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# Characterization of the Interleukin-1β-Converting Enzyme/Ced-3-Family Protease, Caspase-3/CPP32, in Hodgkin's Disease

## *Lack of Caspase-3 Expression in Nodular Lymphocyte Predominance Hodgkin's Disease*

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**Apoptosis (programmed cell death) serves an important role in the normal morphogenesis, immunoregulation, and homeostatic mechanisms in both normal and neoplastic cells. Caspase-3/CPP32, a member of the ICE/Ced-3-family of cysteine proteases, is an important downstream mediator of several complex proteolytic cascades that result in apoptosis in both hematopoietic and nonhematopoietic cells. Previous studies have demonstrated that caspase-3 is commonly expressed in classical Hodgkin's disease (CHD); however, the biological significance of its expression in Hodgkin's disease is unknown. In this report, the expression of caspase-3 in nodular lymphocyte predominance Hodgkin's disease (NLPHD) was evaluated by immunohistochemistry; in addition, we investigated the role of caspase-3 in CD95 (Fas)-mediated apoptosis in three CHD cell lines. Formalin-fixed, paraffin-embedded tissue sections from 11 cases of NLPHD were immunostained for caspase-3 using a polyclonal rabbit antibody that detects both the 32-kd zymogen and the 20-kd active subunit of the caspase-3 protease. Only 1/11 cases of NLPHD demonstrated caspase-3 immunopositivity in lymphocytic/histiocytic cells. Caspase-3 expression was also evaluated in three CHD cell lines, HS445, L428, and KMH2. Whereas caspase-3 expression was detected in HS445 and L428 cell lines, no expression was found in KMH2 cells by immunohistochemical staining. Treatment of HS445 and L428 cell lines for 72 hours with agonistic CD95 monoclonal antibody induced marked apoptosis that was significantly inhibited by pretreatment with the caspase-3 inhibitor, DEVD-FMK, as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay and flow cytometric analysis of 7-amino-actinomycin D staining. In addition, a significant increase in caspase-3 activity as determined by an enzyme colorimetric assay was detected in HS445 and L428 cells after 48 hours of CD95 stimulation. In marked contrast, treatment of caspase-3-deficient KMH2 cells with anti-CD95 mAb did not demonstrate an increase in caspase-3 activity or induce apoptosis. These data demonstrate caspase-3 is important for CD95-mediated apoptosis in CHD cell lines. In addition, the majority of NLPHD cases examined in this study failed to express detectable levels of caspase-3, suggesting these tumor cells may be resistant to apoptotic stimuli dependent on caspase-3 activity. Furthermore, these data suggest the differential expression of caspase-3 noted between NLPHD and CHD may provide additional evidence that each is a unique disease entity.** ***(Am J Pathol 1999, 154:1439–1447)***

Increased understanding of the physiological and pathological processes of programmed cell death, or apoptosis, at the molecular level will provide insights into carcinogenesis and potentially create new opportunities for development of novel prognostic markers and therapeutic tools for the treatment of various neoplasms. One of the earliest cell death-regulating genes to be identified was the proto-oncogene Bcl-2, an apoptosis inhibitor that appears to block a step in an evolutionarily conserved pathway involved in apoptosis.[1–2] Subsequent investigations led to the isolation of a homologue of Bcl-2 in the nematode *Caenorhabitis elegans*. This homologue, called Ced-9, is necessary for the survival of all cells in this organism.[3] Ced-9 opposes the actions of two cell death-

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promoting genes, Ced-3 and Ced-4, which are critical for apoptosis in *C. elegans*.[4] The gene product of Ced-3 demonstrates homology to the mammalian interleukin-1$\beta$-converting enzymes (ICE), a group of cysteine proteases.[5] Ced-4 is thought to be homologous to Apaf-1, a mammalian protein that can associate with several death proteases to promote apoptosis.[6]

To date at least 13 members of the ICE/Ced-3 family (caspases) have been identified, the majority of which, on activation, are involved in the induction and execution phases of apoptosis.[7,8] Of these cysteine proteases, caspase-3 (CPP32, Yama, apopain) is believed to be one of the most commonly involved in the execution of apoptosis in various cell types.[7] On cleavage by other caspases, caspase-3 gives rise to two active subunits with molecular masses of 17–20 kd and 10–12 kd.[7,9] These subunits assemble to form an enzymatically active tetrameric complex.[9] Activation of caspase-3 has been described in a number of cell types undergoing apoptosis induced by a variety of stimuli, including CD95 (Fas/Apo-1) signaling.[10,11]

CD95, a cell surface protein receptor belonging to the tumor necrosis factor (TNF)/nerve growth factor receptor family, is an important molecule in the induction of apoptosis in both hematopoietic and nonhematopoietic cells.[9–15] Mutations in the gene that codes for CD95 have been linked to the development of autoimmune disease and lymphoproliferative disorders in both humans and animal models.[16–18] Previous studies demonstrated that crosslinking of the CD95 receptor on the cell surface by agonistic antibody or by its ligand, CD95L, induced apoptosis that was dependent on caspase activation.[19–23] Furthermore, the inhibition of CD95-mediated apoptosis by blocking proteolysis of caspase-3 by viral proteins is suggested to play a role in the pathogenesis of various neoplasms.[10,11,14,15]

The role of caspases, including caspase-3, applied to apoptotic processes in Hodgkin's disease is currently undefined. In this report, we demonstrate caspase-3 plays an important role in CD95-mediated apoptosis in classical Hodgkin's disease (CHD) cell lines. Furthermore, we demonstrate that nodular lymphocyte predominance Hodgkin's disease (NLPHD) lacks caspase-3 expression by immunophenotypic analysis. The lack of caspase-3 expression in NLPHD may contribute to the development and pathogenesis of this disease by imbuing tumor cells with resistance to caspase-3-dependent apoptotic pathways.

## Materials and Methods

### Case Selection, Histological Examination, and Immunohistochemistry of NLPHD

Formalin-fixed, paraffin-embedded tissue sections from 11 cases of NLPHD were selected from the surgical pathology files of Loyola University Medical Center and the University of Michigan Medical School for immunohistochemical determination of caspase-3. Diagnosis of NLPHD was performed using established criteria on lymph node biopsy histology and immunohistochemistry.[24,25] NLPHD was diagnosed by the finding of typical nodular architecture and lymphocytic/histiocytic (L&H) cells with the appropriate CD20- and CD45RB-positive immunophenotype.

Table 1. Antibodies Used in Immunohistochemical Staining of NLPHD

| Antibody | Source | Dilution |
|---|---|---|
| LCA (PD7/26/16 and 2B11) | DAKO (Carpinteria, CA) | 1:50 |
| CD30 (Ber-H2) | DAKO | 1:40 |
| CD20 (L26) | DAKO | 1:100 |
| EMA (E29) | DAKO | 1:100 |
| CD15 (Leu-M1) | Becton Dickinson, (San Jose, CA) | 1:50 |
| CD45RO (A6) | Zymed Laboratories (San Francisco, CA) | 1:50 |

Morphology assessment of NLPHD cases was performed on 4-$\mu$m tissue sections with hematoxylin-eosin. Immunoperoxidase staining of lymph node sections with the antibodies listed in Table 1 was performed using a Ventana 320 automated stainer (Ventana Medical Systems, Tucson, AZ) and a streptavidin/horseradish peroxidase detection kit (Ventana), with microwave antigen retrieval and trypsin pretreatment used as necessary. The chromogen was 3,3′-diaminobenzidine tetrahydrochloride (DAB).

### Cell Lines

The CHD cell lines KMH2, L428, and HS445 were used in this study. KMH2 and L428 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). HS445 and Jurkat cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured in RPMI 1640 (Gibco-BRL, Grand Island, NY) supplemented with 20% (v/v) heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 mmol/L L-glutamine (Gibco-BRL), 25 mmol/L Hepes (Sigma), and antibiotic-antimycotic solution (Sigma). All cell lines were maintained at 37°C in a humidified incubator at 5% $CO_2$.

### Immunohistochemical Analysis for Caspase-3 Expression

Four-micron-thick formalin-fixed, paraffin-embedded tissue sections from each case of NLPHD were deparaffinized in xylene, hydrated in graded alcohol, and pretreated for antigen retrieval in 10 mmol/L citrate buffer, pH 6.0, for 10 minutes. Cytospins from CHD cell lines were fixed in a 1:1 mixture of acetone and methanol for 10 minutes. Staining was performed using polyclonal rabbit anti-human CPP32 (1:200 titer, DAKO Corp., Carpinteria, CA) and a Vectastain ABC peroxidase, rabbit IgG detection kit (Vector Laboratories, Burlingame, CA) with

3-amino 9-ethyl carbazole (AEC) as the chromogen. The chromogen DAB was used for paraffin-embedded specimens. Formalin-fixed, paraffin-embedded tissue sections from three cases of caspase-3-positive nodular sclerosis Hodgkin's disease and a reactive tonsil were used as positive controls for caspase-3 staining.

### *Apoptosis Induction and Detection*

For apoptosis assays, $1 \times 10^6$ cells from each cell line were cultured in 24-well tissue culture plates (Falcon, Lincoln Park, NJ) and incubated with 500 ng/ml of agonistic anti-CD95 monoclonal antibody (mAb) (clone CH11, mouse IgM, Upstate Biotechnology, Lake Placid, NY) for indicated time periods, with or without 1 hour preincubation with 10 $\mu$mol/L caspase-3 peptide inhibitor Ac-Asp-Glu-Val-Asp-fluoromethyl ketone (DEVD-FMK, Clontech, Palo Alto, CA).

Detection of apoptosis in CHD cell lines by terminal deoxynucleotidyl tranferase-mediated dUTP nick end-labeling (TUNEL) was quantitated using the ApopTag *in situ* apoptosis peroxidase detection kit (Oncor, Gaithersburg, MD). Cytospin preparations of cells were fixed in 1% formaldehyde for 15 minutes followed by 1 hour fixation in 70% ethanol at −20°C. After a brief wash in FA buffer (Difco Laboratories, Detroit, MI), each slide was incubated at room temperature (RT) for 10 minutes with equilibration buffer followed by 1 hour incubation at 37°C with TdT enzyme (or deionized water ($dH_2O$) for negative controls) diluted with the reaction buffer. The TdT reaction was stopped with stop/wash buffer and each specimen was briefly washed with FA buffer before 30 minute incubation with anti-digoxigenin-peroxidase at RT. After a series of washes with FA buffer, each slide was developed with DAB/hydrogen peroxide (Sigma) color substrate for 6 minutes at RT. All slides were counterstained with hematoxylin. A CD95-sensitive Jurkat T cell line was used as a positive control for apoptosis. A positive reaction for apoptosis was characterized by brown/black coloration of the nuclear or perinuclear region of the cell. Apoptotic cells were quantitated by 1000-cell count at 400× magnification.

The 7-Amino Actinomycin D (7-AAD) staining method to measure cell viability was performed per manufacturer's protocol using Via-Probe 7-AAD (PharMingen, San Diego, CA). Briefly, anti-CD95 mAb-treated and untreated cells ($1 \times 10^6$ cell/ml) were washed twice in cold PBS and resuspended in 1× binding buffer (10 mmol/L Hepes/NaOH (pH 7.4), 140 mmol/L NaCL, and 2.5 mmol/L $CaCl_2$). Resuspended cells were then incubated for 20 minutes at 20–25°C in the dark with 5 $\mu$l of 7-AAD. Samples (30,000 events per sample) were then quantitated on an Epics XL-MCL flow cytometer (Coulter, Miami Lakes, FL), recorded in LIST mode, and registered on logarithmic scales. 7-AAD emission was detected in the FL-3 channel (>650 nm). Analysis was performed using Coulter System II software.

### *Determination of Caspase-3 Activity in Cell Lines*

Caspase-3 activity was determined using the ApoAlert CPP32/Caspase-3 colorimetric assay kit (Clontech). After a 48-hour incubation with anti-CD95 mAb, duplicate samples of untreated and treated cells ($2 \times 10^6$ cells) were washed in cold PBS, resuspended in 50 $\mu$l cell lysis buffer, and incubated on ice for 10 minutes. Cell lysates were pelleted, followed by transfer of the supernatants to microcentrifuge tubes. Fifty microliters of 2× reaction buffer with 5 mmol/L DTT and 5 $\mu$l of 1 mmol/L DEVD-p-nitroanilide (pNA)-conjugated CPP32 substrate were added to each tube, followed by 1 hour incubation in a water bath at 37°C. A control reaction of treated cells without DEVD-pNA was included. Optical density (OD) for each specimen was determined at 405 nm using the EL 312e microplate reader (Bio-Tek Instruments, Winooski, VT). For quantification of protease activity, sample values were plotted on a calibration curve derived from the OD values obtained from each of five standards (range: 0–20 nmole pNA). For each sample, units of CPP32 activity were determined by the following formula:

$$\text{Units of CPP32 activity} = (\Delta OD) \times (\text{calib. Curve slope})^{-1} \times (\text{nmole pNA/OD})$$

where $\Delta$OD is the change in optical density from the control reaction without conjugated substrate.

## *Results*

### *Histology and Immunohistochemical Characterization of NLPHD Cases*

All 11 specimens demonstrated architectural changes consistent with NLPHD and were composed predominantly of large nodules with focal areas of diffuse effacement. Typical nodules contained characteristic L&H cells intermixed in a background of small lymphocytes and occasional epithelioid histiocytes separated by compressed intervening paracortical areas composed of small lymphocytes and scattered plasma cells.

In all specimens, L&H cells demonstrated positive staining for CD20 and LCA. In seven specimens, L&H cells expressed EMA, with one case also expressing CD30. In all cases, L&H cells were negative for CD15 and CD45RO.

### *Most NLPHD Cases Failed to Express Caspase-3 by Immunohistochemistry*

In 10 of 11 cases of NLPHD, including the case which expressed CD30, L&H cells were negative for caspase-3 expression by immunohistochemical staining as represented in Figure 1A. In one case, caspase-3 immunopositivity was detected in the cytoplasm in a few scattered L&H cells (Figure 1B). In contrast, caspase-3 expression was demonstrated both in Hodgkin Reed-Sternberg

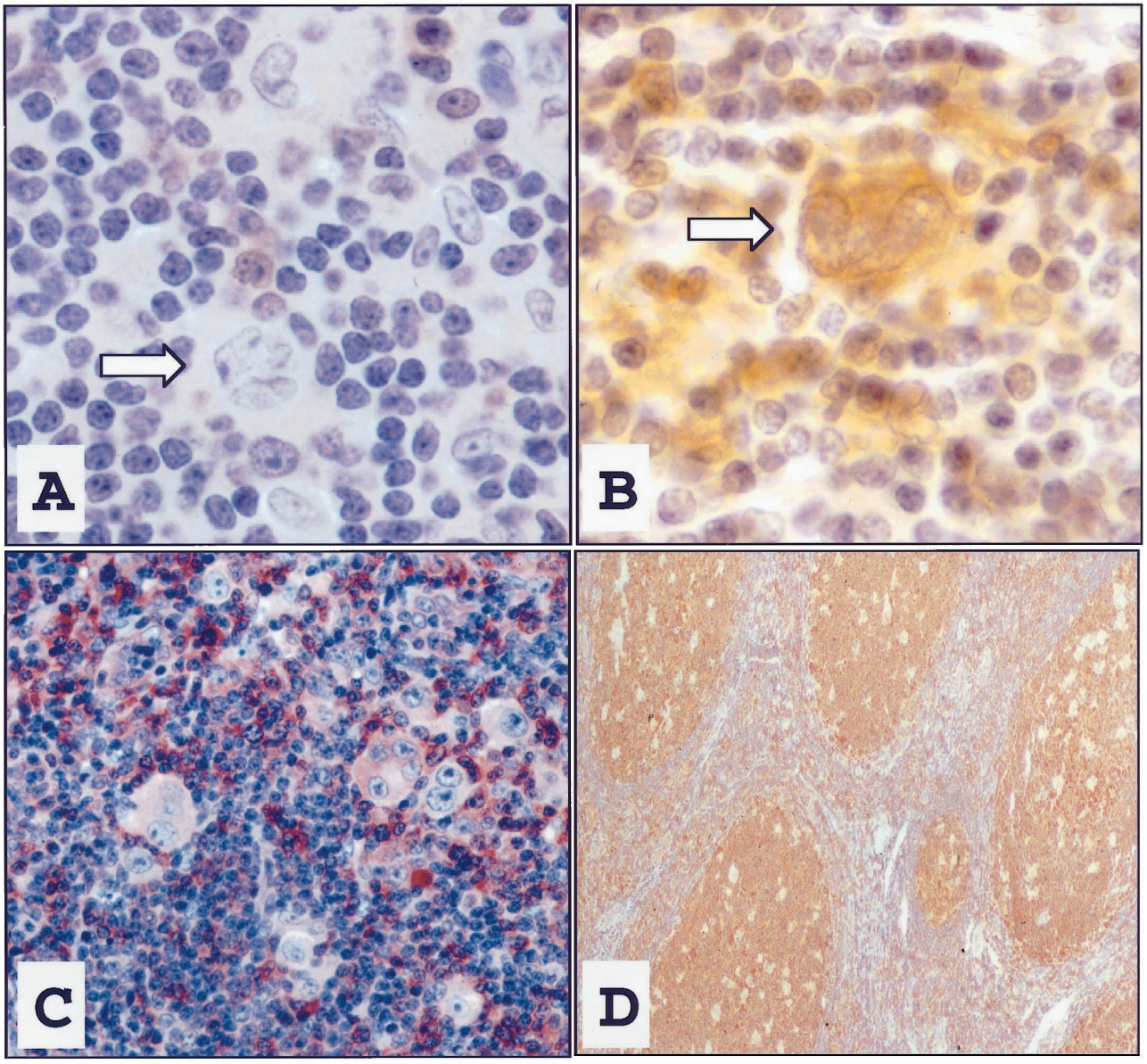


Figure 1. This L&H cell (arrow), as seen in the majority of NLPHD cases, was immunohistochemically negative for caspase-3, whereas scattered plasma cells and lymphocytes expressed caspase-3 (A, DAB ×1000). A single L&H cell (arrow) from one case of NLPHD displayed cytoplasmic expression of caspase-3 (B, DAB ×1000). Control cases of nodular sclerosis Hodgkin's disease demonstrated diffuse caspase-3-immunopositivity of HRS cells and intense positive immunostaining of lymphocytes and plasma cells within the surrounding infiltrate (C, DAB ×400). Reactive follicular centers in tonsil controls also displayed intense positive staining for caspase-3 (D, DAB, ×200).

(HRS) cells and in background lymphocytes in three cases of nodular sclerosis Hodgkin's disease (Figure 1C). In addition, tonsil tissue positive controls demonstrated caspase-3 immunopositivity concentrated predominantly in germinal center cells of secondary follicles (Figure 1D).

### *Caspase-3 Was Detectable in CHD Cell Lines*

Three CHD cell lines (HS445, L428, and KMH2) were analyzed for caspase-3 expression by immunohistochemistry. HS445 and L428 consistently demonstrated substantial cytoplasmic immunostaining for caspase-3 (Figure 2 and data not shown). However, in contrast, repeated immunohistochemistry assays failed to detect expression of caspase-3 in the KMH2 cell line (Figure 2).

### *Caspase-3 Was Proteolytically Cleaved and Activated during CD95-Mediated Apoptosis in Caspase-3-Positive CHD Cell Lines*

Activation of the CD95 receptor by ligand or agonistic mAb is known to induce apoptosis with concomitant proteolytic cleavage and activation of caspases, including caspase-3, in CD95-positive neoplasms.[19–23] To investigate the effect of CD95 stimulation with potential activation of caspase-3 in Hodgkin's disease, we examined the

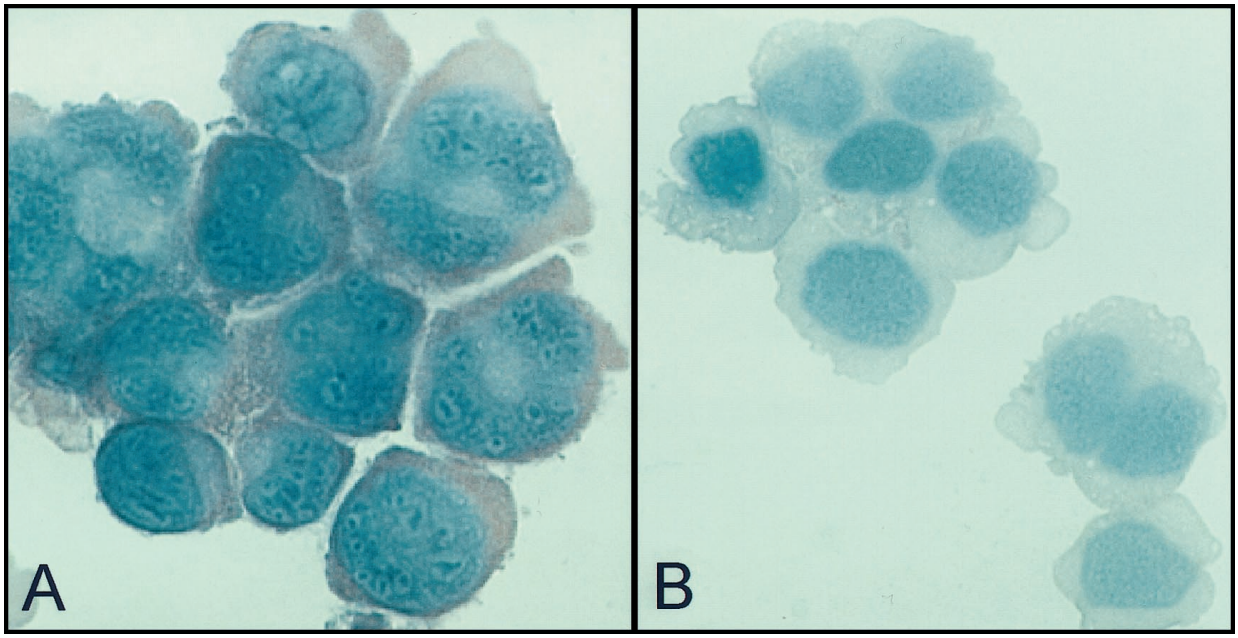


Figure 2. Immunohistochemical detection of caspase-3 on cytospin preparations of the L428 cell line (**A**) displayed strong cytoplasmic positive staining for caspase-3 (AEC, ×400); however, the KMH2 cell line (**B**) failed to express caspase-3 (AEC, ×400). Isotype control antibody staining was negative (data not shown).

effect of agonistic CD95 mAb on CHD cell lines. The HS445 and L428 cell lines displayed a significant increase in apoptosis after 72 hours' treatment with anti-CD95 mAb as quantitated by both flow cytometric analysis with 7-AAD (Figure 3) staining and the TUNEL assay (Figure 4, Table 2). CD95-induced apoptosis was significantly inhibited in these cells by the caspase-3 peptide inhibitor, DEVD-FMK, as demonstrated by the decrease in number of apoptotic cells to nearly background levels (Figures 3 and 4, Table 2). In contrast, the KMH2 cell line demonstrated consistent resistance to anti-CD95 mAb treatment with no effect by the addition of DEVD-FMK (Figure 3 and Table 2). Resistance of KMH2 cells to CD95-mediated apoptosis was not due to lack of CD95 expression, as all three cell lines expressed similarly high levels of CD95 as determined by flow cytometric staining.[26]

The significant inhibition of apoptosis by a caspase-3 inhibitor in HS445 and L428 cells, and the lack of apoptosis induced by caspase-3-deficient KMH2 cells suggests caspase-3 is important for CD95-mediated apoptosis in CHD cell lines. To further substantiate caspase-3 cleavage and activation in CD95-mediated apoptosis in CHD, each cell line was evaluated for changes in caspase-3 activity before and after treatment with anti-CD95 mAb using an enzyme colorimetric assay (Figure 5). Forty-eight-hour treatment revealed approximately tenfold increases in caspase-3 activity in HS445 and L428 cells in contrast to no difference detected in treated KMH2 cells. Positive control CD95-sensitive Jurkat T cells displayed a fivefold increase in caspase-3 activity after 24 hours of treatment with anti-CD95 mAb.

## *Discussion*

Among the caspases identified in humans thus far, caspase-3 is probably one of the most relevant and best studied as regards to apoptosis in hematopoietic cells. Caspase-3 (CPP32, Yama, apopain) has been shown to be a key effector molecule in the downstream execution of various apoptotic stimuli.[9–11,27–30] Activated caspase-3 cleaves and inactivates many vital cellular proteins during apoptosis including kinases and proteins associated with cellular structure, cell cycle, and DNA repair. One such well characterized caspase-3 death substrate is poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair, genome surveillance, and integrity.[31,32] In addition, caspase-3 appears to indirectly activate endonucleases implicated in internucleosomal DNA cleavage by removing the negative regulatory effect of PARP.[31]

The cleavage and activation of caspase-3 during apoptosis has been well documented in neoplastic cells. Caspase-3 activation and subsequent cleavage of its substrates, protein kinase C-δ (PKC-δ) and PARP, was demonstrated by chemotherapeutic drug treatment in human leukemic cell lines.[33] Cross-resistance to CD95- and chemotherapeutic drug-induced apoptosis due to lack of caspase activation including caspase-3 was demonstrated in a human acute T-cell leukemia line, CEM.[34] Also, MCF breast carcinoma cells lacking expression of caspase-3 were resistant to apoptotic stimuli.[35] Thus, the expression and activation of caspase-3 appears to be critical for the execution of various apoptotic stimuli in neoplasms.

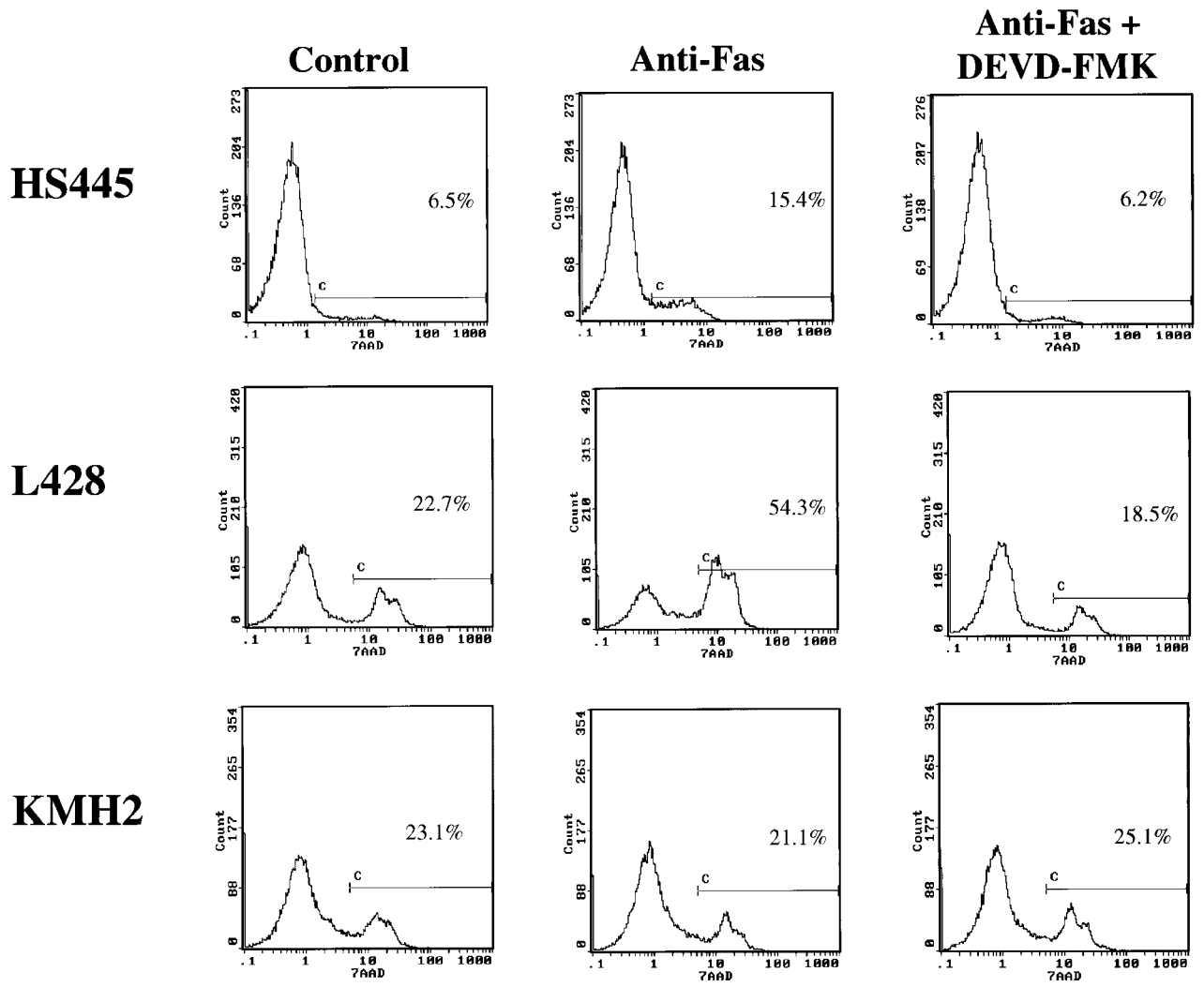


Figure 3. Flow cytometric analysis of 7-AAD staining in anti-CD95 (Fas)-treated CHD cell lines. Increased cell death was observed after 72 hours of treatment with 500 ng/ml agonistic CD95 mAb (CH11) in HS445 and L428 cell lines compared to untreated control cells. Pretreatment of cells with caspase-3 peptide inhibitor, DEVD-FMK, significantly decreased cell death in anti-CD95-treated cells to near background levels. In contrast, no significant increase in cell death was observed after anti-CD95 treatment of KMH2 cells as compared to untreated cells. The *x* axis represents fluorescence intensity (log scale) and the *y* axis represents relative cell number. These data are representative of at least three separate experiments performed.

Hodgkin's disease accounts for 14% of malignant lymphomas. Currently, one-third of advanced Hodgkin's disease patients are resistant to conventional therapies.[36] Our knowledge of the expression and function of apoptosis-related proteins such as caspases and how they may contribute to the pathogenesis and treatment of this malignancy is limited. Previous immunohistochemical studies *in situ* demonstrated that caspase-3 is commonly expressed in CHD.[37] However, the examination of caspase-3 expression in NLPHD has been limited. Furthermore, the overall biological significance of caspase-3 in Hodgkin's disease is unknown. Therefore, in this study we examined the expression of caspase-3 in NLPHD and determined its functional significance in CHD cell lines.

We first examined the *in situ* expression of caspase-3 in NLPHD. By immunohistochemistry, we identified caspase-3 immunopositivity in scattered L&H cells from only 1 of 11 cases of NLPHD. These findings are in agreement with the study of Chhanabhai and colleagues, who found no expression of caspase-3 in L&H cells from 6 cases of NLPHD.[37] In addition, these authors observed the HRS in the majority of cases of CHD were positive for caspase-3 expression.[37] These latter observations differ from the immunohistochemical findings of Xerri et al in which only 3 of 16 cases of HRS of CHD (nodular sclerosis and mixed cellularity type) were caspase-3-immunopositive.[38] The reason for the difference in caspase-3 expression in CHD noted between these groups is presently unclear.

Our immunohistochemical analysis of HRS cells of three CHD cell lines revealed substantial expression of caspase-3 in HS445 and L428, but only weak expression in KMH2 cells. These findings concur with Western blot analysis of these cell lines, which revealed expression of the 32-kd zymogen form of caspase-3 in HS445 and L428, but virtually undetectable expression in KMH2 cells.[26]

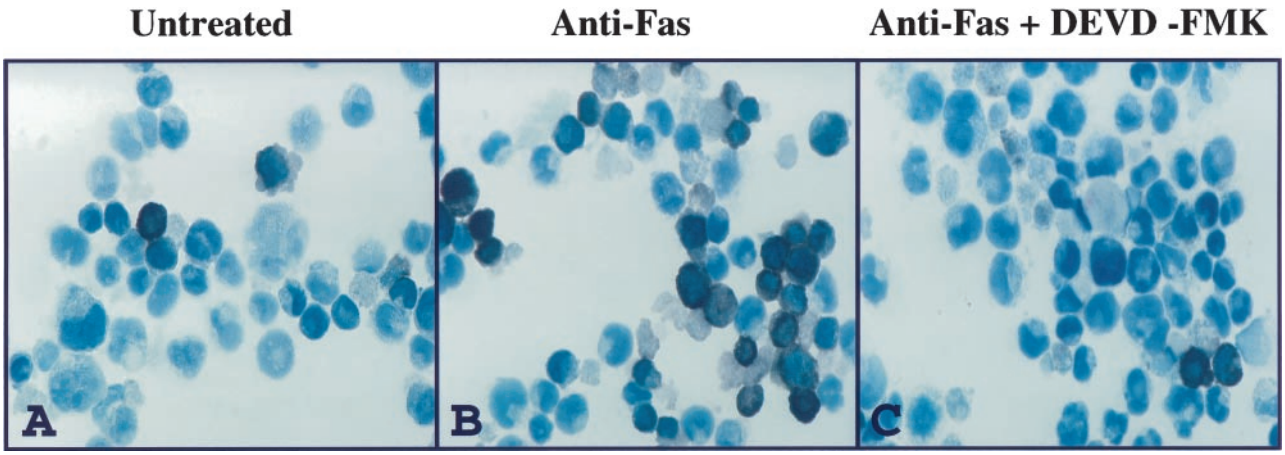


Figure 4. TUNEL assay for apoptosis. Compared to untreated cells (A), the L428 cell line displayed a considerable increase in apoptosis (dark brown/black cells) after 72 hour incubation with 500 ng/ml agonistic CD95 (Fas) mAb (B). Pretreatment of L428 cells with 10 $\mu$M DEVD-FMK decreased the number of apoptotic cells to near baseline levels (C). Stimulation of KMH2 cells with anti-CD95 mAb with or without DEVD-FMK pretreatment showed no increase in apoptosis compared to untreated cells (data not shown).

To address the biological significance of caspase-3 in Hodgkin's disease, we investigated the role of caspase-3 in CD95-mediated apoptosis in CHD lines. After stimulation of the CD95 receptor by agonistic CD95 mAb, significant apoptosis was induced in caspase-3 positive cell lines HS445 and L428 by TUNEL and 7-AAD assays. However, KMH2 cells, which virtually failed to express caspase-3 by immunohistochemistry, were consistently resistant to CD95 stimulation, suggesting that resistance to CD95-mediated apoptosis in this cell line may be due to a deficiency of caspase-3[26] (manuscript in preparation).

Table 2. Apoptosis Rates Induced in Anti-CD95 mAb Treated or Untreated CHD Cell Lines as Determined by TUNEL Assay

| | Apoptosis* | | |
|---|---|---|---|
| CHD cell line | Untreated | Anti-CD95 | Anti-CD95+ DEVD-FMK |
| HS445 | 7.5% | 20.5% | 9.9% |
| L428 | 7.8% | 30.2% | 10.8% |
| KMH2 | 1.0% | 1.3% | 1.0% |

*1 $\times$ $10^6$ cells were untreated or treated with 500 ng/ml anti-CD95 mAb (CH11) for 72 hours with or without pretreatment with 10 $\mu$M caspase-3 peptide inhibitor, DEVD-FMK. Apoptosis was quantitated by TUNEL staining as described in Materials and Methods.

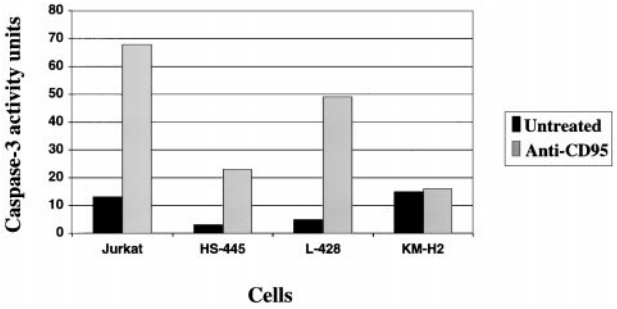


Figure 5. Caspase-3 activity in anti-CD95-treated CHD cell lines. Positive control for caspase-3 activity is demonstrated in Jurkat T cells by fivefold induction of caspase-3 activity after 24 hours of agonistic CD95 mAb treatment. HS445 and L428 cell lines had approximately ten-fold increases in caspase-3 activity after 48 hours' treatment with anti-CD95 mAb compared to untreated controls. In contrast, no significant increase in caspase-3 activity was detected in anti-CD95-treated KMH2 cells when compared to untreated cells. These data are representative of three separate experiments.

To establish caspase-3 as a key mediator in CD95-induced apoptosis in CHD cell lines, enzyme assays specific for caspase-3 activity were performed. Approximately tenfold increases in caspase-3 activity were observed in HS445 and L428 after 48 hours' incubation with anti-CD95 mAb, compared to no increase in KMH2 cells. In addition, we pretreated each cell line with the caspase-3 peptide inhibitor DEVD-FMK before CD95 activation. Previous studies in other experimental systems have demonstrated that DEVD inhibitors have specificity for caspase-3 by bearing similarities to the cleavage site of the caspase-3 substrate, PARP.[9,11,27–31,39–41] The addition of DEVD-FMK to cultures of HS445 and L428 significantly decreased CD95-mediated apoptosis; however, there was no effect on KMH2 cells. These findings in CHD lines correlate with previous studies which demonstrated caspase-3 is proteolytically cleaved and activated and plays a key role in CD95-mediated apoptosis in other experimental systems.[9–11,27–30,42] However, it should be noted that CD95-induced apoptosis may occur without activation of caspase-3, suggesting the existence of alternate apoptosis execution pathways in response to CD95 signaling.[43]

Most investigations related to apoptosis in Hodgkin's disease have focused on the expression of mitochondrial apoptosis regulatory proteins Bcl-2, Bcl-x, and Bax.[44–49] These studies revealed variable expression of Bcl-2[44–48] but frequent expression of the pro-apoptotic protein Bax[47] and the apoptosis antagonist Bcl-$x_L$.[48–49] Previous investigations of CD95 expression by HRS cells have been limited; however, these studies revealed that CD95 is expressed on HRS cells in the majority of cases of CHD.[50–53] In this report, we demonstrate that CHD cell lines expressing CD95 can undergo apoptosis by CD95 stimulation. A recent study assayed CD95-induced apoptosis in fresh tissue samples with Hodgkin's disease; however, the HRS cells were not specifically analyzed.[51]

Because HRS cells usually constitute less than 1% of involved tissue, it is difficult to assay CD95 stimulation of HRS cells directly without selective separation.

The absence of caspase-3 expression in L&H cells is similar to that seen in several indolent B-cell NHLs, most notably follicular center lymphoma (FCL), grade I.[38,54] Recent studies have noted clinical similarities between NLPHD and indolent B-cell NHL.[55–57] However, NLPHD differs from the majority of low-grade B-cell NHLs with respect to treatment response. Most NLPHD patients are cured and rarely show progressive disease, in contrast in the majority of FCL patients. Furthermore, although bcl-2 is commonly overexpressed in FCLs, NLPHD typically lacks expression of this protein.[24,46,58] The combined high and low expression of bcl-2 and caspase-3 protein, respectively, in low-grade FCL suggests the incurability of many of these lymphomas may be directly related to the overexpression of anti-apoptotic proteins (eg, Bcl-2) combined with the lack of downstream apoptotic mediators such as caspase-3. Furthermore, overexpression of Bcl-2 and Bcl-$x_L$ in cell lines can also block cleavage and activation of caspase-3.[27,29,59–61]

The lack of caspase-3 expression in NLPHD may also be an important mechanism of resistance to apoptosis. Furthermore, the differential expression of caspase-3 between CHD and NLPHD suggests that each may be a distinct disease entity, and may account for some of the clinical differences between these two disorders. Additional studies to define the expression and function of caspases and their relationship to other apoptosis-related proteins may provide novel insights into the pathogenesis and treatment resistance of this malignancy.

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