UPR is necessary for not only ER homeostasis but also persistent infection by, and replication of, many viruses. However, the precise roles and regulation of the UPR in KSHV infection remain poorly understood. Here, we found that IRE1α and PERK were significantly downregulated in PEL cells cultured under normal conditions, compared with KSHV-uninfected B-lymphoma cells. IRE1α and PERK mRNA levels were decreased in PEL cells, and KSHV-encoded LANA and v-cyclin D led to suppressed IRE1α transcription. Thapsigargin-induced ER stress activated the UPR and increased the mRNA levels of UPR-related molecules, including IRE1α and PERK, in PEL cells. However the IRE1α and PERK mRNA levels in PEL cells were lower than those in KSHV-uninfected cells. Furthermore, ER stress induced by brefeldin A and thapsigargin dramatically reduced the viability of PEL cells, compared with KSHV-uninfected cells, and induced apoptosis of PEL cells via the pro-apoptotic UPR through expression of CHOP and activation of caspase-9. In addition to the pro-apoptotic UPR, thapsigargin-induced ER stress enhanced transcription of lytic genes, including RTA, K-bZIP and K8.1, and viral production in PEL cells resulted in induction of the lytic cycle. Thus, we demonstrated downregulation of IRE1α and PERK in PEL cells, transcriptional suppression of IRE1α by LANA and v-cyclin D, apoptosis induction in PEL cells by ER stress, and potentiation of lytic replication by ER stress.

1. Introduction

The ER functions in the folding and maturation of newly synthesized proteins. However, ER machinery and integrity can be disrupted by production of unfolded or misfolded proteins, which leads to the unfolded protein response (UPR) [1]. The UPR induces the expression of ER chaperones to reinforce the UPR and abate ER stress. However, high levels of ER stress induce activation of the pro-apoptotic UPR, causing apoptosis [2]. The UPR consists of three pathways: those involving IRE1α, PERK, and ATF6α [1,2]. Under normal conditions, GRP78/Bip binds and inactivates IRE1α, PERK, and ATF6α, while in the presence of ER stress, protein unfolding results in dissociation of GRP78, leading to activation of the three UPR pathways to reduce ER stress and the protein load therein. In an ER stress-dependent manner, ATF6α is transported to the Golgi apparatus, where it is cleaved. Cleaved ATF6α (known as p50ATF6α) is translocated into the nucleus, where it activates transcription of GRP78 and ERAD components (EDME). IRE1 has an endoribonuclease domain, and activated IRE1α removes the 26-nucleotide intron from the mRNA of XBP1, and this resulting spliced transcript is translated into the XBP1 protein, which induces transcription of GRP78 and EDME. Activated PERK induces eIF2α phosphorylation, which inhibits translation. Thus, the UPR abates ER stress by increasing production of ER chaperones and decreasing

Abbreviations: ATF6, activating transcription factor-6; CHOP, CCAAT/enhancer-binding protein-homologous protein; ER, endoplasmic reticulum; GADD34, growth arrest and DNA damage gene-34; GRP78, 78 kDa glucose-regulated protein; IRE1, inositol-requiring enzyme-1; KSHV, Kaposi's sarcoma-associated herpesvirus; LANA, latency associated nuclear antigen; PERK, Protein kinase-like ER kinase; PEL, primary effusion lymphoma; Tg, thapsigargin; UPR, unfolded protein response; XBP1, X-box binding protein-1.

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protein synthesis within the ER. However, in the presence of severe or prolonged ER stress, which prevents restoration of homeostasis, the pro-apoptotic UPR is activated [1,2]. The PERK–eIF2α pathway increases the translation of ATF4, which activates expression of CHOP. CHOP induces expression of the pro-apoptotic factor Bim [3] and suppresses expression of the anti-apoptotic Bcl-2 [4], causing apoptosis. The proapoptotic UPR also induces activation of human caspase-4 (mouse caspase-12 homolog) through an unknown mechanism. Caspase-4 directly activates caspase-9 independently of cytochrome c release from mitochondria [5,6].

PEL is a malignant B-cell lymphoma caused by KSHV in immunosuppressed patients [7]. In general, PEL cells are derived from post-germinal center B cells and are latently infected with KSHV. PEL is a subtype of non-Hodgkin's lymphoma that is resistant to chemotherapy regimens, such as CHOP and R-CHOP [8]. KSHV has two life cycles: latency and lytic replication. The KSHV genome forms circularized double-stranded DNA in the nucleus during latent infection. To establish a latent infection, KSHV expresses latent genes, including LANA, v-FLIP, v-cyclin (human cyclin D2 homolog), and microRNAs, in PEL or Kaposi's sarcoma cells [9]. These viral molecules manipulate NF-κB, Akt, Wnt, and Erk signaling to maintain the malignant phenotype [9,10]. KSHV alternates between lytic replication and latency by expression of RTA/Orf50, which induces lytic replication from the latency cycle. During lytic replication, viral genomes and proteins are synthesized, and assembled progeny viruses are released from the cell. That is, abundant viral proteins are maturated and glycosylated in the ER, which perturbs ER homeostasis. Hence, the UPR seems to be necessary for efficient replication and survival of KSHV [11–14]. Additionally, two observations have been reported: the UPR activates KSHV lytic replication [11,12], while the UPR represses lytic replication [15]. Because the precise roles and regulation of the UPR in KSHV infection remain unclear, we investigated the relationship between the UPR and KSHV, dysregulation of the UPR by KSHV, and the underlying molecular mechanism(s).

2. Materials and methods

2.1. Cells, compounds, plasmids, and antibodies

KSHV-positive PEL cell lines (BC2, HBL6, BC3, JSC1, JSC-PEL, and BCBL1) and KSHV-negative B-lymphoma cell lines (Ramos, BJAB, Raji, and DG75) were maintained in RPMI medium. Thapsigargin (Tg; Cayman Chemical, MI, USA), tunicamycin (Tm), brefeldin A (BFA; Osaka, Japan), and salubrinal (Sal; Focus Biomolecules, PA, USA) were dissolved in DMSO. For eukaryotic expression, LANA and LANA-dCR were amplified from Dy52 and MF24 [10], respectively. v-Flip and v-cyclin D were amplified from the KSHV genome in BC3 cells. Amplified ORFs were cloned into a pCIneo-based vector (Promega, WI, USA). The PERK (ID: 21814) and IRE1α (ID: 13009) plasmids were purchased from Addgene (MA, USA). Primary antibodies used in western blotting included those against IRE1α, PERK, ATF6α, p53, PCNA, and β-Actin (Santa Cruz Biotechnology, CA, USA), Ser32/36-phospho-IκBα, Ser473-phopho-Akt, Akt, caspase-3, caspase-7, caspase-9, PARP (Cell Signaling, MA, USA), Bip, IκBα, Erk1, Thr202/Tyr204-phospho-Erk1/2 (BD, NJ, USA).

2.2. Protein stability assay

HeLa cells (8×10^6) were transfected with a plasmid harboring PERK or IRE1α by the calcium-phosphate method [16] and cultured for 48 h. Cells were lysed in 4 ml lysis buffer containing 20 mM Tris-HCl (pH 7.8), 1% NP-40, 0.5 mM EDTA, 150 mM NaCl, and 1 mM DTT and homogenized by sonication for 5 s. PEL cells (4×10^6) were lysed in 1.5 ml lysis buffer and then sonicated. HeLa lysates (300 μl) and 200 μl PEL lysates were mixed and incubated at 37 °C. Reaction mixtures were subjected to Western blotting.

2.3. Reverse transcription (RT)-PCR

Preparation of total RNA and cDNA synthesis by reverse transcriptase were performed using a method described previously [17]. The PCR products were subjected to electrophoresis in 2–2.5% agarose gels. The sequences of the primers used are as follows: IRE1α-forward 5′-GGGTCTGAGGAAGGTGATGC-3′, IRE1α-reverse 5′-CAGTGGGGTTTCATGGTGTC-3′, PERK-forward 5′-TGAGACAGAG TTGCGACCG-3′, PERK-reverse 5′-GCCAACACTGAAATTCCACTTCTC-3′, ATF6α-forward 5′-CTTTACTAGGCCACCGTCTCG-3′, ATF6α-reverse 5′-CAAATCCAACTCCTCAGGAAC-3′, GAPDH-forward 5′-TGACCACAGTCC ATGCCATC-3′, GAPDH-reverse 5′-GGGGAGATTCAGTGTGGTGG-3′, CHOP-forward 5′-GGTACCTATGTTTCACCTCCTG-3′, CHOP-reverse 5′-GAGCCGTTCATTCTCTTCAGC-3′, GADD34-forward 5′-CGAGGAA-GAGGGAAGTTGCTG-3′, and GADD34-reverse 5′-CTCCATCCTTCT-CAGCTGCC-3′.

2.4. Real-time reverse transcription (RT)-PCR

Preparation of cDNA and real-time PCR were performed as described previously [18]. The sequences of the primers used are as follows: IRE1α-forward 5′-GGCCTCGGGATTTTTGGAAGTAC-3′, IRE1α-reverse 5′-TGCAAACTTCCATCCAGCGTT-3′, PERK-forward 5′-GCGCGGCAGGTCATTAGT-3′, PERK-reverse 5′-TGCTAAGGCTGGAT-GACACC-3′, sXBP1-forward 5′-CTGAGTCCGCAGCAGGTGCA-3′, sXBP1-reverse 5′-GGTCCAAGTTGTCCAGAATGCCCAA-3′, uXBP1-forward 5′-ACTCAGACTACGTGCACCTC-3′, and uXBP1-reverse 5′-GTCAATACCGCCAGAATCCA-3′. A GAPDH primer set (GAPDH-forward 5′-GAGTCAACGGATTTGGTCGT-3′, GAPDH-reverse 5′-GACAAGCTTCCCGTTCTCAG-3′) was used as an internal control for normalization. The expression levels were normalized to that of GAPDH for quantification.

3. Results and discussion

3.1. IRE1α and PERK expression are suppressed in PEL cells but induced transcriptionally by ER stress

First, we evaluated whether the ER-stress inducer Tg, which inhibits the ER Ca^{2+} ATPase, upregulated expression of IRE1α, PERK, GRP78, ATF6α, p50ATF6α (cleaved ATF6α), unspliced XBP1, and spliced XBP1 in PEL cells. When KSHV-infected PEL cell lines (BC3, BCBL1, JSC1, JSC-PEL, and HBL6) and KSHV-uninfected cells (BJAB, Ramos and DG75) were exposed to Tg-induced ER stress, the levels of IRE1α, PERK, and p50ATF6α were increased in KSHV-uninfected cells (Fig. 1a, b). However, ER stress did not increase the levels of IRE1α and PERK, and only slightly increased that of p50ATF6α, in PEL cells. In contrast, ER stress slightly upregulated expression of GRP78 in both PEL and KSHV-uninfected cells (Fig. 1a). Since IRE1α and PERK protein levels were not increased in PEL cells exposed to ER stress, we examined the mRNA levels of IRE1α, PERK, ATF6α, unspliced XBP1 (uXBP1), and spliced XBP1 (sXBP1) in PEL cells cultured under either normal or ER stress conditions (Fig. 1c, d). We also measured the mRNA levels of those genes in PEL cells (BCBL1) and KSHV-uninfected cells (Ramos) cultured under normal conditions (Fig. 1e). Under normal conditions, transcription of IRE1α, PERK, and sXBP1 was markedly suppressed in PEL cells as compared with KSHV-uninfected cells (Fig. 1c, e), while mRNA levels of ATF6α (Fig. 1c) and uXBP1 (Fig. 1e) were not reduced in either cell type. In contrast to normal conditions, mRNA levels of IRE1α, PERK, ATF6α, sXBP1 and uXBP1 were increased by ER stress in both cell types (Fig. 1c, d). However, the mRNA levels of IRE1α,

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