Resistance to Fas-mediated apoptosis is restored by cycloheximide through the downregulation of cellular FLIP_L in NK/T-cell lymphoma

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Extranodal NK/T-cell lymphoma (NKTL), nasal type, is a highly aggressive neoplasm and is strongly associated with Epstein–Barr virus (EBV). In this study, we demonstrate that EBV-positive NKTL cell lines, namely, Hank-1, NK-YS, and NK-L, are resistant to Fas-mediated apoptosis induced by anti-Fas antibodies despite high levels of Fas surface expression and no mutation in the Fas gene. Fas stimulation of Hank-1 and NK-YS cells showed little processing of caspase 8, caspase 3, or bid, although the proximal signaling molecules of the death-inducing signaling complex, namely, Fas, Fas-associated protein with a death domain, caspase 8, and bid were present in these cells. Consistent with previous reports on the hypermethylation of death associated protein (DAP) kinase in NKTLs, the promoter of DAP kinase was methylated and its mRNA not detected in Hank-1 cells. However, the restoration of DAP kinase expression by 5-aza-2′-deoxycytidine did not sensitize Hank-1 to Fas-mediated apoptosis, indicating that DAP kinase deficiency does not contribute to resistance to Fas-mediated apoptosis. Since etoposide-induced apoptosis involved caspase 3 activation in Hank-1 and NK-YS cells, the caspase 3-dependent apoptotic machinery appears to be intact. Interestingly, cotreatment of Hank-1 with cycloheximide, a protein synthesis inhibitor, markedly sensitized cells to Fas-mediated apoptosis along with caspase 8 activation and c-FLIP_L (cellular FLICE inhibitory protein long form) downregulation. Moreover, immunohistochemistry on paraffin-embedded tissue revealed c-FLIP expression in 39% (14 of 36) of NKTL patients. Taken together, these findings indicate that c-FLIP_L-mediated resistance to Fas contributes to the development and progression of NKTLs. This study also suggests that agents capable of downregulating c-FLIP_L could be used to treat NKTL.
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Fas (CD95/Apo-1), a member of the tumor necrosis factor (TNF) receptor superfamily, is a well-known member of the death receptor pathway. Fas stimulation by Fas ligand or an agonistic antibody leads to Fas oligomerization and the recruitment of FADD (Fas-associated protein with a death domain) and caspase 8/FLICE (FADD-like IL-1β-converting enzyme) to the intracellular death domains of Fas, which results in the formation of the death-inducing signaling complex (DISC). DISC formation then triggers the sequential activations of caspase 8 and caspase 3, either directly or through a mitochondrial pathway.[1,2] Fas-mediated apoptosis plays an important role in immune system surveillance as evidenced by its ability to induce tumor cell death, and thus is regarded as a tumor suppressor mechanism.[3] However, many tumors escape the immune system by developing diverse ways to bypass normal Fas activation. One example is provided by somatic mutation of the Fas gene, which has been demonstrated in various lymphoid and nonlymphoid malignancies, including NK/T-cell lymphoma

(NKTL).[4,5] Mutations of the cytoplasmic death domain of Fas might block effective DISC formation,[6] and defects in its transmembrane domain can result in the secretion of soluble Fas, which acts as a decoy receptor for Fas ligand.[7] Ineffective DISC formation, despite of intact Fas signaling molecules, could also endow Fas resistance to lymphoblastoid cells.[8] Apoptosis can also be evaded via the overexpression of inhibitory signaling molecules involved in Fas-mediated cell death, and these include cellular FLICE-inhibitory protein (c-FLIP). c-FLIP contains a 'death effector domain' and binds to FADD and caspase 8 in DISC, thereby blocking caspase 8 activation and the resultant apoptotic cascade.[9,10] Abnormally high c-FLIP expression has been reported in many malignancies, and it has also been reported that the downregulation of c-FLIP sensitizes variable tumor cells to Fas-mediated apoptosis.[11–14]

NKTL is a neoplasm that originates from natural killer (NK) cells and rarely from cytotoxic T cells. Epstein–Barr virus (EBV) is latently infected in nearly all NKTLs irrespective of ethnic or geographic origin, and thus EBV is believed to be an important pathogenic component. Although some patients respond to chemotherapy and radiotherapy, many are resistant to conventional chemotherapy and succumbed to this disease.[15,16] Latent EBV infections are classified into three types according to the genes expressed, and NKTL is committed to type 2 latency expressing Epstein–Barr virus nuclear antigen 1 (EBNA1) and latent membrane protein 1 (LMP1). LMP1 functions as a constitutively activated TNF receptor, and is essential for the transformation and immortalization of EBV-infected cells.[17,18] Moreover, LMP1 is known to exhibit extensive oncogenic properties associated with its activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways.[19,20] Recently, it has been reported that LMP1 also activates the PI3K/Akt pathway to promote cell survival in several cell lines.[21,22] The biologic aggressiveness of NKTL is due to the low cell death rates of NKTL cells and/or their high rates of proliferation. This low cell death rate may be the result of several possible defects in apoptosis, whereas the high proliferation rate may be due to various cell-cycle regulation abnormalities. Some of these changes arise from the involvement of specific proteins such as LMP1, or defects in signaling pathways. Interestingly, tumors with defects in death receptor pathways often exhibit resistance to chemotherapy and radiotherapy. Therefore, the elucidation of these abnormal apoptotic pathways provides information helpful for the development of new and more efficacious treatment modalities.

In this study, we demonstrate that NKTL cell lines are resistant to Fas-mediated apoptosis mainly due to defects in proximal signaling, and that this occurs despite the expression of intact molecules involved in Fas-mediated apoptosis. Death associated protein

(DAP) kinase methylation, although present, was not found to be substantially involved in the Fas resistance of Hank-1. However, cycloheximide (CHX), a protein synthesis inhibitor, restored Fas sensitivity in Hank-1, an NKTL cell line with high LMP1 expression, by suppressing c-FLIP$_L$. The inhibition of Fas-mediated apoptosis probably through c-FLIP is presumed to be an important mechanism in the development and progression of NKTL.

Materials and methods

Cell Lines and Reagents

Three NKTL cell lines, namely, Hank-1, NK-YS, and NK-L were maintained in a 5% CO_2 atmosphere at 37°C. Hank-1 and NK-YS are two IL-2-dependent cell lines that were established from nasal-type NKTLs latently infected with EBV.[23,24] Hank-1 cells were kindly provided by Dr Kagami and Dr Seto (Aichi Cancer Center, Nagoya, Japan) and cultured in COS medium (Cosmo Bio, Tokyo, Japan) containing 5% human plasma and 100 U/ml of human recombinant IL-2 (Chiron-France, Suresnes, France). NK-YS cells were kindly provided by Dr Tsuchiyama and Dr Yoshino (Okayama University Medical School, Okayama, Japan) and maintained in Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY, USA) containing 10% FBS (Gibco, Gaithersburg, MD, USA), 1% kanamycin and 100 U/ml of IL-2. NK-L cells were kindly provided by Dr Seto (Aichi Cancer Center, Nagoya, Japan) and cultured in RPMI 1640 medium containing 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine, and 200 U/ml of IL-2. The human T-cell lymphoma cell line, Jurkat, and the EBV-infected lymphoblastoid cell line, SKW6.4 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in RPMI 1640 medium containing 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mM L-glutamine. Mouse anti-human Fas monoclonal antibody (IgM clone 7C11; Coulter-Immunotech, Marseilles, France) was used up to 200 ng/5 × 10^5/ml to induce Fas-mediated apoptosis in the different cell lines. Etoposide, CHX (Sigma, St Louis, MO, USA), dexamethasone, actinomycin D, and staurosporin (BioVision, Mountain View, CA, USA) were added directly to the culture media at different concentrations.

Cell Proliferation and Viability Assay

Hank-1, NK-YS, NK-L, Jurkat, and SKW6.4 cells were seeded at a density of 1–4 × 10^4/well in 96-well tissue culture plates. The cells were treated with anti-Fas monoclonal antibody (7C11) or with other apoptotic inducers for 8–24 hours (h), and then incubated with MTS [3-(4,5-dimethylthiazol-2-yl)-

5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, WI, USA) at 37°C for an additional hour. Sample spectrophotometric absorbance at 490 nm was determined using an Ultra Multifunctional Microplate Reader. Each experiment was performed in triplicate and results were averaged.

Flow Cytometry

To determine apoptosis, treated cells were harvested and incubated for 10 min at room temperature (RT) with 3 μl of fluorescein isothiocyanate (FITC)-conjugated annexin V reagent (Pharmingen, San Diego, CA, USA) and 1 μl of 50 μg/ml propidium iodide (PI) in binding buffer. Cells were analyzed using a FACScan (Epics XL, Coulter, Marseille, France). Annexin V positive and PI positive cells were both deemed apoptotic. Data are presented as means $\pm$ standard deviation (s.d.), and the significances of differences were determined using the Mann–Whitney *U*-test in SPSS 11.0.1 (Stanford version).

To evaluate Fas cell surface expression, about 2×10^5 cells were washed twice with cold PBS containing 0.1% BSA, and incubated for 30 min on ice with 10 μg/ml FITC-conjugated anti-CD95 monoclonal antibody (BD Pharmingen, San Diego, CA, USA). After washing twice and fixing with 4% paraformaldehyde, 5000 cells were analyzed by flow cytometry.

To measure mitochondrial membrane potential ($\Delta \Psi_m$) disruption, 1×10^5 treated cells were incubated with 20 nM 3,3'-diethyloxacarbocyanine iodide (DiOC$_6$) (Molecular Probes, Eugene, OR, USA) for 20 min at 37°C. $\Delta \Psi_m$ was determined by flow cytometric analysis.

Sequencing of Fas and Caspase 3 Gene Transcripts

Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH, USA), and 2 μg of total RNA was used for cDNA synthesis with oligo(dT) primers and M-MLV reverse transcriptase (Promega, M531A). Fas transcripts were amplified using two pairs of primers designed to amplify the entire coding region. The sequences of the primers used were as follows: F1 5'-TCTTTCACTTCGGAG GATTGCT-3', R1 5'-GAACTTTCTGTTCTGCTGTGT CTTG-3', F2 5'-TGCCAAGAAGGGAAGGAGTA-3', R2 5'-ACCAAGCAGTATTTACAGCCAG-3', which were designed from Fas mRNA (GeneBank accession no. M67454). PCR was performed using a 5 min initial denaturation at 95°C, 31 amplification cycles (denaturation for 1 min at 95°C, annealing for 1 min at 53°C, and extension for 1 min at 72°C) and a 10 min final extension at 72°C. Amplified PCR products were purified using a PCR purification kit (Qiagen GmbH, Hilden, Germany) and directly sequenced from both directions using an ABI PRISM 377 Sequencer (Applied Biosystems, Foster City, CA, USA).

Methylation-Specific PCR and RT-PCR for DAP Kinase

To demethylate CpG islands, Hank-1 cells were incubated with 1–10 μM of 5-aza-2'-deoxycytidine (5-aza) for 6 days with media changes on day 2 and 4. For methylation-specific PCR (MSP), DNA was modified with 1 μg of genomic DNA using a CpGenome™ DNA modification kit (Chemicon, Temecula, CA, USA) according to the manufacturer's protocol. The PCR primers used for the unmethylated reaction were: forward 5'-GGAGGA TAGTTGGATTGAGTTAATGTT-3' and reverse 5'-CAAATCCCTCCCAAACACCAA-3'. And, the primers used for the methylated reaction were: forward 5'-GGATAGTCGGATCGAGTTAACGTC-3' and reverse 5'-CCCTCCCAAACGCCG A-3'. The PCR conditions used were same for both PCRs and are as follows: 5 min of initial denaturation at 95°C, 40 amplification cycles (denaturation for 1 min at 95°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C), and 10 min final extension at 72°C.

For the RT-PCR of DAP kinase, total RNA extraction and cDNA synthesis were carried out as described above. cDNA was amplified using the following primers, forward 5'-GATAGAAATGTCCC CAAACCTCG-3' and reverse 5'-TCTTCTTTGGATCC TTGACCAGAA-3'. The PCR conditions used were 5 min at 95°C, 28 amplification cycles (1 min at 95°C, 1 min at 60°C, and 1 min at 72°C), and 10 min at 72°C. Human β-actin cDNA was used as a control for RT-PCR, and amplified using the primers described above.

Western Blot

After washing, cells were lysed with 2 × sample buffer (20 mM TRIS, pH 8.0, 2 mM EDTA, 2 mM DTT, 1 mM Na$_3$VO$_4$, 2% SDS, and 20% glycerol). Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). In all, 30–50 μg of total cellular proteins were subjected to 8–15% SDS-PAGE and transferred to PVDF (polyvinylidene fluoride) membranes. The membranes were then blocked with 5% nonfat dry milk in TTBS (0.1% Tween 20 in Tris-buffered saline) for 1 h at RT or overnight at 4°C. Membranes were then incubated overnight at 4°C with anti-caspase 3 (1:500, rabbit polyclonal, Santa Cruz, CA, USA), anti-cleaved caspase 3 (1:500, rabbit polyclonal, Cell Signaling, Beverly, MA, USA), anti-caspase 8 (1:100, rabbit polyclonal, Santa Cruz), anti-caspase 9 (1:500, rabbit polyclonal, Santa Cruz), anti-bcl-2 (1:500, mouse polyclonal, Santa Cruz), anti-bcl-xL (1:500, rabbit polyclonal, Santa Cruz), anti-bax (1:500, mouse polyclonal, Santa Cruz), anti-human Fas (APO-1) antibody (1:500, mouse polyclonal, clone APO-1-3, Alexis Corporation, Coger,

Paris, France), anti-human FADD (1:250, mouse, BD Pharmingen, San Diego, CA, USA), anti-FLIP (1:250, rabbit polyclonal, Upstate Biotechnology, NY, USA), anti-LMP1 (1:250, mouse, BD Pharmingen), and anti-DAP kinase (1:500, mouse monoclonal, BD Transduction). After washing with TTBS 3 times, membranes were then incubated with a secondary horseradish peroxidase-conjugated antibody at 1:5000 for 1 h at RT. Blots were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire, England).

Caspase 3 Enzyme Activity Assay

Cells were harvested by centrifugation at $450\,g$ for 10 min at 4°C. After removing supernatant, cell pellets were resuspended in $100\,\mu$l of cell lysis buffer (50 mM HEPES pH 7.5, 1 mM DTT, 0.1 mM EDTA, 0.1% CHAPS), and lysed by repetitive freezing in -70°C and thawing on ice. The cell lysates were cleared by centrifugation at $15\,000\,g$ for 20 min at 4°C and the resulting supernatants were defined as cell extract. In all, $100\,\mu$g of cell extracts was, respectively, added to caspase assay buffer (100 mM HEPES pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM DTT, $200\,\mu$M DEVD-pNA) with or without $100\,\mu$M Boc-D. These mixtures were then incubated at 37°C for 4 h and the absorbance due to pNA (a yellowish color) release was measured using an ELISA reader at 405 nm.

Immunohistochemistry

To detect the expression of c-FLIP in tumor tissues, 39 NKTL patients were randomly selected and immunohistochemical staining was performed using anti-c-FLIP mouse monoclonal antibody (Santa Cruz). After deparaffinization and rehydration, slides were immersed in 1 mM ethylene diamine tetraacetic acid (EDTA) buffer, and boiled in a steamer for 1 h. After blocking endogenous peroxidase activity, primary antibody was applied at a dilution of 1:50 overnight at 4°C. The slides were then incubated in biotinylated goat anti-mouse/rabbit Ig (LSAB Kit, DAKO, Carpinteria, CA, USA) and then in a solution of streptavidin–biotin complex (LSAB Kit, DAKO). Immunoreactivity was visualized using 3,3-diaminobenzidine. A tissue microarray slide containing 50 cases of Hodgkin lymphoma was immunostained at the same time as a positive control for c-FLIP expression.[14]

Results

Resistance to Fas-Mediated Apoptosis in NKTL Cell Lines

To determine the initial sensitivity of NKTL cell lines to Fas-mediated apoptosis, Hank-1, NK-YS, and NK-L cell lines were treated with anti-human Fas mAb (IgM clone 7C11). As a positive control for Fas-mediated apoptosis, Jurkat or the SKW6.4 cells were included in all experiments. Apoptotic cell percentage was defined as the sum of annexin V- and/or PI-positive cells. In addition, cell viability and proliferate activity were assessed by MTS assay. Hank-1 cells exhibited apoptosis only in $7.3\pm5.7\%$ (mean$\pm$s.d.) of cells after treatment with anti-Fas mAb for 8 h and in $17.6\pm9.8\%$ after treatment for 24 h as compared with SKW6.4 cells, which showed $50.3\pm9.8\%$ at 8 h and $64.0\pm11.6\%$ at 24 h, respectively. The significances of the differences in Fas-mediated apoptosis shown by SKW6.4 and Hank-1 cells were estimated using the Mann–Whitney U-test ($P<0.01$). Other NKTL cell lines, namely, NK-YS and NK-L, also exhibited strong resistance to Fas-mediated apoptosis (Figure 1a). Consistent with the results of the apoptosis assay, MTS assays revealed only about 23% growth inhibition in Hank-1 in contrast to marked inhibition (up to 66% of the control level) in Jurkat cells after 24 h of treatment with anti-Fas mAb. Strikingly, NK-YS and NK-L cells showed little impairment of cell viability or proliferation by Fas stimulation (Figure 1b).

During apoptosis, alterations in mitochondrial membrane potential (Ψ_m) are followed by cytochrome c release and caspase activation.[1,2] Thus, we examined Ψ_m changes by measuring the uptake of the mitochondrial specific dye $DiOC_6$ in cells undergoing apoptosis. As shown in Figure 1c, a distinct loss of Ψ_m was observed in SKW6.4 cells after 8 h of anti-Fas mAb treatment, but not in Hank-1 or NK-YS cells, which correlated well with Fas-mediated apoptosis resistance results. These data indicated that Hank-1 and NK-YS cells are resistant to Fas-mediated cell death.

Normal Fas Expression and the Presence of Proximal Fas-Signaling Molecules in NKTL Cell Lines

To exclude the possibility that impaired Fas expression causes resistance to Fas-mediated apoptosis in NKTL cell lines, we evaluated Fas cell surface expression by flow cytometry and total Fas protein levels by Western blot analysis. More than 80% of Hank-1, NK-YS, NK-L, and control Jurkat cells showed Fas cell surface expression (Figure 2a), and similar levels of Fas protein were observed in SKW6.4, Hank-1, and NK-YS cells (Figure 2b). Next, we confirmed the presence of only a single gene transcript of Fas in Hank-1, NK-YS, and Jurkat cell lines by repetitive RT-PCR analyses using two pairs of primers described in Materials and methods (data not shown). Sequencing of the purified RT-PCR products (in duplicate) using the above primers revealed no mutations or deletions of Fas gene transcripts (data not shown). As seen in Figure 2b, Western blot analysis of Hank-1 and NK-YS cells using anti-Fas antibody revealed Fas of expected size (48 kDa) and similar as the control SKW6.4

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cells. Next, we examined the presence of other Fas signaling molecules, such as FADD, procaspase 8, and c-FLIP by Western blot. Immunoblotting data disclosed the presence of these molecules in Hank-1, NK-YS, and control Jurkat cells (Figure 2c). These
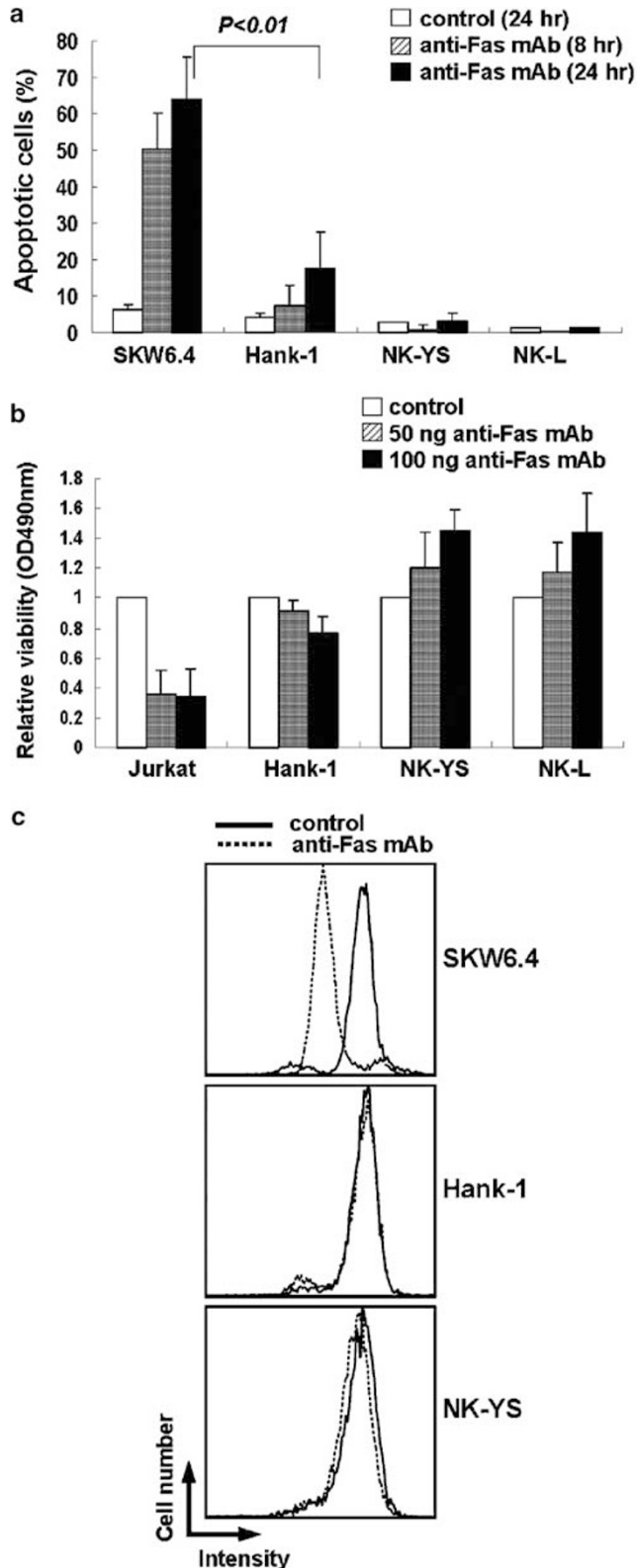
data indicated that normal Fas is expressed on the cell surface and that Fas-associated signaling molecules are present in Hank-1 and NK-YS cells.
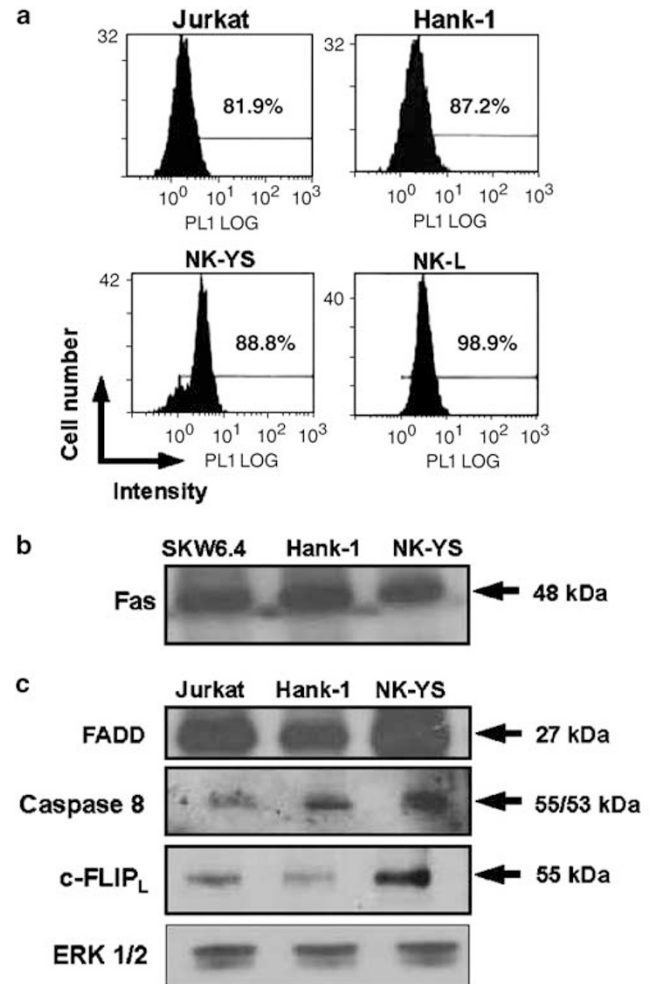

Figure 2 Intact Fas signaling molecules in NKTL cell lines. (**a**) To evaluate cell surface Fas expression, control Jurkat cells and NKTL cell lines, namely, Hank-1, NK-YS, and NK-L (2×10^5 cells) were stained with FITC-conjugated anti-CD95 (Fas) monoclonal antibody and analyzed by flow cytometry. (**b**) In all, $30 \, \mu g$ of total cell lysates from each cell line were separated by SDS-PAGE and subjected to Western blot analysis using anti-Fas antibody. (**c**) In all, $30–50 \, \mu g$ of cell lysates from each cell line were separated by SDS-PAGE and subjected to western blot analysis using anti-FADD, anti-caspase 8, and anti-c-FLIP.

Figure 1 Resistance to Fas-mediated apoptosis in NKTL cell lines. (**a**) Cells were treated with anti-Fas mAb (7C11) $200 \, ng/5 \times 10^5$ cells/ml for 8 or 24 h, stained with annexin V–propidium iodide (PI), and analyzed by flow cytometry. The percentage of apoptotic cells shown is based on the total numbers of annexin V- or PI-positive cells. Data represent means of five separate experiments, and error bars represent standard deviations (s.d.). (**b**) Cells were treated with anti-Fas mAb $50 \, ng/5 \times 10^5$ cells/ml or $100 \, ng/5 \times 10^5$ cells/ml for 24 h, and subjected to MTS assay to evaluate cell viability and proliferation. Data are the average absorbances at $490 \, nm$ of two experiments performed in triplicate. (**c**) To determine mitochondrial membrane potential (Ψ_m) disruption, 1×10^5 cells, were treated for 8 h with anti-Fas mAb, incubated with $20 \, nM$ $3,3'$-diethyloxacarbocyanine iodide (DiOC$_6$) and analyzed by flow cytometry.

Dispensability of DAP Kinase in Fas-Mediated Apoptosis in Hank-1 Cells

DAP kinase, a proapoptotic serine/threonine kinase with a death domain, is involved in the execution of apoptosis mediated by Fas, TNF-α, and interferon-γ in some cells.[25] The downregulation of DAP kinase by its promoter methylation has been suggested associated with Fas resistance, as was found in T- and NK/T-cell lymphoma as well as B-cell lymphoma.[26] Therefore, we evaluated the methylation status of DAP kinase gene and its possible involvement in Fas-mediated apoptosis in Hank-1 cells. DAP kinase was methylated and its mRNA expression was undetected in Hank-1 cells. Although a methylated band of DAP kinase (98 bp) was reduced by 5-aza treatment in Hank-1 cells, and its mRNA (343 bp) and protein expression 160 (160 kDa) appeared vs β-actin (Figure 3a), the recovery of DAP kinase expression per se did not sensitize Hank-1 cells to Fas-mediated apoptosis (Figure 3b). These data suggest that DAP kinase is dispensable in the Fas-mediated apoptosis of Hank-1 cells.

Reduced Activations of Caspase 3, Caspase 8, and bid After Anti-Fas mAb Treatment in NKTL Cell Lines

The transduction and execution of apoptotic signals requires the coordinated action of caspases, which are present in cells as inactive procaspases and activated by the removal of a prodomain and cleavage into large and small subunits.[27] Caspase 3 is the final common pathway in the execution of both the death-receptor and the mitochondrial apoptotic pathways.[2,27] To explore the downstream effects of Fas stimulation, caspase 3 activation was evaluated by Western blot using two different anti-caspase 3 antibodies. The first antibody recognized inactive procaspase 3 (32 kDa), intermediate (22 kDa), and active (19 and 17 kDa) forms of caspase 3, whereas the second antibody (anti-cleaved caspase 3-specific antibody) recognized selectively the intermediate and active forms of caspase 3. In SKW6.4 and Jurkat cells, caspase 3 was converted from its inactive form to its active forms, as evidenced by a marked reduction in the level of the inactive proform (solid arrow) and an increase in the active forms (dash arrows), after only 2 h of anti-Fas mAb treatment (Figure 4a). In contrast, Hank-1 cells showed little detectable active caspase 3 after 8 h of treatment with anti-Fas mAb (Figure 4a). In NK-YS cells, procaspase 3 did not quantitatively change and the active forms of caspase 3 did not appear even after 24 h of Fas stimulation. The pattern of active forms of caspase 3 noted in immunoblots using anti-cleaved caspase 3-specific antibody was in accordance with the above results (Figure 4a). In addition, caspase 3 enzyme activity was increased by 11-fold in control SKW6.4 cells, only by 1.8-fold in Hank-1 cells, and by little in NK-
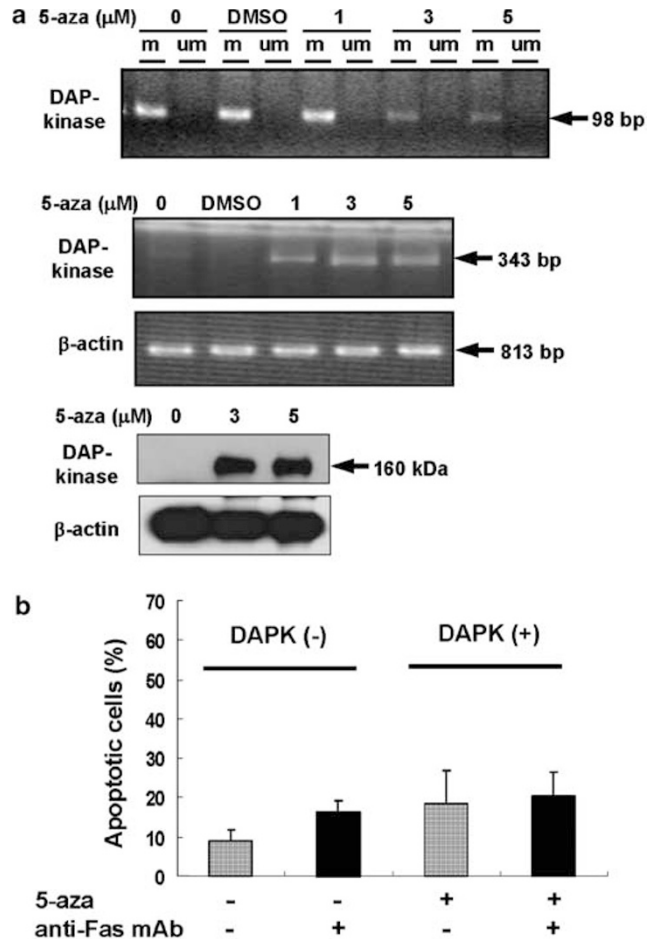

Figure 3 DAP kinase methylation and its effect on Fas-mediated apoptosis in Hank-1 cells. (**a**) Hank-1 cells were incubated with 5-aza at 1, 3, and 5 μM for 6 days, and total DNA and RNA were extracted. Then, methylation-specific PCR (MSP) was performed to detect methylated and unmethylated bands (upper panel). To evaluate demethylation of DAP kinase and its expression, RT-PCR for DAP kinase was also performed using the primers detailed in Materials and methods (middle panel). In all, 30 μg of total cell lysate from each cell line was separated by SDS-PAGE and subjected to Western blot analysis using anti-DAP kinase antibody (lower panel). (**b**) Hank-1 cells were pretreated with or without 5-aza at 5 μM for 6 days and then stimulated with anti-Fas mAb 200 ng/5 × 10^5 cells/ml for 24 h. Fas-mediated apoptosis was evaluated by annexin V–PI staining and flow cytometric analysis.

YS cells (Figure 4b), which was consistent with Western blot results.

To exclude the possibility of functional defects in caspase 3-mediated apoptosis per se in Hank-1 and NK-YS, we examined the cell death induced by etoposide, a prototypic reagent for caspase 3-dependent apoptosis. Hank-1 and NK-YS cells treated with etoposide displayed apoptosis accompanied by caspase 3 activation (Figure 5a,b). These results indicate that the caspase 3-dependent apoptotic machinery, but not the Fas-mediated machinery, is intact in these NKTL cell lines. Next, we examined the proximal Fas-signaling pathways before caspase 3 activation, as represented by
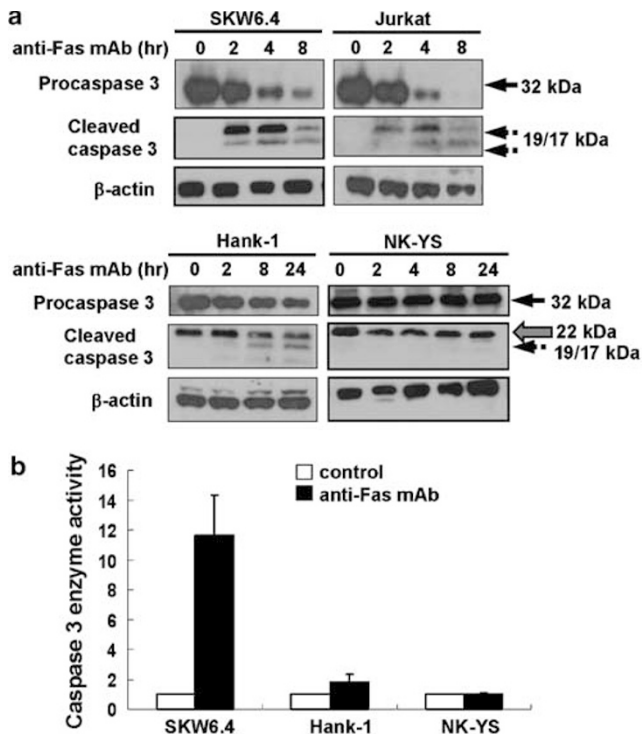
Figure 4 Effect of anti-Fas mAb on caspase 3 activation in Hank-1 and NK-YS cells. (**a**) Cells were treated with $200 \, ng/5 \times 10^5$ cells/ml of anti-Fas mAb for the described hours, and $30 \, \mu g$ of total cellular proteins were subjected to SDS-PAGE. Caspase 3 activation was analyzed by Western blotting using two different antibodies that recognized procaspase 3 (32 kDa, solid arrow) and active cleaved caspase 3 (19 and 17 kDa, dash arrows), respectively. In Hank-1 and NK-YS cells, intermediate caspase 3 (22 kDa, gray arrows) were found to be constitutively expressed. (**b**) Cells treated for 24 h with $200 \, ng/5 \times 10^5$ cells/ml of anti-Fas mAb were resuspended in cell lysis buffer, and $100 \, \mu g$ of cellular extract was added to caspase assay buffer with or without $100 \, \mu M$ of Boc-D as substrate. After incubation at $37^\circ C$ for 4 h, an absorbance of the yellowish color released by the enzymatic action of caspase 3 was measured using an ELISA reader fitted with a 405 nm filter. The results shown are the averages of three separate experiments, and the error bars represent s.d.

caspase 8 and bid processing by Western blot analysis. Following Fas stimulation, procaspase 8 (55/53 kDa) was promptly processed and activated in SKW6.4 cells but not in Hank-1 and NK-YS cells (data not shown). In addition, bid protein was not truncated in Hank-1 or NK-YS cells, whereas the 22 kDa-sized wild-type bid expression was markedly attenuated after Fas stimulation in SKW6.4 cells (data not shown). These data demonstrate that in Hank-1 and NK-YS cells, the Fas-mediated signal might be blocked at a proximal step by inhibitors such as c-FLIP before it reaches the mitochondria or executive caspase 3.

Restoration of Sensitivity to Fas-Mediated Apoptosis by CHX in Hank-1 Cells

Based on reports that the inhibition of Fas-mediated apoptosis by c-FLIP is relieved by protein synthesis inhibitors in various cancer cell lines,[11–14] we
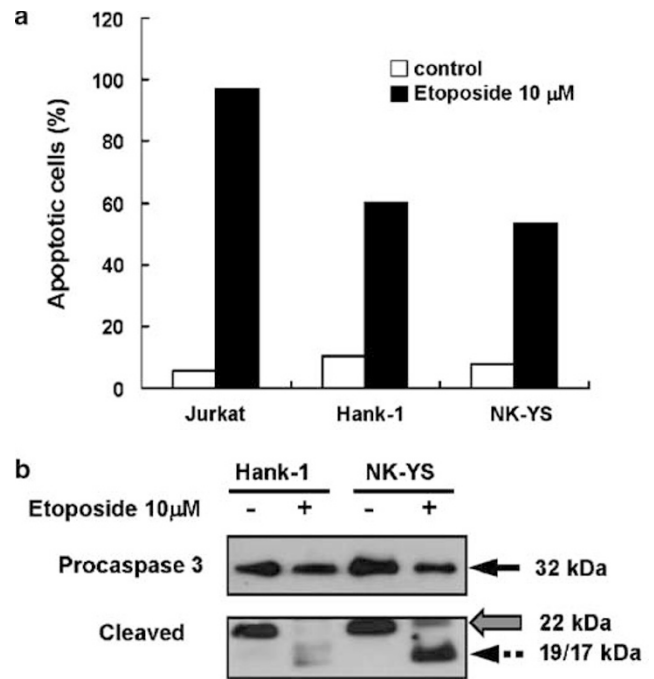

Figure 5 Intact etoposide-induced caspase-3-dependent apoptotic machinery in Hank-1 and NK-YS cells. (**a**) Hank-1 and NK-YS cells were treated with etoposide at $10 \, \mu M/6 \times 10^5$ cells/ml and stained with annexin V and PI. Levels of apoptosis were measured by flow cytometry. The data shown represent the total numbers of annexin V- or PI-positive cells. (**b**) Alterations in caspase 3 levels during etoposide-induced apoptosis were evaluated by Western blotting of cell lysates obtained from the preceding experiment using the two antibodies described in the legend of Figure 3a. Caspase 3 was activated as evidenced by the reduced intensity of procaspase 3 (32 kDa) (solid arrow) and by the increased intensity of the 19 and 17 kDa blots of active caspase 3 (dash arrow).

investigated the influence of the protein synthesis inhibitor, CHX, on Fas-induced apoptosis in Hank-1 cells. Hank-1 cells were treated with nonlethal doses of CHX (5 and $10 \, \mu M$) with or without anti-Fas mAb for 24 h, and then apoptosis levels were measured by flow cytometry. Interestingly, as shown in Figure 6a, treatment of Hank-1 cells with CHX and anti-Fas mAb together dramatically increased apoptosis up to 42.4 ± 20.9 and $66.2 \pm 9.9\%$ (mean $\pm$ s.d.) compared with cells treated with anti-Fas mAb only. The significances of differences were estimated by comparing Fas-induced apoptosis levels between Hank-1 cells treated with and without CHX ($P < 0.01$). In addition, cotreatment of Hank-1 cells with CHX and anti-Fas mAb resulted in an increase in procaspase 8 processing and the mitochondrial membrane potential change (Figure 6b,c). It was likely that CHX enhanced sensitivity to Fas-mediated apoptosis by downregulating inhibitory molecules such as c-FLIP. To test this possibility, we examined the effect of CHX on c-FLIP expression level by immunoblotting. As shown in Figure 6d, the level of $c\text{-FLIP}_L$ was dramatically reduced in CHX-treated Hank-1 cells. However, no marked changes in bcl-2, bcl-xL, or bax expression levels were observed, except for a slight decrease in bax
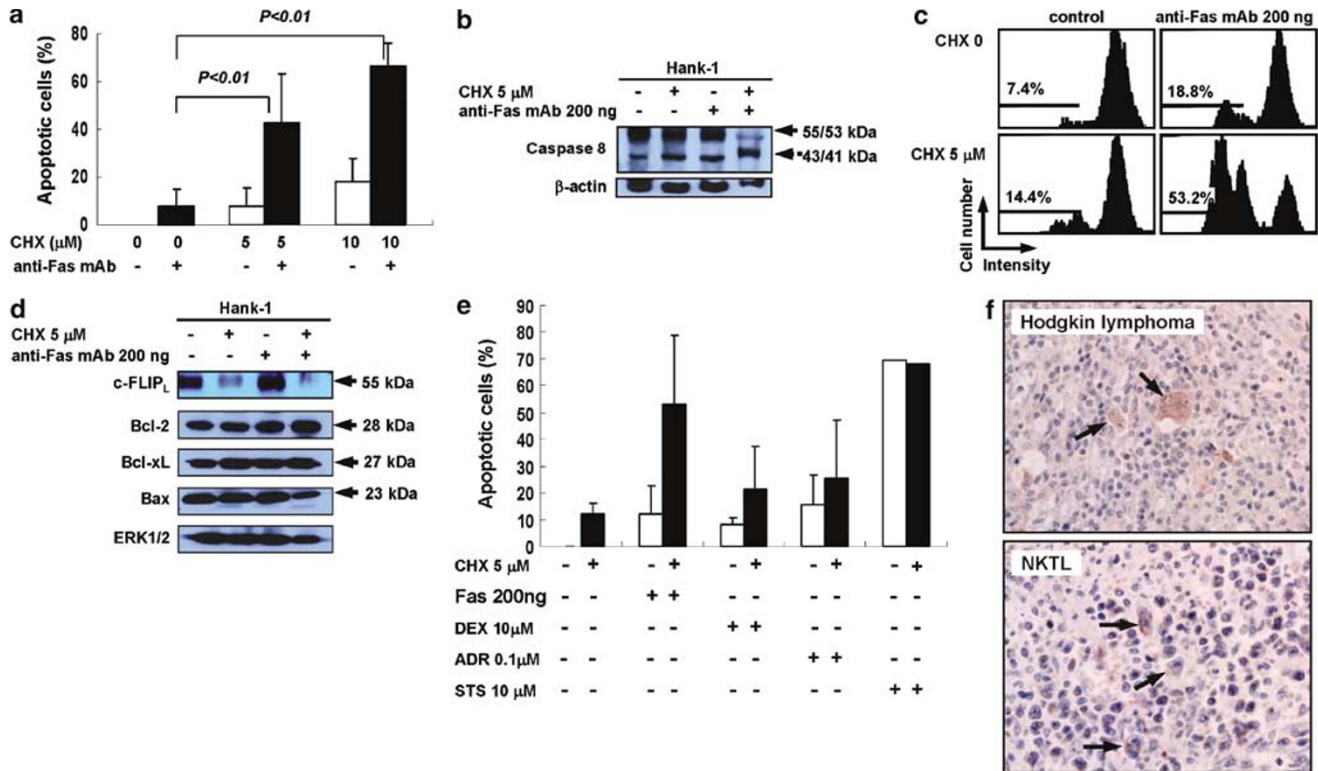
Figure 6 Effect of CHX on Fas-mediated apoptosis in Hank-1 cells. (**a**) CHX at concentrations of 5 or 10 μM was applied to Hank-1 cells for 24 h with or without 200 ng/5 × 10⁵ cells/ml of anti-Fas mAb. Apoptosis was measured by annexin V and PI staining and flow cytometric analysis. The histogram represents the percentage of apoptotic cells based on the total number of annexin V- or PI-positive cells. The results shown are averages of five separate experiments, and the error bars represent s.d.s. (**b**) To examine caspase 8 activation after cotreatment with CHX and anti-Fas mAb, 30 μg of cell lysate from treated cells was subjected to Western blotting using anti-caspase 8 antibody. (**c**) Mitochondrial membrane potentials were also measured by DiOC₆ staining and flow cytometric analysis in Hank-1 cells treated with CHX and anti-Fas mAb for 24 h, respectively or simultaneously. (**d**) To evaluate changes in c-FLIP, bcl-2, bcl-xL, and bax expression, Hank-1 cells were treated with CHX 5 μM and/or 200 ng/5 × 10⁵ cells/ml of anti-Fas mAb, and 30–40 μg aliquots of cell lysate were subjected to Western blot analysis. (**e**) To examine the sensitization effect of other apoptotic inducers of CHX in Hank-1 cells, CHX (5 μM) was added to Hank-1 cells for 24 h without or with dexamethasone (10 μM), adriamycin (0.1 μM), or staurosporin (10 μM). Apoptosis was measured by annexin V or PI staining and flow cytometric analysis. The histogram represents the percentage of apoptotic cells defined based on the total number of annexin V- or PI-positive cells. The results shown are averages of two separate experiments, and the error bars represent s.d.s. (**f**) To examine the *in vivo* expression of c-FLIP in NKTL, immunohistochemical staining was performed on formalin-fixed paraffin-embedded tissue sections of 36 NKTL patients. c-FLIP was immunostained in the cytoplasm with diffuse or granular pattern (arrows) in Hodgkin/Reed-Sternberg cells of Hodgkin lymphoma (upper panel) and in the tumor cells of NKTLs (lower panel).

level. In addition, CHX did not affect Hank-1 cell death induced by other apoptotic agents not involving the Fas death receptor, that is, by dexamethasone (10 μM), adriamycin (0.1 μM), or staurosporin (10 μM) (Figure 6e). Altogether, these data demonstrate that CHX treatment converts Fas-resistant Hank-1 cells to Fas-sensitive cells probably by desensitizing the effects of inhibitory molecules such as c-FLIP$_L$.

Expression of c-FLIP in NKTL Tissues

Finally, to elucidate the biological meaning *in vivo* of the above results obtained from *in vitro* experiments, we investigated c-FLIP expression in NKTL tissues obtained from 36 patients by immunohistochemistry. c-FLIP immunostaining was observed in the cytoplasm as a diffuse or

granular pattern in tumor cells of NKTL and in Hodgkin/Reed-Sternberg cells of Hodgkin lymphoma (Figure 6f). c-FLIP expression was detected in 14 (39%) of the 36 patients. Therefore, it was considered that resistance to Fas-mediated apoptosis by c-FLIP might contribute to NKTL tumor progression.

Discussion

NKTL is common in South-East Asia including Korea, Hong Kong, Japan, and China, and in Central-South America. NKTL is notable for its clinical aggressiveness and resistance to conventional chemotherapy in the advanced stage.[28] The observation that apoptotic rates in non-necrotic viable areas are low despite the common immunohistochemical

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expressions of Fas and Fas ligand in NKTL tumor cells, suggests the possibility of Fas resistance in NKTLs.[29] Frequent Fas gene mutations have been found in studies on NKTL tumor tissues, and these have been suggested to be a major mechanism of resistance to Fas-mediated apoptosis in NKTLs.[4,5] However, the NKTL cell lines utilized in our study, that is, Hank-1 and NK-YS, exhibited resistance to Fas-mediated apoptosis despite high levels of Fas cell surface expression and the absence of Fas gene mutations. Therefore, we presumed that NKTLs have developed another ways of evading Fas-mediated apoptosis.

Fas-mediated apoptosis occurs primarily via two steps, that is, via a proximal signaling pathway after Fas-stimulation and via a subsequent mitochondrial pathway. Cells can be divided into two types according to the main Fas-signaling pathway used. In type I cells, Fas stimulation is followed by large amount of DISC formation and prompt caspase 8 activation, and apoptotic cascades are continued by the direct caspase 3 activation as well as via the mitochondrial pathway. On the other hand, in type II cells, DISC formation is markedly reduced, and caspase activation is preceded by the mitochondrial pathway.[1] Therefore, although undetectable DISC formation after Fas stimulation may be one of the causes of resistance to Fas-mediated apoptosis,[8] a reduced level of DISC formation alone is not enough to explain Fas-insensitivity. In Hank-1 and NK-YS cells, participants in the proximal signaling pathway such as Fas, FADD, caspase 8, and bid protein were all intact, and the nonreceptor-mediated caspase 3-dependent apoptotic machinery provoked by etoposide was also preserved. Nevertheless, Hank-1 and NK-YS cells showed very low rates of Fas-mediated apoptosis. Therefore, we speculated that these cells might resist Fas-mediated apoptosis by blocking efficient proximal signaling.

c-FLIP has two isoforms, c-FLIP long form (c-FLIP$_L$) and c-FLIP short form (c-FLIP$_S$), both of which suppress Fas-mediated apoptosis by interfering with the processing of caspase 8.[9] In vivo, tumors escape host immune surveillance via the c-FLIP-mediated inhibition of the death receptor pathway, and thus c-FLIP is regarded as a tumor progression factor.[30,31] High c-FLIP expression has been found in many malignancies, including aggressive B-cell lymphoma, and Hodgkin lymphoma.[12,30,32–34] In some malignant cells including EBV-infected Burkitt lymphoma, the relative levels of caspase 8 and c-FLIP were reported to determine susceptibility to Fas-mediated apoptosis.[10,35] Moreover, the downregulation of c-FLIP sensitized Hodgkin lymphoma cells,[14] hepatocellular carcinoma cells,[12] Jurkat T-cell lymphoma cells,[11] osteosarcoma cells,[13] and endothelial cells[36] to Fas-mediated apoptosis. c-FLIP is a short-lived protein, and thus the prototypic method used to downregulate c-FLIP is protein synthesis inhibition by CHX. In our study, treatment with sublethal doses of CHX dramatically restored sensitivity to Fas-mediated apoptosis in Hank-1 cells, and this was accompanied by the cleavage of procaspase 8 and an alteration in mitochondrial membrane potential along with the downregulation of c-FLIP$_L$ expression. We tried to downregulate c-FLIP expression selectively by treating with c-FLIP antisense oligonucleotides; however, this failed due to the very low transfection efficiency of Hank-1 cells. The in vivo demonstration of c-FLIP expression in 39% of NKTL patients further suggests that c-FLIP-mediated resistance to Fas might enable tumor cells to escape host immune response and play a role in the pathogenesis of NKTL. c-FLIP expression is regulated by NF-κB and the PI3K/Akt pathway.[37,38] In Hodgkin lymphoma, NF-κB modulated c-FLIP-mediated tumor cell resistance to apoptosis induced by death receptor, which was restored by CHX- and by the small interfering RNA (siRNA)-induced downregulation of c-FLIP.[14] In Hank-1 cells, LMP1 was expressed at high levels as determined by immunoblotting (data not shown). LMP1 induces various antiapoptotic molecules, and promotes cell survival through the activation of NF-κB and PI3K/Akt pathways.[21,22] Recently, it was reported that c-FLIP is one of the proteins induced by LMP1 in an NF-κB-dependent manner in EBV-infected B lymphoblastoid cell lines.[39] Therefore, it is conceivable that c-FLIP might be actively upregulated in Hank-1 cells by the constitutive LMP1-induced activation of the NF-κB and PI3K/Akt pathways. This issue must be progressed by obtaining direct evidence.

Unlike c-FLIP, DAP kinase does not actively participate in DISC, but rather functions further downstream of death receptor complex and upstream of other caspases.[25] Death receptor pathways may convey signals to diverse branches, and DAP kinase functions along some of these branches.[25] Therefore, the functional significance of DAP kinase during death-receptor mediated apoptosis differs in cell lines, and some DAP kinase-negative cell lines commit to TNF-α-induced apoptosis.[40] Despite the absence of DAP kinase expression due to its promoter methylation, the Fas-mediated apoptosis of Hank-1 cells was restored by CHX treatment. However, the recovery of DAP kinase expression by treating with demethylating agent did not sensitize Hank-1 to Fas-mediated apoptosis. Therefore, we conclude that DAP kinase does not play an important role in Fas-mediated apoptosis in Hank-1 cells.

During our apoptotic experiments, the intermediate form of caspase 3 (22 kDa) was found to be present constitutively in Hank-1 and NK-YS cells. Moreover, when RT-PCR products were sequenced, we did not observe either transcripts of the unexpected size or mutations of caspase 3 transcripts (data not shown). During caspase 3 activation, initial cleavage occurs between the small and large procaspase 3 subunits at a site recognized by initiator

caspase 8 and 9, and by granzyme B, which gives rise to a 22 kDa intermediate product. However, this intermediate form of caspase 3 does not have caspase 3 activity in itself, but it must be autocatalytically further processed to 19/17 kDa-sized active forms. NKTLs have abundant cytotoxic granules containing granzyme B. Granzyme B has been reported to be responsible for the partial processing of caspase 3 in activated nonapoptotic CD3-positive cytotoxic T cells.[41] The inhibition of granzyme B serine protease activity by Z-ADD.cmk in Hank-1 and NK-YS cells diminished the intermediate form of caspase 3 without affecting cell viability (data not shown). Inhibitors of apoptosis (IAP) family proteins and the small heat shock protein αB-crystallin have been known to block the autocatalytic processing of intermediate caspase 3;[42,43] however, the physiologic roles of this partial processed caspase 3 remain unknown.

In conclusion, this study revealed that CHX treatment causes the Fas-resistant NKTL cell line, Hank-1, to become Fas-sensitive, probably by relieving c-FLIP$_L$-mediated inhibition of the proximal Fas signaling pathway. Moreover, some NKTL tissues were found to express c-FLIP in tumor cells. Our results suggest that c-FLIP-mediated Fas-induced apoptosis inhibition is a mechanism that underlies the development and progression of NKTL. Thus, the modulation of the Fas-mediated apoptotic pathway by CHX should be viewed as a potential therapeutic target in NKTL.

Acknowledgements

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