The human herpes virus 8–encoded viral FLICE inhibitory protein protects against growth factor withdrawal–induced apoptosis via NF-κB activation

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The human herpes virus 8 (HHV8)–encoded viral FLICE (Fas-associating protein with death domain–like interleukin-1–converting enzyme) inhibitory protein (vFLIP) is believed to protect cells against death receptor–mediated apoptosis. In the present study we demonstrate that expression of HHV8 vFLIP in a growth factor–dependent TF-1 leukemia cell line protects against growth factor withdrawal–induced apoptosis. Unlike vector-expressing cells, those expressing HHV8 vFLIP maintain their mitochondrial membrane potential upon withdrawal from growth factor and also exhibit a block in the activation of caspases. The protective effect of HHV8 vFLIP is associated with its ability to activate the nuclear factor–κ B (NF-κB) pathway and is missing in the vFLIP encoded by equine herpes virus 2 that lacks this activity. Inhibition of the NF-κB pathway by IκB superrepressor, lactacystin, MG132, arsenic trioxide, and phenylarsine oxide reverse the protection against growth factor withdrawal–induced apoptosis conferred by HHV8 vFLIP. HHV8 vFLIP up-regulates the expression of Bcl-xL, an antiapoptotic member of the Bcl2 family, which is a known target of the NF-κB pathway. Collectively, the above results suggest that HHV8 vFLIP–induced NF-κB activation may contribute to cellular transformation seen in association with HHV8 infection by preventing the apoptosis of cells destined to die because of growth factor deprivation. (Blood. 2003;101:1956-1961) © 2003 by The American Society of Hematology

Introduction

Caspase-8 (FLICE [Fas-associating protein with death domain–like interleukin-1 (IL-1)–converting enzyme]/MACH or Mch5) is one of the apical caspases of the extrinsic (death receptor) apoptosis pathway and possesses a prodomain containing 2 homologous death effector domains (DEDs).1,3 In addition to its role in maintaining caspase-8 in its zymogen state, the prodomain of caspase-8 also helps in its recruitment to the aggregated complex of death receptors, which results in its activation via cross-proteolysis. Several viruses also encode proteins containing 2 DEDs, called viral FLICE inhibitory proteins (vFLIPs), which have been postulated to protect against death receptor–induced cell death by blocking the recruitment or activation of caspase-8.4,6 These vFLIPs include the orf-K13 from the human herpes virus 8/Kaposi sarcoma–associated herpes virus (HHV8/KSHV), MC159L, and MC160L from the molluscum contagiosum virus (MCV) and E8 from the equine herpes virus 2 (EHV2).4,6 We have previously demonstrated that the HHV8 vFLIP possesses the ability to activate the nuclear factor–κ B (NF-κB) pathway, a property that is not shared by other vFLIPs.7,8 In this report we describe the ability of HHV8 vFLIP to protect against growth factor withdrawal–induced apoptosis and link this property to its ability to activate the NF-κB pathway. These results have important implications for the pathogenesis of HHV8–associated malignancies and suggest that vFLIPs have functions beyond their known role as inhibitors of caspase-8.

Materials and methods

Cell line and culture

TF-1 cell line (human acute myeloid leukemia) was obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI medium supplemented with 10% fetal calf serum and 2 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF).

Drugs and chemicals

Recombinant human GM-CSF and tumor necrosis factor α (TNFs) were purchased from R&D systems (Minneapolis, MN), Hoechst 33342, tetramethylrhodamine ethyl ester (TMRE), propidium iodide (PI), phenylarsine oxide (PAO), and arsenic trioxide (As2O3) were purchased from Sigma (St Louis, MO). MG132 and lactacystin were purchased from Calbiochem (San Diego, CA) and Biomol (Plymouth, PA), respectively.

Retrovirus and adenovirus constructs

Retrovirus constructs containing C-terminal Flag epitope–tagged HHV8 vFLIP (K13-Flag) and EHV2 vFLIP (E8-Flag) were generated in murine stem cell virus (MSCV) neo-based retroviral vector and amphotropic viruses generated and used for infection as described previously.8 Cells were selected in the presence of 1 mg/mL of G418 (Invitrogen, Carlsbad, CA). Adenoviral vectors encoding β-galactosidase and IκB superrepressor (DN-IκBα) were kindly provided by Dr Richard Gaynor of our institution.

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Supported by a grant from the National Institutes of Health (CA85177).

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Cell viability assay

For the measurement of cell viability, cells from exponentially growing cultures were washed twice with GM-CSF–free medium and plated in a flat-bottom 96-well plate at a density of 20,000 cells/well in the absence and presence of GM-CSF (2 ng/mL). Cell viability was measured after 48 hours using the MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and following the instructions of the manufacturer (Promega, Madison, WI). Absorbance of viable cells was measured at 490 nm with 600 nm as a reference wavelength. Percent cell survival was calculated based on the reading of cells grown in the presence of GM-CSF as 100%.

Western blot analysis

Cells were lysed in a lysis buffer containing 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol supplemented with a protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Indianapolis, IN). Western blot analysis was performed essentially as described previously.8 Quantitative analysis of the Western blots was carried out using the National Institutes of Health (NIH; Bethesda, MD) Image (version 1.61) software package. Primary antibody dilutions used in these experiments were Flag (Santa Cruz Biotechnology, Santa Cruz, CA; sc-807, 1:5000); cleaved caspase-9 (Cell Signaling, Beverly, MA; Asp 330, 1:2000); cleaved caspase-3 (Cell Signaling; Asp 175, 1:2000); caspase-8 (Cell Signaling; IC12, 1:2000); caspase-6 (Cell Signaling; 9762, 1:2000); actin (Santa Cruz Biotechnology; sc-1616, 1:1000); α-ItB (Santa Cruz Biotechnology; SC-371, 1:2000) and p-ItB (Santa Cruz Biotechnology; 9241S, 1:1000); Mcl-1 (Santa Cruz Biotechnology; S-19, 1:1000); and Bcl-x L/S (Santa Cruz Biotechnology; S-18, 1:1000).

Results

Generation and characterization of TF-1 cells expressing vFLIPs

In order to study the effect of HHV8 vFLIP on growth factor withdrawal–induced apoptosis we used retroviral-mediated gene transfer to generate mass population of TF-1 cells with stable expression of Flag epitope–tagged HHV8 vFLIP (K13-Flag) or an empty vector. We selected TF-1 cell line for this study as this CD34+ leukemia cell line is known to be responsive to multiple cytokines such as interleukin-3 (IL-3), erythropoietin, stem cell factor, and GM-CSF.9 In addition to HHV8 vFLIP, we also generated TF-1 cells expressing E8-Flag to serve as a control. Expression of the transduced proteins was confirmed by Western blot analysis on the immunoprecipitated proteins (Figure 1A).

HHV8-encoded vFLIP K13 protects against growth factor withdrawal–induced apoptosis

We next studied the effect of the vFLIPs on GM-CSF withdrawal–induced cell death of TF-1 cells. TF-1 cells expressing the empty vector demonstrated rapid loss of viability when deprived of growth factors and demonstrated massive fragmentation into small apoptotic bodies (Figure 1B-C). In contrast, cells expressing the HHV8 vFLIP K13 demonstrated significant protection against growth factor withdrawal–induced apoptosis (Figure 1B-C). However, no protective effect was observed in cells expressing vFLIP E8, suggesting that this effect was unique to vFLIP K13 (Figure 1B-C).

Protective effect of HHV8 vFLIP on growth factor withdrawal–induced cell death was further characterized by nuclear staining with Hoechst 33342 dye. TF-1 cells expressing the control vector and vFLIP E8 demonstrated the classical nuclear features of apoptosis upon withdrawal from GM-CSF, such as nuclear condensation and fragmentation, which were markedly reduced in vFLIP K13–expressing cells (Figure 1D). Moreover, a DNA-content analysis revealed a significant decrease in the cells with sub-G0/G1 DNA content among vFLIP K13–expressing TF-1 cells compared with those expressing empty vector (Figure 1E). Collectively, the...
above results suggested that expression of the HHV8 vFLIP protected against growth factor withdrawal–induced cell death by blocking key nuclear and cytoplasmic features of apoptosis.

Previous studies have demonstrated the ability of vFLIPs to protect against apoptosis induced by death domain receptors belonging to the tumor necrosis factor receptor family. Therefore, we were interested in checking whether expression of HHV8 vFLIP would also lead to protection against TNFα–induced apoptosis. However, as shown in Figure 2A, we failed to observe a significant difference in cell death induced by treatment with TNFα in the vector and vFLIP K13–expressing TF-1 cells. Similarly, expression of vFLIP K13 failed to protect against anticancer drug–induced apoptosis (Figure 2B). Taken together, the above results suggest that the ability of HHV8 vFLIP to protect against growth factor withdrawal–induced apoptosis does not extend to all forms of cell death.

HHV8 vFLIP blocks loss of mitochondrial membrane potential and resultant caspase activation during growth factor withdrawal–induced apoptosis

Growth factor withdrawal–induced apoptosis is generally believed to be triggered by the loss of mitochondrial membrane potential with the resultant release of cytochrome c, which forms a multiprotein apoptosome complex with Apaf-1 and procaspase-9 in the presence of dATP. Procaspase-9 is activated upon recruitment to this complex and in turn activates the effector caspases, such as caspase-3. In order to understand the mechanism of protective effect of vFLIP K13 against growth factor withdrawal–induced apoptosis, we examined the status of mitochondrial membrane potential after staining with potentiometric dye TMRE. GM-CSF deprivation for 24 hours led to the appearance of a significant subpopulation of TF-1 vector cells with loss of TMRE staining, which was absent in the case of vFLIP K13–expressing cells (Figure 3A). We also examined the status of caspase-9 and -3 in the vector- and vFLIP K13–expressing TF-1 cells upon withdrawal from GM-CSF for various time intervals using antibodies directed against the cleaved form of the above caspases. As shown in Figure 3B, significantly higher amounts of cleaved caspase-9 and -3 were observed in TF-1 vector cells compared with those expressing vFLIP K13, and this difference was most pronounced at 27 hours.

Figure 2. vFLIP K13 fails to protect against TNFα- and anticancer drug–induced apoptosis. (A) TF-1 cells expressing an empty vector or vFLIP K13 were treated with TNFα (10 ng/mL) for 48 hours and cell viability examined using the MTS assay. The values shown are means ± SD of 2 independent experiments performed in triplicate. (B) TF-1 cells expressing an empty vector or vFLIP K13 were treated with the indicated doses of drugs for 48 hours and cell viability examined using the MTS assay. The values shown are means ± SD of 2 independent experiments performed in triplicate.

Figure 3. vFLIP K13 blocks growth factor withdrawal–induced apoptosis by blocking loss of mitochondrial membrane potential and caspase-activation. (A) vFLIP K13–expressing cells maintain their mitochondrial potential in the absence of GM-CSF. TF-1 vector and vFLIP K13–expressing cells were grown in the absence or presence of GM-CSF for 24 hours. Cells were stained with 100 nM TMRE for 30 minutes at 37°C and fluorescence determined using a flow cytometer. (B) Inhibition of caspase-9, -3, and -8 cleavage during GM-CSF withdrawal–induced apoptosis in TF-1 vFLIP K13 cells. TF-1 vector and vFLIP K13 cells were grown in the presence and absence of GM-CSF for the indicated time periods. Cell lysates were analyzed for cleavage of caspase-9, -3, and -8 by Western blot analysis using antibodies that can recognize their cleaved forms. (C) Inhibition of caspase-6 cleavage during GM-CSF withdrawal–induced apoptosis in TF-1 vFLIP K13 cells. The experiment was performed essentially as described for panel B, except expression of caspase-6 was analyzed using an antibody that recognizes its full-length form.
HHV8 vFLIP protects against growth factor withdrawal–induced apoptosis via the NF-κB pathway

In order to test the hypothesis that vFLIP K13 protects TF-1 cells against growth factor withdrawal–induced apoptosis via the activation of the NF-κB pathway, we took advantage of known inhibitors of this pathway. As shown in Figure 5A, infection of TF-1 vector– and vFLIP K13–expressing cells with an adenovirus encoding a phosphorylation-resistant dominant-negative form of IκBα (IκBα superrepressor), which is known to block NF-κB activation via diverse stimuli,24,25 led to an almost complete reversal of the protective effect afforded by vFLIP-K13. Similarly treatement of vector and vFLIP K13 cells with lactacystin and MG132, 2 proteasome inhibitors that block NF-κB activation by blocking the degradation of IκB,26 led to reversal of protective effect afforded by the expression of vFLIP in a dose-dependent fashion (Figure 5B-C). Essentially similar results were obtained upon treatment of TF-1 vector and vFLIP K13 cells with PAO and As2O3. 2 recently identified inhibitors of the NF-κB pathway (Figure 5D-E).27

HHV8 vFLIP activates the NF-κB pathway in TF-1 cells

We and others have previously demonstrated constitutive NF-κB activation in HHV8-infected primary effusion lymphoma (PEL) cell lines.8,21 We have also demonstrated that HHV8-encoded K13 vFLIP can activate the NF-κB pathway while the EHV-encoded E8 vFLIP lacks this property.7 We were therefore interested in checking whether the differential ability of these vFLIPs to protect against growth factor withdrawal–induced apoptosis could be attributed to their differential ability to activate the NF-κB pathway in TF-1 cells. As shown in Figure 4A, an electrophoretic mobility shift assay demonstrated persistent NF-κB activation in TF-1 cells expressing vFLIP K13 compared with those expressing an empty vector or vFLIP E8. NF-κB is usually present in the cytoplasm of cells in association with a family of inhibitory proteins, called IκB.22,23 Cytokine-inducible phosphorylation of the IκB proteins leads to their rapid ubiquitination and proteasome-mediated degradation, which releases NF-κB from its inhibitory influence.22,23 Consistent with the increased NF-κB binding activity in vFLIP K13–expressing TF-1 cells, we also observed an increase in the phosphorylated and a decrease in the total IκB protein in these cells compared with those expressing an empty vector (Figure 4B).
HHV8 vFLIP maintains the expression of several antiapoptotic proteins during growth factor withdrawal–induced apoptosis

In order to understand the mechanism by which vFLIP K13–induced NF-κB activation might protect TF-1 cells against growth factor withdrawal–induced apoptosis we examined the expression of Bcl-xL and Mcl-1 proteins, which are known to be up-regulated by the NF-κB pathway.28-31 The expression of the above proteins was normalized relative to the expression of actin to ensure equal loading of all lanes. As shown in Figure 6, TF-1 cells expressing vFLIP K13 demonstrated a significant increase in the level of Bcl-xL protein compared with the vector-expressing cells, and this difference was maintained up to 48 hours after withdrawal from GM-CSF. We also observed a difference in the level of expression of Mcl-1 protein between vector- and K13-expressing cells grown in the presence of GM-CSF, which was maintained up to 6 hours after withdrawal from GM-CSF. However, this difference had considerably narrowed at the 24 hour time point and the expression of the 2 proteins was not significantly different at 48 hours after GM-CSF withdrawal (Figure 6). Collectively, the above results suggest the involvement of Bcl-xL in the protective effect of vFLIP K13 against growth factor withdrawal–induced apoptosis. However, the role of additional antiapoptotic proteins, including Mcl-1, in this process cannot be ruled out and deserves further study.

Discussion

In addition to Kaposi sarcoma (KS), HHV8 genomes have been frequently detected in PELs, multicentric Castleman disease, angioimmunoblastic lymphadenopathy, and some cases of reactive lymphadenopathies.32-35 However, the exact role played by HHV8 in the pathogenesis of these disorders is still unclear. Although HHV8 is known to encode for homologs of several cytokines and their receptors, none of them is expressed in latently infected PEL cell lines or KS spindle cells.36 HHV8 vFLIP is one of the few viral proteins that is expressed in latently infected KS spindle and PEL cells, making it a prime candidate for the dysregulated cellular proliferation and transformation associated with infection by this virus.36-39

Somatic cells in multicellular organisms are dependent on a continuous supply of survival signal from the neighboring cells, which limits the proliferative autonomy of any single cell.40,41 These survival signals are usually provided in the form of growth and survival factors within discrete somatic environments, thereby effectively trapping the somatic cells in specialized trophic microenvironments and acting as a major defense against metastatic spread.40,41 In the present study, we demonstrate that HHV8-encoded vFLIP can protect cells against growth factor withdrawal–induced apoptosis. Because growth factor independence is considered one of the hallmarks of cancer,42 these results provide a possible mechanism by which HHV8 might contribute to the development of lymphoproliferative disorders seen in association with infection by this virus.

Based on its sequence homology with the promdomain of caspase-8, earlier studies speculated that the main biologic function of HHV8 vFLIP might be to protect against death receptors–induced apoptosis by blocking the recruitment and activation of caspase-8.4-6 However, we recently demonstrated that HHV8 vFLIP can also activate the NF-κB pathway, which is known to protect against apoptosis induced by diverse stimuli.7,8 Consistent with the above observation, the present study demonstrates the ability of HHV8 vFLIP to protect against growth factor withdrawal–induced apoptosis in a factor-dependent cell line. Interestingly, the protective effect of HHV8 vFLIP against growth factor withdrawal–induced apoptosis in the present study was associated with the activation of the NF-κB pathway and not accompanied by the concomitant protection against apoptosis mediated by death receptors or anticancer drugs. Collectively, the above results suggest that the main biologic function of HHV8 vFLIP might be to activate the NF-κB pathway rather than its previously speculated role as an inhibitor of caspase-8 activation during death receptor signaling.

Although the exact mechanism(s) via which HHV8 vFLIP–mediated NF-κB might protect against growth factor withdrawal–induced apoptosis is not entirely clear, our results suggest a role of the Bcl-xL in this process. This antiapoptotic protein is known to be up-regulated by the NF-κB pathway and has been shown to block the processing of caspase-9, -6, and -2.16,28,29 However, in addition to Bcl-xL, the NF-κB pathway is known to induce the expression of a number of other antiapoptotic genes, such as A1, cIAP1, cIAP2, XIAP, and IEX-1.31,43 Thus, it is conceivable that additional genes encoded by the NF-κB pathway might contribute to the protective effect of HHV8 vFLIP against growth factor withdrawal–induced apoptosis.

In the present study we demonstrate the ability of the inhibitors of the NF-κB pathway to reverse the protective effect of HHV8 vFLIP against growth factor withdrawal–induced apoptosis. In addition to HHV-associated PEL, abnormal activation of the NF-κB pathway has been previously implicated in the pathogenesis of several lymphoproliferative disorders such as multiple myeloma, Hodgkin disease, Epstein-Barr virus–mediated Burkitt lymphoma, and human T-cell leukemia virus 1–induced adult T-cell leukemia.13 A number of drugs targeting this pathway are in various stages of clinical development or in clinical use for the treatment of hematologic malignancies.27,44-46 Our study suggests that the inhibitors of the NF-κB pathway also deserve study in the treatment of HHV8-associated lymphoproliferative disorders.

Acknowledgments

We would like to thank Dr Richard Gaynor for providing the adenoviral vectors expressing β-galactosidase and IκB superrepressor and Dr Suwan Sinha for technical assistance.
References


