c-FLIP confers resistance to Fas-mediated apoptosis in anaplastic large cell lymphoma

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ABSTRACT

We hypothesized that inhibition of the FAS-mediated apoptosis pathway by FLICE-like inhibitory protein (c-FLIP) may contribute to oncogenesis in ALK+ anaplastic large cell lymphoma (ALCL). Treatment with increasing concentrations of CH-11 (CD95/Fas agonistic antibody) had no effect on cell viability of two ALK+ ALCL cell lines, Karpas 299 and SU-DHL1, each expressing high levels of c-FLIP. However, inhibition of endogenous c-FLIP expression by specific c-FLIP siRNA in Karpas 299 and SU-DHL1 cells treated with CH-11 resulted in FAS-mediated cell death associated with increased annexin V binding, apoptotic morphology and cleavage of caspase 8. In 26 ALK+ ALCL tumors, assessed for expression of DISC–associated proteins, CD95/Fas and c-FLIP were commonly expressed, in 23/25 (92%) and 21/23 (91%) tumors, respectively. By contrast, CD95L/FasL was expressed in only 3/26 (12%) ALCL although it was strongly expressed by surrounding small reactive lymphocytes. Our findings suggest that overexpression of c-FLIP protects ALK+ ALCL cells from death-receptor induced apoptosis and may contribute to ALCL pathogenesis.
INTRODUCTION

Anaplastic large cell lymphoma (ALCL)\(^1\) frequently carries the t(2;5)(p23;q35)\(^2\) or other 2p23 locus rearrangements\(^3\) that result in overexpression of anaplastic lymphoma kinase (ALK). The t(2;5), the most common abnormality, creates a novel fusion gene, npm-alk. Among other mechanisms, NPM-ALK is believed to mediate its oncogenic potential through activation of phosphatidylinositol 3-kinase PI3K/Akt and STAT3 signaling pathways resulting in downstream anti-apoptotic signals.\(^4\)-\(^8\)

The extrinsic apoptotic pathway is initiated by death surface receptors, a subgroup of the tumor necrosis factor receptor (TNFR) superfamily, that have a characteristic intracytoplasmic domain, designated the death domain (DD). Upon ligation, CD95 (Fas), the best characterized death surface receptor, oligomerizes at the cell membrane and recruits the adapter protein FADD (Fas-associated death domain protein) through DD-DD association. FADD, in turn, recruits pro-caspase 8 by its death effector domain (DED) initiating the formation of the death-inducing signaling complex (DISC). In the DISC, pro-caspase 8 molecules are located in close proximity and are autocatalytically processed to active caspase 8. In “type I” cells, death receptors induce strong caspase 8 activation, directly activating effector caspases, including caspase 3, to ensure apoptosis. In “type II” cells, low amounts of caspase 8 are activated, depending on a mitochondrial amplification mechanism through the cleavage of BID.\(^9\),\(^10\)

Death-receptor induced apoptosis is inhibited by c-FLIP, a death effector domain (DED)-containing protein with a non-functional caspase-like catalytic
domain with antiapoptotic function in the DISC.\textsuperscript{11,12} The death receptor system has been involved in normal and abnormal immune processes,\textsuperscript{13,14} as well as in Hodgkin\textsuperscript{15,16} and non-Hodgkin lymphomagenesis.\textsuperscript{17} and it is currently attracting interest as a possible therapeutic target.\textsuperscript{18,19}

Here we show that overexpression of c-FLIP in ALCL may confer resistance to apoptosis induced by cell surface signals.

**MATERIAL AND METHODS**

**Cell lines**

Two ALK+ (Karpas 299, SU-DHL-1)\textsuperscript{20} cell lines known to carry the t(2;5) were used. As controls, a CD30+ T-cell lymphoma, Mac2a, (a gift from Dr. K. Elenitoba-Johnson, Salt Lake City, UT), a Hodgkin lymphoma cell line, L-1236, (purchased from DSMZ, Braunschweig, Germany) and a mantle cell lymphoma cell line, Mino, (a gift from Dr. R. Ford, Houston, TX) were also used. The cell lines were maintained according to methods reported.\textsuperscript{20} Karpas 299 and SU-DHL1 cells were treated with the PI3K inhibitor LY294002, and the JAK3 inhibitors, WHI-P131 and WHI-P154, at the indicated concentrations. Whole cell lysates were prepared 24 or 48 hours following treatment. Karpas 299 and SU-DHL1 cells also were infected with the HA-tagged, constitutively active, adenomyrAkt adenovirus \textsuperscript{21} at a multiplicity of infection (MOI) of 20, previously shown to significantly increase $\text{Ser}^{473}$p-AKT levels \textit{in vitro} (not shown).
**Induction and Detection of Apoptosis**

Each cell line (1x10⁶ cells/ml) was incubated with increasing concentrations of the anti-CD95/Fas agonistic CH11 antibody (Beckman Coulter, Fullerton, CA), for 24 hours in 6-well plates and a dose response curve of cell viability was generated. A 200 ng/ml concentration of CH-11 produced approximately 60% and 50% cell death 24 hours after treatment in control Mac2a and Mino cells, respectively (see Results). Cell viability was evaluated using trypan blue staining in triplicate. Apoptosis was assessed with Annexin-V staining (BD Biosciences Pharmingen, San Diego, CA) detected by flow cytometry (FACS Calibur, Becton-Dickinson) and DAPI staining detected by immunofluorescence.⁸

**Western Blot Analysis and Immunofluorescence**

Lysates prepared from cells in log-phase growth or after apoptosis induction were collected, washed twice in cold PBS and lysed at 4°C in lysis buffer as previously described.²⁰ Western blot analysis was performed as reported elsewhere.²⁰ The primary antibodies used were as follows: CD95/Fas (sc-715, polyclonal), CD95L/FasL (N-20, polyclonal) and c-FLIP (H-202, polyclonal) (Santa Cruz Biotechnology, Santa Cruz, CA); procaspase 8 (Ab-3, monoclonal) and cleaved caspase 8 (Asp³⁸⁴, 11G10, monoclonal) (Oncogene, La Jolla, CA); and β-actin (Sigma, St Louis, MO).

Cleaved caspase 8 (dilution 1:50, Oncogene) was also detected using an immunofluorescence method. Briefly, cytopsin cell preparations were incubated with the primary antibody overnight. Detection was achieved by incubation with a
fluorescent anti-mouse antibody (Alexa fluor 488, Molecular Probes, Eugene, OR) at a 1:200 dilution for 1 hour. DAPI (Molecular Probes) was used as counterstain.

**Inhibition of c-FLIP Expression by siRNA**

The sequences of c-FLIP small interference (si) RNA targeting the human c-FLIP gene product were purchased from Ambion Inc (Austin, TX) and were: GGGACCUUCUGGAUAUUUUtt (sense) and AAAAUAUCCAGAAGGUCCtg (antisense). Transient transfection of Karpas 299 and SU-DHL1 cells was performed using the Nucleofector solution “T” (Amaxa Biosystems, Gaithersburg, MD). Approximately 2x10^6 cells were transfected with 0, 5 and 10 μg c-FLIP siRNA using 100μl of T solution (Amaxa Biosystems) according to the manufacturer’s protocol. Cells were harvested at 48 hours following transfection and whole cell lysates were prepared for Western blot analysis. In addition, Karpas 299 and SU-DHL1 cells (each 1x10^6 cells/ml) were pre-incubated with 10 μg of c-FLIP siRNA for 24 hours in 6-well plates and subsequently with 200ng/ml of CH11 (Beckman Coulter). Control transfections with two housekeeping gene siRNA, GAPDH and 4EBP1, were also performed.

**Tissue Microarray and Immunohistochemical Methods**

Twenty-six ALK+ ALCL tumors obtained prior to therapy at The University of Texas MD Anderson Cancer Center between 1984 and 2003 were analyzed using immunohistochemistry after antigen retrieval and tissue microarrays as
previously described. The panel of antibodies used included CD95/Fas (C-20, polyclonal, dilution 1:200), CD95L/FasL (N-20, polyclonal, dilution 1:200) and c-FLIP (H-202, polyclonal, dilution 1:50) (Santa Cruz Biotechnology). Lymph nodes with reactive follicular hyperplasia served as internal controls. A 10% cutoff was used to define positivity of all proteins studied.

RESULTS AND DISCUSSION

ALK+ ALCL Cells Are Resistant to FAS-Induced Apoptosis

Induction of the extrinsic apoptotic pathway using an agonistic anti-CD95/Fas antibody demonstrated no cell death in both ALK+ ALCL cell lines (Figure 1A). In contrast, substantial apoptotic cell death was observed after treatment of Mac2a and Mino cells with increasing concentrations of CH-11. Apoptotic death of Mac2a cells after CH-11 treatment was associated with cleavage of caspase 8 and downregulation of c-FLIP (Supplemental Figure 1).

These results are consistent with previously published data by Dirks et al who described resistance to CD95/Fas-induced apoptosis in ALK+ ALCL cells. They also assessed for fas gene mutations as a possible underlying mechanism, but they did not identify mutations in three ALK+ ALCL cell lines, including Karpas 299. Another possibility would be inhibition of DISC function through overexpression of c-FLIP. In both ALK+ ALCL cell lines (Karpas 299, SU-DHL1), the long (c-FLIPL) and short (c-FLIPS) isoforms of c-FLIP were expressed (Supplemental Figure 2). Of note, the short c-FLIP isoform, which has been
shown to totally prevent caspase-8 cleavage, was expressed at a relatively higher level in ALK+ ALCL cells than in control cell lines.

**Specific Inhibition of c-FLIP Expression by siRNA Induces CD95/Fas-Mediated Apoptosis in ALK+ ALCL**

We show here that a major inhibitor of DISC function, c-FLIP, appears to be responsible for resistance of ALK+ ALCL cells to death-receptor-induced apoptosis. Endogenous c-FLIP expression was completely inhibited when 10 μg siRNA was used (Figure 1B). This was associated with a slight increase in the number of apoptotic cells (Figure 1C,D) that may be due to the endogenous FasL expression (Supplemental Figure 2). However, pre-incubation of ALK+ ALCL cells with c-FLIP siRNA and treatment with CH-11 significantly induced apoptosis, associated with apoptotic morphology and cleavage of caspase 8 (Figure 1E,F). Therefore, inhibition of c-FLIP expression using c-FLIP siRNA sensitized ALK+ ALCL cells to FAS-induced apoptosis. In line with our data, two recent reports demonstrated sensitization to CD95/Fas-mediated cell death in Hodgkin lymphoma cell lines using a specific c-FLIP siRNA. However, additional factors contributing to protection of ALK+ ALCL cells from extrinsic apoptotic signals cannot be excluded.

**Expression of Extrinsic Apoptotic Pathway Proteins in ALK+ ALCL Tumors**

Using a 10% cutoff, CD95/Fas and c-FLIP were overexpressed in 23 of 25 (92%) and 21 of 23 (91%) tumors, respectively, (Figure 2A,C). By contrast,
CD95L/FasL was expressed by neoplastic cells in only 3 of 26 (12%) ALCL tumors (Figure 2B). However, FAS-L was always detected at high levels in the reactive small lymphocytes and endothelial cells, suggesting that the microenvironment of these tumors is capable of producing FAS-L. These results concur with previous findings reported by Sigel and Hsi. In a series of 10 ALCL cases (including 2 ALK+) CD95 was expressed in over 90% of tumor cells in all cases, whereas CD95L was expressed only in a subset of tumor cells.

The mechanisms underlying c-FLIP upregulation in ALK+ ALCL cells most likely are complex. Although, it has been suggested that c-FLIP upregulation depends on NF-κB activation in Hodgkin lymphoma, recent evidence shows that NF-κB is not active in ALK+ ALCL. Therefore, NF-κB - independent mechanisms may be involved. One possibility is that c-FLIP is upregulated by the PI3K/Akt pathway, as Akt can be phosphorylated/activated by NPM-ALK. Here, we show that inhibition of PI3K/Akt downregulates c-FLIP and, inversely, forced expression of constitutively active Akt upregulates c-FLIPs splice variant (Figure 2D). Another possibility is that ALK or other kinases interacting with ALK, such as JAK3, upregulates c-FLIP. Indeed, we have shown that inhibition of JAK3 kinase activity, which has been shown to inhibit ALK activity, also downregulates c-FLIP (Figure 2E).

Taken together, our results suggest that ALK+ ALCL cells are resistant to CD95/Fas-induced cell death due to c-FLIP overexpression. Upregulation of c-FLIP may be a downstream target of oncogenic pathways involved in ALCL pathogenesis, thus providing a novel therapeutic target in ALCL patients.
REFERENCES


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FIGURE LEGENDS

Figure 1. ALK+ ALCL Cells Are Resistant to CD95/Fas-Induced Cell Death As a Result of c-FLIP Overexpression

(a) Using trypan blue exclusion assay, cell viability did not change after treatment of two ALK+ ALCL cell lines, Karpas 299 and SU-DHL-1, with increasing concentrations of the CH-11 antibody, an inducer of the extrinsic apoptotic pathway. These experiments were performed in triplicate and the mean percentage of viable cells was calculated. Flow cytometry also showed no change in Anexin V binding after treatment of Karpas 299 and SU-DHL-1 cells with increasing concentrations of the CH-11 (data not shown). By contrast, cell viability of the control CD30+ T-cell lymphoma cell line, Mac2A, decreased significantly after treatment of cells with increasing concentrations of the CH11 antibody. Approximately 60% death of Mac 2a cells was seen at a concentration of 200 ng/ml CH-11. Similar results were obtained for a control B-cell line, Mino (not shown).

(b) Karpas 299 cells were transiently transfected with 5 μg or 10 μg of c-FLIP siRNA and whole cell lysates were prepared at 48 hours after transfection. Immunoblots showed that endogenous c-FLIP expression was completely inhibited when 10 μg siRNA was used.

(c) Transient transfection of Karpas 299 cells with 10 μg of siRNA resulted in increased annexin V binding (from 21 to 39%) as shown by flow cytometry. Control transient transfections of Karpas 299 and SU-DHL1 cells with 10 μg or 20 μg of siRNA (Ambion) of two housekeeping genes, GAPDH and 4EBP1,
were also performed using the Nucleofector system (Amaxa Biosystems). No changes in cell viability and only minimal changes in the fraction of Annexin V-positive cells were observed (not shown). These experiments were performed in triplicate.

**(d)** Karpas 299 cells were initially transfected with 10 μg c-FLIP siRNA and, at 24 hours post-transfection, the cells were treated with 200 ng/ml CH-11. As shown here inhibition of c-FLIP expression significantly sensitized Karpas cells to FAS-mediated apoptosis induced by CH-11. Cell viability was assessed by trypan blue exclusion studies. Bar graphs show results from experiments performed in triplicate using SU-DHL1 cells. Similar results were obtained using Karpas 299 cells.

**(e)** Annexin V / propidium iodide (PI) staining assessed by flow cytometry showed a substantial increase of Annexin-V positive (from 10% to 45%) and PI-positive SU-DHL1 cells.

**(f)** Evidence of apoptotic morphology (nuclear condensation and fragmentation) in SU-DHL1 cells stained with DAPI (**upper panel**). Membranous and cytoplasmic expression of cleaved caspase 8 detected using an immunofluorescence method in SU-DHL1 cells that were treated with CH-11 and c-FLIP siRNA (**lower panel**). Similar findings were seen using Karpas 299 cells.

**(g)** Immunoblots showed that CH-11-induced apoptotic cell death is associated with cleavage of caspase 8 in SU-DHL1 (and Karpas 299) cells treated with c-FLIP siRNA.
Figure 1

A

Viability (%) vs. CH-11 (ng/ml)

Karpas 299
SU-DHL1
Mac2a

B

c-FLIP siRNA (μg)

Karpas 299

c-FLIP
β-actin

C

Control vs. c-FLIP siRNA (10μg)

Annexin V

D

Viability (%) vs. c-FLIP siRNA and CH-11

E

Annexin V vs. c-FLIP siRNA and CH-11

F

CH-11 vs. CH-11 + c-FLIP siRNA

G

Karpas 299

c-FLIP
Caspase 8 (Δ-Δ92 kDa)
Cleaved caspase 8
β-actin
**Figure 2a-c.** Immunohistochemical detection of extrinsic apoptotic pathway proteins in reactive lymph node and ALK+ anaplastic large cell lymphoma (ALCL) tumors.

(a) CD95/Fas was weakly expressed in a subset of centroblasts within the germinal centers (GC) of reactive lymph nodes (*left*). Strong cytoplasmic and membranous CD95/Fas expression was observed in most ALK+ ALCL tumors (*right*).

(b) CD95L/FasL was strongly positive in the mantle and marginal zones of reactive lymph nodes (*left*). CD95L/FasL was not expressed in most ALK+ ALCL tumors (*right*). Infiltrating small reactive lymphocytes strongly expressed CD95L/FasL and served as internal positive controls in all tumor specimens.

(c) c-FLIP expression was restricted in a subset of GC cells (*left*). Strong cytoplasmic expression for c-FLIP was observed in most ALK+ ALCL tumors (*right*).

**Figure 2d-e.** c-FLIP Upregulation May Result From Activation of PI3K/Akt and JAK3/ALK Oncogenic Pathways

(d) (*left panel*) Karpas 299 and SU-DHL1 cells were treated with a PI3K inhibitor, LY294002, at concentrations of 0, 5, or 20 μg/μL. Whole cell lysates were then prepared at 48 hours. Immunoblots showed that serine 473 phosphorylation of Akt is almost completely inhibited at a concentration of 20 μg/μL that is associated with a substantial decrease of c-FLIP levels.
Karpas 299 and SU-DHL1 cells were infected with the constitutively active, adeno-myrAkt adenovirus at a MOI 20 that significantly increases \(\text{Ser}^{473}\)-pAKT levels in these cells. Expression of adeno-myrAkt was confirmed by Western blot analysis using a monoclonal antibody specific for the hemagglutinin (HA) tag. An adeno-\(\beta\)-Gal adenovirus construct expressing \(\beta\)-Gal served as a control in this experiment. Whole cell lysates were prepared from control and infected cells 48 hours after infection. Immunoblots showed a dramatic increase of Akt phosphorylation, which was associated with an increase of c-FLIP\(_S\) splice variant.

(\textbf{e}) ALK+ ALCL cells were treated with two known JAK3 inhibitors, WHI-P131 and WHI-P154, at concentrations previously shown to inhibit JAK3 and ALK enzymatic activity. Whole cell lysates were prepared 24 hours following treatment. Immunoblots demonstrate that c-FLIP levels are decreased after treatment with WHI-P131 or WHI-P154 in a dose dependent manner.
Figure 2

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