XIAP-mediated Caspase Inhibition in Hodgkin's Lymphoma–derived B Cells

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Abstract

The malignant Hodgkin and Reed-Sternberg cells of Hodgkin's lymphoma (HL) and HL-derived B cell lines were previously shown to be resistant to different apoptotic stimuli. We show here that cytochrome *c* fails to stimulate caspases-9 and -3 activation in cytosolic extracts of HL-derived B cells, which is due to high level expression of X-linked inhibitor of apoptosis (XIAP). Coimmunoprecipitation studies revealed that XIAP, apoptosis protease-activating factor–1, and caspase-3 are complexed in HL-derived B cell lysates. Even after stimulation with exogenous cytochrome *c* and dATP, XIAP impairs the proteolytic processing and activation of caspase-3. In cytosolic extracts, inhibition of XIAP by the second mitochondria-derived activator of caspases (Smac)/DIABLO, or immunodepletion of XIAP restores cytochrome *c*–triggered processing and activation of caspase-3. Smac or a Smac-derived agonistic peptide also sensitized intact HL-derived B cells for the apoptotic action of staurosporine. Finally, Hodgkin and Reed-Sternberg cells of primary tumor HL tissues also constitutively and abundantly express XIAP. The results of this paper suggest that high level XIAP expression is a hallmark of HL, which may play a crucial role in resistance to apoptosis.

Key words: cancer • tumor • apoptosis • mitochondria • Smac/DIABLO

Introduction

At least two distinct major apoptotic signaling pathways have been identified. The triggering of death domain containing cell surface receptors of the TNF super family results in the recruitment and activation of the initiator caspase, caspase-8, followed by a rapid cleavage of caspases-3 and -7, which in turn cleave vital substrates in the cell (1). The second apoptotic signaling pathway involves mitochondria and results in the release of proapoptotic factors from mitochondria, such as cytochrome *c* and the second mitochondria-derived activator of caspases (Smac*; references 2, 3). Subsequently, the released cytochrome *c*, the cytosolic apoptosis protease-activating factor–1 (Apaf-1), and procaspase-9 form the apoptosome (4). Activation of caspase-9 is induced by dimerization (5) driven by formation of the multimeric Apaf-1 complex (6). Once activated, caspase-9 can directly process and activate caspases-3 and -7.

Apoptotic pathways converge at the level of caspase-3, which is expressed in cells as an inactive 32-kD precursor. The activation of caspase-3 occurs in two steps, beginning with the initial cleavage by caspase-8 or -9 to form two subunits. After this cleavage, caspase-3 removes its own prodomain in two sequential steps, generating the ultimate p17 active subunit (7).

The activity of the caspases is modulated by another set of proteins, the inhibitors of apoptosis. One of these, the X-linked inhibitor of apoptosis (XIAP), binds to and inhibits caspases-3, -7, and -9, but not caspase-8 (8). Overexpression of XIAP and its family members has been shown to suppress apoptosis induced by a variety of stimuli, including TNF, Fas-L, menadione, staurosporine, etoposide, taxol, and growth factor withdrawal (9, 10). In the presence of XIAP, the initial cleavage of procaspase-3 can occur but not the second autocatalytic cleavage, due to the inhibition of intrinsic caspase-3 activity (9–11).

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**Abbreviations used in this paper:* AFC, 7-amino-4-trifluoromethylcomarin; Apaf-1, apoptosis protease-activating factor–1; HL, Hodgkin's lymphoma; H-RS, Hodgkin and Reed-Sternberg; Smac, second mitochondria-derived activator of caspases; XIAP, X-linked inhibitor of apoptosis.

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Additional regulation is provided by Smac (3). The proform of Smac contains an NH_2-terminal sequence that targets this protein to the intermembrane space of mitochondria. Upon induction of apoptosis, Smac is released into the cytosol and modulates apoptosis (3). Smac promotes apoptosis by repressing the anticaspase activity of XIAP, thereby inducing the enzymatic activity of mature caspase-3.

Resistance to apoptotic stimuli is one of the keys to cancer cell survival. The malignant Hodgkin and Reed-Sternberg (H-RS) cells of Hodgkin's lymphoma (HL) are germinal center B cells with rearranged but nonproductive immunoglobulin genes (12). However, through an as yet unknown mechanism, H-RS cells resist the apoptotic fate characteristic of defective B cells with crippled immunoglobulin genes.

In a previous paper, we showed that all tested HL-derived B cell lines are resistant to staurosporine-induced cell death and identified a defect in Bax activation (13). These HL-derived B cell lines are also resistant to CD95-mediated apoptosis, suggesting that additional defects of the apoptotic pathway may exist (14). In the present work, we show that all HL-derived B cell lines have a defect in caspase-3 activation, which is apparently secondary to high level expression of XIAP.

Materials and Methods

Cell Culture. The establishment and culturing of the Hodgkin and control B cell lines have been described previously (13). Cells were transfected with 0.5 μg Smac NH_2-terminal peptide, H-AVPIAQK-OH, (Calbiochem), Smac protein, or β-galactosidase (119-kD subunit) as a control using the Chariot protein transfection kit according to the instructions of the manufacturer (Active Motif). Apoptosis was induced by 1 μM staurosporine (Qbiogene) and cell death was examined using trypan blue exclusion.

Sample Preparation and Immunoblotting. Whole cell extracts were prepared by lysing cells in CHAPS lysis buffer (14). Cytosolic extracts were prepared in buffer A (20 mM Pipes, pH 7.0, 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, and 1 mM dithiothreitol) as described previously (13). Equal amounts (100 μg) of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane (0.2 μm Protran; Schleicher & Schuell). Rabbit polyclonal antisera specific for human caspase-3, Apaf-1, human Bax, and mouse anti-XIAP mAb were obtained from BD Biosciences. Polyclonal antiserum specific for caspase-9 was generated as described previously (11).

Caspase Activation. For initiating caspase activation, either 10 μM horse heart cytochrome *c* together with 1 mM dATP or 100 nM of purified recombinant active caspase-8 (15) or 60 μg granzyme-B (Sigma-Aldrich) were added to 20 μl of cell extracts or buffer A as a control and incubated for 1 h at 30°C. To determine the effect of Smac on caspase activation, cytosolic extracts were incubated with 10 μM horse heart cytochrome *c* together with 1 mM dATP and 1 μM recombinant Smac protein for 1 h at 30°C. Reactions were initiated by the addition of 100 μM Ac-DEVD–7-amino-4-trifluoromethylcomarin (AFC), and caspase activity was assayed and presented as arbitrary fluorescence units per minute (FU/min); 1 FU is equivalent to 0.65 pmol released AFC (13).

Immunoprecipitation. Equal amounts (30 μl) of 20 μg/μl cytosolic extracts were adjusted to a final volume of 500 μl with buffer A. Samples were rotated for 6 h at 4°C with 1 μg of mouse anti–caspase-3 mAb, mouse anti-Bax mAb (BD Biosciences) or rat anti–Apaf-1 mAb (Chemicon). Antigen–antibody complexes were immobilized by rotation for 2 h at 4°C with GammaBind-G Sepharose (Amersham Biosciences). The complexes were centrifuged (1 min, 14,000 *g* at 4°C) and the supernatant was removed. After three washes with buffer A, samples were subjected to SDS-PAGE and immunoblotted as described in Sample Preparation and Immunoblotting section above.

Immunodepletion of XIAP. 20 μl (250 μg/ml) mouse anti-XIAP mAb was added to 100 μl GammaBind-G Sepharose in 500 μl PBS and rotated at 4°C for 3 h. The beads were collected and the supernatant was removed. The beads were washed once with 1 ml buffer A and incubated with 300 μl cytosolic extract (20 μg/μl) for 6 h on a rotator at 4°C. The beads were subsequently pelleted and the resulting supernatant was used as XIAP-immunodepleted cytosolic extract.

Expression and Purification of Recombinant Smac Protein. The cDNA encoding the amino acids 56–239 of Smac was cloned from a human thymus cDNA library by PCR and cloned into the NdeI and XhoI sites of the pET-23b vector (Novagen), resulting in a COOH-terminal His-6–tagged Smac. Expression of recombinant Smac was induced with 0.5 mM IPTG for 5 h at 30°C. The protein was purified on Nichelate sepharose (Amersham Biosciences) from the soluble fraction of sonicated cells.

Immunohistology. Primary tumor tissues from 12 cases of HL in Danish patients were selected from the archives of the Institute of Pathology, Aarhus University Hospital. Histological diagnosis and subtyping of HL was based on accepted morphological and immunophenotypical criteria (16). All cases had been examined for the presence of EBV sequences using EBV-encoded small RNA (EBER) in situ hybridization. In addition, two tonsils and three lymph nodes showing benign reactive lymphoid hyperplasia were included as controls for immunohistology. All tissues had been routinely fixed in formalin and embedded in paraffin. Formalin-fixed and paraffin-embedded tissues were cut at 4 μm onto silanized slides. Antigen retrieval was performed by microwave superheating in TEG buffer (10 mM TRIS and 0.5 mM EGTA) at pH 9.0. Sections were incubated with primary mouse anti-XIAP mAb for 30 min (dilution 1:50). Reactions were detected using a standard, highly sensitive EnVison™ horseradish peroxidase staining system (DakoCytomation) developed with diaminobenzidine.

Results

Failure of Caspase Activation in HL-derived B Cells. HL-derived B cell lines have been shown to be resistant to apoptosis induced by CD95 and staurosporine (13, 14). To investigate if caspases are functional in these cells, we used a cell-free system and exogenously added cytochrome *c* to induce proteolytic processing and activation of procaspases-9 and -3 (4, 11). In untreated cell extracts, caspase-9 is detected as its 46-kD proform in all four HL-derived B cell lines (L1236, L591, L428, and KMH2) and in the control B cell line L1309 (Fig. 1 A). The addition of cytochrome *c* to the cytosolic extracts of L1309 leads to autocatalytical cleavage of procaspase-9, producing the characteristic p35 fragment. A second cleavage, mediated by subsequently ac-

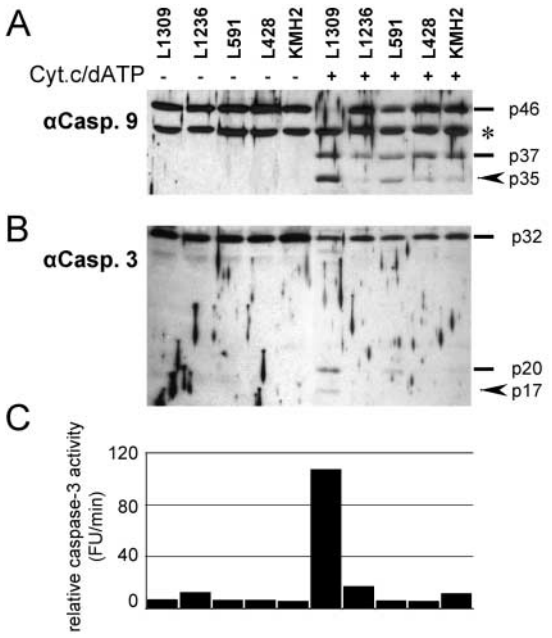
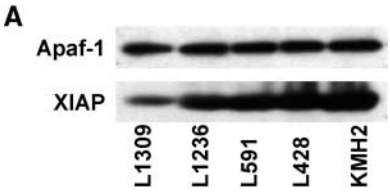


Figure 1. Failure of cytochrome *c* to induce caspase activation in cytosolic extracts of HL-derived B cell lines. Cytosolic extracts of L1236, L591, L428, and KMH2 cells, and of control B cell L1309 were prepared and equal amounts of protein were incubated with or without cytochrome *c*/dATP for 1 h at 30°C. Cytosolic extracts were resolved by SDS-PAGE and subjected to Western blot analysis. (A) Procaspase-9 (p46) and its fragments (p37, p35) were detected by polyclonal rabbit anti–caspase-9 antibody. (B) Procaspase-3 (p32) and its fragments (p20, p17) were detected by polyclonal anti–caspase-3 antibody. (C) Measurement of relative caspase activity using DEVD-AFC. Samples were normalized for total cytosolic protein content. Asterisk indicates nonspecific bands recognized by polyclonal antibodies.



B

No.	Age (yr)	Sex	Diagnosis	EBV	XIAP positivity
1.	33	Male	HL-LP	neg	+
2.	37	Male	HL-LP	neg	++
3.	20	Male	HL-MC	pos	+++
4.	88	Male	HL-MC	pos	+++
5.	67	Female	HL-MC	pos	++++
6.	23	Male	HL-NS	pos	++++
7.	78	Male	HL-NS	pos	++++
8.	42	Female	HL-NS	neg	+++
9.	29	Male	HL-NS	neg	++++
10.	20	Female	HL-NS	neg	+++
11.	40	Male	HL-NS	neg	++++
12.	60	Male	HL-NS	neg	++++

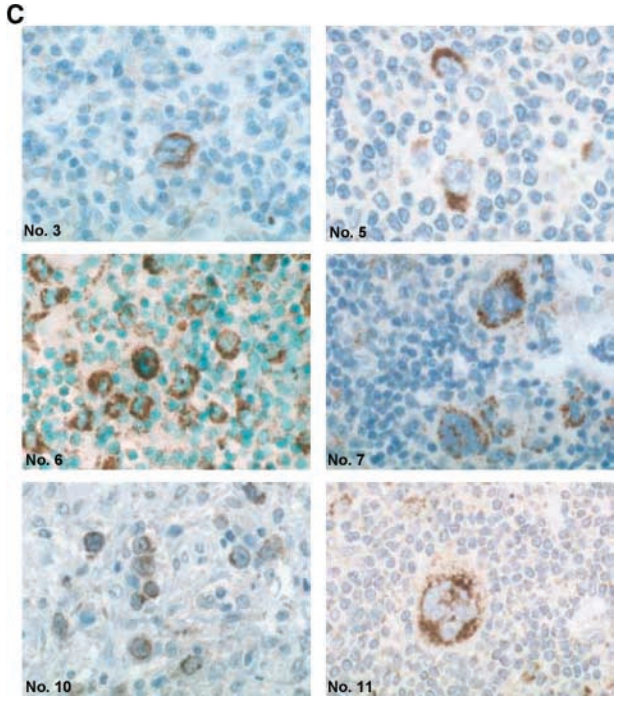


Figure 2. Expression of caspase activators and inhibitors in HL-derived B cell lines. (A) Equal amounts of proteins from total cell lysate of L1236, L591, L428, and KMH2 cells and of control B cells L1309 were subjected to SDS-PAGE and Western blot analysis. Proteins were detected using antibodies against Apaf-1 and XIAP. (B) XIAP expression in primary Hodgkin's lymphoma (HL) tissues. XIAP positivity in H-RS cells: + = very weak; ++ = weak; +++ = moderate; ++++ = strong staining. (C) Paraffin section immunohistochemistry of cases 3, 5, 6, 7, 10, and 11 of classical HL cases listed in Fig. 2 B. XIAP was stained using anti-XIAP specific mAb. There are strong (Nos. 5, 6, 7, and 11) or moderate (Nos. 3 and 10) granular intracytoplasmic staining for XIAP in essentially all morphologically recognizable Hodgkin or Reed-Sternberg cells. Background lymphocytes are negative for XIAP staining.

tivated caspase-3, results in the p37 fragment of caspase-9 (17). Concomitantly, the initial p20 fragment and the ultimate p17 subunit of caspase-3 were detected (Fig. 1 B). In contrast, autocatalytic processing of caspase-9 was impaired in HL-derived B cell lines, in that only small amounts of the p35 fragment were generated upon the addition of cytochrome *c* to cytosolic extracts (Fig. 1, A and B). Similarly, caspase-3 processing was not detected. In addition, cytochrome *c* induced caspase activity in the control B cell line but not in the HL-derived B cell lines, which indicated a defective caspase-3 activation cascade (Fig. 1 C).

The examination of proteins involved in caspase-3 activation downstream of mitochondria showed that the level of expression of the adaptor protein, Apaf-1, was comparable in all cell lines. In contrast, the antiapoptotic protein XIAP appeared to be expressed at significantly higher levels in the HL-derived B cell lines compared with the control B cell line L1309 (Fig. 2 A).

Moderate or strong immunohistological expression of XIAP was also seen in H-RS cells in cases of biopsies from patients suffering HL (Fig. 2 B). Although the two cases of lymphocytic predominance-HL (Nos. 1 and 2) showed weak and very weak XIAP staining in their "L and H" (lymphocytic and histiocytic) Reed-Sternberg variant cells respectively, the strong staining was seen in both EBV-positive and EBV-negative classical HL cases. Staining was predominantly intracytoplasmic, with a striking granularity (Fig. 2 C). Control tissues and nonneoplastic lymphoid

components within the HL tumors showed mostly no XIAP staining with few exceptions, where focal weak staining was observed. However, in some tumors, stronger staining was detected in background lymphocytes, both within germinal centers, and in the interfollicular regions (unpublished data).

XIAP Association with Caspase-3 in HL-derived B Cell Lines. It has been shown previously by Bratton et al. that XIAP associates with oligomerized Apaf-1, binds processed caspase-3 produced within the apoptosome, and sequesters it within the complex (18). To characterize XIAP action in HL-derived B cells, coimmunoprecipitation studies combined with Western blot analysis were performed (Fig. 3). In the control B cell line, XIAP did not coimmunoprecipitate with caspase-3 in activated lysates upon the addition of dATP/cytochrome *c*, and caspase-3 was fully processed to form the enzymatically active p17 fragment, which is consistent with previous papers. In contrast, XIAP coimmunoprecipitated with caspase-3 in lysates from HL-derived B cells. Upon addition of cytochrome *c*, caspase-3 was only partially processed to form the p24–20 fragments and failed to remove its own prodomain, which can be sensed as a "footprint" for XIAP-mediated inhibition of caspase-3 (Fig. 3 B; reference 11). The incomplete processing of caspase-3 was accompanied by partial cleavage of XIAP, which led to formation of a p48 XIAP fragment. Both intact XIAP and the p48 fragments remained associated with caspase-3 in activated extracts of HL-derived B cells. The coimmunoprecipitation of Apaf-1 with caspase-3 demonstrated the efficient formation of apoptosome complexes in all cell lines after cytochrome *c* addition.

XIAP, Apaf-1, and caspase-3 also coimmunoprecipitated in unactivated lysates of HL-derived B cell lines (Fig. 3, B and C), indicating the preformation of XIAP–apoptosome complexes. In these lysates, XIAP did not coimmunoprecipitate with Bax (Fig. 3 D), which excludes immunoprecipitation conditions favoring nonspecific binding.

Caspase-8 and Granzyme-B Partially Bypass the XIAP-mediated Inhibition of Caspase-3 in HL-derived B Cell Lines. Caspase-3 is not only activated within the apoptosome but alternatively by caspase-8 and granzyme-B (19, 20). To determine whether XIAP action in HL-derived B cells extends to caspase-3 activation outside the apoptosome, recombinant active caspase-8 or granzyme-B were added to the cytosolic extracts of HL-derived B cells. As shown in Fig. 4, both of these proteases cleaved caspase-3, which resulted in the p20 fragment, characteristic of the initial cleavage of caspase-3 (19, 20). Notably, the autocatalytic maturation generating the p17 fragment proceeded to a lesser degree in the cytosolic extracts of HL-derived B cell lines compared with the control B cell line. We conclude that in HL-derived B cells, XIAP does not prevent the initial cleavage of caspase-3 induced by caspase-8 or granzyme-B, but rather inhibits the subsequent autocatalytic processing to the mature p17 fragment. Incomplete caspase-3 processing corresponds with decreased caspase-3 activity in HL-derived B cell extracts (Fig. 4).

Physical or Functional Depletion of XIAP Restores Cytochrome c–induced Caspase-3 Activity in HL-derived B Cells. If XIAP was the key inhibitor of apoptosis in HL-derived

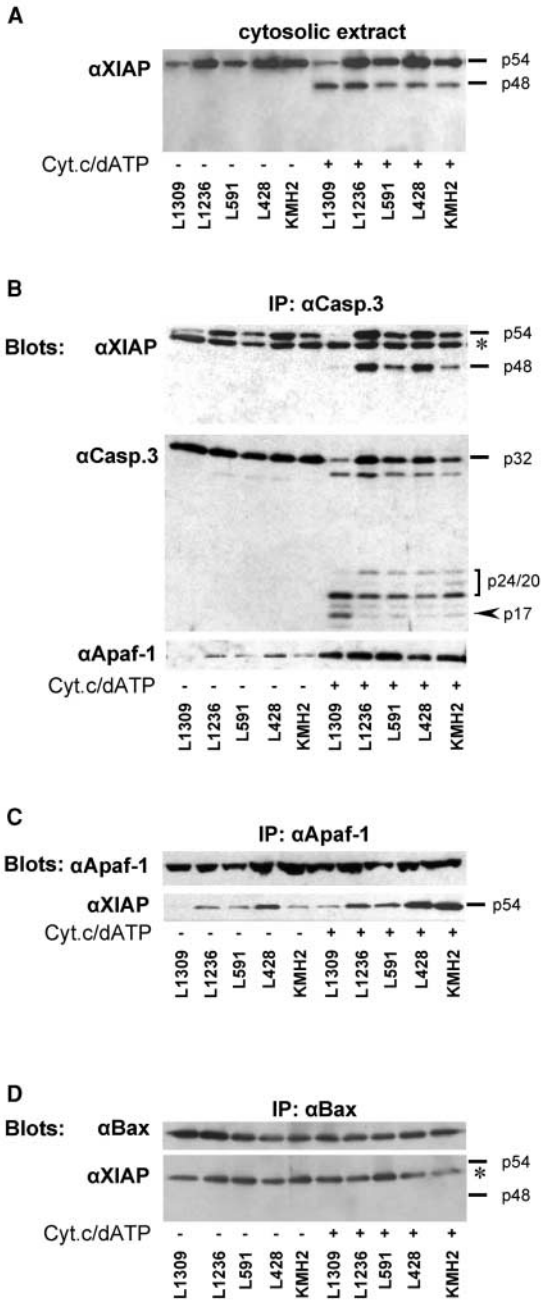


Figure 3. XIAP coimmunoprecipitates with activated caspase-3. Cytosolic extracts of L1236, L591, L428, and KMH2 cells and of control B cells L1309 were prepared, and equal amounts of protein were incubated with or without cytochrome *c*/dATP for 1 h at 30°C. (A) 100 μg samples were resolved by SDS-PAGE and subjected to Western blot analysis. XIAP was detected by mouse anti-XIAP mAb. (B–D) Caspase-3, Apaf-1, and Bax were immunoprecipitated by mouse anti–caspase-3, rat anti–Apaf-1, and mouse anti-Bax mAb in 600-μg cytosolic extracts and subjected to SDS-PAGE and Western blotting. XIAP, caspase-3, Apaf-1, and Bax were detected by mouse anti-XIAP mAb, polyclonal rabbit anti–caspase-3, polyclonal rabbit anti–Apaf-1, and polyclonal rabbit anti-Bax antibodies. Asterisk indicates mouse IgG.

B cells, removal of XIAP should relieve the inhibition of caspase-3 activation. Indeed, in the presence of Smac, a mitochondrial inhibitor of XIAP, the ability of cytochrome *c* was restored to trigger processing and activation of caspase-3 in HL-derived B cells (Fig. 5, A and B). In addition, immunodepletion of XIAP using a mouse mAb anti-XIAP antibody resulted in an enhancement of caspase-3 activity (Fig. 5 C), providing further evidence for a central role of XIAP in inhibition of caspase-3 activation in HL-derived B cells. Finally, transfection of intact HL-derived B cells with Smac or a Smac agonistic peptide sensitized these cells for the proapoptotic activity of staurosporine (Fig. 5 D), confirming that high level expression of XIAP constitutes a state of apoptotic resistance in HL.

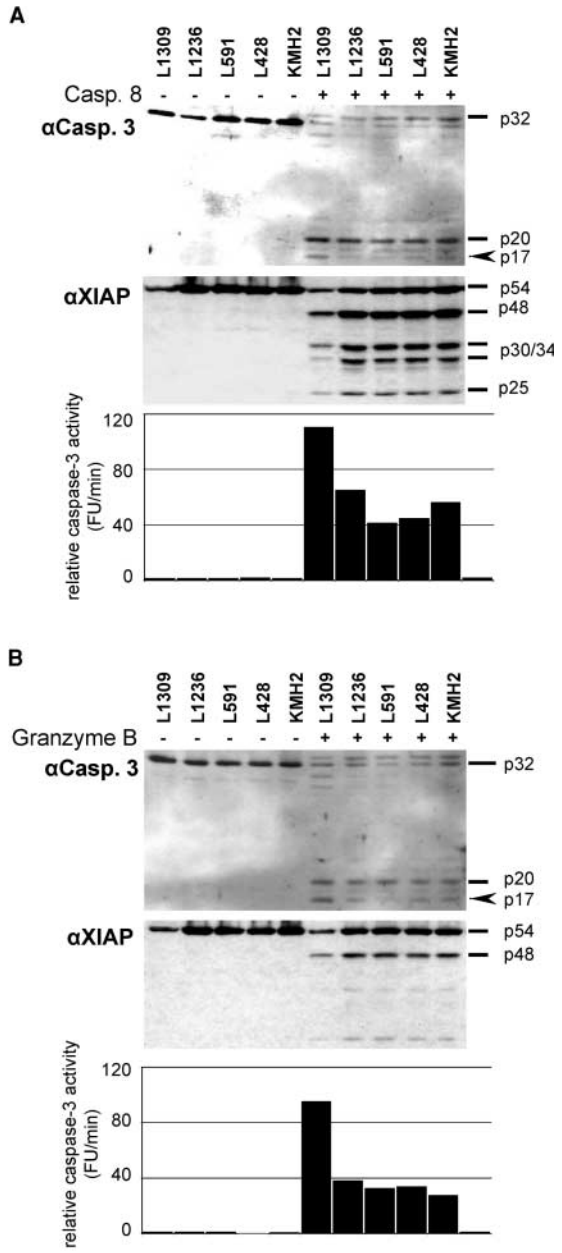


Figure 4. Caspase-3 activation by caspase-8 and granzyme-B. Cytosolic extracts of L1236, L591, L428, and KMH2 cells and of control B cells L1309 were prepared, and equal amounts of protein were left untreated or incubated for 1 h with recombinant active caspase-8 (A) and granzyme-B (B) at 30°C. Cytosolic extracts were resolved by SDS-PAGE and subjected to Western blot analysis. Caspase-3 and XIAP were detected by polyclonal rabbit anti–caspase-3 antibody and mouse anti-XIAP mAb. Relative caspase activity was measured using DEVD-AFC after incubation of cytosolic extracts with recombinant active caspase-8 (A)and granzyme-B (B). Samples were normalized for total cytosolic protein content.

Discussion

Primary H-RS cells and HL-derived B cell lines have been shown to be resistant to different apoptotic stimuli (13, 14). We show here that XIAP, a potent inhibitor of caspase-3, is expressed constitutively and at high levels in HL-derived B cells as well as in H-RS cells of tumor biopsies. Correspondingly, cytochrome *c*, as well as caspase-8– or granzyme-B–induced activation of caspase-3 is severely impaired in lysates of HL-derived B cell lines. Functional neutralization of XIAP by Smac restored the apoptotic response in lysates as well as in intact HL-derived B cells, suggesting that XIAP is a main mediator of apoptotic resistance in HL.

Upon the addition of cytochrome *c*, caspase-9 is thought to be activated without cleavage (18, 21), and ultimately processed by an autocatalytic mechanism generating a p35 fragment (4). It has been suggested that the processing of caspase-9 to p35 is the signal for inhibition by XIAP, due to the unmasking of a neoepitope in the small subunit (17). Thus, XIAP may also inhibit the unprocessed form of caspase-9, as described previously (11). The incomplete caspase-9 processing in cytochrome *c*–treated cytosolic extracts of HL-derived B cells (Fig. 1 A) is fully consistent with the mechanism described for XIAP-mediated caspase-9 inhibition.

Our finding that XIAP is coimmunoprecipitated with both caspase-3 and Apaf-1 in lysates of HL-derived B cells deserves consideration. With regard to the formation of apoptosomes, the sequence of events has been established previously. Accordingly, XIAP does not bind to Apaf-1 apoptosome in unactivated lysates but does so after cytochrome *c*–triggered oligomerization of Apaf-1 (18). Within the apoptosome, XIAP binds to caspases-9 and -3 simultaneously (18). However, XIAP does not bind to unactivated caspase-3. The coimmunoprecipitation of XIAP with caspase-3 in the apparent absence of caspase-3 processing (Fig. 3 B) could be of an indirect nature because of XIAP association with Apaf-1 (Fig. 3 C), which in turn associates with both processed and unprocessed caspase-3 (18). The coprecipitation of XIAP, Apaf-1, and caspase-3 in untreated cytosolic extracts of HL-derived B cells raises the question whether high-level expression of XIAP leads to spontaneous association with Apaf-1 apoptosome. However, only little if any association of XIAP with caspase-3 has been observed in unactivated lysates (18). Alternatively, very low amounts of cytochrome *c*, accidently released during the preparation of cytosolic extracts, could have induced the formation of Apaf-1 apoptosome–XIAP complexes, which

are particularly detectable in XIAP-overexpressing cells. In this context, it is important to note that Apaf-1, caspases-9, and -3 were expressed at the same levels and that neither caspase-3 and nor XIAP revealed mutations in the primary structure (unpublished data).

As an executioner caspase, caspase-3 represents a target of converging caspase cascades including caspase-8, caspase-9, and granzyme-B. When added to cytosolic extracts of HL-derived B cells, caspase-8 and granzyme-B induced protease activity of caspase-3, although at reduced levels. This is due to the initial cleavage of caspase-3 into large and small subunits, whereas autocatalytic processing is apparently inhibited by XIAP (8, 11, 20). In contrast to caspase-8 and granzyme-B, caspase-9–induced caspase-3 activation was nearly completely abrogated (Fig. 1, B and C).

Overexpression of XIAP has been shown to effectively inhibit a variety of cell death programs (9–10, 22). These observations suggest that XIAP may play a key role in the regulation of apoptosis in cancer cells, such as human melanoma (22). The XIAP gene can be up-regulated by NF-κB (23). Constitutive activation of NF-κB appeared to be an unique feature of H-RS cells, which has been suggested previously to play an important role in the pathogenesis of H-RS cells (24). The overexpression of XIAP, a target gene for NF-κB, is in line with these observations and provides a plausible link for NF-κB–mediated resistance to apoptosis in HL-derived B cells. The finding that Smac or Smac agonistic peptides sensitize HL-derived B cells for staurosporine-induced apoptosis may have clinical implications. Further work will be required to determine whether neutralization of XIAP can be developed into a novel modality to sensitize HL cells for chemotherapeutic agents.

HL is unusual among neoplasms in that the malignant H-RS cells are typically rare in the affected tissues. This has hampered research into the pathogenesis of the disease for many years. The use of HL-derived B cell lines has helped many investigators to analyze this tumor at the molecular level. Our observation that XIAP is consistently overexpressed in H-RS cells of primary HL tumors provides another example, suggesting that molecular mechanisms defined in HL B cell lines may hold true for HL tumor cells in vivo.

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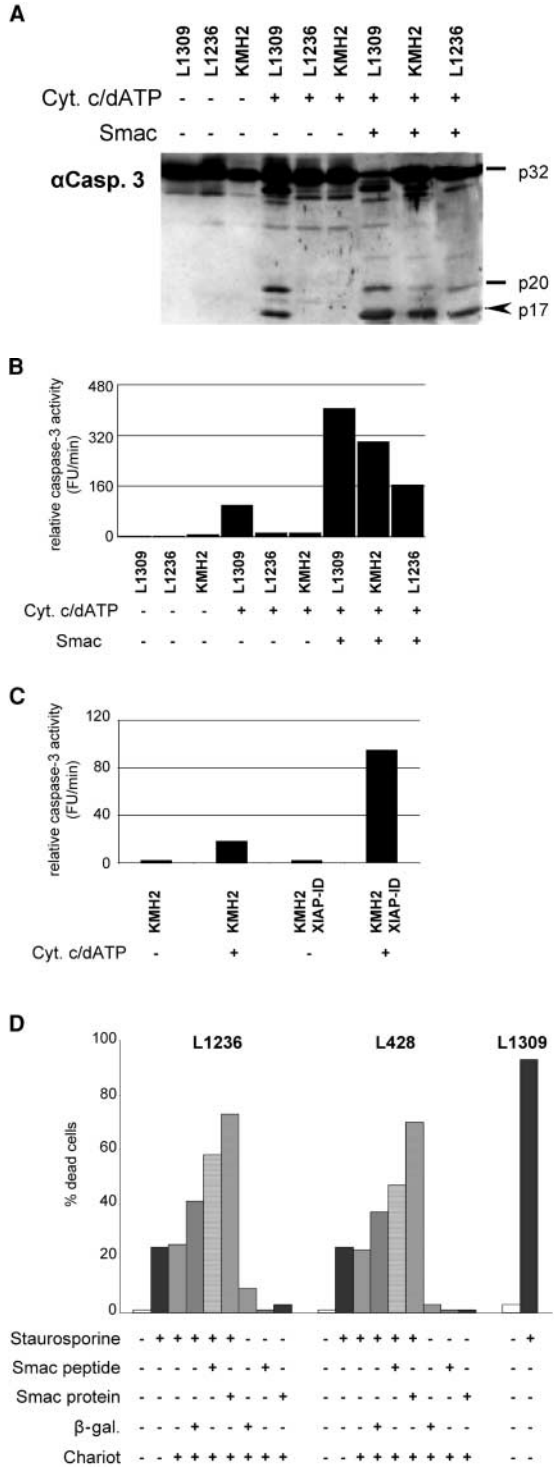

Figure 5. Depletion of XIAP restores caspase-3 processing and activity. Cytosolic extracts of L1236 and KMH2 cells and of control B cell L1309 were prepared, and equal amounts of protein were incubated with or without cytochrome *c*/dATP in the absence and presence of Smac protein for 1 h at 30°C. (A) Cytosolic extracts were resolved by SDS-PAGE and subjected to Western blot analysis. Caspase-3 was detected by polyclonal rabbit anti–caspase-3 antibody. (B) Relative caspase activity was measured by hydrolysis of DEVD-AFC. Samples were normalized for total cytosolic protein content. (C) XIAP was immunodepleted by mouse anti-XIAP mAb. Cytosolic extracts of KMH2 cells with or without XIAP were incubated with or without cytochrome *c*/dATP for 1 h at 30°C. Samples were normalized for total cytosolic protein content and relative caspase-3 activity was measured by DEVDase activity. (D) Cells were transfected with vehicle, β-galactosidase, Smac N7 peptide, or Smac protein and treated for 24 h with 1 μM staurosporine. Cell death was determined by trypan blue exclusion. Each value represents the average of results from two independent experiments.

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