Constitutive Expression of c-FLIP in Hodgkin and Reed-Sternberg Cells

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Crosslinking of the transmembrane receptor CD95/Fas leads to activation of a signaling cascade resulting in apoptosis. c-FLIP is a recently described protein that potently inhibits Fas-mediated apoptosis and has been shown to be a key factor in germinal center B cell survival. Because Hodgkin and Reed-Sternberg cells in classical Hodgkin's disease (cHD) are also resistant to Fas-mediated apoptosis we studied the role of c-FLIP in classical HD. High levels of c-FLIP protein were identified in two Fas-resistant Hodgkin-derived cell lines. In contrast to other tumor cells, inhibition of protein synthesis by cycloheximide did not lead to down-regulation of c-FLIP protein in these HD cell lines. Furthermore, Fas-mediated apoptosis was only partially restored suggesting that normal regulation of c-FLIP was disrupted. The *in vivo* relevance of these findings was supported by demonstration of significant c-FLIP expression by immunohistochemistry in 18 of 19 evaluable cases of primary HD. Taken together, c-FLIP is constitutively expressed in HD and may therefore be a major mechanism responsible for Fas-resistance in HD. (*Am J Pathol* 2002, 160:1521–1528)

CD95/Fas protein is a 45-kd transmembrane protein that belongs to the tumor necrosis factor superfamily type of receptors.¹ Crosslinking of the receptor leads to the clustering of an oligomolecular signaling platform, termed death-inducing signaling complex (DISC) that consists of the death domain of the intracellular part of Fas, the adapter molecule Fas-associated-death domain, and the cysteine protease caspase-8. Autoproteolytic cleavage of caspase-8 at the DISC leads to activation of the caspase cascade resulting in the cleavage of DNA and finally, cell death.^{2,3} CD95/Fas plays a critical role in the elimination of autoreactive T and B cells and inactivation of Fas by mutation or deletion in humans results in the

production of autoreactive antibodies, accumulation of activated lymphocytes, splenomegaly, and a high risk for the development of B cell neoplasms.^{4–6}

One downstream key player in CD95/Fas-mediated apoptosis is a protein that has recently been described as the cellular homologue of the viral protein v-Flip termed c-FLIP.^{7,8} A long and a short splice variant of c-FLIP protein are synthesized, c-FLIP_l and c-FLIP_s, respectively.⁸ In cells expressing high levels of c-FLIP_l, Fas-mediated apoptosis is blocked by inhibition of the recruitment of caspase-8 to the DISC, thus preventing its autoproteolytic cleavage and subsequent activation of downstream caspases.^{8,9} c-FLIP overexpression thereby causes resistance to Fas-mediated apoptosis *in vitro* and *in vivo* leading to the accumulation of autoreactive T cells and the development of autoimmune disease.¹⁰ Furthermore, high-level expression of the c-FLIP protein has recently been shown to contribute to a more aggressive phenotype of B lymphoma cells *in vivo* and could be correlated with tumor progression.^{11,12} More recently, evidence has emerged that c-FLIP plays a role in the regulation of apoptosis in naïve B cells.^{13,14} Current work suggests that c-FLIP may be the central factor for survival of germinal center (GC) B cells.^{15,16}

Hodgkin/Reed-Sternberg (HRS) cells represent the malignant cell population in classical Hodgkin's disease (cHD). In most cases, they derive from GC or post-GC B cells.¹⁷ Physiologically, B cells are selected for expression of high-affinity antibody (Ab) in the GC. GC B cells with self-reactive or low-affinity antibody die by CD95/Fas-mediated apoptosis, whereas cells that express immunoglobulin (Ig) with increased affinity for the corresponding antigen are stimulated to proliferate and exit the GC as memory B cells or plasma cells.^{18–20} In contrast, although HRS cells harbor rearranged Ig genes, they do not express a B cell receptor (BCR), partly because of crippling mutations in their somatically mutated Ig gene rearrangements leading to a nonfunctional rearrangement, or by loss of transcription factors important for Ig-transcription, namely Oct2 and Bob1.^{21–24} Thus, HRS cells are crippled GC B cells that physiologically are

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to be eliminated during the GC reaction. Instead, they survive, clonally expand, and lead to disseminated tumor growth and clonal relapse.^{25,26}

Several studies have shown CD95/Fas expression by HRS cells.^{27,28} However, cultured HRS cells are resistant to Fas-mediated apoptosis.²⁹ Mutations in the Fas gene occur only rarely in cHD and because these mutations are observed in GC B cells, too, it is likely that they merely reflect the GC origin of HRS cells.^{30,31} Consequently, it might be conceivable that the defect that rescues HRS cells from apoptosis in the GC is located downstream of the CD95/Fas receptor.

Here, we show that c-FLIP is expressed in the HRS cells in 18 of 19 primary cases of cHD. Using two Fas-resistant HD cell lines as a model, we also demonstrate significant c-FLIP expression and show that treatment with the protein synthesis inhibitor cycloheximide (CHX) fails to down-regulate c-FLIP protein. Consequently, the known Fas-sensitizing effect of CHX was not observed.

Materials and Methods

Cell Lines

L1236 is an Epstein-Barr virus (EBV)-negative cell line that has been derived from the HRS cells of a patient with mixed cellularity subtype of HD.^{32,33} L428 is an EBV-negative cell line.³⁴ L428 cells are CD15- and CD30-positive and harbor clonally rearranged and mutated Ig genes, making a HRS cell derivation probable.^{35,36} Both cell lines are Fas-resistant, although they express wild-type Fas mRNA.²⁹ The human T-lymphoblastic leukemia cell line Jurkat, known to be sensitive toward CD95/Fas-mediated apoptosis, served as a positive control in apoptosis assays. K562, a myeloid cell line derived from a patient with chronic myeloid leukemia during blast crisis is known to express c-FLIP.³⁷ BJAB is a B cell lymphoma cell line that has been shown to down-regulate c-FLIP protein by incubation with CHX. All cell lines were grown in RPMI 1640 (Gibco, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μ g/ml), and glutamine (2 mmol/L) at 37°C in an atmosphere containing 5% CO₂ under sterile conditions.

Induction of Apoptosis

For induction of apoptosis, cells were seeded in 24-well plates at a concentration of 5×10^5 cells/well, suspended in 1 ml of medium supplemented with various concentrations of a mouse anti-Fas monoclonal antibody (mAb) (clone CH11; Coulter Immunotech, Marseille, France) or an isotype-matched control mAb (mouse IgM; Alexis Corp., San Diego, CA). CHX (Sigma Aldrich, St. Louis, MO) was added, depending on the respective experiment. For dose finding of Fas-agonistic antibody, CH11 was used in the following concentrations: 50 ng/ml, 100 ng/ml, 200 ng/ml, and 500 ng/ml. In the subsequent experiments, CH11 was used at 100 ng/ml. For dose finding of CHX, the following concentrations were used: 1

μ g/ml, 10 μ g/ml, and 100 μ g/ml. In the following analyses CHX was used at 10 μ g/ml on L428 and L1236, and at 1 μ g/ml for treatment of Jurkat cells. Cells were incubated overnight and apoptosis was measured after 24 hours of incubation, or at various time points, depending on the experiment performed.

Measurement of Apoptosis

Apoptosis was detected by fluorescence-activated cell sorting (FACS) analysis using phycoerythrin-coupled Annexin-V (Pharmingen, BD, Heidelberg, Germany) and Propidium iodide on a FACScan flow-cytometer (BD). Analyses were performed using CellQuest software (BD).

Western Blot

Cells were incubated with or without various doses of CHX. For extraction of proteins, 1×10^6 cells were harvested, washed twice in ice-cold phosphate-buffered saline, and then lysed in 50 μ l of RIPA buffer. Forty μ g of protein per slot were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gel containing 10% acrylamide. After blotting onto nitrocellulose filters (Hybond C Extra; Amersham-Pharmacia, Freiburg, Germany), a 1-hour incubation with blocking reagent was done to inhibit unspecific binding of antibodies. The blots were incubated overnight with the polyclonal rabbit anti-human c-FLIP₁ antibody (raised against the C-terminus of human c-FLIP₁ protein, concentration 1:1000; Sigma) or with a monoclonal mouse anti-human actin antibody (Chemicon, Hofheim, Germany), to document equal loading of the gel. Subsequently, the blots were washed three times with Tris-buffered saline containing 0.05% Tween and a second goat anti-rabbit antibody coupled to horseradish peroxidase (concentration 1:2000; DAKO, Hamburg, Germany) was added. The Enhanced Chemiluminescence system (Amersham-Pharmacia) was used for development of the blots, according to the manufacturer's instructions.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

mRNA was extracted from cultured cells using the μ Mac^s mRNA Isolation Kit (Miltényi Biotec, Bergisch Gladbach, Germany), following the recommendations of the manufacturer. cDNA synthesis was performed using an oligo-dT oligonucleotide and Superscript reverse transcriptase (Life Technologies, Karlsruhe, Germany). Oligonucleotides were intron-spanning to differentiate between amplified genomic DNA and cDNA sequences; they were designed to hybridize to the 3'-end of the human c-FLIP₁ transcript (c-FLIP₁.S.: acagttcaccgagaagctgact; c-FLIP₁.AS.: tccttg-cagaaactctgctgt). Amplification was performed in a 50- μ l assay containing 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 200 μ mol/L of each dNTP, and 25 pmol of each oligonucleotide. c-FLIP templates were amplified in 35 cycles of denaturation, annealing and synthesis (95°C for

Table 1. Characteristics of Pathological Specimen

Case	HD subtype*	Age	Presentation	Localization	c-FLIP
1	NS	17	First	Supraclavicular	+
2	NS	28	First	Axillar	+
3	NS	64	First	Abdominal	++
4	NS	28	First	Supraclavicular	n.inf.
5	NS	32	First	Supraclavicular	n.inf.
6	NS	18	First	na	++
7	NS	36	Relapse	Abdominal	+
8	NS	34	Relapse	na	—
9	NS	35	Relapse	Abdominal	++
10	NS	21	Relapse	Axillar	+
11	NS	52	Relapse	Cervical	n.inf.
12	NS	33	Relapse	Abdominal	++
13	NS	72	Relapse	Mediastinal	++
14	NS	49	Relapse	Mediastinal	++
15	NS	41	Prim. progr.	Supraclavicular	++
16	NS	26	Prim. progr.	Cervical	++
17	NS	17	Prim. progr.	Supraclavicular	n.inf.
18	MC	26	First	Supraclavicular	++
19	MC	16	First	Supraclavicular	+
20	MC	29	First	na	+
21	MC	72	Relapse	Cervical	++
22	MC	37	Relapse	Abdominal	++
23	LD	17	Relapse	Axillar	+

*NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depleted.

c-FLIP positive were classified as followed: ++, >75% positive HRS cells; +, 25 to 75% positive cells; —, no reactivity.

Prim. progr., primary progressive; na, not assessed/not applicable.

30 seconds; 61°C for 30 seconds; 72°C for 60 seconds). After a final extension step at 72°C for 6 minutes, products were cooled to 10°C, analyzed by agarose gel electrophoresis, and visualized by ethidium bromide staining and UV light. Representative bands were excised, extracted using the Jetsorb kit (Genomed, Bad Oeynhausen, Germany), and directly sequenced using the Ready Reaction Dye Terminator cycle-sequencing kit (Perkin Elmer, Weiterstadt, Germany) on an automated sequencing apparatus (ABI 377, Applied Biosystems/Perkin Elmer). Sequences were compared to published c-FLIP sequences applying the BLAST software from the National Center for Biotechnology Information.

Pathological Specimen

Twenty-three primary cases of classical HD, two nonneoplastic lymph nodes, and one specimen of striated muscle tissue infiltrated by a B cell non-Hodgkin's lymphoma were analyzed by immunohistochemistry. All cases were classified according to the World Health Organization classification and diagnoses were reviewed by the pathologist reference panel of the German Hodgkin's Lymphoma Study Group. Characteristics are listed in Table 1.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections and two nonneoplastic lymph nodes used as positive controls were stained. Rabbit-anti-human c-FLIP polyclonal antibody (Sigma), directed against the long isoform of c-FLIP or the monoclonal mouse anti-human CD30 antibody

Ber-H2 (DAKO) were used in these experiments. Six- μ m sections were mounted on glass slides, deparaffinized in xylene, rehydrated in graded alcohol, and washed in water. The slides were stained following standard procedures. The antibody reaction was detected using avidin-biotin-complex (ABC)-bound alkaline phosphatase (DAKO) and FastRed (DAKO) or NBT (Sigma) as chromogen. After immunostaining, slides were counterstained with hemalaun (Merck, Darmstadt, Germany). Sections from hyperplastic tonsils, striated muscle tissue, reactive lymph nodes containing GCs, and endothelial cells in all analyzed specimen served as external and internal positive controls, respectively. The percentage of c-FLIP-positive cells was estimated by comparing serial sections of most cases, stained either with an anti-CD30 mAb or the polyclonal anti-c-FLIP antibody. Four cases could not be evaluated because of lack of CD30-positive HRS cells in the control sections or overstaining, and were therefore categorized as "not informative." However, it cannot be excluded that the HRS cells in the "not informative" cases did not express c-FLIP protein. A case was categorized as + when 25 to 75% of HRS cells showed at least weak to moderate c-FLIP staining, as compared to CD30-positive cells. When 75 to 100% of HRS cells showed a moderate to strong staining, a case was categorized as ++.

Results

HRS Cells Are Resistant to Fas-Mediated Apoptosis

The two HRS cell lines, L1236 and L428, and the T-cell leukemia cell line Jurkat have recently been shown to express wild-type Fas mRNA and protein.²⁹ These cell lines were incubated with the agonistic anti-Fas mAb CH11 or isotype control at concentrations ranging from 50 to 500 ng/ml and apoptotic cells were determined after 24 hours using Annexin-V and propidium iodide staining. As shown in Figure 1, significant apoptosis was only induced in Jurkat cells (mean $\pm$ SD = 80.5 $\pm$ 8.7% of three independent experiments) whereas the portion of apoptotic cells for L1236 and L428 did not differ significantly when cells were treated with CH11 mAb or isotype control. These data clearly demonstrate that the HRS cell lines L1236 and L428 are resistant to Fas-mediated apoptosis.

c-FLIP_l mRNA and Protein Are Highly Expressed in Fas-Resistant Cultivated HRS Cells

To elucidate potential mechanisms for the observed Fas resistance in HRS cells we studied the expression of c-FLIP by RT-PCR and Western blotting. K562 cells and freshly isolated CD77⁺-CD38⁺ GC B cells served as positive controls. RT-PCR from L428, L1236, and controls using intron-spanning c-FLIP-specific primers yielded a strong signal of the expected size of 192 bp (Figure 2).

Figure 1. HD cell lines L428 and L1236 are resistant to Fas-mediated apoptosis. Jurkat, L1236, and L428 cells (5×10^5) were incubated for 24 hours with 100 ng/ml of agonistic anti-Fas mAb CH11 or the IgM-isotype control mAb. Apoptosis was assessed after 24 hours of incubation as described in Materials and Methods. Results are shown as percentages of apoptotic cells (MV of three independent experiments; bars, SD).

Using identical PCR conditions, attempts to amplify c-FLIP sequences from genomic DNA of L428 and L1236 cells failed because of a large intron between the two primer binding sites (data not shown).

Direct sequencing of the 192-bp RT-PCR fragment and comparison with published c-FLIP₁ sequences under www.ncbi.nlm.nih.gov/blast/ confirmed c-FLIP₁ amplification corresponding to bp 1605 to 1707 of accession no. u97074 (data not shown). Thus, c-FLIP₁ m-RNA is expressed in Fas-resistant cultured HRS cells L428 and L1236, similar to CD77⁺-CD38⁺ centroblasts. Western blotting of cell lysates of L1236, L428, and K562 cells was performed and yielded bands of the expected size of 55

Figure 2. c-FLIP mRNA is expressed in Fas-resistant HRS cell lines L1236 and 428, and in their physiological counterpart, the GC B cells. cDNAs from L1236, L428, and freshly isolated GC B cells were submitted to 35 cycles of RT-PCR amplification using oligonucleotides specific for the long isoform of c-FLIP. Products were analyzed by agarose gel electrophoresis and ethidium bromide staining. cDNA from K562 was co-amplified as a positive control. Equal amounts of cDNAs were verified by amplifying GAPDH transcripts.

Figure 3. c-FLIP protein is not down-regulated in cultured HRS cells after treatment with CHX. Cells were incubated with 1 or 10 $\mu\text{g/ml}$ of CHX. At time points of 0 hours, 12 hours, and 24 hours, 1×10^6 cells were harvested, lysed, and cell lysates were submitted to Western blotting using polyclonal c-FLIP₁ antibody. Time points are indicated. **A:** Western blots from BJAB, L428, and L1236 cells at various time points of treatment with 1 $\mu\text{g/ml}$ of CHX. The 55-kd band represents c-FLIP₁, the 42-kd band represents β -actin. **B:** Western blots from BJAB, L428, and L1236 cells at various time points of treatment with 10 $\mu\text{g/ml}$ of CHX.

kd for all cell lines, thereby demonstrating c-FLIP protein expression of cultured HRS cells (data not shown).

Blockage of Protein Synthesis in Fas-Resistant HRS Cell Lines by CHX Does Not Lead to Down-Regulation of c-FLIP Protein

To determine whether c-FLIP expression is normally regulated in HRS cells, protein synthesis was blocked using CHX because CHX blockade was recently described to down-regulate c-FLIP expression in several cell lines.³⁷ Western blot analyses of lysates of cell lines L1236 and L428 were performed to study c-FLIP protein levels in L428 and L1236 cells treated with doses from 1 to 10 $\mu\text{g/ml}$ of CHX. Cell lysates were prepared of L428 and L1236 cells at different time points (0 hours, 12 hours, 24 hours) of CHX treatment and submitted to Western blotting. Cell lysates prepared from BJAB cells served as controls because down-regulation of c-FLIP by CHX in these cells has been documented.³⁸ Surprisingly, c-FLIP levels in both L428 and L1236 cells remained unaltered throughout the whole time period (24 hours) of CHX treatment whereas c-FLIP levels decreased in BJAB cells at a CHX dose of 1 $\mu\text{g/ml}$ (Figure 3). Augmentation of the CHX dose up to 10 $\mu\text{g/ml}$ had no effect on c-FLIP protein levels in L428 and L1236 cells. Thus, CHX fails to down-regulate anti-apoptotic c-FLIP protein in L428 and L1236 cells.

Fas-Sensitivity Is Moderately Restored by CHX Treatment in L428 but Not in L1236 Cells

CHX is known to sensitize tumor cells to Fas-mediated apoptosis.^{38,39} To test whether CHX treatment would in-

Figure 4. Treatment with 10 $\mu\text{g/ml}$ of the protein synthesis inhibitor CHX leads to a moderate sensitization to Fas-mediated apoptosis in L428 cells. Jurkat, L1236 and L428 cells (5×10^5) were incubated for 24 hours with varying concentrations of CHX (1 $\mu\text{g/ml}$ for Jurkat cells, 10 $\mu\text{g/ml}$ for both L1236 and L428 cells), 100 ng/ml of agonistic anti-Fas mAb CH11 or the IgM-isotype control mAb. Apoptosis was assessed at indicated time points as described in Materials and Methods. Results are shown as percentages of apoptotic cells (MV of three independent experiments; bars, SD). $\square$, Jurkat, 1 $\mu\text{g/ml}$ CHX, 100 ng/ml isotype; $\blacksquare$, Jurkat, 1 $\mu\text{g/ml}$ CHX, 100 ng/ml CH11; $\triangle$, L428, 10 $\mu\text{g/ml}$ CHX, 100 ng/ml isotype; $\blacktriangle$, L428, 10 $\mu\text{g/ml}$ CHX, 100 ng/ml CH11; $\circ$, L1236, 10 $\mu\text{g/ml}$ CHX, 100 $\mu\text{g/ml}$ isotype; $\bullet$, L1236, 10 $\mu\text{g/ml}$ CHX, 100 ng/ml CH11.

crease Fas-mediated apoptosis in HD cells, L1236 and L428 cells were incubated with the Fas-agonistic mAb CH11 (100 ng/ml) or an isotype control antibody in the presence of increasing concentrations of CHX. Induction of apoptosis was assessed between 12 and 24 hours. As expected, significant apoptosis was induced in the positive control cell line Jurkat as early as 12 hours after treatment with the Fas agonistic mAb in the presence of 1 $\mu\text{g/ml}$ of CHX (82.4%; Figure 4) and stayed similarly high throughout the observation period (Figures 4 and 5). Higher concentrations of CHX showed toxic effects as demonstrated by increased apoptotic cells in the isotype control cultures (data not shown). In contrast, neither L1236 nor L428 showed a significant increase in the percentage of apoptotic cells compared to incubation with isotype control mAb under these experimental conditions (data not shown). Only when increasing the concentration of CHX to 10 $\mu\text{g/ml}$ did the number of apoptotic L428 cells increase to 35.1% at 12 hours and 58.9% at 24 hours (Figures 4 and 5). L1236 were still insensitive to Fas-mediated apoptosis under these conditions. Further increasing the concentration of CHX revealed a toxic effect because apoptotic cells increased similarly in the isotype control cultures. Thus, Fas-mediated apoptosis is not induced in L1236 and shows a delayed onset and is of much lower magnitude in L428 cells as compared to Jurkat cells after blocking protein synthesis with CHX.

c-FLIP Protein Is Expressed in HRS Cells of Primary Cases of cHD

While demonstrating expression and altered regulation of c-FLIP in HD cell lines it was critical to demonstrate expression of c-FLIP protein in primary HRS cells by immunohistochemistry. Of the 19 informative cases (see Materials and Methods), 11 cases showed a strong cy-

Figure 5. FACS analyses of Jurkat, L428, and L1236 cells after 24 hours of combined treatment with CHX and Fas-agonistic mAb CH11. Jurkat, L1236, and L428 cells (5×10^5) were incubated for 24 hours with varying concentrations of CHX (1 $\mu\text{g/ml}$ for Jurkat cells, 10 $\mu\text{g/ml}$ for both L1236 and L428 cells), 100 ng/ml agonistic anti-Fas mAb CH11 or the IgM-isotype control mAb. The cells were incubated with phycoerythrin-labeled Annexin V stained with propidium iodide and submitted to FACS analyses. 10,000 events were recorded. Twenty percent of dots are shown. Percentages of apoptotic cells are indicated. **A:** Jurkat cells after a 24-hour treatment with 1 $\mu\text{g/ml}$ of CHX and an isotype mAb. **B:** Jurkat cells after a 24-hour treatment with 1 $\mu\text{g/ml}$ of CHX and 100 ng/ml of CH11. **C:** L428 cells after a 24-hour treatment with 10 $\mu\text{g/ml}$ of CHX and an isotype mAb. **D:** L428 cells after a 24-hour treatment with 10 $\mu\text{g/ml}$ of CHX and 100 ng/ml of CH11. **E:** L1236 cells after a 24-hour treatment with 10 $\mu\text{g/ml}$ of CHX and an isotype-matched control mAb. **F:** L1236 cells after a 24-hour treatment with 10 $\mu\text{g/ml}$ of CHX and 100 ng/ml of CH11.

toplasmic c-FLIP staining in more than 75% of the HRS cells (Figure 6), whereas 7 showed a positive cytoplasmic staining in 25 to 75% of HRS cells. Only one case was found to be negative for c-FLIP protein expression. No correlation was found between histological subtype, clinical characteristics, and c-FLIP expression levels (data not shown). As expected, GC B cells in hyperplastic tonsils and in reactive lymph nodes, striated muscle cells, as well as vascular endothelial cells in the diseased

Figure 6. c-FLIP protein is expressed in HRS cells in HD-involved tissue. Serial sections from HD-involved tissues were stained either with a polyclonal anti-c-FLIP antibody or an anti-CD30 mAb to show HRS cells and then counterstained with hemalaun. The number of c-FLIP⁺ cells was estimated and compared to the number of CD30⁺ cells. Cases with 75 to 100% of HRS cells being positive for c-FLIP were categorized as ++, whereas cases with 25 to 75% of c-FLIP⁺ HRS cells were classified as +. Striated muscle tissue and GCs served as positive controls. **A:** Primary HD case stained with c-FLIP Ab showing one HRS cell. **B:** Primary HD case stained with a polyclonal c-FLIP Ab showing two HRS cells surrounded by lymphoid cells. **C:** Specimen of striated muscle tissue infiltrated by a B cell non-Hodgkin's lymphoma stained with polyclonal c-FLIP Ab. **D:** Reactive lymph node with three GCs and vascular endothelial cells stained with polyclonal c-FLIP Ab. Original magnifications: $\times 200$ (**A**); $\times 400$ (**B**); $\times 100$ (**C** and **D**).

tissue were also positive, thereby demonstrating sensitivity and specificity of the immunohistochemistry procedure used. Thus, c-FLIP protein was synthesized by HRS cells of HD-involved tissue in 18 of 19 informative cases analyzed.

Discussion

We demonstrate here that the anti-apoptotic factor c-FLIP is expressed at high levels in HRS cells in the majority of patients with HD. Furthermore, in contrast to normal GC B cells c-FLIP is likely to be dissociated from normal regulatory circuits in cultivated HRS cells. This was demonstrated by treatment of HRS cells *in vitro* with CHX, which is known to inhibit protein synthesis in eukaryontae thereby significantly reducing the levels of c-FLIP protein. In contrast to other tumor cells c-FLIP protein levels remained unaltered in the HRS cell lines L428 and L1236

upon treatment with CHX, which was also associated with unaltered resistance to Fas-mediated apoptosis. Constitutive expression of c-FLIP might therefore be an important mechanism for the survival advantage of HRS cells.

The normal counterparts of HRS cells, namely GC B cells, have recently been demonstrated to enter the GC with an activated apoptosis program.^{15,16} Activation of the Fas pathway and subsequent clustering of the DISC seem to be critical events in this process. Although it is clearly demonstrated that HRS cells derive from crippled GC B cells, the mechanisms by which these cells are prevented from apoptotic elimination remain elusive. The high-level expression of c-FLIP in HRS cells might play a central role because other mechanisms of inactivation of the CD95/Fas pathway such as mutation or deletion of the CD95/Fas receptor do not seem to be a dominant feature of HRS cells.^{29–31} p53 mutations or bcl-2 rearrangements in HRS cells, both of which could explain Fas resistance

have also not been closely associated with the pathogenesis of HD.^{40,41}

Although both HD cell lines tested showed high levels of c-FLIP expression, and a stringent correlation between Fas resistance and c-FLIP protein levels was eminent in L1236 cells, we were able to demonstrate a residual but delayed Fas sensitivity of L428 cells, however only when challenged with close to toxic concentrations of CHX. It is therefore not ruled out that additional unknown pathways also contribute to resistance to apoptosis in HD.

Whether the dissociation of c-FLIP regulation in HRS cells is because of exogenous or endogenous signals will be an important question for further investigation. Because c-FLIP levels in isolated GC B cells decrease rapidly after the isolation procedure, a stimulatory surrounding might be essential for the maintenance of c-FLIP levels. Survival of GC B cells is restricted to those cells that bear high-affinity BCR on their cell surface. Because HRS cells do not present a BCR on their cell surface, c-FLIP protein expression in these cells is likely disconnected from this physiological regulatory pathway. Another exogenous signal could be delivered via CD40. Primary HRS cells express CD40 in most if not all cases and are surrounded by CD40L-expressing T cells.^{42,43} One might therefore argue that the CD40 pathway is active in at least primary HRS cells. Indeed, normal human and murine B cells up-regulate c-FLIP on stimulation via the BCR and CD40.^{13–15} However in normal primary human B cells c-FLIP expression on separate or concomitant CD40 and BCR signaling is transient and begins to disappear after 24 hours of stimulation.¹⁴ In the absence of BCR expression on HRS cells and CD40L in the culture system, an autocrine signaling loop leading to prolonged c-FLIP expression through chronic stimulation can be excluded.^{42,43} Therefore, other mechanisms must account for the high-level expression in up to 100% of primary HRS cells demonstrated here.

Constitutive NF κ B expression by HRS cells could explain activation of HRS cells that are destined to die.^{44,45} I κ B α mutations in primary and cultured HRS cells have been reported and might underlie activation of NF κ B in a minority of cases, rescuing the HRS cells from programmed cell death.^{46,47} However, a percentage of cases remains for which the causes of apoptosis resistance have to be elucidated. NF κ B activation may be explained by I κ B α mutations or by Epstein-Barr virus-encoded latent membrane proteins (LMP1 and LMP2a). Because c-FLIP is also a potent NF κ B activator, it is tempting to speculate that this represents a putative mechanism for constitutive NF κ B expression.⁴⁸ The precise interaction, however, between c-FLIP and NF κ B in HRS cells remains to be elucidated.

In summary, we demonstrate constitutive expression of anti-apoptotic c-FLIP protein in a panel of primary cHD cases. Furthermore, we demonstrate the failure of two Fas-resistant HRS cell lines to down-regulate c-FLIP in response to CHX treatment. We conclude that constitutive c-FLIP expression may be a central mechanism rescuing HRS cells from Fas-mediated apoptosis.

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References

1. Nagata S, Golstein P: The Fas death factor. *Science* 1995, 267:1449–1456
2. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko ANJ, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM: FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996, 85:817–827
3. Medema JP, Scaffidi C, Kischkel FC, Shevchenko ANJ, Mann M, Krammer PH, Peter ME: FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J* 1997, 16: 2794–2804
4. Fisher GH, Rosenberg FJ, Straus SE, Dale JK, Middleton LA, Lin AY, Strober W, Lenardo MJ, Puck JM: Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative disorder. *Cell* 1995, 81:935–946
5. Drappa J, Vaishnav AK, Sullivan KE, Chu JL, Elkon KB: Fas gene mutations in the Canale-Smith syndrome, an inherited lymphoproliferative disorder associated with autoimmunity. *N Engl J Med* 1996, 335:1643–1649
6. Bettinardi A, Brugnani D, Quiros-Roldan E, Malagoli A, La Grutta S, Corra A, Notarangelo LD: Missense mutations in the Fas gene resulting in autoimmune lymphoproliferative syndrome: a molecular and immunological analysis. *Blood* 1997, 89:902–909
7. Thome M, Schneider P, Hofmann K, Fickenscher H, Meinel E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M, Scaffidi C, Krammer PH, Peter ME, Tschopp J: Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 1997, 386: 517–521
8. Irmier M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J: Inhibition of death receptor signals by cellular FLIP. *Nature* 1997, 388:190–195
9. Scaffidi C, Schmitz I, Krammer PH, Peter ME: The role of c-FLIP in modulation of CD95-induced apoptosis. *J Biol Chem* 1999, 274: 1541–1548
10. Van Parijs L, Refaelli Y, Abbas AK, Baltimore D: Autoimmunity as a consequence of retrovirus-mediated expression of C-FLIP in lymphocytes. *Immunity* 1999, 11:763–770
11. Medema JP, de Jong J, van Hall T, Melief CJ, Offringa R: Immune escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. *J Exp Med* 1999, 190:1033–1038
12. Dierbi M, Screpanti V, Catrina AI, Bogen B, Biberfeld P, Grandien A: The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J Exp Med* 1999, 190:1025–1032
13. Wang J, Lobito AA, Shen F, Hornung F, Winoto A, Lenardo MJ: Inhibition of Fas-mediated apoptosis by the B cell antigen receptor through c-FLIP. *Eur J Immunol* 2000, 30:155–163
14. Hennino A, Berard M, Casamayor-Palleja M, Krammer PH, Defrance T: Regulation of the Fas death pathway by FLICE-inhibitory protein in primary human B cells. *J Immunol* 2000, 165:3023–3030
15. Hennino A, Berard M, Krammer P, Defrance T: Flice-inhibitory protein is a key regulator of germinal center B cell apoptosis. *J Exp Med* 2001, 193:447–458
16. van Eijk M, Medema JP, de Groot C: Cutting edge: cellular Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein protects germinal center B cells from apoptosis during germinal center reactions. *J Immunol* 2001, 166:6473–6476
17. Küppers R, Rajewsky K: The origin of Hodgkin and Reed/Sternberg cells in Hodgkin's disease. *Annu Rev Immunol* 1998, 16:471–493
18. Rajewsky K: Clonal selection and learning in the antibody system. *Nature* 1996, 381:751–758
19. Rothstein TL, Wang JK, Panka DJ, Foote LC, Wang Z, Stanger B, Cui H, Marshak-Rothstein A: Protection against Fas-dependent Th1 me-

- diated apoptosis by antigen receptor engagement in B-cells. *Nature* 1995, 374:163–165
20. Schattner E, Friedman SM: Fas expression and apoptosis in human B cells. *Immunol Res* 1996, 15:246–257
21. Kanzler H, Küppers R, Hansmann ML, Rajewsky K: Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells. *J Exp Med* 1996, 184:1495–1505
22. Marafioti T, Hummel M, Foss HD, Laumen H, Korbjuhn P, Anagnostopoulos I, Lammert H, Demel G, Theil J, Wirth T, Stein H: Hodgkin and Reed-Sternberg cells represent an expansion of a single clone originating from a germinal center B-cell with functional immunoglobulin gene rearrangements but defective immunoglobulin transcription. *Blood* 2000, 95:1443–1450
23. Re D, Müschen M, Ahmadi T, Wickenhauser C, Staratschek-Jox A, Holtick U, Diehl V, Wolf J: Oct-2 and Bob-1 deficiency in Hodgkin and Reed-Sternberg cells. *Cancer Res* 2001, 61:2080–2084
24. Stein H, Marafioti T, Foss HD, Laumen H, Hummel M, Anagnostopoulos I, Wirth T, Demel G, Falini B: Down-regulation of BOB1/OBF1 and Oct2 in classical Hodgkin disease but not in lymphocyte predominant Hodgkin disease correlates with immunoglobulin transcription. *Blood* 2001, 97:496–501
25. Jox A, Zander T, Kornacker M, Kanzler H, Küppers R, Diehl V, Wolf J: Detection of identical Hodgkin-Reed-Sternberg cell specific immunoglobulin gene rearrangements in a patient with Hodgkin's disease of mixed cellularity subtype at primary diagnosis and in relapse two and a half years later. *Ann Oncol* 1998, 9:283–287
26. Jox A, Zander T, Diehl V, Wolf J: Clonal relapse in Hodgkin's disease. *N Engl J Med* 1997, 337:499
27. Xerri L, Carbuccia N, Parc P, Hassoun J, Birg F: Frequent expression of FAS/APO-1 in Hodgkin's disease and anaplastic large cell lymphomas. *Histopathology* 1995, 27:235–241
28. Metkar SS, Naresh KN, Redkar AA, Soman CS, Advani SH, Nadkarni JJ: Expression of Fas and Fas ligand in Hodgkin's disease. *Leuk Lymphoma* 1999, 33:521–530
29. Re D, Hofmann A, Wolf J, Diehl V, Staratschek-Jox A: Cultivated H-RS cells are resistant to CD95L-mediated apoptosis despite expression of wild-type CD95. *Exp Hematol* 2000, 28:31–35
30. Muschen M, Re D, Brauning A, Wolf J, Hansmann ML, Diehl V, Küppers R, Rajewsky K: Somatic mutations of the CD95 gene in Hodgkin and Reed-Sternberg cells. *Cancer Res* 2000, 60:5640–5643
31. Muschen M, Re D, Jungnickel B, Diehl V, Rajewsky K, Küppers R: Somatic mutation of the CD95 gene in human B cells as a side-effect of the germinal center reaction. *J Exp Med* 2000, 192:1833–1840
32. Wolf J, Kapp U, Bohlen H, Kornacker M, Schoch C, Stahl B, Mücke S, von Kalle C, Fonatsch C, Schaefer HE, Hansmann ML, Diehl V: Peripheral blood mononuclear cells of a patient with advanced Hodgkin's lymphoma gives rise to permanently growing Hodgkin-Reed-Sternberg cells. *Blood* 1996, 87:3418–3428
33. Kanzler H, Hansmann ML, Kapp U, Wolf J, Diehl V, Rajewsky K, Küppers R: Molecular single cell analysis demonstrates the derivation of a peripheral blood-derived cell line (L1236) from the Hodgkin/Reed-Sternberg cells of a Hodgkin's lymphoma patient. *Blood* 1996, 87:3429–3436
34. Schaadt M, Fonatsch C, Kirchner H, Diehl V: Establishment of a malignant, Epstein-Barr-virus (EBV)-negative cell-line from the pleural effusion of a patient with Hodgkin's disease. *Blut* 1979, 38:185–190
35. Messineo C, Coupland R, Bakhshi A, Raffeld M, Irving SG, Bagg A, Cossman J: Rearrangement, hypermutation, and possible preferential use of a VH5 gene, VH32, in a Hodgkin's cell line. *Hematopathol Mol Hematol* 1997–1998, 11:19–28
36. Falk MH, Tesch H, Stein H, Diehl V, Jones DB, Fonatsch C, Bornkamm GW: Phenotype versus immunoglobulin and T-cell receptor genotype of Hodgkin-derived cell lines: activation of immature lymphoid cells in Hodgkin's disease. *Int J Cancer* 1987, 40:262–269
37. Hu S, Vincenz C, Ni J, Gentz R, Dixit VM: I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. *J Biol Chem* 1997, 272:17255–17257
38. Fulda S, Meyer E, Debatin KM: Metabolic inhibitors sensitize for CD95 (APO-1/Fas)-induced apoptosis by down-regulating Fas-associated death domain-like interleukin 1-converting enzyme inhibitory protein expression. *Cancer Res* 2000, 60:3947–3956
39. Natoli G, Ianni A, Costanzo A, De Petrillo G, Ilari I, Chirillo P, Balsano C, Levrero M: Resistance to Fas-mediated apoptosis in human hepatoma cells. *Oncogene* 1995, 11:1157–1164
40. Montesinos-Rongen M, Roers A, Küppers R, Rajewsky K, Hansmann ML: Mutation of the p53 gene is not a typical feature of Hodgkin and Reed-Sternberg cells in Hodgkin's disease. *Blood* 1999, 94:1755–1760
41. Gravel S, Delsol G, Al Saati T: Single-cell analysis of the t(14;18)(q32;q21) chromosomal translocation in Hodgkin's disease demonstrates the absence of this translocation in neoplastic Hodgkin and Reed-Sternberg cells. *Blood* 1998, 91:2866–2874
42. Gruss HJ, Hirschstein D, Wright B, Ulrich D, Caligiuri MA, Barcos M, Strockbine L, Armitage RJ, Dower SK: Expression and function of CD40 on Hodgkin and Reed-Sternberg cells and the possible relevance for Hodgkin's disease. *Blood* 1994, 84:2305–2314
43. Carbone A, Ghoghini A, Gattei V, Aldinucci D, Degan M, De Paoli P, Zagonel V, Pinto A: Expression of functional CD40 antigen on Reed-Sternberg cells and Hodgkin's disease cell lines. *Blood* 1995, 85:780–789
44. Bargou RC, Leng C, Krappmann D, Emmerich F, Mapara MY, Bommer K, Royer HD, Scheidereit C, Dorken B: High-level nuclear NF-kappa B and Oct-2 is a common feature of cultured Hodgkin/Reed-Sternberg cells. *Blood* 1996, 87:4340–4347
45. Bargou RC, Emmerich F, Krappmann D, Bommer K, Mapara MY, Arnold W, Royer HD, Grinstein E, Greiner A, Scheidereit C, Dorken B: Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* 1997, 100:2961–2969
46. Emmerich F, Meiser M, Hummel M, Demel G, Foss HD, Jundt F, Mathas S, Krappmann D, Scheidereit C, Stein H, Dorken B: Overexpression of I kappa B alpha without inhibition of NF-kappaB activity and mutations in the I kappa B alpha gene in Reed-Sternberg cells. *Blood* 1999, 94:3129–3134
47. Jungnickel B, Staratschek-Jox A, Brauning A, Spieker T, Wolf J, Diehl V, Hansmann ML, Rajewsky K, Küppers R: Clonal deleterious mutations in the I kappa B alpha gene in the malignant cells in Hodgkin's lymphoma. *J Exp Med* 2000, 191:395–402
48. Hu WH, Johnson H, Shu HB: Activation of NF-kappaB by FADD, Casper, and caspase-8. *J Biol Chem* 2000, 275:10838–10844