The Inhibitor of Death Receptor Signaling, FLICE-inhibitory Protein Defines a New Class of Tumor Progression Factors

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Summary

Death receptor-mediated apoptosis can be modulated by several antiapoptotic proteins, such as the FLICE (FADD [Fas-associated death domain]-like IL-1β-converting enzyme)-inhibitory proteins (FLIPs). The FLIP family includes both cellular and viral members. The Kaposi's sarcoma-associated herpesvirus protein (KSHV)-FLIP is expressed by human herpesvirus 8 (HHV-8), which is associated with malignancies such as Kaposi's sarcoma and certain lymphomas. In this paper, we demonstrate that KSHV-FLIP protects cells from Fas-mediated apoptosis by inhibiting caspase activation and permits clonal growth in the presence of death stimuli in vitro. Furthermore, we show that KSHV-FLIP can act as a tumor progression factor by promoting tumor establishment and growth in vivo. When injected into immunocompetent recipient mouse strains, murine B lymphoma cells (A20) transduced with KSHV-FLIP rapidly develop into aggressive tumors showing a high rate of survival and growth. The tumor-progressive activity of KSHV-FLIP is mediated by prevention of death receptor-induced apoptosis triggered by conventional T cells. Consequently, inhibitors of death receptor signaling can be regarded as a new class of tumor progression factors, and HHV-8-associated tumors may represent naturally occurring examples of the tumorigenic effect of such inhibitors.

Key words: apoptosis • Fas receptor • antiapoptotic proteins • herpesvirus • lymphoma

Apoptosis is a highly regulated mechanism that plays a pivotal role in the physiology of organisms and possibly participates in the defence against pathogens and cancer. Cell death can be induced through engagement of the death receptors belonging to the TNFR superfamily (TNFR-1, Fas/Apo1/CD95, DR3/Apo3, DR4, and DR5/Apo2) (1). Upon death receptor ligation, intracellular adapter molecules, such as Fas-associated death domain (FADD)/MORT-1 (mediator of receptor-induced toxicity), TRADD (TNFR1-associated death domain protein), RIP (receptor interacting protein), and RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain) are recruited via death domain interactions (1, 2). These molecules can in turn associate with and activate the upstream caspases through death effector domain (DED) or caspase recruitment domain (CARD) interactions, leading to the activation of the downstream caspases and thus resulting in effective cell death (2, 3).

Excessive or impaired cell death strongly correlates to many disorders (4). Therefore, in normal physiology, the apoptotic machinery must be strictly controlled at several levels to avoid dysregulated cell death. Intracellularly, this control is exerted through several antiapoptotic proteins, many of them targeting the caspases. Interestingly, some viruses have adopted similar strategies to prevent cell death by expressing viral counterparts to these modulators of apoptosis, allowing extended viral replication (5). Viral FLICE (FADD [Fas-associated death domain]-like IL-1β-converting enzyme)-inhibitory proteins (FLIPs), found in several herpesviruses (5, 6), and their cellular homologues (cFLIPs) (7) constitute a novel class of antiapoptotic proteins that prevent the association of the upstream caspases 8 and 10 with the adapter molecule FADD through DED-DED interactions.

Another major pathway for induction of cell death, caused by various types of cellular stress, involves the Bcl-2
family (8). The antiapoptotic protein Bcl-2 was originally discovered as an oncogenic protein in a follicular B cell lymphoma (9). The dual role of Bcl-2 as both an inhibitor of cell death and an oncogenic protein could perhaps apply to other regulators of apoptosis, for instance the proteins counteracting death receptor signaling. We therefore decided to test the hypothesis that proteins that interfere with death receptor-mediated apoptosis can be involved in tumor development. We chose to study the human herpesvirus 8 (HHV-8) gene FLIP, also referred to as Kaposi’s sarcoma-associated herpesvirus FLIP (KSHV-FLIP), K131, or ORF71 for the following reasons. First, KSHV-FLIP is postulated to elicit antiapoptotic activities (6). Second, HHV-8 has been implicated in Kaposi’s sarcoma pathogenesis as well as primary effusion lymphoma or body cavity-based B cell lymphoma (BCBL) and multicentric Castleman’s syndrome in HIV-infected patients (10). Hence, we sought to determine the possible involvement of KSHV-FLIP in tumor establishment and growth.

Materials and Methods

Cell Lines and Mice. Mouse B and T lymphoma cell lines A20 and L5178Y (mock and FasL transfectant) and the human retroviral packaging cell line phoenix-ampho (available at http://www.uib.no/mlb/nolan/NL-phoenix.html) were grown as described (11, 12). A20 is derived from a spontaneous reticulum cell neoplasm found in an old BALB/c mouse (11). Sex- and age-matched (4–6 wk old) inbred BALB/c, (BALB/c × C57BL/6) F1, C57BL/6, and C.B-17SCID mice were obtained from Charles River Labs. Mouse B and T lymphoma cell lines A20 and L5178Y (mock and FasL transfectant) and the human retroviral packaging cell line phoenix-ampho (available at http://www.uib.no/mlb/nolan/NL-phoenix.html) were grown as described (11, 12).

Expression Vectors. Cell Transduction, and Cloning. KSHV-FLIP was amplified by PCR from the BCBL-1 cell line, established from a BCBL (13) using the oligonucleotides K13eou 5’-ACTGGAATTTGACGGCACTTACGAGGTTCTCTGG-3’ and K13bamL 5’-CATGGATCCCCTATGGTGTATGGCGATAGTGTTG-3’. The fragment was inserted into the EcoRI and BamHI sites of the retroviral expression vector pLXSN (14) and then used to transiently transfect the phoenix-ampho packaging cell line. Supernatants containing recombinant viral particles were used for retroviral transduction of A20 cells. Stable G418-resistant clones were obtained by limiting dilution. Mock and KSHV-FLIP-expressing clones were identified by RT-PCR, and the presence of helper virus was excluded by PCR amplification of viral env using the primers 5’-ACCTGAGAGTTACCAACAC-3’ and 5’-TACTTTGAGAGTCTGAGC-3’.

Apoptosis and Limiting Dilution Assays. Sensitivity of mock and KSHV-FLIP clones to Fas-mediated apoptosis was assessed by treating 10^6 cells with 40 ng/ml of the anti–mouse Fas mAb Jo2 (PharMingen) for 24 h at 37°C. Alternatively, the human retroviral packaging cell line phoenix-ampho was transfected with the FasL-hCD8v/sPSG5 vector encoding the soluble mouse FasL, and 5 × 10^5 A20 cells were cultured with 1:16 diluted soluble FasL supernatant or 1:2 diluted supernatant from nontransfected A20 cells in a total volume of 1 ml for 24 h at 37°C. Apoptosis was also assessed with membrane-bound FasL by mixing mock- or mouse FasL-transfected L5178Y cells with 2 × 10^5 A20 cells at an E/T ratio of 1:4 for 24 h at 37°C. Cells were then stained with propidium iodide and annexin-V–fluos (Boehringer Mannheim) according to the manufacturer’s instructions, and apoptosis was monitored by flow cytometry analysis. The mock- and KSHV-FLIP–transduced A20 cell lines and clones were treated with 200 ng/ml of the Fas antibody in a limiting dilution assay for 12 d at 37°C, and the frequency of clonal growth was determined by visual inspection on day 12 and calculated as described (15).

Flow Cytometry Analysis. The expression of Fas on the mock- and KSHV-FLIP–transduced A20 clones was evaluated by incubating 10^6 cells with 1:100 PE-conjugated hamster anti–mouse Fas (Jo2) or 1:100 of an isotype-matched control in the presence of 1:100 anti–mouse CD16/CD32 (Fc-block). Dead cells and debris were excluded from the analysis by gating in forward and side scatter. Determination of Caspase Activity. 6 × 10^6 cells of mock and KSHV-FLIP clones (c)11 and 17 were subjected to 40 ng/ml of the anti–mouse Fas mAb Jo2 for 20, 40, 60, or 120 min at 37°C. Cells were then washed twice in PBS and immediately frozen in liquid nitrogen and stored at −80°C. DEVD- (caspase-3), IETD- (caspase-8), and LEHD-AMC (aminomethyl-coumarin; caspase-9) cleavage was measured using a protocol adopted from Nicholson et al. (16). DEVD- and IETD-AMC were obtained from the Peptide Institute, Inc., and LEHD-AMC was purchased from Enzyme Systems Products. Cell lysates (10^6) and 50 μM substrate were mixed in a reaction buffer (100 mM HEPES for DEVD- and IETD-AMC and 100 mM 2-(N-morpholino)ethanesulfonic acid for LEHD-AMC, 10% sucrose, 0.1% [3-(3-cholamidopropyl) dimethylammonio] propane-1-sulfonic acid [CHAPS], 5 mM dithiothreitol, and 10–40% NP-40, pH 7.2 for DEVD- and IETD-AMC and pH 6.8 for LEHD-AMC) and dispensed in duplicate in a microtiter plate. The cleavage of the fluorogenic peptide substrates was monitored by AMCA, AMCA, and AMCA, and dispensed in duplicate in a microtiter plate. The cleavage of the fluorogenic peptide substrates was monitored by AMCA, AMCA, and AMCA, and dispensed in duplicate in a microtiter plate. The cleavage of the fluorogenic peptide substrates was monitored by AMCA, AMCA, and AMCA, and dispensed in duplicate in a microtiter plate. The cleavage of the fluorogenic peptide substrates was monitored by AMCA, AMCA, and AMCA, and dispensed in duplicate in a microtiter plate. The cleavage of the fluorogenic peptide substrates was monitored by AMCA, AMCA, and AMCA, and dispensed in duplicate in a microtiter plate. The cleavage of the fluorogenic peptide substrates was monitored by AMCA, AMCA, and AMCA, and dispensed in duplicate in a microtiter plate. The cleavage of the fluorogenic peptide substrates was monitored by AMCA, AMCA, and AMCA, and dispensed in duplicate in a microtiter plate.

Tumor samples were obtained using a caliper, and the square root of each tumor area was calculated. Mice were killed with CO2 when the tumors had reached the maximal allowed size of ~1 cm, as decided by the Stockholm Ethical Committee for Animal Experiments, or when the experiment was terminated. Tumor samples were obtained by surgical excision and frozen at −70°C or fixed in a 4% solution of paraformaldehyde and then processed to paraffin blocks. All material was cut into 6-μm-thick sections. The frozen material was mounted on Super Frost microscope slides (Menzel-Glaeser), fixed in cold acetone for 10 min, and stored at −20°C until use for immunostaining. Paraffin sections were mounted on glass slides and used for morphological evaluation after hematoxylin and eosin staining or after immunohistochemistry.

Immunofluorescence and TdT-Mediated dUTP-Biotin Nick-End Labeling Assays. Frozen sections were immunostained for mouse antigens using rat primary antibodies specific for B cell (ID3, B220), cytotoxic T cell (Lyt 2), and macrophage markers (Mac 3), and an avidin–biotin complexes-peroxidase detection system was used ac-
Results and Discussion

To assess the antiapoptotic property of KSHV-FLIP in vitro, KSHV-FLIP was cloned into the retroviral expression vector pLXSN, followed by transduction of a Fas-sensitive subclone of the B lymphoma cell line A20. Two clones (KSHV-FLIPc11 and -c17) and a mock clone were chosen for further studies and tested for sensitivity to apoptosis induced by the agonistic anti-Fas mAb Jo2 or by soluble or membrane-bound Fas ligand. KSHV-FLIP conferred almost complete protection against Fas-mediated apoptosis, whereas the mock clone was as sensitive as the wild-type A20 cell line (Fig. 1). The expression level of the Fas receptor (Fig. 2) and the proliferation rate (data not shown) were similar between sensitive and resistant clones, indicating that the difference in Fas sensitivity is not due to differential expression of Fas and that KSHV-FLIP confers no growth advantage to the cells in vitro. Limiting dilution analysis showed that KSHV-FLIP-positive cells were able to form clones in the presence of anti-Fas mAb at a frequency between 1/11 and 1/37, whereas the corresponding frequencies for the mock-transduced cells were between 1/5,000 and 1/27,000 (Table I). Previous reports have established that the FLIP proteins prevent death receptor-mediated apoptosis by impeding the activity of the upstream caspases 8 and 10 (6, 7), resulting in a disruption of the signal through the caspase cascade and, consequently, a suppression of the activity of the downstream caspases.

The mechanism of action of KSHV-FLIP in the KSHV-FLIP clones 11 and 17 was therefore investigated by monitoring IETD-like activity (caspase-8), LEHD-like activity (caspase-9), and DEVD-like activity (caspase-3) in vitro in a continuous fluorometric assay. The mock and KSHV-FLIP clones 11 and 17 were challenged with 40 ng/ml of the mouse anti–Fas mAb Jo2 and collected at different time points for measurement of AMC release (Fig. 3). The activity of caspase-8 was almost completely blocked in the presence of KSHV-FLIP (Fig. 3 A). Similar results were obtained measuring caspase-9- and caspase-3–like activities (Fig. 3, B and C). Some caspase-3–like activity was, however, observed in the KSHV-FLIP clones but was significantly lower and delayed in time (Fig. 3 C). We have thus shown that KSHV-FLIP can act as an antiapoptotic protein in vitro by interfering with caspase activity, as anticipated from previous reports on other members of the FLIP family (6, 7). Furthermore, KSHV-FLIP allowed clonal cell growth in the continuous presence of death stimuli. As KSHV-FLIP presumably inhibits apoptosis through interaction with death effector domain–containing proteins, it is likely that it can interfere with death signals from other death receptors as well.

We wanted to test the role of KSHV-FLIP in tumor progression in vivo. Therefore, the mock and KSHV-FLIP A20 clones 11 and 17 were injected subcutaneously into two recipient mouse strains, BALB/c (syngeneic) and (BALB/c × C57BL/6) F1 (semiallogeneic), and tumor growth was monitored for 110 d. The semiallogeneic system was chosen to assess whether expression of KSHV-
FLIP would be involved in the so-called hybrid resistance to parental tumors, possibly comprising NK cells (17). The results are shown in Table II and summarized in Fig. 4. The frequency of tumor appearance in BALB/c recipient mice was dramatically higher for KSHV-FLIP– than for mock-transduced cells (90 and 89% for c17 and c11 versus 32% for mock; Fig. 4 A). A similar pattern was seen in the semiallogeneic mice (88 and 65% for c17 and c11 versus 17% for mock; Fig. 4 B). In addition, tumor appearance was delayed in mice injected with mock-transduced cells compared with mice injected with KSHV-FLIP–transduced clones. This holds true both in the syngeneic and semiallogeneic systems (Fig. 4, A and B). Compared with the mock tumors, the KSHV-FLIP tumors reached the maximally allowed size of 1 cm with a higher frequency in both recipient strains (67 and 50% for c11 and c17 versus 11% for mock in BALB/c; 81 and 60% for c17 and c11 versus 11% for mock in (BALB/c × C57BL/6) F1). Furthermore, the KSHV-FLIP tumors grew faster: ~45 d after injection, most KSHV-FLIP tumors had progressed to a size of 1 cm and mice had to be killed. In contrast, the few progressive mock tumors attained a similar size first after 70–90 d after injection, as shown in Fig. 4, C and D. These data strongly suggest that KSHV-FLIP promotes tumor establishment and progression in vivo and significantly increases uncontrolled growth of the tumor. The tumor-promoting property of KSHV-FLIP was, however, not sufficient to allow tumor establishment upon injection of 4 × 106 cells in a completely allogeneic tumor–host system.

In contrast to the tumor cells transduced with KSHV-FLIP, most mock cells were rejected shortly after appearance in both mice strains. This prompted us to determine the nature of the immune response that is triggered and accounts for the elimination of the A20 tumor cells, against which KSHV-FLIP confers protection. Tumor samples were therefore stained for cell markers of B and T cells and macrophages. Histologically, the tumors appeared to be homogeneous growing tumors of large, undifferentiated lymphoid cells staining positively for B cell markers. The percentage of apoptotic cells by TUNEL was higher in mock-transduced (2%) than in KSHV-FLIP tumors (0.5%) (data not shown). To investigate the role of conventional T cells and NK-like cells in tumor clearance, KSHV-FLIP–transduced cells were injected into BALB/c congenic C.B-17ScID mice lacking both the B and T cell compartments due to a recombination deficiency. KSHV-FLIP– as well as mock-transduced cells developed tumors with similar kinetics and size in all mice injected (Fig. 5, A and B). As B cells are normally not involved in the rejection of solid tumors (18), we conclude that conventional T cells, rather than NK-like cells, are necessary for the rejection of A20

### Table I. Results from Limiting Dilution Assays

| Cell line                  | Frequency of clonal cells in the presence of mAb anti-Fas (Jo2) |
|----------------------------|---------------------------------------------------------------|
| A20 pLXSN (mock)           | 1/27,000                                                      |
| A20 KSHV-FLIP              | 1/37                                                          |
| A20 clone 1 (mock)         | 1/5,000                                                       |
| A20 KSHV-FLIP clone 11     | 1/11                                                          |
| A20 KSHV-FLIP clone 17     | 1/29                                                          |

KSHV-FLIP allows clonal growth in the continuous presence of death stimuli. All A20 cell clones and the mock- and KSHV-FLIP–transduced cell lines were treated with 200 ng/ml of Fas antibody in a limiting dilution assay for 12 d at 37°C, and the frequency of clonal growth was determined by visual inspection on day 12 and calculated as described (15).

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**Figure 3.** KSHV-FLIP inhibits caspase-3, caspase-8, and caspase-9-like activities in Fas-triggered A20 cells. Mock (□) and KSHV-FLIP-c11 (●) and -c17 (▲) were treated with the anti–mouse Fas mAb Jo2 and cleavage of the peptide substrates IETD-, LEHD-, and DEVD-AMC was measured using a fluorogenic assay according to the procedure described in Materials and Methods. Time course of (A) IETD-AMC–like (caspase-8), (B) LEHD-AMC–like (caspase-9), and (C) DEVD-AMC–like (caspase-3) processing in Fas-stimulated mock- and KSHV-FLIP–transduced A20 cells is shown. Means and SE of duplicate samples have been calculated.
tumor cells. The results suggest that T cells may have a role in immunosurveillance of death receptor–positive tumors. However, the observation that the frequency of tumor growth was somewhat lower in the semiallogeneic situation (Fig. 4, A and B) suggests that NK-like cells may participate in the rejection of tumor cells lacking KSHV-FLIP.

Our work formally demonstrates that KSHV-FLIP can function as a tumor progression factor by blocking signaling through death receptors and thereby protecting the tumors against rejection mediated by conventional T cells. Mechanistically, this inhibition of apoptosis is shown by our data to be mediated through direct prevention of caspase-8 activation and subsequent inhibition of caspases 3 and 9. The kinetics of caspase-8 induction is very rapid, and caspase-3 activation is delayed as expected. Interestingly, caspase-9 activity is appearing as quickly as caspase-8 activity. This finding may suggest an involvement of the mitochondrial pathway in Fas-mediated apoptosis induced in A20 cells. In fact, it has previously been described that

| Cell type     | A20 mock | A20 clone 11 | A20 clone 17 |
|---------------|----------|-------------|-------------|
| Recipient strain | N. of cells | N. of mice with tumors/ no. of injected mice |
| BALB/c        | 4 × 10⁶  | 2/4         | 3/3         | 4/4         |
|               | 10⁶      | 2/8         | 8/8         | 6/8         |
|               | 2.5 × 10⁵| 2/7         | 6/8         | 8/8         |
| (BALB/c × C57BL/6) F₁ | 4 × 10⁶  | 0/2         | 4/4         | 4/4         |
|               | 10⁶      | 1/8         | 5/8         | 3/5         |
|               | 2.5 × 10⁵| 2/8         | 5/8         | 8/8         |

**Figure 4.** KSHV-FLIP promotes tumor establishment and progression in vivo. Groups of four to eight BALB/c or (BALB/c × C57BL/6) F₁ mice were injected subcutaneously in the interscapular region with three different concentrations of mock- or KSHV-FLIP-expressing A20 cells (KSHV-FLIPc11 and -c17). Occurrence of tumors was monitored every second day for 110 d. Tumor sizes were measured using a caliper, and the square root of the tumor area was calculated. Mice were killed when tumors had reached the maximal allowed size of ~1 cm. The number of BALB/c (A) and (BALB/c × C57BL/6) F₁ (B) mice developing tumors and the number of BALB/c (C) and (BALB/c × C57BL/6) F₁ (D) mice killed due to large tumor size is shown.

**Figure 5.** KSHV-FLIP- and mock-transduced A20 tumor cells grow equally well in C.B-17ScID mice. Two groups of six mice were injected subcutaneously in the interscapular region with mock- or KSHV-FLIP-expressing A20 cells (KSHV-FLIPc11). Occurrence of tumors was monitored every day for 25 d. Tumor sizes were measured using a caliper, and the square root of the tumor area was calculated. Mice were killed when tumors had reached the maximal allowed size of ~1 cm. The number of mice developing tumors (A) and the number of killed mice due to large tumor size (B) are plotted against time.
Fas-induced apoptosis through caspase-8 can be enhanced by release of cytochrome c and the induction of the caspase-9 pathway (19). Therefore, the classification of cells into types I and II based on the apoptotic pathway (via the death-inducing signaling complex or mitochondria) preferentially used by cells undergoing Fas-induced cell death (20) cannot be applied to the A20 B lymphoma cells used in this study.

The tumor-progressive activity of KSHV-FLIP not only provides insight into the role of this protein in pathogenesis of KSHV infection but also defines the family of inhibitors of death receptor signaling as a new class of tumor progression factors. To date, this family includes viral and cellular FLIPs and is likely to grow. It is expected from our data that dysregulated expression of inhibitors of death receptor signaling, such as the cellular FLIPs, will also be involved in tumor formation and progression in humans. Interestingly, the long form of cellular FLIP (cFLIPs) is reported to be upregulated in human metastatic melanoma tumors (7), compatible with a role for FLIP proteins in nonviral tumorigenesis. In addition, the apoptosis inhibitor survivin, which is structurally similar to the baculovirus inhibitor of apoptosis protein, has been shown to be highly expressed in cells of several common human cancers such as lung, colon, pancreas, prostate, and breast cancer and in ~50% of high-grade non-Hodgkin's lymphomas (21).

The newly discovered TRAIL (TNFR-related apoptosis-inducing ligand) receptors 1 and 2 (TRAILR-1/DR4 and TRAILR-2/DR5) have been reported to play an important role in the clearance of tumors (22). As the FLIPs have been reported to inhibit TRAIL signaling (6, 7), this sensitivity to TRAIL-induced apoptosis can be due to the fact that tumor cells express lower levels of FLIP, as observed in human melanoma cells (23). This would predict that tumors expressing high levels of FLIP would be particularly aggressive. In fact, the inhibition of death receptor signaling by loss of Fas receptor expression on certain tumors could enable and enhance metastatic progression (24).

Further knowledge of the role of inhibitors of death receptor signaling in tumorigenesis is needed to comprehend the complex process that leads to malignancy so as to develop efficient diagnostic and therapeutic strategies.

We thank Dr. H. Yagita for providing the FasL-hCD8α/pSG5 vector encoding the soluble murine Fas ligand and for Fas ligand expressing L5178Y cells and Dr. G.P. Nolan for the human retroviral packaging cell line phoenix-ampho. We are very grateful to Dr. Boris Zhivotovsky for helping us to perform the caspase activity assay. We also thank Drs. G. Möller and G. Klein for stimulating discussions and critical reading of the manuscript.

This work was supported by grants from the Jeansson Research Foundations and from the Swedish Medical Research Council (grant no. 16X-12215). M. Djerbi is supported by the National Network and Graduate School for Inflammation Research and V. Screpanti by the Blanceflor Ludovisi-Boncompagni (née Bildt Foundation).

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Submitted: 4 March 1999 Revised: 11 June 1999 Accepted: 30 July 1999

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