Defective apoptotic pathways
in
Hodgkin and Reed-Sternberg cells

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dedicated to my parents

Contents

Abbreviations

AIF	apoptosis inducing factor
Akt	protein-serine/threonine kinase
Apaf-1	apoptotic protease-activating factor-1
BH	Bcl-2 homology
BIR	baculoviral IAP repeat
c-FLIP	cellular FLICE-inhibitory protein
DEVD-AFC	DEVD-7-amino-4-trifluoromethyl coumarin
DFF45	DNA fragmentation factor-45
DISC	death-inducing signaling complex
EBV	Epstein-Barr Virus
EGTA	Ethylene Glycol-bi α-aminoethyl Ether
FLICE	FADD-like interleukin-1beta-converting enzyme
GrB	granzyme B
HD B cells	Hodgkin derived B cells
H-RS	Hodgkin and Reed-Sternberg
IAP	inhibitor of apoptosis
LD	lymphocyte depletion
MHC	major histocompatibility complex
MC	mixed cellularity
NS	nodular sclerosis
OMM	outer mitochondrial membrane
PBS	phosphate buffered saline
PI3 Kinase	phosphoinositide 3-OH kinase
PMSF	Phenylmethylsulfonyl Fluoride
Smac/DIABLO	second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI
TNF	Tumour necrosis-factor
XIAP	X-linked inhibitor of apoptosis

Introduction

Apoptosis; Life requires Death. Programmed cell death, or apoptosis, is currently one of the most active areas of bio-medicinal research. Apoptosis describes the orchestrated collapse of a cell, which manifests in membrane blebbing, cell shrinkage, protein processing, chromatin condensation and DNA degradation followed by rapid engulfment of corpses by neighbouring cells. Programmed cell death has been discovered and rediscovered several times by various developmental biologists and cytologists, and has been named differently over the past two centuries (Vaux et al. 1996). The term finally adopted is apoptosis, coined by Wyllie and colleagues in 1972 to describe a common type of programmed cell death that the authors repeatedly observed in various tissues and cell types (Kerr et al. 1972). The authors noticed that these dying cells shared many morphological features, which were distinct from those observed in cells undergoing necrotic cell death, and they suggested that these shared morphological features might be the result of an underlying common, conserved, endogenous cell death programme (Wyllie et al. 1980) (Fig. 1).

Apoptosis is an essential part of life for any multicellular organism. It is necessary to purge the body of pathogen-infected cells, to shape the organs during development, but it is also needed to eliminate activated or auto-aggressive immune cells. Apoptosis has to be tightly regulated because too little or too much of cell death may lead to pathologic phenomena, including developmental defects, autoimmune diseases, neurodegeneration or cancer.

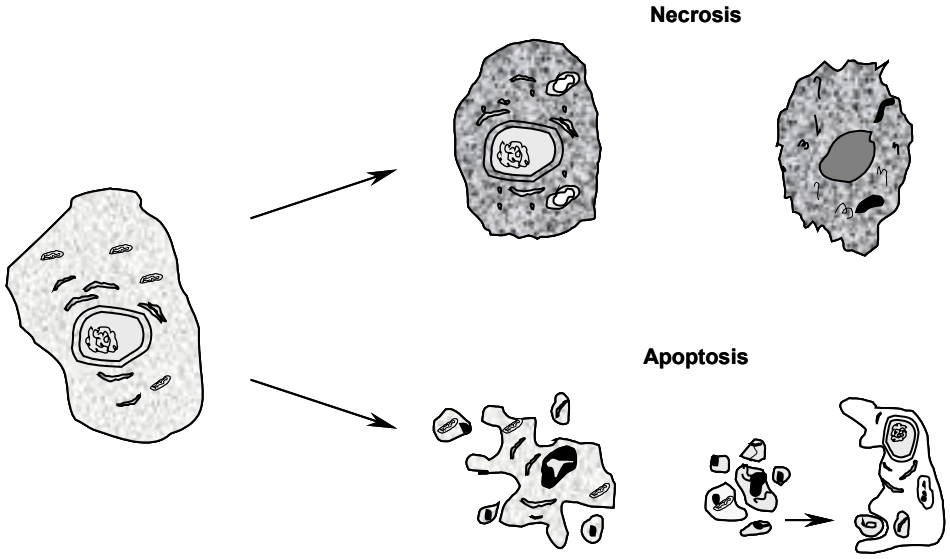


Fig. 1. Apoptosis or cell suicide. Upon apoptosis cells activate an intracellular death program and kill themselves in a controlled way. In contrast to necrotic cells, apoptotic cells shrink and form apoptotic bodies, which are rapidly up taken by neighbouring cells, before there is any leakage of their contents (Willie et al. 1980).

Central effectors of apoptosis. Apoptosis, the regulated destruction of a cell, is a complicated process. The decision to die depends on the activity of many molecules that determine a cell's likelihood of activating its self-destruction programme. Most of the specific morphological changes that were observed in apoptotic cells are caused by a set of cystein proteases upon apoptotic stimuli. These death proteases are homologous to each other, and are part of a large protein family known as caspases (Alnemri et al. 1996). Caspases are highly conserved throughout evolution, and can be found from humans to insects, nematodes and hydra (Budihardjo et al. 1999, Cikala et al. 1999 and Earnshaw et al. 1999). Over a dozen caspases have been identified in humans, and most of these have been suggested to fulfill distinct functions in apoptosis (Thornbery et al. 1998 and Rano et al. 1997).

All caspases are cystein containing aspartate-specific endoproteases. Caspases display distinct substrate specificity, determined by the four residues amino-terminal

to the cleavage site (Asp-Xxx). Caspases are activated upon various apoptotic stimuli that occur via cleavage of the proenzyme at a specific aspartate, which is followed by the dimerization of cleaved products to generate the active enzyme (Alnemri et al. 1997). The active enzyme can then cleave numerous cellular proteins necessary for cellular homeostasis and results in the typical morphological hallmarks of apoptosis (Thornbery et al. 1998).

Two apoptotic signaling pathways. Two major distinct apoptotic signaling pathways have been identified (Fig. 2). The first route can be initiated by the oligomerization, most probably the trimerization, of tumor necrosis factor receptor (TNF-R) family members, such as CD95, induced by ligation of their specific ligand. Receptor oligomerization results in formation of a complex of proteins associated with activated receptors. The so-called death-inducing signaling complex (DISC) is the first initiator complex after apoptotic induction. The death signal is propagated by a caspase cascade initiated by the activation of caspase-8, also called FLICE (FADD-like interleukin-1beta-converting enzyme), at the DISC followed by a rapid cleavage of executioner caspases, like caspase-3 and other caspases, which eventually cleave vital substrates in the cell (Krammer 2000, Krammer 1999, Kischkel et al. 1995, Peter et al. 1998, Muzio et al. 1996, Scaffidi et al. 1998 and Ashkenazi et al. 1999). The second apoptotic signaling pathway involves mitochondria. The mitochondrial apoptotic pathway is normally activated intrinsically. For example DNA damage can activate the mitochondrial apoptotic pathway through an unknown p53 dependent mechanism without the use of death receptors (Dubrez et al. 2001 and Henry et al. 2002). Once this pathway is activated, the mitochondria release pro-apoptotic factors from its intermembrane space, such as cytochrome c (Reed et al. 1998 and Jürgensmeier et al. 1998). The released cytochrome c together with the apoptosis

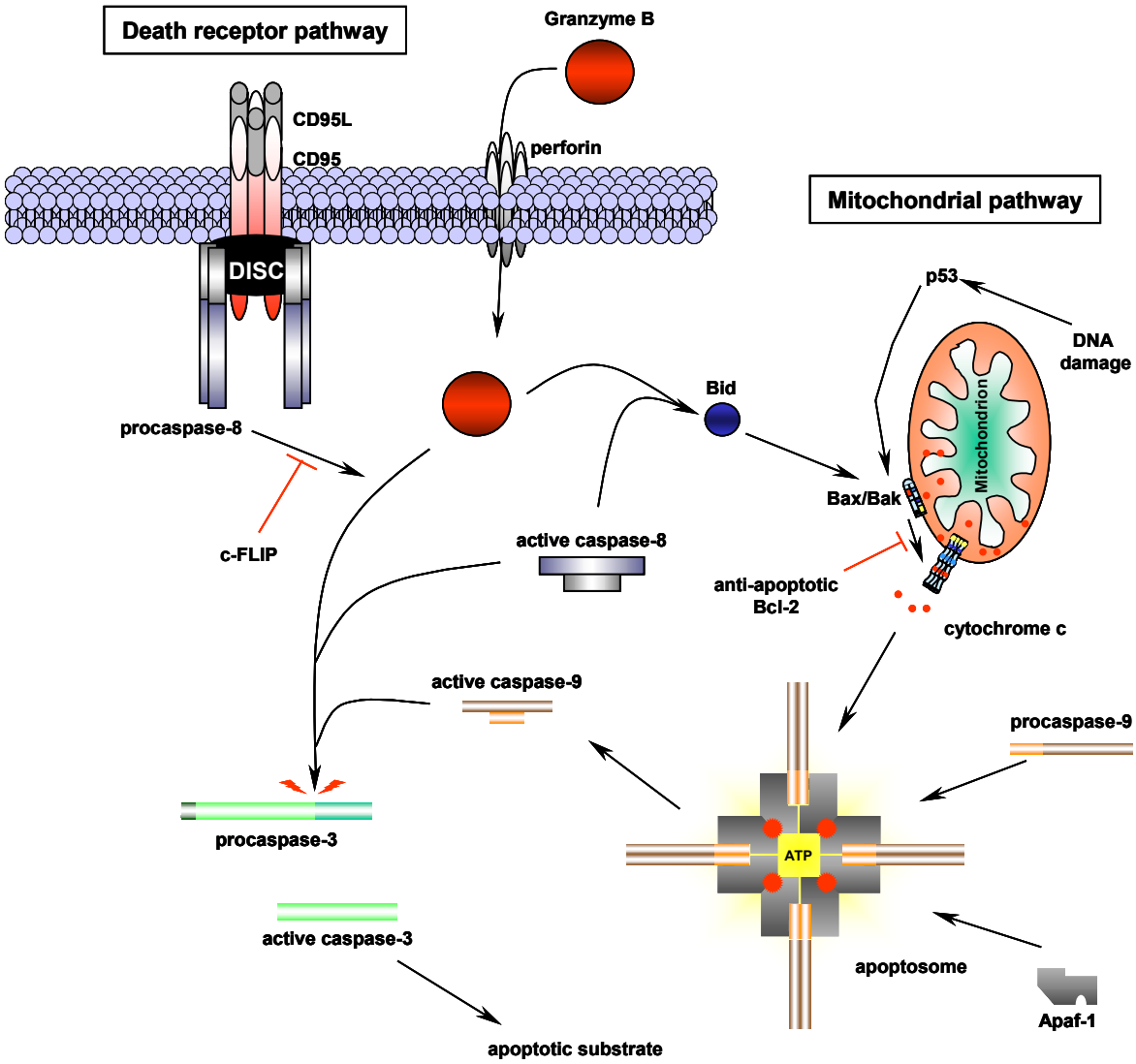
protease-activating factor (Apaf-1) and procaspase-9 in the cytoplasm forms the apoptosome, the other initiator complex of apoptosis (Li et al. 1997 and Saleh et al. 1999). This apoptosome complex in turn activates downstream executioner caspases, like caspase-3, -6 and -7 (Li et al. 1997, Reed et al. 1998 and Green et al. 1998). In addition, activated caspase-8 can also cleave Bid, a member the pro-apoptotic Bcl-2 family. Cleavage of Bid greatly increases its pro-death activity, and results in translocation of Bid to mitochondria, where it promotes cytochrome c release (Hengartner 2000). In most situations, the cross-talk between the death-receptor and mitochondrial pathways is minimal and probably depends on the amount of caspase-8 (Krammer 2000), therefore the two pathways operate largely independently of each other (Hengartner 2000, Gross et al. 1999-a and Yin et al. 1999).

Fig. 2. Apoptotic pathways in mammalian cells.

The death-receptor pathway is triggered by members of the death-receptor superfamily (such as CD95 and tumour necrosis factor receptor I). Binding of CD95 ligand to CD95 induces receptor clustering and formation of the DISC. The DISC recruits multiple procaspase-8 molecules, resulting in caspase-8 processing and activation. Caspase-8 activation can be blocked by recruitment of the caspase homologue c-FLIP (cellular FLICE-inhibitory protein) (Irmler et al. 1997). The mitochondrial pathway is used extensively in response to extracellular cues and internal insults such as DNA damage. These diverse response pathways converge on mitochondria, often through the activation of a pro-apoptotic member of the Bcl-2 family, such as Bid. Pro- and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria, where they compete to regulate cytochrome c release by a mechanism that is still debated. If the pro-apoptotic proteins are dominant, an array of molecules is released from the mitochondrial compartment. Principal among these is cytochrome c, which associates with Apaf-1 and then procaspase-9 (and possibly other proteins) to form the apoptosome. The death-receptor and mitochondrial pathways converge at the level of caspase-3 activation. Downstream of caspase-3, the apoptotic programme branches into a multitude of subprogrammes, the sum of which results in the ordered dismantling and removal of the cell.

Cross-talk and integration between the death-receptor and mitochondrial pathways is provided by Bid, a pro-apoptotic Bcl-2 family member. Caspase-8-mediated cleavage of Bid greatly increases its pro-death activity, and results in its translocation to mitochondria, where it promotes cytochrome c release. Additional death-inducing pathways are mediated by granzyme B, which enters the cells through a

pore-forming protein, perforin. Cytosolic granzyme B can then promote both, mitochondrial mediated or direct activation of caspase-3.



The mitochondrion in apoptosis. The mitochondrion is not only the cell's powerhouse, it is also its arsenal. The mitochondrion acts as a pivotal decision center in many types of apoptotic responses, because it releases death-promoting factors from its intermembrane space into the cytosol. Release of cytochrome c from mitochondria couples these organelles to a pathway for caspase activation and apoptosis induction (Reed et al. 2000 and Matsuyama et al. 2000). Cytochrome c, which normally shuttles electrons between protein complexes of the respiratory chain in the mitochondria, can trigger formation of the apoptosome in the cytoplasm in the

presence of dATP. Apoptosis is therefore depending on ATP production. If mitochondria are damaged and fail to produce ATP early during apoptosis, the apoptosome can not form, and caspase-9 is not activated and cells die by necrosis. This emphasizes the pro-apoptotic role of functional mitochondria (Desagher et al. 2000, Nicotera et al. 1999 and Chautan et al. 1999). Cytochrome c is only one of the mitochondrial pro-death molecules. Also present in mitochondria and released upon induction of apoptosis are Smac/DIABLO, which antagonizes the inhibitory effect of the inhibitors of apoptosis (IAP) (Du et al. 2000 and Verhagen et al. 2000), apoptosis inducing factor (AIF), a flavoprotein with potent but relatively mysterious apoptotic activity (Lorenzo et al. 1999) and Omi/HtrA2, a serin protease which interacts and inhibits the X-linked inhibitor of apoptosis (XIAP) and enhances caspase activity (Hegde et al. 2002).

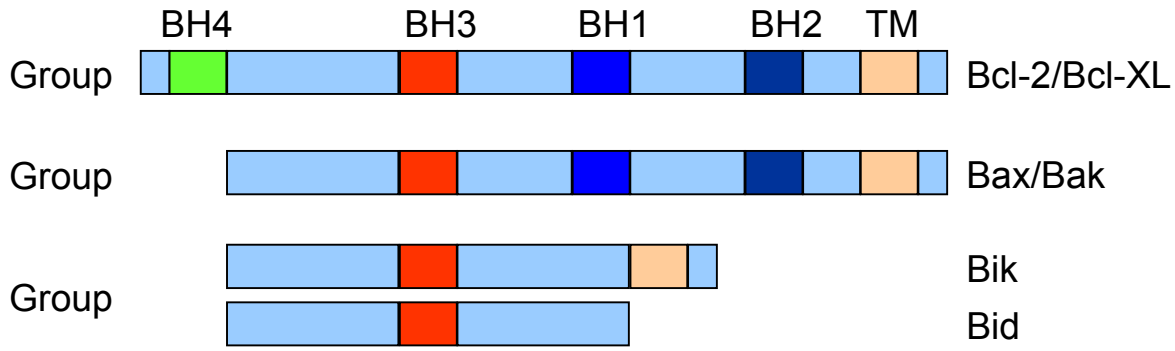
The outer mitochondrial membrane (OMM) becomes permeable to apoptogenic factors through different apoptotic stimuli. Permeabilization of the OMM is controlled by members of the Bcl-2 family (Desagher et al. 2000).

Bcl-2 family, regulators of apoptosis. Each step of the apoptotic signaling cascade is under stringent control. Important regulators of apoptotic signaling are the proteins of the Bcl-2 family. This protein family was named after the founding member of the family, which was isolated as a gene involved in follicular B cell lymphoma (Tsujimoto et al. 1985). The Bcl-2 family is comprised of well over a dozen proteins, which have been classified into three functional groups (Adams et al. 1998, Antonsson et al. 2000 and Hengartner 2000). Members of the first group, such as Bcl-2 and Bcl-x_L, are characterized by four short, conserved Bcl-2 homology (BH) domains (BH1–BH4) (Fig. 3). They also possess a C-terminal hydrophobic tail, which localizes the proteins to the outer surface of mitochondria, and occasionally of the endoplasmic reticulum

(Foyouzi-Youssefi et al. 2000 and Hacki et al. 2000), with the bulk of the protein facing the cytosol (Reed et al. 1998). The key feature of group I members is that they all possess anti-apoptotic activity and protect cells from death. In contrast, group II consists of Bcl-2 family members with pro-apoptotic activity. Members of this group, which includes Bax and Bak, have a similar overall structure to group I proteins, containing the hydrophobic tail and all but the most N-terminal, BH4 domain (Adams et al. 1998 and Antonsson et al. 2000). Structure/function studies suggest that anti- versus pro-apoptotic activity is determined by relatively large regions of the protein, including two large α-helices that have been proposed to participate in membrane insertion. Group III consists of a large and diverse collection of proteins, whose only common feature is the presence of the 12–16-amino-acid BH3 domain (Adams et al. 1998). Although some members of group III, including Bid, are indeed divergent homologues of Bcl-2 and Bax (McDonnell et al. 1999 and Chou et al. 1999), others share little sequence or structural similarity with group I and II, suggesting that the BH3 domain in these proteins has arisen through convergent evolution (Gross et al. 1999-b). Classification of such proteins as Bcl-2 family members is thus more a matter of convenience than a statement of presumed evolutionary relationship (Hengartner 2000).

Fig. 3. Bcl-2 family protein.

The Bcl-2 family is comprised of well over a dozen proteins, which have been classified into three functional groups. Members of the first group, such as Bcl-2 and Bcl-x_L, are anti-apoptotic and characterized by four short, conserved Bcl-2 homology domains and a C-terminal hydrophobic tail. In contrast, group II consists of Bcl-2 family members with pro-apoptotic activity, including Bax and Bak, have a similar overall structure to group I proteins, but the most N-terminal, BH4 domain. Group III consists of a large and diverse collection of proteins, including Bid and Bik, whose only common feature is the presence of the ~12–16-amino-acid BH3 domain.



How do Bcl-2 family members regulate cell death? Many Bcl-2 family members can homodimerize, but more importantly, pro- and anti-apoptotic members can form heterodimers (Adams et al. 1998, Antonsson et al. 2000 and Reed 1997). To a first approximation, heterodimerization can simply be thought as resulting in mutual neutralization of the bound pro- and anti-apoptotic proteins. Thus, cells with more pro-death proteins are sensitive to death, cells with an excess of protective family members are usually resistant (Hengartner 2000).

Based on the structural similarity of Bcl-x_L to the pore-forming subunit of diphtheria toxin (Muchmore et al. 1996), it has been suggested that Bcl-2 proteins might act by undergoing a conformational change, followed by insertion into the outer mitochondrial membrane, where they could form channels or even large pores (Hengartner 2000). Bcl-2 family members indeed can insert into synthetic lipid membrane and mitochondrial outer membrane, oligomerize, and form channels with discrete conductances (Reed 1997).

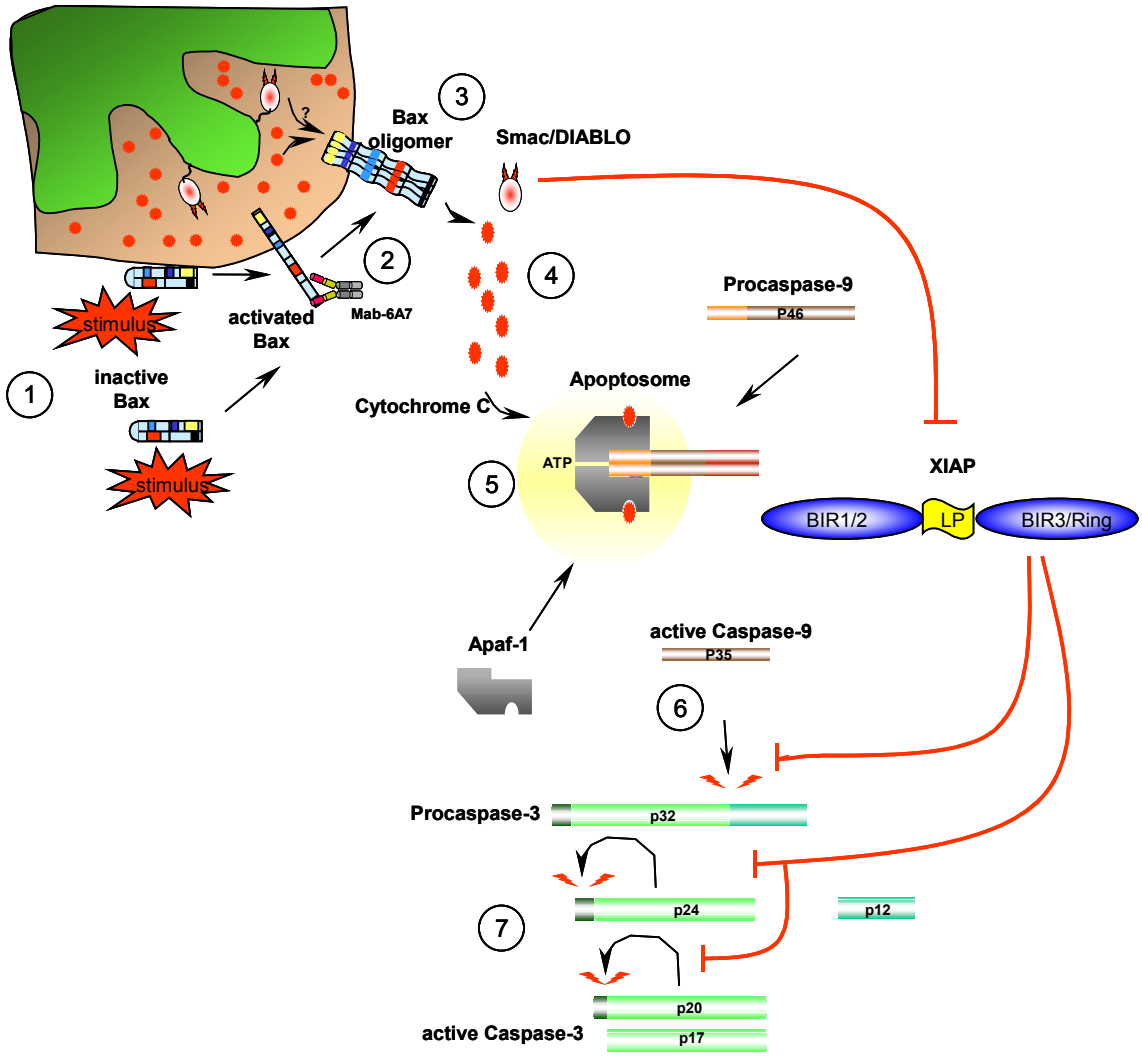
One of the most investigated molecular cross talks among the Bcl-2 family proteins is the Bid-mediated oligomerization of Bax and Bak (Wei et al. 2001, Wei et al. 2000 and Korsmeyer et al. 2000). The connection and integration between the death-receptor and mitochondrial pathways is apparently provided by Bid. Caspase-8

mediated cleavage of Bid greatly increases its pro-death activity and results in its translocation to mitochondria, where Bid either promotes cytochrome c release directly (Zhai et al. 2000 and Kim et al. 2000) or via interaction with other pro-apoptotic Bcl-2 proteins such as Bax and Bak. Cleaved Bid binds to Bax and Bak and stimulates an allosteric activation of these molecules resulting in oligomerization and formation of the proposed pores for cytochrome c efflux (Wei et al. 2000, Desagher et al. 1999 and Eskes et al. 2000).

Conformational change of Bax: a question of life or death. Bax is a pro-apoptotic Bcl-2 protein that in healthy cells is 'dormant', located in the cytosol or loosely associated with mitochondria (Roucou et al. 2001). Upon induction of apoptosis, Bax can induce the release of cytochrome c from mitochondria and represents an important relay of the mitochondrial apoptotic pathway and subsequent activation of caspases (Kluck et al. 1997, Reed et al. 1998 and Jürgensmeier et al. 1998). In order to induce cytochrome c release, Bax moves from the cytosol to the mitochondria, undergoes a conformational change, leading to exposure of its N- and C-termini which appears to be required for insertion into the mitochondrial outer membrane. Allosteric activated Bax can now form oligomers and gain its pro-apoptotic activity (Wolter et al. 1997, Nechushtan et al. 1999 and Murphy et al. 2000). The recent resolution of the 3D structure of full length monomeric Bax has brought new insights into the requirement for structural modifications but the underlying mechanisms for the conformational change and the activation of Bax are not yet known (Fig. 4).

Fig. 4. Mitochondrial apoptotic pathway. Bax, either cytosolic or loosely associated with mitochondria (1), undergoes a conformational change upon apoptotic stimulation. The conformational change leads to exposure of its N- and C-termini with subsequent insertion in the mitochondrial outer membrane. Only the relaxed, flexible N-termius of Bax in its new conformation can be detected by

mouse anti-Bax 6A7 (2). Allosteric activated Bax can now form oligomers (3) and promote release of apoptotic proteins, such as cytochrome c and Smac/DIABLO (4). Cytosolic cytochrome c induced Apaf-1 oligomerization, which in turn induces recruiting and processing of pro-caspase-9 (p46) in the apoptosome by an intrinsic autocatalytic activity of procaspase-9 itself, generates active caspase-9 (p35) (5). Active caspase-9 cleaves and activates procaspase-3 (p32) generating the large (p24) and small (p12) subunits (6). Autocatalytic processing of p24 subunit of caspase-3 occurs in two sequential steps, removing its own prodomain generating the mature p17 fragment (7). The initiation of the caspase cascade by the apoptosome can be inhibited by XIAP at two distinct levels, at the level of caspase-9 activation and at the level of caspase-3 activation by inhibiting the autocatalytical processing. The inhibitory effect of XIAP can be abrogated by cytosolic Smac.



Apoptotic signaling downstream of mitochondria. A critical step in the induction of the caspase cascade downstream of mitochondria is the activation of the apoptotic initiator caspase-9. How the formation of the apoptosome complex leads to caspase-

9 activation is not yet clear but it might be induced by dimerization which is mediated by multimeric Apaf-1 complex (Acehan et al. 2002 and Renatus et al. 2001). Apaf-1 induces processing of procaspase-9 by an intrinsic autocatalytic activity generating active caspase-9 (Srinivasula et al. 1998) (Fig. 4). Once activated, caspase-9 can initiate a caspase cascade involving downstream executioners such as caspase-3, -6, and -7. Caspase-3 is expressed in cells as an inactive 32-kDa precursor from which the 17-kDa and 12-kDa subunits of the mature caspase-3 are proteolytically generated during apoptosis (Han et al. 1997). The processing of caspase-3 occurs in two steps, beginning with a cleavage by other site specific proteases between what will be the large and small subunits. Following this cleavage, the large domain of caspase-3 removes its own prodomain in two sequential steps generating the mature p17 fragment (Martin et al. 1996, Deveraux et al. 1999-a, Deveraux et al. 1998, Deveraux et al. 1997 and Fernandes-Alnemri et al. 1996).

Regulation of caspase activation. The function of the caspases is modulated by a set of proteins, the IAPs (inhibitor of apoptosis) (Deveraux et al. 1999-b). The IAP gene products play an evolutionary conserved role in regulating apoptosis in diverse species ranging from insects to humans (Deveraux et al. 1998). Members of the IAP family, originally identified in baculoviruses (Crook et al. 1993), contain one or more modules called baculoviral IAP repeat (BIR) (Deveraux et al. 1999-b). Some family members also contain a C-terminal RING finger through which they are targeted for ubiquitination and subsequent proteolysis (Huang et al. 2000).

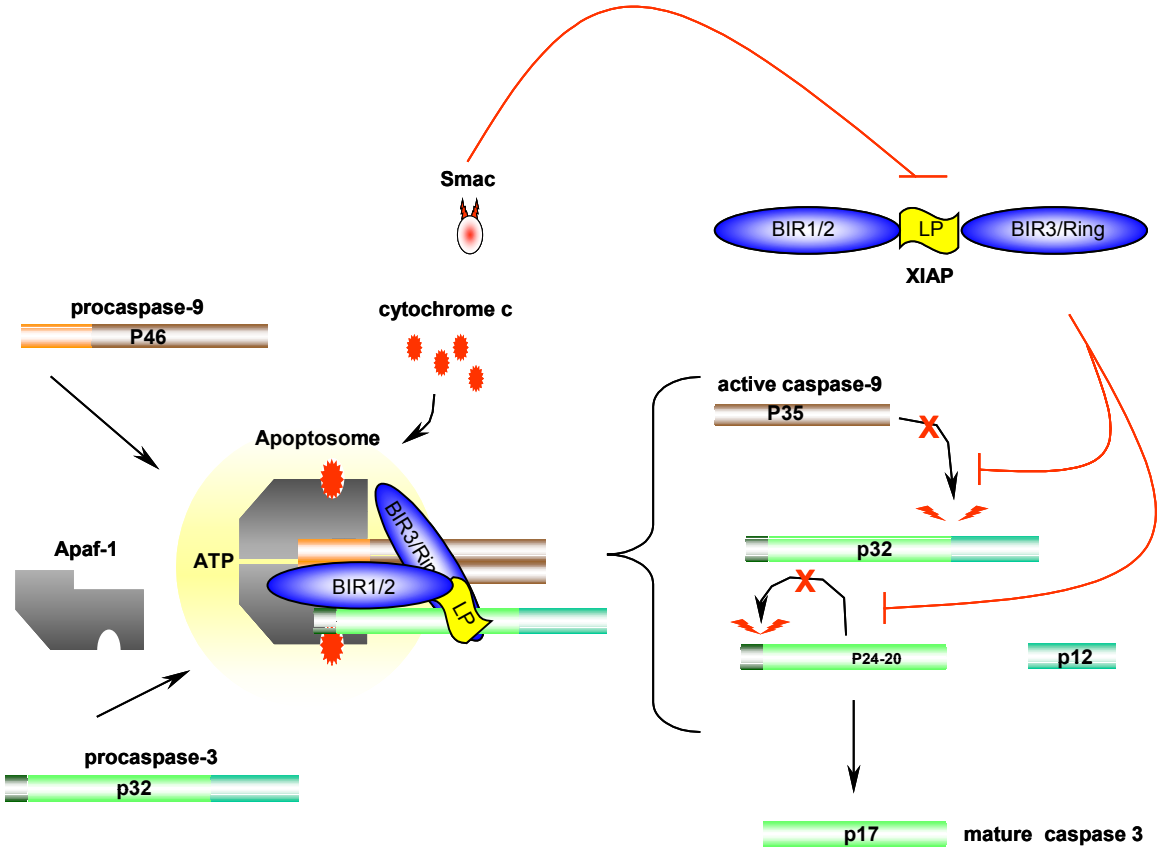
One of these, XIAP, binds to and inhibits the protease activity of caspase-9, and thereby blocks the apoptotic process at this point (Deveraux et al. 1998). It also binds to and inhibits caspase-3 and –7, but not caspase-8 (Takahashi et al. 1998 and Riedl et al. 2001). Caspase binding and inhibition is mediated by the BIR domains of IAPs,

which are necessary and sufficient for their function. The inhibition of caspase-3 by XIAP has an unique characteristic. If XIAP inhibits caspase-3, the first cleavage can occur but the second will not, due to the inhibition of caspase-3 activity (Deveraux et al. 1999-a, Deveraux et al. 1998 and Deveraux et al. 1997 and Fernandes-Alnemri et al. 1996). The removal of the prodomain is not required for caspase-3 activity, but this partial cleavage might serve as a "footprint" of XIAP inhibition of caspase-3 function (Deveraux et al. 1999-a, Deveraux et al. 1998, Deveraux et al. 1997 and Green 2000). The RING domain of XIAP acts as an ubiquitin ligase and promotes the degradation of XIAP and any caspase bound to it. Highly expressed XIAP can promote ubiquitination and proteosomal degradation of active-form of caspase-3 and enhances its anti-apoptotic effect (Suzuki et al. 2001). In summary, XIAP, and probably other IAP molecules, block the apoptotic process by binding, inhibiting, and degrading caspases.

Additional regulation is provided by Smac (second mitochondria-derived activator of caspases) or its murine homolog DIABLO (direct IAP-binding protein with low pI), which binds to IAPs and abrogates caspase inhibition (Du et al. 2000 and Verhagen et al. 2000). The proform of Smac contains a N-terminal sequence that targets this protein to the intermembrane space of mitochondria. Upon induction of apoptosis, Smac is released into the cytosol together with cytochrome c and modulates apoptosis (Verhagen et al. 2000 and Stephan et al. 2001). In addition to regulation of apoptosis-associated mitochondrial cytochrome c release, Bcl-2 family proteins also regulate the Smac release, suggesting that both molecules may escape via the same route (Adrain et al. 2001, Deng et al. 2002 and Roucou et al. 2001). Smac promotes apoptosis through at least two mechanisms: by inducing the proteolytic activation of procaspase-3 and by promoting the enzymatic activity of mature caspase-3. Both functions depend on the ability of Smac to interact physically with IAPs. The N-

terminal residues of Smac share significant homology with the conserved class of IAP-binding motifs (Srinivasula et al. 2001). It is likely that the N-terminus of the Smac protein simply displaces the bound caspase and releases the activated enzyme (Adrain et al. 2001 and Chai et al. 2000) (Fig. 4 and 5).

Fig. 5. Inhibition of the caspase cascade in the apoptosome by XIAP.
Apaf-1 forms a cytochrome c/dATP dependent oligomer and recruits procaspase-9 (p46) to the apoptosome and promotes capase-9 autocatalytic activity. Caspase-9 in a complex with Apaf-1 recruits caspase-3 and promotes its initial cleavage followed by its autocatalytical processing. XIAP is also attracted to the apoptosome and can bind to and inhibit caspase-9 through its BIR3 domain. It can simultaneously bind to and inhibit caspase-3. The binding to caspase-3 is mediated by LP (linker peptide) part of XIAP, which binds to the catalytic groove of caspase-3 and might provide allosteric inhibition for the substrate binding site of caspase-3, which requires BIR1/2 domains. Additional regulation is provided by Smac, which binds to XIAP and abrogates caspase inhibition. Smac promotes caspase-3 activation through two mechanisms: promoting caspase-9 activation which leads to initial cleavage of caspase-3, and promoting the caspase-3 proteolytic activity which leads to maturation of caspase-3 generating the proteolytically active p17 fragment.



Granzyme B as an initiator and executioner of apoptosis. Whereas some cytotoxic T lymphocytes kill their target cells by turning on the death receptor, others use perforin and granzyme B (GrB). The major mechanism of cytotoxic lymphocyte killing involves the directed release of granules containing perforin and a number of proteases onto the target cell membrane. One of these proteases, GrB, has an unusual substrate site preference for Asp residues, a property which is shared with members of the caspase family (Martin et al. 1996). With the help of perforin, GrB finds its way into the target cell and can kill it by 1) cleaving and activating caspase-8 (Medema et al. 1997), 2) cleaving caspase-3 between the large and small subunits of proenzyme and converting it to its active form (Martin et al. 1996 and Fernandes-Alnemri et al. 1996), 3) cleaving of Bid and activation of mitochondrial apoptotic pathway (Heibein et al. 2000, Sutton et al. 2000 and Pinkoski et al. 2001), or 4) caspase-independent direct cleavage of death substrates, such as the human DNA fragmentation factor-45 (DFF45) (Sharif-Askari et al. 2001) (Fig. 2).

Cancer is a disease of deregulated Apoptosis. Cancer is a plethora of conditions characterized by unscheduled and uncontrolled cellular proliferation. The causes of cancer are many and varied, and include genetic predisposition, environmental influences, infectious agents and ageing. In almost all instances, deregulated cell proliferation associated with suppressed apoptosis provide the underlying platform for neoplastic progression and carcinogenesis (Evan et al. 2001). The study of the molecular basis of apoptosis is leading to the discovery of molecular cross talks, some of which are already used in clinical trials, and promise for more refined and more effective cancer therapies.

Discovery of Reed-Sternberg cells. In 1832, Thomas Hodgkin (Hodgkin 1832) described the anatomic findings of seven patients with enormous lymph node swellings. Independently, Samuel Wilks published an article in 1856 summerizing ten cases having the same anatomic features (Wilks 1856). In the following years, Wilks collected additional 15 cases and interpreted them as pathognomonic for a new disease entity (Wilks 1865). Although it was Wilks who separated Hodgkin's disease from other diseases associated with lymph node swellings, he was sufficiently magnanimous to name the disease after Hodgkin in acknowledgment of Hodgkin's first report of some cases. In 1898, Carl Sternberg (Sternberg 1898) and in 1902, Dorothy Reed (Reed 1902) published, independently of each other, a more detailed description of the cytological features of the multinucleated giant cells, which have since been known as Reed-Sternberg cells. The mononucleated blasts were later designated Hodgkin cells. Combined histological and immunohistological studies of the last 25 years characterized Hodgkin and Reed-Sternberg cells (H-RS) as a distinct immunotype of atypical blasts in Hodgkin's disease with constant expression of CD30, frequent expression of CD15, and constant absence of the J chain of immunoglobulin. In view of these findings, the international Lymphoma Study Group proposed to subsume all Hodgkin's disease histotypes with this distinct immunotype, including nodular sclerosis (NS), mixed cellularity (MC), and lymphocyte depletion (LD), under the generic term classical Hodgkin's disease (Harris et al. 1994 and Mauch et al. 1999).

Permanent Hodgkin's disease cell lines. Because H-RS are rare in the tissues affected by Hodgkin's disease, research has been hampered for decades. Immunohistological methods allowed the specific detection of certain molecules in the tissues at the single-cell level but for functional analysis and crosstalks between

different molecules permanent cell lines are needed. Attempts were made to establish permanent cell lines derived from Hodgkin and Reed-Sternberg cells in last 30 years. The first permanent cell line, designated L428, was established in Diehl's laboratory in 1979 (Schaadt et al. 1979). The L428 line was established from a patient with advanced-stage Hodgkin's disease and grew out from a pleural effusion. All additional cell lines were also established from body fluids (bone marrow, pleural effusion and peripheral blood) of patients with advanced-stage disease (Table 1, materials and methods). These cell lines resemble in situ H-RS cells as confirmed by i) morphology, ii) ability to bind T cells to form rosettes, iii) expression of major histocompatibility complex (MHC) class II molecules, and iv) the absence of immunoglobulin and macrophages characteristic enzymes (Mauch et al. 1999).

Aim of the work. Apoptosis in the immune system is a fundamental process regulating lymphocyte maturation, receptor repertoire selection and homeostasis. Death by apoptosis is as essential for the function of lymphocytes as growth and differentiation. The ability to avoid apoptosis is one of the keys to cancer cell survival and expansion. The Reed-Sternberg cell of Hodgkin's disease (H-RS) is a malignant germinal center B cell with rearranged but nonproductive immunoglobulin genes (Küppers et al. 1998 and Cossman et al. 1999). However, through yet unknown mechanisms, H-RS cells resist the apoptotic fate normally suffered by defective B cells with crippled immunoglobulin genes. Four Hodgkin derived B cell lines (HD B cell lines) uniformly proved resistant to apoptosis induced by CD95L (Re et al. 2000) suggesting the existence of defective apoptotic pathways in these cell lines. While emerging evidence suggests a proximal defect in CD95 signaling in H-RS cells due to c-FLIP (Thomas et al. 2001) little is known about possible defects of mitochondrial apoptotic pathway. This work is focused on the mitochondrial apoptotic pathway, and

aimed to unravel the intracellular mechanisms that contribute to resistance to apoptosis. HD B cell lines were stimulated with staurosporine, that induces apoptosis selectively involving the intrinsic mitochondrial pathways. Special emphasis was devoted to the action of Bcl-2 family members and the apoptosome, in order to obtain new insights into possible molecular defects up- and downstream of mitochondria. The detailed analysis revealed two distinctive defects in mitochondrial apoptotic pathway in Hodgkin and Reed-Sternberg cells at the level of activation of Bax, upstream of mitochondria, and caspase activation, downstream of mitochondria.

Materials and Methods

Cell culture

The establishment of the Hodgkin B-cell lines L591, L428, L1236 and KMH2 has been described elsewhere (Schaadt et al 1979, Diehl et al 1982, Kanzler et al 1996 and Kamesaki et al 1986) (Table 1). The control B cell lines were established by immortalisation of human primary B cells by EBV. The cell lines were cultured in VLE RPMI 1640 (Biochrom) supplemented with 10% FCS (Biochrom), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. Apoptosis was induced by incubating the cells in the presence of 1 µM staurosporine (Alexis). Cell death was examined using trypanblue exclusion. All chemicals were purchased from Sigma unless indicated otherwise.

Table 1. Immunophenotype and genotype of cell lines established from tissues affected with Hodgkin's disease.

Cell lines	CD30	CD70	CD15	CD68	B-AG	T-AG	rearanged gene	chain Expression	EBV
L428	+	+	+	-	-	-	IgH, L	-	-
L591	+	+	+/-	-	CD19, 20	CD2	IgH, L	IgAλ	+
L1236	+	+	+	-	-	-	IgH, L	-	-
KMH2	+	+	+	-	-	-	IgH, L	-	-

CD: cluster of differentiation, AG: Antigen, EBV: Epstein Barr virus, Ig: Immunoglobulin,

- The HD B cell lines and L1309, L1311 were a gift of Prof. Dr. V. Diehl, University of Köln, Germany.
- LCL6 control B cell line was a gift of Dr. M. Kochanek, University of Köln, Germany.

- C28 control B cell line was a gift of Dr. H. Abken, University of Köln, Germany.

Sample Preparation and Immunoblotting

For isolation of cytosolic extracts for western blot analysis, 10^7 cells were washed twice with PBS at 4 °C. Cells were resuspended in 50 µl of buffer A and 1 mM dithiothreitol and incubated for 20 min on ice for swelling. After addition of mannitol and sucrose to a final concentration of 220 mM and to 68 mM respectively, cells were cracked by passing through a 27-gauge needle. Cell breakage was verified microscopically using trypanblue exclusion. Membranes were pelleted at 14,000 x g for 20 min at 4 °C, and the resulting supernatants recovered (cytosolic extract). Whole cell extracts were prepared by lysing 10^7 cells in 1 ml of CHAPS lysis buffer on ice for 30 min. The crude lysate was then centrifuged at 14,000 x g for 20 min at 4 °C and the supernatant stored at -80 °C. Equal volumes of cytosol and whole cell extract (same cell number) were separated by SDS-PAGE and transferred to nitrocellulose membrane (Protran 0.2 µm; Schleicher and Schuell). Blots were incubated with Blocking buffer for 30 min. and subsequently incubation with primary antibodies shown in table 2. Horseradish peroxidase conjugates of anti-rabbit and anti-mouse IgG (Biorad) were used as secondary antibodies and signals were detected by ECL (Amersham).

- Polyclonal anti-sera specific for Bcl-2 and Bcl-X_L were a gift from Dr. John C. Reed, La Jolla, USA.
- Polyclonal anti-serum specific for caspase-9 was a gift from DR. Guy Salvesen La Jolla, USA.

Table 2. Antibodies used in western blotting.

antigen	isotype	immunogene	supplier	dilution	Catalog No.
hApaf-1	polyclonal/rabbit	aa 12-28	BD Pharmingen	1/2000	559683
m,hβactin	monoclonal/mouse	N-terminal 16 aa	Sigma	1/5000	A-5541
h,m,rBak	polyclonal/rabbit	aa 14-33	BD Pharmingen	1/2000	65401A
hBax	polyclonal/rabbit	aa 43-61	BD Pharmingen	1/2500	13666E
hBcl-2	polyclonal/rabbit	-	Dr. JC Reed	1/2500	-
hBcl-XL	polyclonal/rabbit	-	Dr. JC Reed	1/2500	-
Caspase-3	polyclonal/rabbit	full length	BD Pharmingen	1/4000	556425
Caspase-9	polyclonal/rabbit	-	Dr. G. Salvesen	1/3000	-
Cytochrome c	monoclonal/mouse	aa 1-80, 66-104	BD Pharmingen	1/2500	556433
hSmac	polyclonal/rabbit	aa 199-212,226-239	Alexis Biochemicals	1/1500	210-788-c100
hILP/XIAP	monoclonal/mouse	aa 268.426	BD Transduction lab.	1/2500	H62120

aa: amino acid, h: human, m: mouse, r: rat

Table 3. Reagents for western blotting

CHAPS lysis buffer	10 mM HEPES, pH 7.4, 150 mM NaCl, 1% CHAPS, protease complete cocktail
Buffer A	50 mM PIPES, pH 7.0, 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 µM cytochalasin B, protease complete cocktail (15 µg/ml pancreatic extract; 15 µg/ml pronase; 0.8 µg/ml thermolysin; 1.5 µg/ml chymotrypsin; 0.2 mg/ml trypsin; 1 mg/ml papain; Roche Diagnostics)
5x loading buffer	60 mM Tris-HCl (pH 6,8), 14,4 mM SDS, 25% Glycerol solution, 1% Bromphenol blue
10x Running buffer	3% Tris base, 14,4 Glycine, 1% SDS
Transfer buffer	25 mM Tris/Glycine, 20% Methanol and 0.05% SDS pH 8,3
10xWashing buffer (STAN PBS)	1,2 M NaCl, 0,1 M NaH_2PO_4, 0,3 M K_2HPO_4 pH7,6

Immunoprecipitation of activated Bax

Equal volumes of whole CHAPS cell lysates (10^7 cells/ml) were used for immunoprecipitation. The KCl concentration of the cell lysates was adjusted to 150 mM, and all samples were brought to a final volume of 500 µl with CHAPS lysis buffer. Samples were rotated for 12 h at 4 °C with 6 µg of monoclonal anti-Bax 6A7 antibody. Antigen-antibody complexes were immobilized by rotation for 2 h at 4 °C with GammaBind G Sepharose (Pharmacia Biotech). The complexes were pelleted (1 min, 14000 g) and the supernatant removed. The complexes were then washed 3 times with the same buffer used for the immunoprecipitation at 4°C and subjected to SDS-PAGE and immunoblotted as described above.

Table 4. Antibodies for immunoprecipitation.

antigen	isotype	immunogene	supplier	Catalog No.
h,m,rBax	monoclonal/mouse (6A7)	aa 12-24	BD Pharmingen	66241A

Immunofluorescence and Fluorescence Microscopy

Cells were treated with 1 µM staurosporine for 0, 2, 4, 6 and 12 h, harvested and washed twice with cold PBS. Cells were then fixed with 3 % paraformaldehyde in PBS for 20 min, permeabilized in washing buffer for 10 min, and blocked with blocking buffer for 30 min. For immunostaining, cells were incubated with primary mouse anti-Bax 6A7 antibody for 1 h, washed 3x with washing buffer and then incubated with goat anti-mouse antibody conjugated with Alexaflour 568 (Molecular Probes) (diluted: 1/1000) for 30 min. Nuclei were counterstained with Hoechst 33258 (Sigma) (10 µg/ml PBS) and mounted on glass slides and examined under a fluorescence microscope.

Table 5. Antibodies for Immunofluorescence

antigen	isotype	immunogene	supplier	dilution	Catalog No.
h,m,rBax	monoclonal/mouse (6A7)	aa 12-24	BD Pharmingen	1/1000	66241A

Table 6. Reagents for Immunofluorescence staining

Blocking buffer	0.1% Saponin, 3% BSA (fraction V), 4 µl/ml Gelatine (Teleostean Gelatine) in PBS
Washing buffer	0.1 % Saponin in PBS

Preparation of cytosolic extracts and caspase activation

For preparation of cell free lysates the procedure described by Ellerby et al., 1997, and Stennicke et al., 1998 was used with minor modifications. Cells were harvested, washed twice in phosphate-buffered saline at 4 °C, pelleted for 5 min at 1200 g, resuspended in HEB and allowed to swell on ice for 20 min. After addition of PMSF to 100 µM, cells were cracked by passing through a 27-gauge needle and pelleted at 14,000 x g for 20 min at 4 °C. The resulting supernatant (cytosolic extract) was recovered. Protein concentration was determined by the bicinchroninic acid assay method (Pierce) using BSA as a standard and were adjusted to 20 mg/ml.

Caspase activation by exogenously added cytochrome c/dATP. 10 µM horse heart cytochrome c together with 1 mM dATP, were added to 20 µl of cell extracts and incubated for 1 h at 30°C.

Caspase activation by exogenously added caspase-8. 100 nM of purified recombinant active caspase-8 (Stennike et al 1997) was added to 20 µl of cell extracts or HEB as a control and incubated for 1 h at 30°C.

Caspase activation by exogenously added granzyme B. 60 µg granzyme B (Sigma) was added to 20 µl of cell extracts or HEB as a control and incubated for 1 h at 30°C.

Caspase activation by cytochrome c/dATP and further addition of Smac. 1 µM recombinant Smac protein without the mitochondrial targeting sequence together with 10 µM horse heart cytochrome c and 1 mM dATP were added to 20 µl of cell extracts and incubated for 1 h at 30°C.

Proteolytic activity of exogenously added mature caspase-3. 100 ng (1µl) recombinant active caspase-3 (BD Bioscience) was added to 40 µg (1µl) cytosolic extract or 1 µl of HEB and pre-incubated for 15 min. on ice followed by 30 min. incubation at 30°C.

1 µl of resulting cytosolic extracts and HEBs were added to 99 µl caspase buffer and reactions were initiated by addition of 100 µM Ac-DEVD-AFC (Ac-DEVD-7-amino-4-trifluoromethyl coumarin) (Stennicke et al 1997). Caspase activity was assayed by release of 7-amino-4-trifluoromethyl-coumarin (AFC) (Alexis) from DEVD containing synthetic peptides using continuous-reading plate reader (Wallac victor ^{2}TM multilabel counter 1420) thermostated at 30°C at 400 /505 nm excitation and emission respectively.

Table 7. Reagents for extraction of cytosolic fraction and caspase activation.

HEB	(20 mM PIPES, pH 7.0, 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol)
Caspase buffer	(20 mM Pipes, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10 % sucrose, 10 mM dithiothreitol)

- Recombinant active caspase-8 and Smac protein were a gift from DR. Guy Salvesen La Jolla, USA.

Immunoprecipitation of caspase-3

Equal amounts of cytosolic extracts were adjusted to a final volume of 500 µl with HEB. Samples were rotated for 6 h at 4 °C with 1 µg of anti-caspase-3 antibody. Antigen-antibody complexes were immobilized by rotation for 2 h at 4 °C with GammaBind G Sepharose (Pharmacia Biotech). The complexes were pelleted (1 min, 14000 g) and the supernatant removed. After 3 cold washing steps with the same buffer used for the immunoprecipitation, samples were subjected to SDS-PAGE and immunoblotted as described above.

Table 8. Antibodies for immunoprecipitation.

antigen	description	organism	immunogene	supplier	Catalog No.
hcaspase-3	Monoclonal	Mouse	aa 1-219	BD Transduction Lab.	610322

Immunodepletion of XIAP.

20 µl (250 µg/ml) of anti-XIAP Mab (transduction laboratories) were added to 100 µl of GammaBind G Sepharose (Pharmacia Biotech) in 500 µl of PBS and rotated at 4°C for 3 h. The beads were collected by centrifugation (1 min at 14000 g 4°C). After removal of the supernatant, the beads were washed once with 1 ml of HEB and incubated with 300 µl of cytosolic extract (20 µg/µl) (KCl concentration was adjusted to 70 mM) for 6 h in a rotator at 4°C. The beads were subsequently pelleted by centrifugation (1 min. at 14000 g 4°C). The resulting supernatant was the cytosolic extract immunodepleted of XIAP.

Table 9. Antibodies for immunoprecipitation

antigen	description	organism	immunogene	supplier	Catalog No.
hILP/XIAP	monoclonal/mouse	aa 268.426	BD Transduction lab.	1/2500	H62120

Cloning and sequencing of XIAP and caspase-3

Total RNA from 10^7 L1309, L1236, L591, L428 and KMH2 cells were isolated using RNeasy Mini Kit (Qiagen). First-strand cDNAs were synthesized using SuperScript II for RT-PCR (Gibco BRL). The cDNAs have been used for amplification of ORF (open reading frame) of XIAP and caspase-3 genes by using Pfu DNA polymerase (Promega) . The primers used were made by MWG-Biotech AG.

Table 10. Primers used for PCR amplification of caspase-3 and XIAP.

primers	Sequence 5'-3'
XIAP-5'	5'-ATG ACT TTT ACC AGT TTT GAA-3'
XIAP-3'	5'-TTA AGA CAT AAA AAT TTT TTG-3'
Caspase-3-5'	5'-ATG GAG AAC ACT GAA AAC TCA-3'
Caspase-3-3'	5'-TTA GTG ATA AAA ATA GAG TTC-3'

The PCR products were clonned in PCR-2.1 vector (Invitrogene) and sequenced by using forward and reverse M13 primers. The complete procedure was done in duplicate and sequences were analyzed by Vector NTI program.

Results

Induction of pro-apoptotic mitochondrial signaling by staurosporine

Staurosporine has been described as a potent inducer of apoptosis through the mitochondrial pathway. Not only nontransformed cells, but also most tumor cells undergo apoptosis when treated with staurosporine (Stepczynska et al. 2001). The characteristic cascade of staurosporine-induced signaling is exemplified by HeLa cells as shown in Fig.6. As an immediate-early event, Bax activation/conformational change can be detected by a N-terminal epitope-specific antibody, 6A7, within 30 min. of staurosporine treatment. The 6A7 monoclonal antibody recognizes Bax in a conformation with an exposed N-terminus, but not native Bax (Hsu et al. 1998, Hsu et al. 1997, Perez et al. 2000, Nechushtan et al. 1999 and Murphy et al. 2000). Immunoprecipitations were performed with cell lysates obtained by extraction with 1% CHAPS. Consistent with published reports, lysis with CHAPS did not induce a Bax conformational change in untreated HeLa cells but Bax was in its native conformation and therefore was not immunoprecipitated by anti-Bax 6A7 antibody (Hsu et al. 1998, Hsu et al. 1997, Nechushtan et al. 1999 and Murphy et al. 2000). The N-terminal epitope of Bax became accessible for 6A7 binding after treatment of HeLa cells with staurosporine. Notably, the total amount of Bax protein remained unchanged, indicating that the increasing fraction of active Bax protein does not correspond to newly synthesized protein. Concomitantly, cytochrome c was released from mitochondria into the cytosol. Activation of caspase-3 occurred with slightly delayed kinetics, indicated by the processing into the p20/p17 fragments. These results are in line with the previously proposed sequence of events in mitochondrial apoptosis, where cytochrome c release is induced by the activation of Bax, which eventually results in the activation of caspase-3 as an executioner caspase.

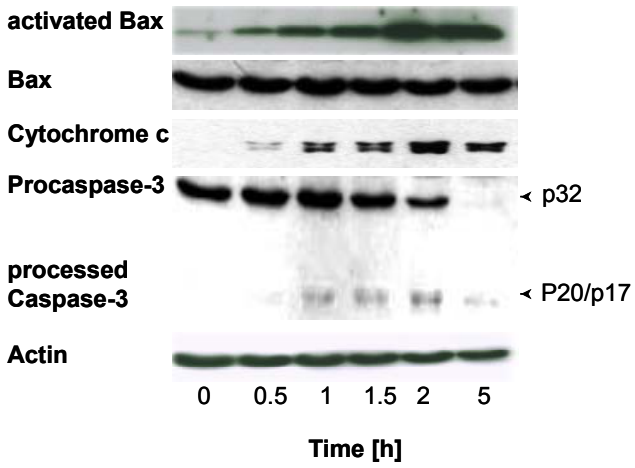


Fig. 6: Bax activation, cytochrome c release and caspase activation in HeLa cells. Cells were treated for 5 h with 1 µM staurosporine, aliquots of cell lysates were prepared at the timepoints indicated. Total cell extracts and cytosolic extracts were subjected to SDS-PAGE and western blotting. Activated Bax was immunoprecipitated in the whole cell extracts with the conformation specific antibody 6A7 and subsequently detected by human Bax antiserum and compared to the total amount of Bax in the lysates. Cytochrome c and caspase-3 were detected in cytosolic extracts. Reprobing for Actin ensured equal loading.

Staurosporine resistance of Hodgkin derived B cell lines

Previously published reports have shown that most tumor cell lines investigated proved sensitive to staurosporine (Stepczynska et al. 2001). However, when HD B cells were investigated, L428, L591, L1236 and KMH2 showed a marked resistance to staurosporine in comparison to control cell lines (Fig. 7). In the first attempt, the expression of proteins involved in the mitochondrial apoptosis pathway was examined by western blot analysis (Fig. 8). Bcl-X_L, one of the major anti-apoptotic proteins, was expressed in all HD B cell lines tested. In contrast, another anti-apoptotic protein, Bcl-2, was expressed at lower levels in HD B-cell lines compared to control B-cells. The expression of Bax and caspase-3 was comparable in all cell lines, whereas the Hodgkin B cells contained slightly less Bak protein compared to

the control cells. Taken together, HD B cells did not reveal an uniform expression pattern of pro- or anti-apoptotic proteins that would explain the staurosporine-resistant phenotype.

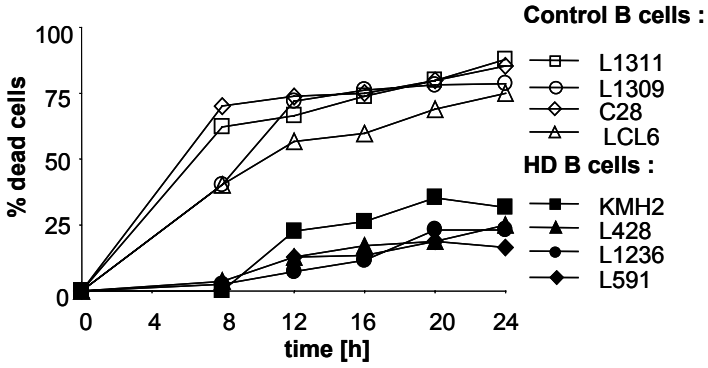


Fig. 7: Induction of cell death by staurosporine. Cells were treated for 0-24 h with 1 µM staurosporine. Cell death was determined by trypanblue exclusion. Each time point represents the average of duplicate experimental values. The figure represents one representative experiment out of three.

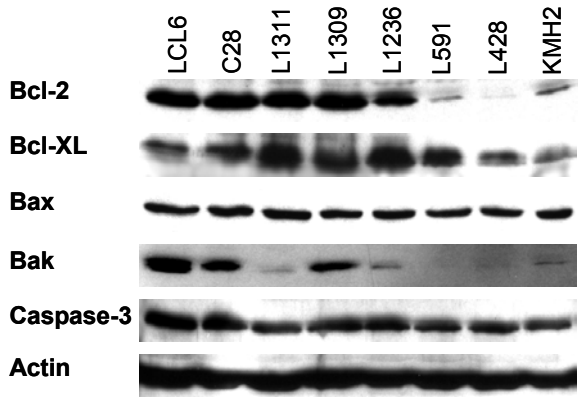


Fig. 8: Expression of pro- and antiapoptotic proteins in Hodgkin cell lines. Equal amounts of total cell lysates from the cells indicated were subjected to SDS-PAGE and western blotting. Proteins were detected after incubation with antibody specific for Bcl-2, Bcl-X_L, Bax, Bak, or Caspase-3 followed by secondary antibody and ECL as described in material and methods. Equal loading was confirmed by detection of Actin on the same membranes.

Defective Bax-activation in HD B cell lines

The mere presence or absence of members of the apoptotic pathway does not allow conclusions with respect to intact or defective mitochondrial signaling. This is because rather complex interactions of the proteins of the Bcl-2 family and caspases involving homo- and heterodimerization, conformational changes, and post-translational processing, seem to regulate the mitochondria-associated apoptosis.To test for putative defects in the mitochondrial apoptotic pathway, HD B cell lines were therefore scrutinized for the characteristic sequence of events shown in Fig. 6. As shown in Fig. 9, staurosporine-treated L591, L1236, L428 and KMH2 cells do neither show activation of Bax, nor cytochrome c release from the mitochondria or processing of caspase-3. In contrast, an intact mitochondrial signaling including Bax conformational change, cytochrome c release and caspase-3 processing was observed in L1309, L1311, LCL6 and C28 cells, EBV transformed non-malignant B cell lines used as control.

To confirm the lack of Bax activation in the HD B-cells, immunofluorescence staining of Bax with anti-Bax 6A7 antibody, recognizing activated Bax protein, were performed. Fig. 10 shows the Bax staining (red) merged with nuclei staining (blue). Consistent with our immunoprecipitation results, untreated L1309, L591 and KMH2 cells were negative for staining with 6A7. Treatment of L1309 cells with staurosporine induced a conformational change in Bax, resulting in a punctuated staining pattern (Murphy et al. 2000) as early as 2 h of treatment, while nuclear fragmentation was observed at later time points in a significant proportion of cells. Neither Bax staining nor nuclear fragmentation was observed in L591 and KMH2 cells after staurosporine treatment up to 12 h, indicating a lack of activated Bax protein and apoptosis.

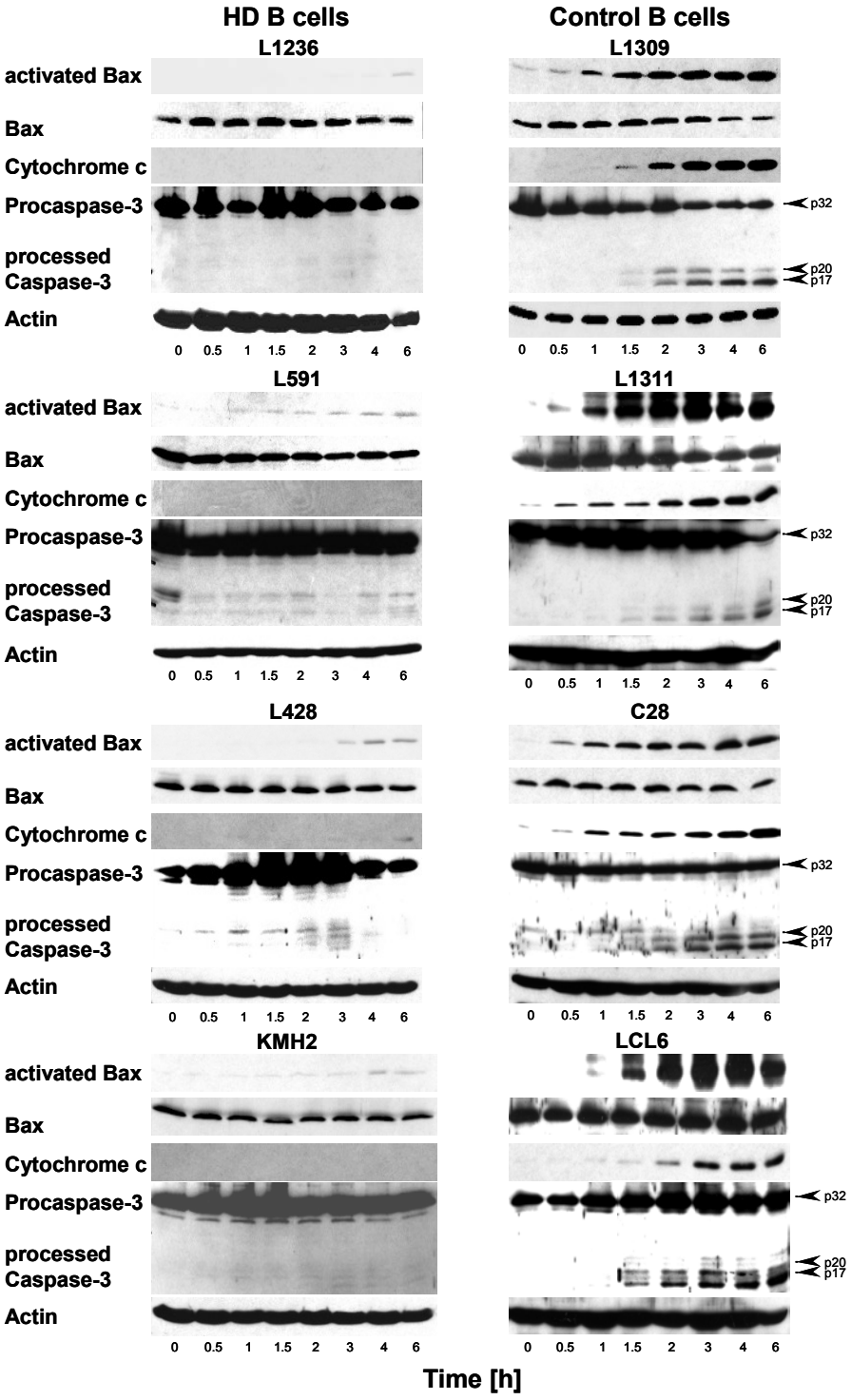


Fig. 9: Bax activation, cytochrome c release and caspase activation in Hodgkin and control cell lines. Cells were treated for 6 h with 1 µM staurosporine, aliquots of cell lysates were prepared at the timepoints indicated. Total cell extracts and cytosolic extracts were subjected to SDS-PAGE and western blotting. Activated Bax was immunoprecipitated in the whole cell extracts with the conformation specific antibody 6A7 and subsequently detected by human Bax antiserum and compared to the total amount of Bax in the lysates. Cytochrome c and Caspase-3 were detected in cytosolic extracts. Reprobing for Actin ensured equal loading.

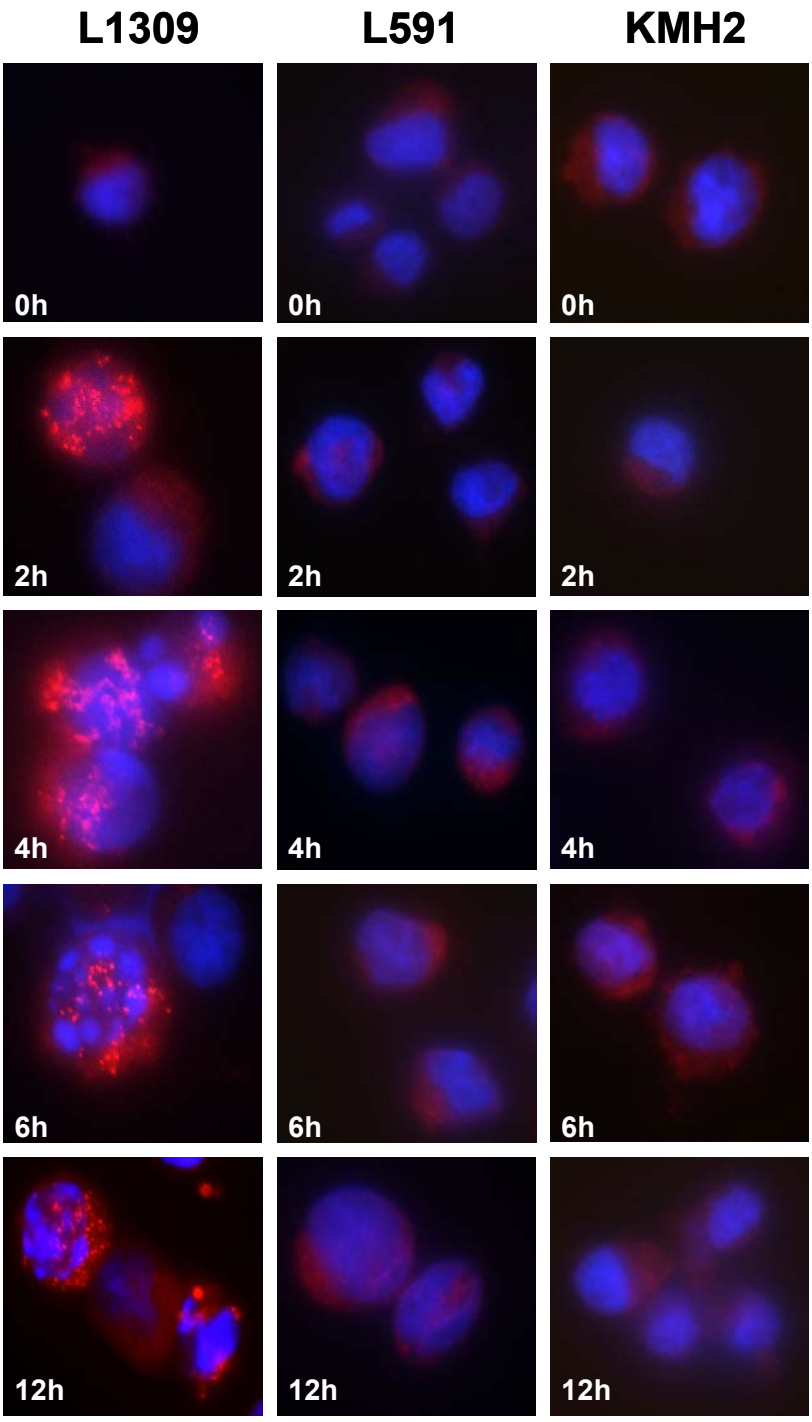


Fig. 10: Bax activation by staurosporine-treatment. L1309, L591 and KMH2 cells were treated for 0, 2, 4, 6 and 12 h with 1 µM staurosporine, and immunostained using 6A7 antibody detecting exposure of the Bax N-terminus. Bax N-terminus staining is seen as red fluorescence, while nuclei are stained blue after counterstaining with Hoechst 33258.

Lack of caspase-3 activity in HD B cells

Induction of apoptosis and activation of the caspase cascade leads to processing and enzymatic activity of the executioner caspase, caspase-3, at the distal end of this cascade. The caspase-3 antibody stained some low molecular weight proteins in the cytosolic extracts of L428, L591 and KMH2 cells. These bands do not represent the right size of properly processed caspase-3 and do not suggest enzymatic activity of caspase-3. To rule out the remote possibility of aberrant caspase activation, the activity of caspase-3 was measured by an independent assay system. Caspase activity of cytosolic extracts from L591, L1236, L428, KMH2, L1309, L1311, LCL6, and C28 control cells treated with staurosporine for the times indicated, was measured using the fluorogenic substrate DEVD-AFC. As shown in Fig.11, staurosporine-induced caspase activity was only observed in L1309, L1311, LCL6 and C28 control B cells with a maximum at 6 hours of treatment. No significant activation of caspases could be measured in staurosporine treated L591, L1236, L428 and KMH2 cells, confirming the results obtained by western blot analysis.

The possibility of delayed cytochrome c release and caspase-3 processing in HD B cells compared to the control B cell line L1309 was ruled out by extended kinetic analysis of cytosolic cytochrome c and caspase-3 processing by western blotting in parallel to measurements of caspase-3 activation using the fluorogenic substrate DEVD-AFC (Fig. 12). In the control cell line L1309 the peak activity of caspase-3 was observed 6 hours after induction of apoptosis with staurosporine, decreasing thereafter due to cell death and degradation of the apoptotic cell fragments. Western blotting for proteolytic cleavage during the activation of caspase-3 confirmed the peak of caspase-3 activity at 6 hours of staurosporine treatment. In the staurosporine resistant cell lines, neither cytochrome c release nor proteolytic cleavage and subsequent activity of caspase-3 could be detected within 24 hours of treatment with

staurosporine, indicating that cytochrome c release and caspase-3 activation do not occur at all.

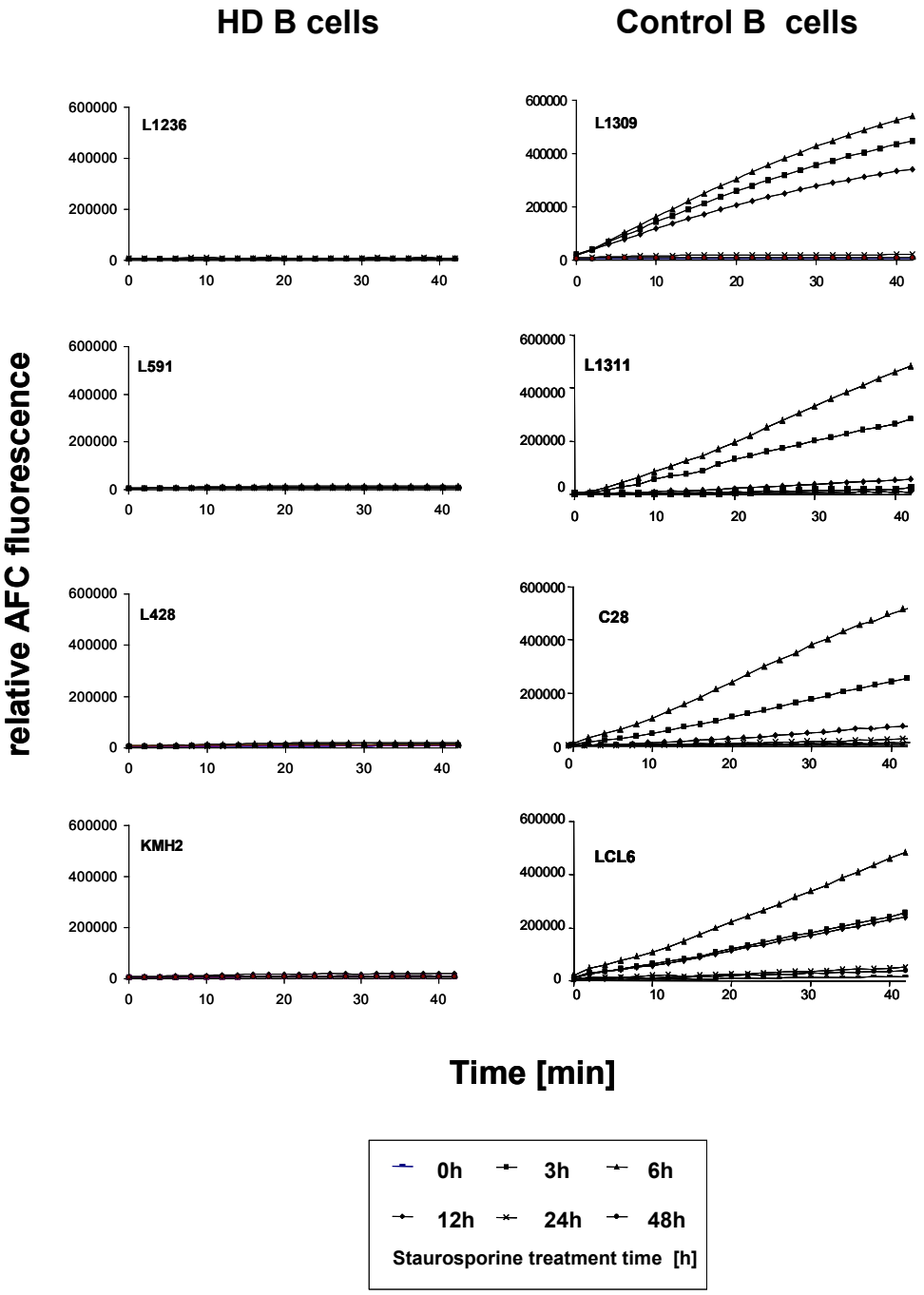


Fig. 11: Staurosporine-induced caspase activity. Cells were treated with 1 µM staurosporine. Cytosolic extracts were prepared after 0, 3, 6, 12, 24, and 48 h of treatment. Caspase activity was measured by hydrolysis of DEVD-afc. Samples were normalized for total cytosolic protein content.

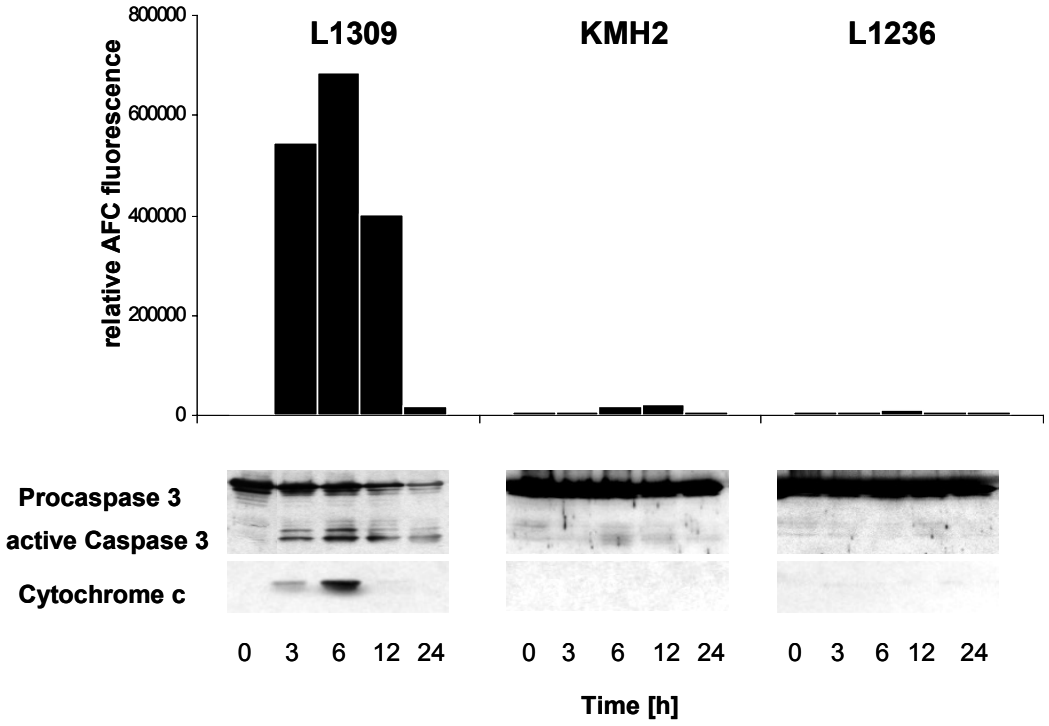


Fig. 12: Staurosporine-induced caspase processing and activity. L1236, KMH2, and L1309 control cells were treated with 1 µM staurosporine. Cytosolic extracts were prepared after 0, 3, 6, 12 and 24 h of treatment. Caspase activity was measured by hydrolysis of DEVD-afc. Samples were normalized for total cytosolic protein content. Cytosolic extracts were also subjected to SDS-PAGE and western blotting. Cytochrome c and Caspase-3 were detected in cytosolic extracts.

Failure of processing and activation of caspase-9 and -3 in HD B cells

So far, the data provided showed that HD B cell lines have a defect in the mitochondrial apoptotic pathway upstream of mitochondria, which contributes to resistance to apoptosis induction by staurosporine. In order to investigate if caspases are functional in these cells, a cell-free system based on the ability of exogenously added cytochrome c to induce proteolytic processing of procaspase-9 and -3 was employed (Liu et al. 1996, and Deveraux et al. 1998). First, the sequential activation of caspase-9 and caspase-3 after addition of cytochrome c to cytosolic extracts was analyzed. The four HD B cell lines, L1236, L591, L428, KMH2, and the control B cell line L1309 were subjected to this analysis. As shown in Fig. 13A, in untreated cell

extracts caspase-9 is detected as its 46-kDa proform. Addition of cytochrome c to the cytosolic extracts of L1309 leads to autocatalytical cleavage of procaspase-9, producing the characteristic p35 fragment of caspase-9. A second cleavage, mediated by subsequently activated caspase-3, results in the p37 fragment of caspase-9 (Srinivasula et al. 1998). Concomitantly, the initial p20 and ultimate p17 fragments of caspase-3 were detected (Fig. 13B). In contrast to the control B cell line, cytochrome c failed to induce any processing of caspase-9 in cytosolic extracts of HD B cell lines. Similarly, caspase-3 remained in its inactive form after addition of cytochrome c to the cytosolic extracts of all HD B cell lines (Fig. 13A, B). Addition of cytochrome c to cytosolic extracts induced caspase-3 enzymatic activity in the control B cell line but not in the HD B cell lines which corresponds to the defective processing of both, caspase-9 and caspase–3 (Fig. 13C).

The defective caspase activation in the HD B cell lines prompted us to examine the expression of proteins involved in this process downstream of mitochondria. As shown in Fig. 14A and B, Apaf-1, cytochrome c and Smac/DIABLO are expressed in all cell lines at comparable levels. Notably, the lack of detection of cytochrome c and Smac in the cytosolic fractions excluded that a possible contamination of cytosolic fractions by mitochondrial proteins would have caused caspase activation in the control B cell line. In contrast, the anti-apoptotic protein XIAP appeared to be significantly overexpressed in the HD B cell lines L1236, L591, L428, and KMH2, compared to the control B cell line L1309.

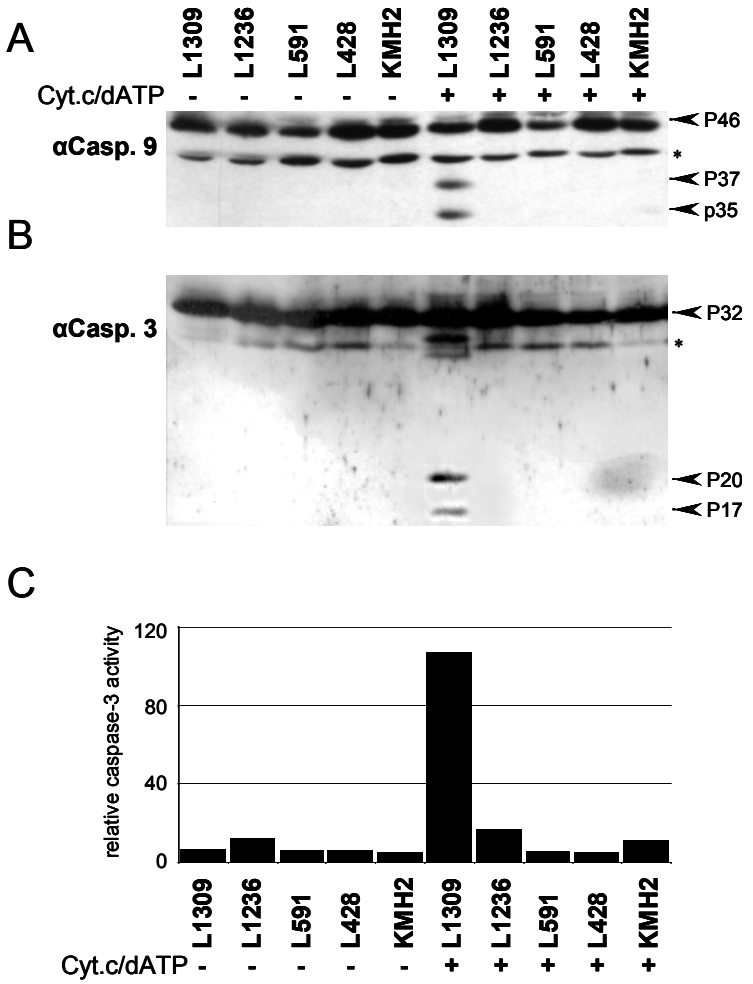


Fig.13. Failure of cytochrome c to induce caspase activation in cytosolic extracts of HD B cell lines. Cytosolic extracts of L1309, L1236, L591, L428, and KMH2 cells were prepared and equal amounts of protein were incubated with or without cytochrome c/dATP for 1h at 30°C. Cytosolic extracts were resolved by SDS/PAGE and subjected to western blot analysis. A) Procaspase-9 (p46) and the processed fragments (p37, p35) were detected by polyclonal rabbit anti-caspase-9 antibody. B) Procaspase-3 (p32) and the processed fragments (p20, p17) were detected by polyclonal anti-caspase-3 antibody. C) Measurement of relative caspase-3 activity was performed, using DEVD-AFC. Samples were normalized for total cytosolic protein content. Asterisks (∗) indicate nonspecific bands recognized by polyclonal antibodies.

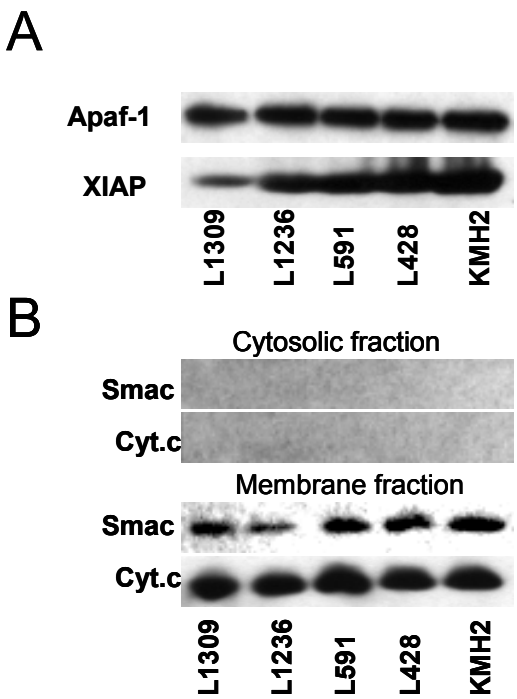


Fig. 14. Expression of caspase activators and inhibitors in HD B cell lines. Equal amounts of proteins from total cell lysate, cytosolic and membrane fractions (including mitochondria) of L1309, L1236, L591, L428, and KMH2 cells were subjected to SDS/PAGE and western blot analysis. Proteins were detected by antibodies against Apaf-1, XIAP, Smac, and Cytochrome c, followed by secondary antibody and ECL as described in material and methods.

XIAP association with caspase-3 in HD B cell lines

XIAP inhibits the catalytic activity of caspases through physical interaction (Deveraux et al. 1998, and Srinivasula et al. 2001). The observation that XIAP is abundantly expressed in HD B cell lines prompted us to examine whether XIAP is associated with caspase-3 in these cell lines. As shown in Fig. 15A, XIAP was detected in cytosolic extracts of control and HD B cell lines at about 54 kDa. Addition of cytochrome c appeared to induce partial degradation of XIAP in the control and in HD B cell cytosolic extracts into one detectable fragment at about 48 kDa. Immunoprecipitation of caspase-3 co-precipitated large amounts of XIAP in cysolic extracts of HD B cell lines but not in that of the control B cell line. That indicates a physical interaction of XIAP with caspase-3, a hallmark for the aberrant antiapoptotic function of XIAP in HD B cells (Fig. 15B).

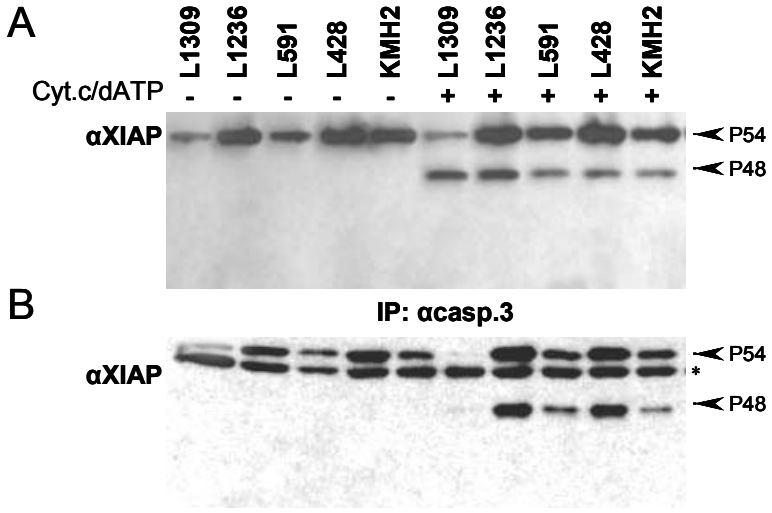


Fig.15. XIAP co-immunoprecipitates with caspase-3 in immunoprecipitation analysis. Cytosolic extracts of L1309, L1236, L591, L428, and KMH2 cells were prepared and equal amounts of protein were incubated with or without cytochrome c/dATP for 1h at 30°C. A) Samples were resolved by SDS/PAGE and subjected to western blot analysis. XIAP was detected by mouse anti-XIAP antibody. B) Caspase-3 was immunoprecipitated by mouse anti-caspase-3 antibody in cytosolic extracts and subjected to SDS/PAGE and western blotting. Co-precipitated XIAP was detected by mouse anti-XIAP antibody. The asterisk (∗) indicates Mouse IgG.

Caspase-8 and granzyme B partially bypass the XIAP-mediated inhibition of caspase-3 in HD B cell lines.

XIAP is known to suppress apoptosis induced by stimulation of both, death receptor and mitochondrial pathways (Deveraux et al. 1998, and Deveraux et al. 1997). To detect the target of XIAP action, caspase-3 was activated by the other initiator proteases caspase-8 or granzyme B (Martin et al. 1996 and Muzio et al. 1997). Indeed, when recombinant active caspase-8 or granzyme B were added to the cytosolic extracts of HD B cells, these two proteases stimulated the cleavage of caspase-3 resulting in the p20 fragment, characteristic of the initial cleavage of caspase-3 (Martin et al. 1996, Deveraux et al. 1998 and Deveraux et al. 1997) (Fig. 16A and 17A). Notably, the autocatalytic maturation generating the p17 fragment,

proceeds to a lesser degree in the cytosolic extracts of HD B cell lines compared to the control B cell line. We conclude that in HD 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. As shown in Fig. 16B and 17B, incomplete caspase-3 processing corresponds with decreased caspase-3 activity in HD B cell extracts.

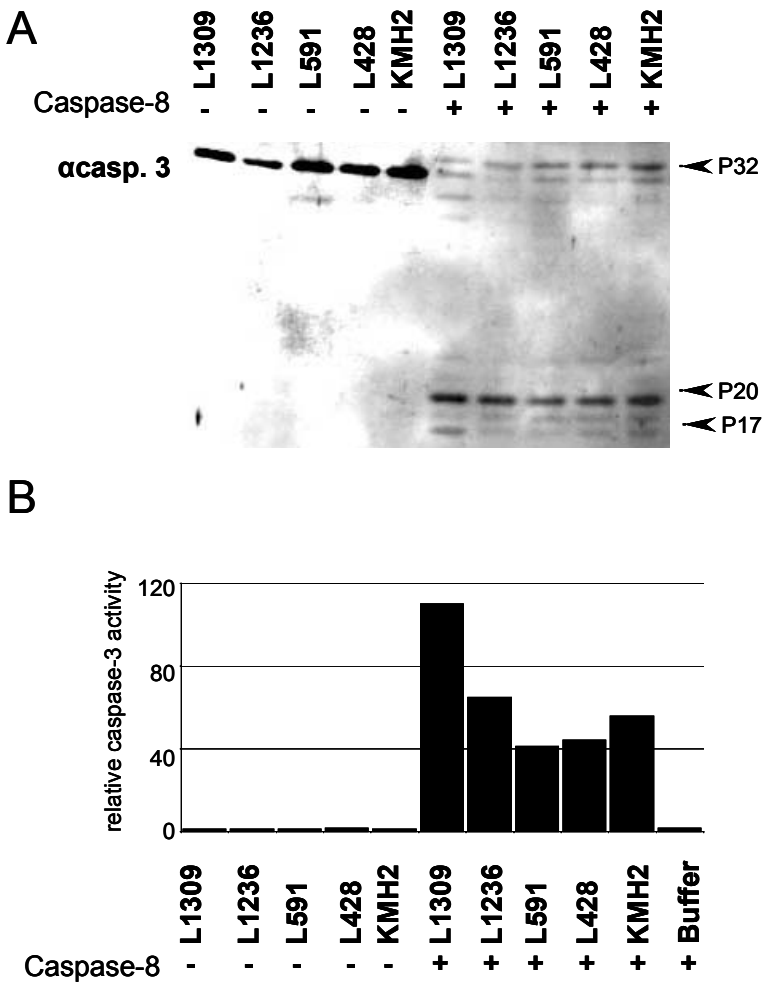


Fig.16. Caspase-3 activation by caspase-8. Cytosolic extracts of L1309, L1236, L591, L428, and KMH2 cells were prepared and equal amounts of protein were left untreated or incubated for 1h with recombinant active caspase-8 at 30°C. A) Cytosolic extracts were resolved by SDS/PAGE and subjected to western blot analysis. Caspase-3 was detected by polyclonal rabbit anti-caspase-3 antibody. B) Relative caspase-3 activity was measured, using DEVD-AFC after incubation with recombinant active caspase-8. Samples were normalized for total cytosolic protein content.

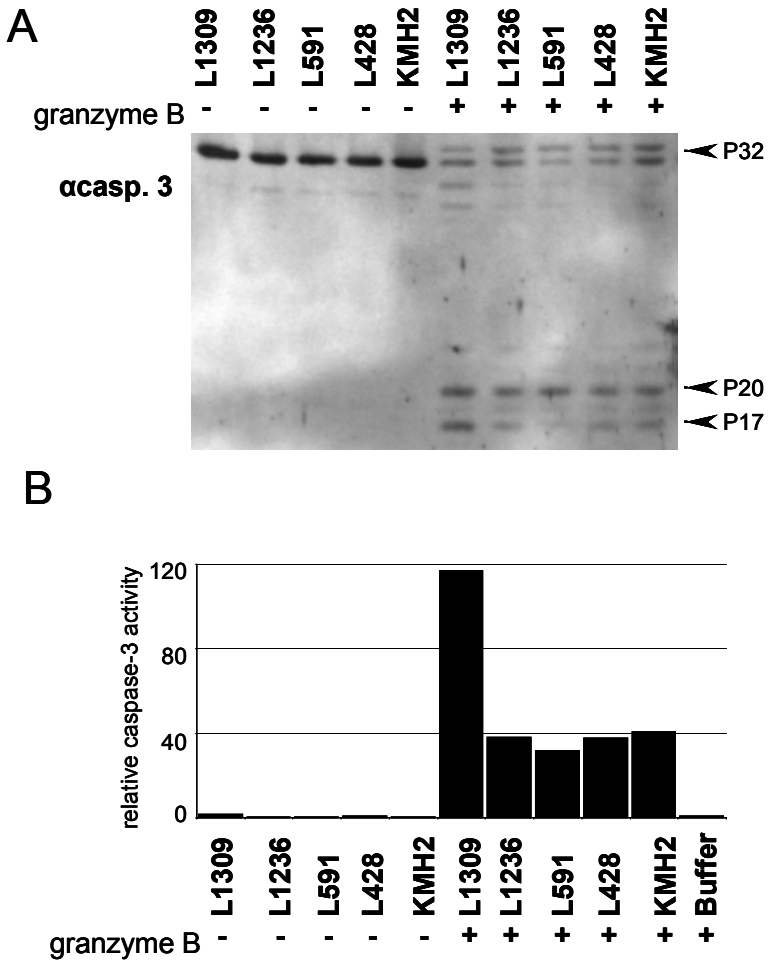


Fig.17. Caspase-3 activation by granzyme B. Cytosolic extracts of L1309, L1236, L591, L428, and KMH2 cells were prepared and equal amounts of protein were left untreated or incubated for 1h with recombinant granzyme B at 30°C. A) Cytosolic extracts were resolved by SDS/PAGE and subjected to western blot analysis. Caspase-3 was detected by polyclonal rabbit anti-caspase-3 antibody. B) Relative caspase-3 activity was measured, using DEVD-AFC after incubation with recombinant active granzyme B. Samples were normalized for total cytosolic protein content.

The observation that caspase-8 overcomes caspase-3 inhibition by XIAP to a greater extent than does granzyme B, might be explained partly by its ability to cleave XIAP into different fragments, which results in the depletion of the full length XIAP (Fig. 18A, B) and is accompanied by the loss of its inhibitory activity (Deveraux et al. 1999-a).

Taken together, the data in Fig. 16 and 17 suggest that caspase-3 in HD B cells can be processed by addition of initiator proteases like caspase-8 and granzyme B, but that its activity is checked by XIAP. The competence of caspase-3 in HD B cell lines was confirmed by sequence analyses of caspase-3, that revealed a lack of mutations in this gene (appendix).

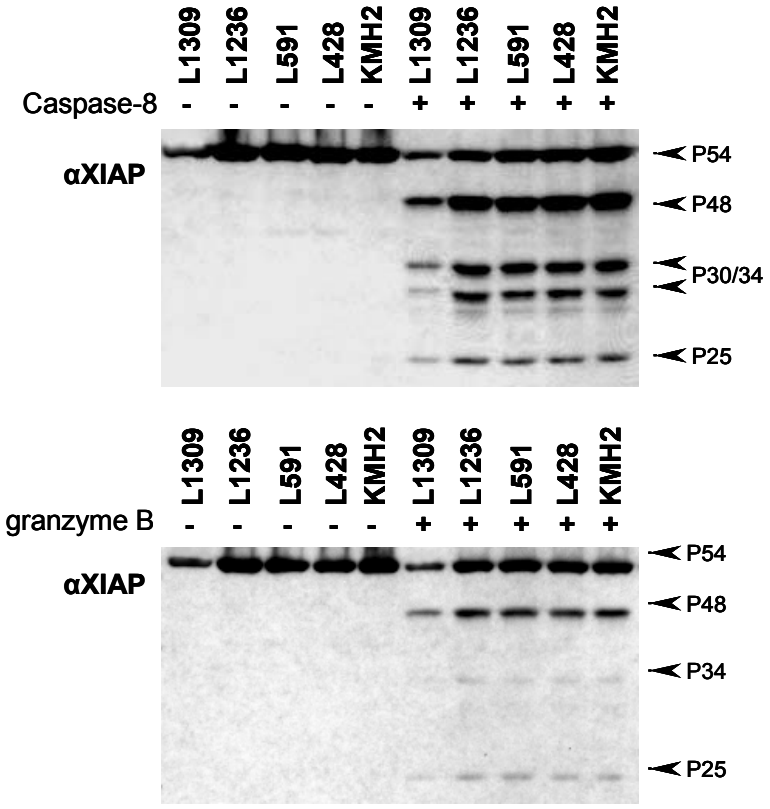


Fig.18. Degradation of XIAP by caspase-8. Cytosolic extracts of L1309, L1236, L591, L428 and KMH2 cells were prepared and equal amounts of proteins were left untreated or incubated for 1h at 30°C with A) recombinant active caspase-8 or B) Granzyme B. Cytosolic extracts were resolved by SDS/PAGE and subjected to western blot analysis. XIAP (p54) and XIAP fragments (p48, p30/34, p25) were detected by mouse anti-XIAP antibody.

Physical or functional depletion of XIAP restores cytochrome c induced caspase-3 activity in HD B cells.

So far our data show that in HD B cell lines XIAP is overexpressed, inhibits the initial cleavage of caspase-3 mediated by the caspase-9 in the apoptosome and prevents caspase-3 activation. Moreover, XIAP may also act at the level of caspase-3 to shut

down the caspase-8 mediated death receptor pathway and the granzyme B pathway. If XIAP was the key inhibitor of apoptosis in HD B cells, removal of XIAP should relieve the inhibition of caspase-3 activation. To provide further evidence for the effect of XIAP on the mitochondrial apoptotic pathway in HD B cells, XIAP was removed from cytosolic extracts by two independent methods. First, a mitochondrial inhibitor of XIAP, Smac, was added to cytosolic extracts, pretreated with cytochrome c. As shown in Fig. 19A recombinant Smac protein restores the ability of cytochrome c to trigger processing and activation of caspase-3 in HD B cells. Corresponding to the processing of caspase-3 into the p20 and the ultimate p17 fragments, addition of Smac to cytosolic extracts of HD B cell lines restored the enzymatic activity of caspase-3 (Fig. 19B). As a second approach, XIAP was immunodepleted from cytosolic extracts of KMH2 cells, using a mouse monoclonal anti-XIAP antibody. As shown in Fig. 19C, immunodepletion of XIAP resulted in an enhancement of caspase-3 activity, providing further evidence for a central role of XIAP in inhibition of caspase-3 activation in HD B cells.

To distinguish whether activation or proteolytic activity of caspase-3 is inhibited in HD B cells, we pre-incubated active mature caspase-3 in cytosolic extracts of HD and control B cell lines and subsequently measured the DEVDase activity of caspase-3. As shown in Fig. 20, the activity of exogenously added mature caspase-3 in cytosolic extracts of HD B cell lines was not inhibited, indicating that it is the caspase-3 activation pathway which is blocked in HD B cell lines.

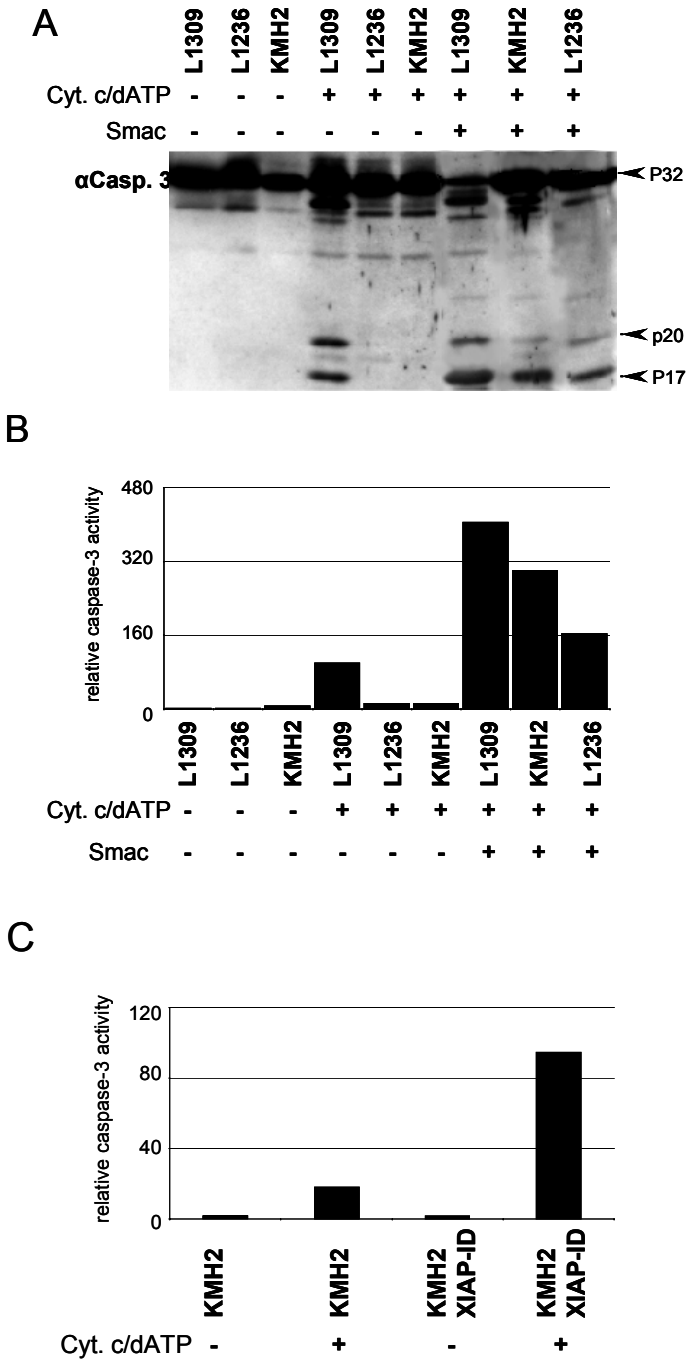


Fig.19. Depletion of XIAP restores caspase-3 processing and activity. Cytosolic extracts of L1309, L1236, and KMH2 cells were prepared and equal amounts of protein were incubated with or without cytochrome c/dATP in the absence and presence of Smac protein for 1h at 30°C. A) Cytosolic extracts were resolved by SDS/PAGE and subjected to western blot analysis. Caspase-3 was detected by polyclonal rabbit anti-caspase-3 antibody. B) Relative caspase-3 activity was measured by hydrolysis of DEVD-AFC. Samples were normalized for total cytosolic protein content. C) XIAP was immunodepleted by mouse anti-XIAP antibody. Cytosolic extracts of KMH2 cells with or without XIAP were incubated in the presence or absence of cytochrome c/dATP for 1h at 30°C. Samples were normalized for total cytosolic protein content and relative caspase-3 activity was measured by DEVDase activity.

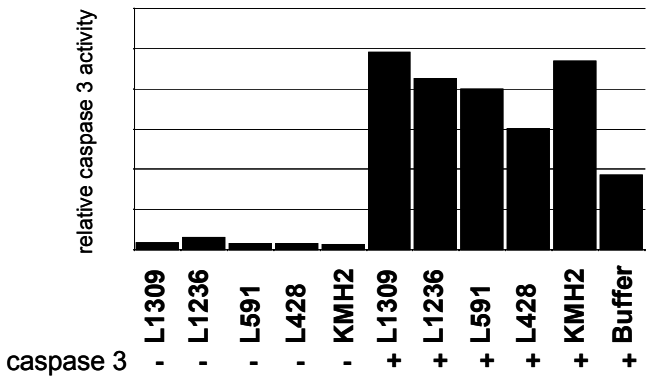


Fig.20. Mature caspase-3 is not inhibited in cytosolic extracts of HD B cell lines. Cytosolic extracts of L1309, L1236, L591, L428 and KMH2 cells were prepared and equal amounts of protein were pre-incubated with or without exogenously added mature caspase-3. Samples were normalized for total cytosolic protein content and relative caspase-3 activity was measured by DEVDase activity.

As shown in Fig. 21, addition of Smac protein to cytosolic extracts of L1236 and KMH2 cells also promoted caspase-9 processing into p35 and p37 fragments. The additional inhibition of XIAP by Smac also restored the processing of caspase-9, suggesting that XIAP not only prevents maturation of caspase-3 but more likely inhibits the caspase-9 mediated initial cleavage of caspase-3 by inhibiting caspase-9 activation.

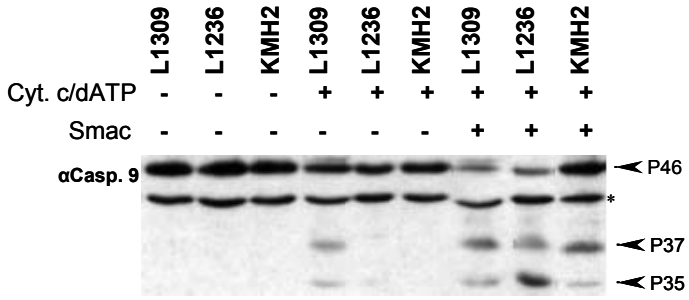


Fig.21. Smac restores caspase-9 processing in HD B cell line. Cytosolic extracts of L1309, L1236, and KMH2 cells were prepared and equal amounts of proteins were left untreated or incubated for 1h at 30°C with cytochrome c/dATP in the presence or absence of Smac protein. Cytosolic extracts were resolved by SDS/PAGE and subjected to western blot analysis. Caspase-9 was detected by polyclonal rabbit anti-caspase-3 antibody.

Discussion

Four neoplastic B cell lines established from patients with Hodgkin's disease were shown to be resistant to staurosporine induced cell death. The results of this study revealed that these HD B cell lines have developed a resistance to apoptosis by alteration of the mitochondrial signaling pathway, which manifests in the failure to trigger the release of cytochrome c from mitochondria and subsequent caspase activation. By detailed analysis of the mitochondrial apoptosis pathway, one signaling defect was localized upstream of mitochondria at the level of Bax activation. A further defect in apoptotic signaling has been identified and localized in the caspase cascade downstream of mitochondria. The data provided by using a cell-free system indicate that the cytochrome c induced activation of caspase-9 and –3 is severely impaired in HD B cell lines. The evidence provided suggests that XIAP is overexpressed in HD B cells, constitutively binds to caspase-3, and plays a key role in the inhibition of caspase-3 processing and activation.

Hodgkin's disease is a lymphoid neoplasm characterized by low-frequent malignant tumor giant cells, known as H-RS cells, in a microenvironment of abundant non-neoplastic inflammatory cells. The HD cell lines used in this study, L428, L591, L1236, and KMH2, are well characterized with regard to their morphology and immunophenotype and display typical B cell associated markers (Drexler 1993 and Mauch et al. 1999). For example, immunoglobulin rearrangements were detected in L1236 cells, proving that this cell line is of clonal B cell origin (Kanzler et al. 1996). Like primary H-RS cells, Hodgkin derived B cell lines have been shown to be resistant to apoptotic stimuli like CD95 (Re et al. 2000). The PKC-inhibitor staurosporine is a classical inducer of the mitochondrial apoptotic pathway in a broad

spectrum of nontransformed and tumor cells. All HD B cells were resistant to cell death induction by staurosporine and showed a defect in Bax activation, whereas their nonmalignant B cell counterparts displayed an intact mitochondrial apoptosis pathway. In the HD B cell lines Bax activation could be disturbed by a variety of possible mechanisms. The pro-apoptotic protein Bax can not only form homodimers but has also been shown to interact with a variety of other proteins (Murphy et al. 2000, Korsmeyer 1995, and Martinou 1999). For example, Bax could be bound to anti-apoptotic Bcl-2 family members or to presently unknown proteins, masking Bax and preventing its activation. Overexpression of Bcl-2 and Bcl-X_L, for example, can inhibit Bax function and cytochrome c release from mitochondria, which subsequently leads to a failure to activate the executioner caspases.

Like HD B cell lines, most HD biopsies however, stained positive for Bax and/or Bak (Reed et al. 1996, Brousset et al. 1996, Schlaifer et al. 1997, Chu et al. 1999, and Kanavaros et al. 2000). This leads to the conclusion that the cell death promoting activity of Bax might be neutralized by other apoptosis regulating proteins (Brousset et al. 1996). In many tumors, resistance to apoptosis was shown to be secondary to high levels of anti-apoptotic Bcl-2 like proteins and/or low level expression of their pro-apoptotic counterparts. Besides Bcl-2, HD B cells expressed Bcl-X_L, another anti-apoptotic member of the Bcl-2 family. Bcl-X_L expression has been previously observed by immunohistochemical analysis of HD biopsies. However, Bcl-X_L is also expressed by nonmalignat B cells. Furthermore, Bcl-X_L has not been shown to be a target for staurosporine. HD B cells provide the first example for a tumor entity, where resistance to mitochondrial apoptosis seems to be brought about by a regulatory defect controlling Bax activation. Clearly, the molecular mode of staurosporine action and, in particular, its failure in HD B cells has yet to be elucidated.

Apoptosis pathways consist of a cascade of signaling proteins before the final irreversible decision to die is made. The whole pathway is tightly regulated not only by the members of the Bcl-2 family, but also by the inhibitors of apoptosis (IAPs), and the Smac/DIABLO proteins. Therefore, resistance to apoptosis can be acquired by regulation of different steps in the pathway.

In the cytosolic extracts of HD B cell lines cytochrome c/dATP addition failed to activate caspase-9 and –3. Correspondingly, neither the initial cleavage of caspase-9 through autocatalysis nor any subsequent proteolytic cleavage mediated by caspase-3 was observed. When complexed with Apaf-1 and cytochrome c, caspase-9 is cleaved and converted into an active protease. Activated caspase-9 then cleaves and activates caspase-3 (Li et al. 1997).

It is important to note that Apaf-1, caspase-9 and –3 were expressed at the same levels (Fig.13, 14) and caspase-3 did not reveal any mutations in the primary structure. From these data can be inferred that one of the caspase inhibitors might prevent caspase processing and activation in HD B cells. Indeed, XIAP was found to be overexpressed in all HD B cells (Fig. 14A). XIAP is the most potent caspase inhibitor of all known IAPs, which suppress apoptosis induced by different stimuli (Deveraux et al. 1998 and Deveraux et al. 1997). XIAP inhibits the catalytic activity of caspases by physically interacting with them. The finding that XIAP could be co-immunoprecipitated with caspase-3 in HD B cells but not in control B cells, supported the hypothesis that XIAP binds constitutively to caspase-3. That the binding of XIAP to caspase-3 results in inhibition of this protease was demonstrated by functional and physical removal of XIAP from cytosolic extracts.

Addition of recombinant Smac protein, an IAP inhibitor that is able to displace caspases from the complex with XIAP, restores the activation of caspase–3 induced

by cytochrome c/dATP. Caspase-3 activation was also restored when XIAP was immunodepleted from cytosolic extracts of the HD B cell lines.

In conclusion, four lines of evidence suggest that XIAP inhibits caspase-3 in HD B cell lines i) XIAP is overexpressed, ii) XIAP co-immunoprecipitates with caspase-3, iii) the XIAP inhibitor Smac restores caspase-3 activity, and iv) physical removal of XIAP by immunodepletion promotes caspase-3 activation.

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 HD B cells, caspase-8 and granzyme B induced protease activity of caspase-3, although at reduced levels. This is due to the initial cleavage into large and small subunits, while autocatalytic processing is still inhibited by XIAP (Martin et al. 1996, Deveraux et al. 1998 and Deveraux et al. 1997). In contrast to caspase-8 and granzyme B, cytochrome c/dATP induced caspase-3 activation was completely abrogated. This is explained by the observation that XIAP inhibits activation of caspase-9. Thus the inhibitory activity of XIAP is not restricted to caspase-3, but rather involves the level of the apoptosom. Upon addition of cytochrome c/dATP, Apaf-1 forms oligomers which recruit caspase-9 and caspase-3 sequentially. Interestingly, XIAP appears to be recruited to the apoptosom through a caspase-9 and -3 independent but dATP dependent binding to Apaf-1. In the Apoptosome XIAP simultaneously binds to and inhibits both, caspase-9 and caspase-3 activation (Bratton et al. 2001).

Overexpression of XIAP has been shown to effectively inhibit a variety of cell death programs (Deveraux et al. 1999-b). These observations suggest that XIAP may play a key role in the regulation of apoptosis in cancer cells, as also shown in melanoma (Zhang et al. 2001). The XIAP gene obviously can be upregulated by NF-κB (Stehlik et al. 1998 and Lee et al. 2001). Interestingly, H-RS cells strongly express CD30 and

CD40, two members of the TNF receptor family that both can activate NF-κB. Constitutive activation of NF-κB appears to be an unique feature of H-RS cells, which is suggested to play an important role in the pathogenesis of H-RS cells (Bargou et al. 1997, Hinz et al. 2001, Izban et al. 2001 and Fiumara et al. 2001). The overexpression of XIAP as 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 HD B cells.

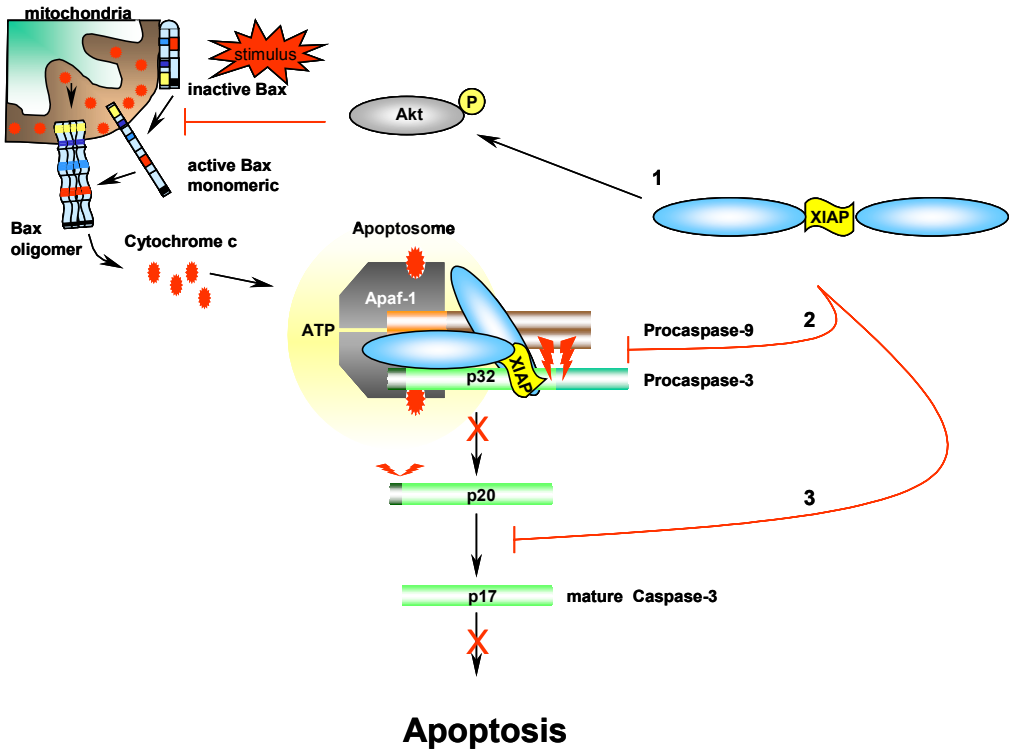


Fig.22. XIAP mediated inhibition of apoptosis in HD B cell lines. Upstream of mitochondria, XIAP prevents activation of Bax possibly through phosphorylation and activation of Akt (1). Since activated Bax is a potent trigger of cytochrome c release, the inhibition of Bax activation impairs the release of proapoptotic factors from mitochondria. Through binding to caspase-9 in the apoptosome XIAP prevents the conversion of caspase-9 into the active protease and thereby blocks the initial cleavage of caspase-3 (2). Finally, XIAP directly binds to caspase-3 and blocks the autocatalytic maturation of caspase-3 (3).

Recent reports indicate that XIAP can prevent apoptosis through a PI3/Akt (phosphoinositide 3-OH kinase /protein-serine/threonine kinase) dependent inhibition of the caspase cascade. Apparently, XIAP can stimulate Akt phosphorylation and activation (Asselin et al. 2001). Akt/PI3 Kinases in turn have been reported to regulate apoptotic events by modulating the function of Bcl-2 family members (Deng et al. 2000, Yamaguchi et al. 2001 and Ito et al. 1997). Specially, Bax conformational change can be inhibited by Akt activation (Yamaguchi et al. 2001). Thus, overexpression of XIAP would be one possible explanation for the lack of Bax activation in HD B cells. These observations can be reconciled in a model, where overexpressed XIAP exerts its anti-apoptotic effects at three levels of the mitochondrial apoptotic pathway as shown in Fig. 22. Overexpressed XIAP in HD B cells might inhibit the activation of Bax by enhancing phosphorylation of Akt and thereby blocks the efflux of cytochrome c and probably Smac/DIABLO into the cytosol. The attraction of XIAP to the apoptosome after addition of cytochrome c results in spatial proximity of XIAP to both, caspase-9 and caspase-3, where it fulfills its dual caspase inhibitory function by simultaneously binding to these two caspases via distinct separate motives.

Certain classes of signaling proteins and pathways are targeted frequently by oncogenic mutations resulting in cancer (Blume-Jensen et al. 2001). The PI3 Kinase and some of its downstream targets, such as the Akt, are crucial effectors in oncogenic kinase signaling. Protein kinases are important regulators of intracellular signal-transduction pathways mediating development and multicellular communication. Their activity is normally tightly controlled and regulated. Deregulated kinase activity has been shown to inhibit apoptosis and lead to malignant transformation (Franke et al 1997). Recent reports suggest that phosphorylation of Bcl-2 enhances its anti-apoptotic activity and inhibits the release

of cytochrome c from mitochondria. The phosphorylation of Bcl-2 occurs to be a result of different kinase activities including those of the IL-3 activated MAP-kinase pathway. Furthermore, staurosporine can only partially inhibit IL-3 stimulated Bcl-2 phosphorylation, suggesting the existence of IL-3 activated, staurosporine resistant kinases (SRKs) in the cells (Deng et al. 2000). Interestingly, recent report suggested that over 90% of H-RS cells in tissues and all tested HD B cell lines strongly expressed the IL-3 receptor, which could serve as a kinase activator in these cells (Aldinucci et al. 2002). The resistance to staurosporine, the blocked Bax activity and the overexpression of both, the IL-3 receptor and XIAP in HD B cell lines provide several lines of evidence that suggest an Akt or Akt-like hyperactivity in HD B cell lines. Further investigation of protein kinase pathways in these cells with the emphasis on the evaluation of possible linkages to apoptotic pathways will raise new opportunities to understand the mode of resistance to apoptosis in HD B cell lines.

Survival of cancer cells requires the continuous input of survival signals to suppress apoptosis. The mitochondrial apoptotic pathway is thought to be the principal target of survival signaling pathways, which act by stabilizing mitochondrial function and integrity and suppressing the release of cytochrome c. A particularly potent driving force for the suppression of apoptosis in tumor cells is the deregulation of the mitochondrial apoptotic pathway, a phenomenon exemplified by overexpression of members of the anti-apoptotic Bcl-2 protein family, overexpression of IAPs or loss of Apaf-1 in several tumor types (Deng et al. 2002, Soengas et al. 2001 and Evan et al. 2001). Understanding the functions and molecular interactions in the mitochondrial apoptotic pathway is the key to the breadth of therapeutic opportunities. Apoptosis-modulating therapeutics, which are mostly converged at the mitochondrial site of apoptotic pathways, are now in human clinical trials or are on the brink, having

shown efficacy in preclinical animal models. Thus apoptosis research may propel significant progress in cancer therapy.

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Appendix

XIAP Alignment

Section 1
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ATGACTTTTAACAGTTTTGAAGGATCTAAAACTTGTGTACCTGCAGACATCAAT L1309 (1)
ATGACTTTTAACAGTTTTGAAGGATCTAAAACTTGTGTACCTGCAGACATCAAT L1236 (1)
ATGACTTTTAACAGTTTTGAAGGATCTAAAACTTGTGTACCTGCAGACATCAAT L591 (1)
ATGACTTTTAACAGTTTTGAAGGATCTAAAACTTGTGTACCTGCAGACATCAAT L428 (1)
ATGACTTTTAACAGTTTTGAAGGATCTAAAACTTGTGTACCTGCAGACATCAAT KMH2 (1)
ATGACTTTTAACAGTTTTGAAGGATCTAAAACTTGTGTACCTGCAGACATCAAT 7159760 (1)
ATGACTTTTAACAGTTTTGAAGGATCTAAAACTTGTGTACCTGCAGACATCAAT Consensus (1)
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Section 2
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AAGGAAGAAGAATTTGTAGAAGAGTTTAATAGATTAAAAACTTTTGCTAATTTT L1309 (55)
AAGGAAGAAGAATTTGTAGAAGAGTTTAATAGATTAAAAACTTTTGCTAATTTT L1236 (55)
AAGGAAGAAGAATTTGTAGAAGAGTTTAATAGATTAAAAACTTTTGCTAATTTT L591 (55)
AAGGAAGAAGAATTTGTAGAAGAGTTTAATAGATTAAAAACTTTTGCTAATTTT L428 (55)
AAGGAAGAAGAATTTGTAGAAGAGTTTAATAGATTAAAAACTTTTGCTAATTTT KMH2 (55)
AAGGAAGAAGAATTTGTAGAAGAGTTTAATAGATTAAAAACTTTTGCTAATTTT 7159760 (55)
AAGGAAGAAGAATTTGTAGAAGAGTTTAATAGATTAAAAACTTTTGCTAATTTT Consensus (55)
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Section 3
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CCAAGTGGTAGTCCTGTTTCAGCATCAACACTGGCACGAGCAGGGTTTCTTTAT L1309 (109)
CCAAGTGGTAGTCCTGTTTCAGCATCAACACTGGCACGAGCAGGGTTTCTTTAT L1236 (109)
CCAAGTGGTAGTCCTGTTTCAGCATCAACACTGGCACGAGCAGGGTTTCTTTAT L591 (109)
CCAAGTGGTAGTCCTGTTTCAGCATCAACACTGGCACGAGCAGGGTTTCTTTAT L428 (109)
CCAAGTGGTAGTCCTGTTTCAGCATCAACACTGGCACGAGCAGGGTTTCTTTAT KMH2 (109)
CCAAGTGGTAGTCCTGTTTCAGCATCAACACTGGCACGAGCAGGGTTTCTTTAT 7159760 (109)
CCAAGTGGTAGTCCTGTTTCAGCATCAACACTGGCACGAGCAGGGTTTCTTTAT Consensus (109)
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Section 4
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ACTGGTGAAGGAGATACCGTGCGGTGCTTTAGTTGTCATGCAGCTGTAGATAGA L1309 (163)
ACTGGTGAAGGAGATACCGTGCGGTGCTTTAGTTGTCATGCAGCTGTAGATAGA L1236 (163)
ACTGGTGAAGGAGATACCGTGCGGTGCTTTAGTTGTCATGCAGCTGTAGATAGA L591 (163)
ACTGGTGAAGGAGATACCGTGCGGTGCTTTAGTTGTCATGCAGCTGTAGATAGA L428 (163)
ACTGGTGAAGGAGATACCGTGCGGTGCTTTAGTTGTCATGCAGCTGTAGATAGA KMH2 (163)
ACTGGTGAAGGAGATACCGTGCGGTGCTTTAGTTGTCATGCAGCTGTAGATAGA 7159760 (163)
ACTGGTGAAGGAGATACCGTGCGGTGCTTTAGTTGTCATGCAGCTGTAGATAGA Consensus (163)
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Section 5
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TGGCAATATGGAGACTCAGCAGTTGGAAGACACAGGAAAGTATCCCCAAATTGC L1309 (217)
TGGCAATATGGAGACTCAGCAGTTGGAAGACACAGGAAAGTATCCCCAAATTGC L1236 (217)
TGGCAATATGGAGACTCAGCAGTTGGAAGACACAGGAAAGTATCCCCAAATTGC L591 (217)
TGGCAATATGGAGACTCAGCAGTTGGAAGACACAGGAAAGTATCCCCAAATTGC L428 (217)
TGGCAATATGGAGACTCAGCAGTTGGAAGACACAGGAAAGTATCCCCAAATTGC KMH2 (217)
TGGCAATATGGAGACTCAGCAGTTGGAAGACACAGGAAAGTATCCCCAAATTGC 7159760 (217)
TGGCAATATGGAGACTCAGCAGTTGGAAGACACAGGAAAGTATCCCCAAATTGC Consensus (217)
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Section 6
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AGATTTATCAACGGCTTTTATCTTGAAAATAGTGCCACGCAGTCTACAAATTCT L1236 (271)
AGATTTATCAACGGCTTTTATCTTGAAAATAGTGCCACGCAGTCTACAAATTCT L591 (271)
AGATTTATCAACGGCTTTTATCTTGAAAATAGTGCCACGCAGTCTACAAATTCT L428 (271)
AGATTTATCAACGGCTTTTATCTTGAAAATAGTGCCACGCAGTCTACAAATTCT KMH2 (271)
AGATTTATCAACGGCTTTTATCTTGAAAATAGTGCCACGCAGTCTACAAATTCT 7159760 (271)
AGATTTATCAACGGCTTTTATCTTGAAAATAGTGCCACGCAGTCTACAAATTCT Consensus (271)
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XIAP Alignment

Section 7
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GGTATCCAGAATGGTCAGTACAAAGTTGAAAACTATCTGGGAAGCAGAGATCAT L1309 (325)
GGTATCCAGAATGGTCAGTACAAAGTTGAAAACTATCTGGGAAGCAGAGATCAT L1236 (325)
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GGTATCCAGAATGGTCAGTACAAAGTTGAAAACTATCTGGGAAGCAGAGATCAT L591 (325)
GGTATCCAGAATGGTCAGTACAAAGTTGAAAACTATCTGGGAAGCAGAGATCAT L428 (325)
GGTATCCAGAATGGTCAGTACAAAGTTGAAAACTATCTGGGAAGCAGAGATCAT KMH2 (325)
GGTATCCAGAATGGTCAGTACAAAGTTGAAAACTATCTGGGAAGCAGAGATCAT 7159760 (325)
GGTATCCAGAATGGTCAGTACAAAGTTGAAAACTATCTGGGAAGCAGAGATCAT Consensus (325)
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Section 8

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TTTGCCTTAGACAGGCCATCTGAGACACATGCAGACTATCTTTTGAGAACTGGG L1236 (379)
TTTGCCTTAGACAGGCCATCTGAGACACATGCAGACTATCTTTTGAGAACTGGG L591 (379)
TTTGCCTTAGACAGGCCATCTGAGACACATGCAGACTATCTTTTGAGAACTGGG L428 (379)
TTTGCCTTAGACAGGCCATCTGAGACACATGCAGACTATCTTTTGAGAACTGGG KMH2 (379)
TTTGCCTTAGACAGGCCATCTGAGACACATGCAGACTATCTTTTGAGAACTGGG 7159760 (379)
TTTGCCTTAGACAGGCCATCTGAGACACATGCAGACTATCTTTTGAGAACTGGG Consensus (379)
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Section 9

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CAGGTTGTAGATATATCAGACACCATATACCCGAGGAACCCTGCCATGTATAGT L1309 (433)
CAGGTTGTAGATATATCAGACACCATATACCCGAGGAACCCTGCCATGTATAGT L1236 (433)
CAGGTTGTAGATATATCAGACACCATATACCCGAGGAACCCTGCCATGTATAGT L591 (433)
CAGGTTGTAGATATATCAGACACCATATACCCGAGGAACCCTGCCATGTATAGT L428 (433)
CAGGTTGTAGATATATCAGACACCATATACCCGAGGAACCCTGCCATGTATAGT KMH2 (433)
CAGGTTGTAGATATATCAGACACCATATACCCGAGGAACCCTGCCATGTATAGT 7159760 (433)
CAGGTTGTAGATATATCAGACACCATATACCCGAGGAACCCTGCCATGTATAGT Consensus (433)
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Section 10

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GAAGAAGCTAGATTAAAGTCCTTTCAGAACTGGCCAGACTATGCTCACCTAACC L1309 (487)
GAAGAAGCTAGATTAAAGTCCTTTCAGAACTGGCCAGACTATGCTCACCTAACC L1236 (487)
GAAGAAGCTAGATTAAAGTCCTTTCAGAACTGGCCAGACTATGCTCACCTAACC L591 (487)
GAAGAAGCTAGATTAAAGTCCTTTCAGAACTGGCCAGACTATGCTCACCTAACC L428 (487)
GAAGAAGCTAGATTAAAGTCCTTTCAGAACTGGCCAGACTATGCTCACCTAACC KMH2 (487)
GAAGAAGCTAGATTAAAGTCCTTTCAGAACTGGCCAGACTATGCTCACCTAACC 7159760 (487)
GAAGAAGCTAGATTAAAGTCCTTTCAGAACTGGCCAGACTATGCTCACCTAACC Consensus (487)
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Section 11

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CCAAGAGAGTTAGCAAGTGCTGGACTCTACTACACAGGTATTGGTGACCAAGTG L1309 (541)
CCAAGAGAGTTAGCAAGTGCTGGACTCTACTACACAGGTATTGGTGACCAAGTG L1236 (541)
CCAAGAGAGTTAGCAAGTGCTGGACTCTACTACACAGGTATTGGTGACCAAGTG L591 (541)
CCAAGAGAGTTAGCAAGTGCTGGACTCTACTACACAGGTATTGGTGACCAAGTG L428 (541)
CCAAGAGAGTTAGCAAGTGCTGGACTCTACTACACAGGTATTGGTGACCAAGTG KMH2 (541)
CCAAGAGAGTTAGCAAGTGCTGGACTCTACTACACAGGTATTGGTGACCAAGTG 7159760 (541)
CCAAGAGAGTTAGCAAGTGCTGGACTCTACTACACAGGTATTGGTGACCAAGTG Consensus (541)
```

Section 12

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CAGTGCTTTTGTTGTGGTGGAAAACTGAAAAATTGGGAACCTTGTGATCGTGCC L1309 (595)
CAGTGCTTTTGTTGTGGTGGAAAACTGAAAAATTGGGAACCTTGTGATCGTGCC L1236 (595)
CAGTGCTTTTGTTGTGGTGGAAAACTGAAAAATTGGGAACCTTGTGATCGTGCC L591 (595)
CAGTGCTTTTGTTGTGGTGGAAAACTGAAAAATTGGGAACCTTGTGATCGTGCC L428 (595)
CAGTGCTTTTGTTGTGGTGGAAAACTGAAAAATTGGGAACCTTGTGATCGTGCC KMH2 (595)
CAGTGCTTTTGTTGTGGTGGAAAACTGAAAAATTGGGAACCTTGTGATCGTGCC 7159760 (595)
CAGTGCTTTTGTTGTGGTGGAAAACTGAAAAATTGGGAACCTTGTGATCGTGCC Consensus (595)
```

XIAP Alignment

Section 13

```
TGGTCAGAACACAGGCGACACTTTCCTAATTGCTTCTTTGTTTTGGGCCGGAAT L1309 (649)
TGGTCAGAACACAGGCGACACTTTCCTAATTGCTTCTTTGTTTTGGGCCGGAAT L1236 (649)
TGGTCAGAACACAGGCGACACTTTCCTAATTGCTTCTTTGTTTTGGGCCGGAAT L591 (649)
TGGTCAGAACACAGGCGACACTTTCCTAATTGCTTCTTTGTTTTGGGCCGGAAT L428 (649)
TGGTCAGAACACAGGCGACACTTTCCTAATTGCTTCTTTGTTTTGGGCCGGAAT KMH2 (649)
TGGTCAGAACACAGGCGACACTTTCCTAATTGCTTCTTTGTTTTGGGCCGGAAT 7159760 (649)
TGGTCAGAACACAGGCGACACTTTCCTAATTGCTTCTTTGTTTTGGGCCGGAAT Consensus (649)
```

Section 14

```
CTTAATATTCGAAGTGAATCTGATGCTGTGAGTTCTGATAGGAATTTCCCAAAT L1309 (703)
CTTAATATTCGAAGTGAATCTGATGCTGTGAGTTCTGATAGGAATTTCCCAAAT L1236 (703)
```

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CTTAATATTCGAAGTGAATCTGATGCTGTGAGTTCTGATAGGAATTTCCCAAAT L591 (703)
CTTAATATTCGAAGTGAATCTGATGCTGTGAGTTCTGATAGGAATTTCCCAAAT L428 (703)
CTTAATATTCGAAGTGAATCTGATGCTGTGAGTTCTGATAGGAATTTCCCAAAT KMH2 (703)
CTTAATATTCGAAGTGAATCTGATGCTGTGAGTTCTGATAGGAATTTCCCAAAT 7159760 (703)
CTTAATATTCGAAGTGAATCTGATGCTGTGAGTTCTGATAGGAATTTCCCAAAT Consensus (703)
```

Section 15

```
TCAACAAATCTTCCAAGAAATCCATCCATGGCAGATTATGAAGCACGGATCTTT L1309 (757)
TCAACAAATCTTCCAAGAAATCCATCCATGGCAGATTATGAAGCACGGATCTTT L1236 (757)
TCAACAAATCTTCCAAGAAATCCATCCATGGCAGATTATGAAGCACGGATCTTT L591 (757)
TCAACAAATCTTCCAAGAAATCCATCCATGGCAGATTATGAAGCACGGATCTTT L428 (757)
TCAACAAATCTTCCAAGAAATCCATCCATGGCAGATTATGAAGCACGGATCTTT KMH2 (757)
TCAACAAATCTTCCAAGAAATCCATCCATGGCAGATTATGAAGCACGGATCTTT 7159760 (757)
TCAACAAATCTTCCAAGAAATCCATCCATGGCAGATTATGAAGCACGGATCTTT Consensus (757)
```

Section 16

```
ACTTTTGGGACATGGATATACTCAGTTAACAAGGAGCAGCTTGCAAGAGCTGGA L1309 (811)
ACTTTTGGGACATGGATATACTCAGTTAACAAGGAGCAGCTTGCAAGAGCTGGA L1236 (811)
ACTTTTGGGACATGGATATACTCAGTTAACAAGGAGCAGCTTGCAAGAGCTGGA L591 (811)
ACTTTTGGGACATGGATATACTCAGTTAACAAGGAGCAGCTTGCAAGAGCTGGA L428 (811)
ACTTTTGGGACATGGATATACTCAGTTAACAAGGAGCAGCTTGCAAGAGCTGGA KMH2 (811)
ACTTTTGGGACATGGATATACTCAGTTAACAAGGAGCAGCTTGCAAGAGCTGGA 7159760 (811)
ACTTTTGGGACATGGATATACTCAGTTAACAAGGAGCAGCTTGCAAGAGCTGGA Consensus (811)
```

Section 17

```
TTTTATGCTTTAGGTGAAGGTGATAAAGTAAAGTGCTTTCACTGTGGAGGAGGG L1309 (865)
TTTTATGCTTTAGGTGAAGGTGATAAAGTAAAGTGCTTTCACTGTGGAGGAGGG L1236 (865)
TTTTATGCTTTAGGTGAAGGTGATAAAGTAAAGTGCTTTCACTGTGGAGGAGGG L591 (865)
TTTTATGCTTTAGGTGAAGGTGATAAAGTAAAGTGCTTTCACTGTGGAGGAGGG L428 (865)
TTTTATGCTTTAGGTGAAGGTGATAAAGTAAAGTGCTTTCACTGTGGAGGAGGG KMH2 (865)
TTTTATGCTTTAGGTGAAGGTGATAAAGTAAAGTGCTTTCACTGTGGAGGAGGG 7159760 (865)
TTTTATGCTTTAGGTGAAGGTGATAAAGTAAAGTGCTTTCACTGTGGAGGAGGG Consensus (865)
```

Section 18

```
CTAACTGATTGGAAGCCCAGTGAAGACCCTTGGGAACAACATGCTAAATGGTAT L1309 (919)
CTAACTGATTGGAAGCCCAGTGAAGACCCTTGGGAACAACATGCTAAATGGTAT L1236 (919)
CTAACTGATTGGAAGCCCAGTGAAGACCCTTGGGAACAACATGCTAAATGGTAT L591 (919)
CTAACTGATTGGAAGCCCAGTGAAGACCCTTGGGAACAACATGCTAAATGGTAT L428 (919)
CTAACTGATTGGAAGCCCAGTGAAGACCCTTGGGAACAACATGCTAAATGGTAT KMH2 (919)
CTAACTGATTGGAAGCCCAGTGAAGACCCTTGGGAACAACATGCTAAATGGTAT 7159760 (919)
CTAACTGATTGGAAGCCCAGTGAAGACCCTTGGGAACAACATGCTAAATGGTAT Consensus (919)
```

XIAP Alignment

Section 19

```
CCAGGGTGCAAATATCTGTTAGAACAGAAGGGACAAGAATATATAAACAATATT L1309 (973)
CCAGGGTGCAAATATCTGTTAGAACAGAAGGGACAAGAATATATAAACAATATT L1236 (973)
CCAGGGTGCAAATATCTGTTAGAACAGAAGGGACAAGAATATATAAACAATATT L591 (973)
CCAGGGTGCAAATATCTGTTAGAACAGAAGGGACAAGAATATATAAACAATATT L428 (973)
CCAGGGTGCAAATATCTGTTAGAACAGAAGGGACAAGAATATATAAACAATATT KMH2 (973)
CCAGGGTGCAAATATCTGTTAGAACAGAAGGGACAAGAATATATAAACAATATT 7159760 (973)
CCAGGGTGCAAATATCTGTTAGAACAGAAGGGACAAGAATATATAAACAATATT Consensus (973)
```

Section 20

```
CATTTAACTCATTCACTTGAGGAGTGTCTGGTAAGAACTACTGAGAAAACACCA L1309 (1027)
CATTTAACTCATTCACTTGAGGAGTGTCTGGTAAGAACTACTGAGAAAACACCA L1236 (1027)
CATTTAACTCATTCACTTGAGGAGTGTCTGGTAAGAACTACTGAGAAAACACCA L591 (1027)
CATTTAACTCATTCACTTGAGGAGTGTCTGGTAAGAACTACTGAGAAAACACCA L428 (1027)
CATTTAACTCATTCACTTGAGGAGTGTCTGGTAAGAACTACTGAGAAAACACCA KMH2 (1027)
CATTTAACTCATTCACTTGAGGAGTGTCTGGTAAGAACTACTGAGAAAACACCA 7159760 (1027)
CATTTAACTCATTCACTTGAGGAGTGTCTGGTAAGAACTACTGAGAAAACACCA Consensus (1027)
```

Section 21
TCACTAACTAGAAGAATTGATGATACCATCTTCCAAAATCCTATGGTACAAGAA L1309 (1081)
TCACTAACTAGAAGAATTGATGATACCATCTTCCAAAATCCTATGGTACAAGAA L1236 (1081)
TCACTAACTAGAAGAATTGATGATACCATCTTCCAAAATCCTATGGTACAAGAA L591 (1081)
TCACTAACTAGAAGAATTGATGATACCATCTTCCAAAATCCTATGGTACAAGAA L428 (1081)
TCACTAACTAGAAGAATTGATGATACCATCTTCCAAAATCCTATGGTACAAGAA KMH2 (1081)
TCACTAACTAGAAGAATTGATGATACCATCTTCCAAAATCCTATGGTACAAGAA 7159760 (1081)
TCACTAACTAGAAGAATTGATGATACCATCTTCCAAAATCCTATGGTACAAGAA Consensus (1081)

Section 22
GCTATACGAATGGGGTTCAGTTTCAAGGACATTAAGAAAATAATGGAGGAAAAA L1309 (1135)
GCTATACGAATGGGGTTCAGTTTCAAGGACATTAAGAAAATAATGGAGGAAAAA L1236 (1135)
GCTATACGAATGGGGTTCAGTTTCAAGGACATTAAGAAAATAATGGAGGAAAAA L591 (1135)
GCTATACGAATGGGGTTCAGTTTCAAGGACATTAAGAAAATAATGGAGGAAAAA L428 (1135)
GCTATACGAATGGGGTTCAGTTTCAAGGACATTAAGAAAATAATGGAGGAAAAA KMH2 (1135)
GCTATACGAATGGGGTTCAGTTTCAAGGACATTAAGAAAATAATGGAGGAAAAA 7159760 (1135)
GCTATACGAATGGGGTTCAGTTTCAAGGACATTAAGAAAATAATGGAGGAAAAA Consensus (1135)

Section 23
ATTCAGATATCTGGGAGCAACTATAAATCACTTGAGGTTCTGGTTGCAGATCTA L1309 (1189)
ATTCAGATATCTGGGAGCAACTATAAATCACTTGAGGTTCTGGTTGCAGATCTA L1236 (1189)
ATTCAGATATCTGGGAGCAACTATAAATCACTTGAGGTTCTGGTTGCAGATCTA L591 (1189)
ATTCAGATATCTGGGAGCAACTATAAATCACTTGAGGTTCTGGTTGCAGATCTA L428 (1189)
ATTCAGATATCTGGGAGCAACTATAAATCACTTGAGGTTCTGGTTGCAGATCTA KMH2 (1189)
ATTCAGATATCTGGGAGCAACTATAAATCACTTGAGGTTCTGGTTGCAGATCTA 7159760 (1189)
ATTCAGATATCTGGGAGCAACTATAAATCACTTGAGGTTCTGGTTGCAGATCTA Consensus (1189)

Section 24
GTGAATGCTCAGAAAGACAGTATGCCAGATGAGTCAAGTCAGACTTCATTACAG L1309 (1243)
GTGAATGCTCAGAAAGACAGTATGCCAGATGAGTCAAGTCAGACTTCATTACAG L1236 (1243)
GTGAATGCTCAGAAAGACAGTATGCCAGATGAGTCAAGTCAGACTTCATTACAG L591 (1243)
GTGAATGCTCAGAAAGACAGTATGCCAGATGAGTCAAGTCAGACTTCATTACAG L428 (1243)
GTGAATGCTCAGAAAGACAGTATGCCAGATGAGTCAAGTCAGACTTCATTACAG KMH2 (1243)
GTGAATGCTCAGAAAGACAGTATGCAAGATGAGTCAAGTCAGACTTCATTACAG 7159760 (1243)
GTGAATGCTCAGAAAGACAGTATGCCAGATGAGTCAAGTCAGACTTCATTACAG Consensus (1243)

XIAP Alignment

Section 25
AAAGAGATTAGTACTGAAGAGCAGCTAAGGCGCCTGCAAGAGGAGAAGCTTTGC L1309 (1297)
AAAGAGATTAGTACTGAAGAGCAGCTAAGGCGCCTGCAAGAGGAGAAGCTTTGC L1236 (1297)
AAAGAGATTAGTACTGAAGAGCAGCTAAGGCGCCTGCAAGAGGAGAAGCTTTGC L591 (1297)
AAAGAGATTAGTACTGAAGAGCAGCTAAGGCGCCTGCAAGAGGAGAAGCTTTGC L428 (1297)
AAAGAGATTAGTACTGAAGAGCAGCTAAGGCGCCTGCAAGAGGAGAAGCTTTGC KMH2 (1297)
AAAGAGATTAGTACTGAAGAGCAGCTAAGGCGCCTGCAAGAGGAGAAGCTTTGC 7159760 (1297)
AAAGAGATTAGTACTGAAGAGCAGCTAAGGCGCCTGCAAGAGGAGAAGCTTTGC Consensus (1297)

Section 26
AAAATCTGTATGGATAGAAATATTGCTATCGTTTTTGTTCCTTGTGGACATCTA L1309 (1351)
AAAATCTGTATGGATAGAAATATTGCTATCGTTTTTGTTCCTTGTGGACATCTA L1236 (1351)
AAAATCTGTATGGATAGAAATATTGCTATCGTTTTTGTTCCTTGTGGACATCTA L591 (1351)
AAAATCTGTATGGATAGAAATATTGCTATCGTTTTTGTTCCTTGTGGACATCTA L428 (1351)
AAAATCTGTATGGATAGAAATATTGCTATCGTTTTTGTTCCTTGTGGACATCTA KMH2 (1351)
AAAATCTGTATGGATAGAAATATTGCTATCGTTTTTGTTCCTTGTGGACATCTA 7159760 (1351)
AAAATCTGTATGGATAGAAATATTGCTATCGTTTTTGTTCCTTGTGGACATCTA Consensus (1351)

Section 27
GTCACTTGTAAACAATGTGCTGAAGCAGTTGACAAGTGTCCCATGTGCTACACA L1309 (1405)
GTCACTTGTAAACAATGTGCTGAAGCAGTTGACAAGTGTCCCATGTGCTACACA L1236 (1405)
GTCACTTGTAAACAATGTGCTGAAGCAGTTGACAAGTGTCCCATGTGCTACACA L591 (1405)
GTCACTTGTAAACAATGTGCTGAAGCAGTTGACAAGTGTCCCATGTGCTACACA L428 (1405)
GTCACTTGTAAACAATGTGCTGAAGCAGTTGACAAGTGTCCCATGTGCTACACA KMH2 (1405)
GTCACTTGTAAACAATGTGCTGAAGCAGTTGACAAGTGTCCCATGTGCTACACA 7159760 (1405)
GTCACTTGTAAACAATGTGCTGAAGCAGTTGACAAGTGTCCCATGTGCTACACA Consensus (1405)

Section 28
GTCATTACTTTCAAGCAAAAAATTTTTATGTCTTAA L1309 (1459)
GTCATTACTTTCAAGCAAAAAATTTTTATGTCTTAA L1236 (1459)
GTCATTACTTTCAAGCAAAAAATTTTTATGTCTTAA L591 (1459)
GTCATTACTTTCAAGCAAAAAATTTTTATGTCTTAA L428 (1459)
GTCATTACTTTCAAGCAAAAAATTTTTATGTCTTAA KMH2 (1459)
GTCATTACTTTCAAGCAAAAAATTTTTATGTCTTAA 7159760 (1459)
GTCATTACTTTCAAGCAAAAAATTTTTATGTCTTAA Consensus (1459)

Caspase-3 Alignment

Section 1
ATGGAGAACACTGAAAACTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCA L1309 (1)
ATGGAGAACACTGAAAACTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCA L1236 (1)
ATGGAGAACACTGAAAACTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCA L591 (1)
ATGGAGAACACTGAAAACTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCA L428 (1)
ATGGAGAACACTGAAAACTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCA KMH2 (1)
ATGGAGAACACTGAAAACTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCA XM_003524 (1)
ATGGAGAACACTGAAAACTCAGTGGATTCAAAATCCATTAAAAATTTGGAACCA Consensus (1)

Section 2
AAGATCATACATGGAAGCGAATCAATGGACTCTGGAATATCCCTGGACAACAGT L1309 (55)
AAGATCATACATGGAAGCGAATCAATGGACTCTGGAATATCCCTGGACAACAGT L1236 (55)
AAGATCATACATGGAAGCGAATCAATGGACTCTGGAATATCCCTGGACAACAGT L591 (55)
AAGATCATACATGGAAGCGAATCAATGGACTCTGGAATATCCCTGGACAACAGT L428 (55)
AAGATCATACATGGAAGCGAATCAATGGACTCTGGAATATCCCTGGACAACAGT KMH2 (55)
AAGATCATACATGGAAGCGAATCAATGGACTCTGGAATATCCCTGGACAACAGT XM_003524 (55)
AAGATCATACATGGAAGCGAATCAATGGACTCTGGAATATCCCTGGACAACAGT Consensus (55)

Section 3
TATAAAATGGATTATCCTGAGATGGGTTTATGTATAATAATTAATAATAAGAAT L1309 (109)
TATAAAATGGATTATCCTGAGATGGGTTTATGTATAATAATTAATAATAAGAAT L1236 (109)
TATAAAATGGATTATCCTGAGATGGGTTTATGTATAATAATTAATAATAAGAAT L591 (109)
TATAAAATGGATTATCCTGAGATGGGTTTATGTATAATAATTAATAATAAGAAT L428 (109)
TATAAAATGGATTATCCTGAGATGGGTTTATGTATAATAATTAATAATAAGAAT KMH2 (109)
TATAAAATGGATTATCCTGAGATGGGTTTATGTATAATAATTAATAATAAGAAT XM_003524 (109)
TATAAAATGGATTATCCTGAGATGGGTTTATGTATAATAATTAATAATAAGAAT Consensus (109)

Section 4
TTTCATAAAAGCACTGGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCA L1309 (163)
TTTCATAAAAGCACTGGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCA L1236 (163)

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TTTCATAAAAGCACTGGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCA L591 (163)
TTTCATAAAAGCACTGGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCA L428 (163)
TTTCATAAAAGCACTGGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCA KMH2 (163)
TTTCATAAAAGCACTGGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCA XM_003524 (163)
TTTCATAAAAGCACTGGAATGACATCTCGGTCTGGTACAGATGTCGATGCAGCA Consensus (163)
```

Section 5

```
AACCTCAGGGAAACATTCAGAAACTTGAAATATGAAGTCAGGAATAAAAATGAT L1309 (217)
AACCTCAGGGAAACATTCAGAAACTTGAAATATGAAGTCAGGAATAAAAATGAT L1236 (217)
AACCTCAGGGAAACATTCAGAAACTTGAAATATGAAGTCAGGAATAAAAATGAT L591 (217)
AACCTCAGGGAAACATTCAGAAACTTGAAATATGAAGTCAGGAATAAAAATGAT L428 (217)
AACCTCAGGGAAACATTCAGAAACTTGAAATATGAAGTCAGGAATAAAAATGAT KMH2 (217)
AACCTCAGGGAAACATTCAGAAACTTGAAATATGAAGTCAGGAATAAAAATGAT XM_003524 (217)
AACCTCAGGGAAACATTCAGAAACTTGAAATATGAAGTCAGGAATAAAAATGAT Consensus (217)
```

Section 6

```
CTTACACGTGAAGAAATTGTGGAATTGATGCGTGATGTTTCTAAAGAAGATCAC L1309 (271)
CTTACACGTGAAGAAATTGTGGAATTGATGCGTGATGTTTCTAAAGAAGATCAC L1236 (271)
CTTACACGTGAAGAAATTGTGGAATTGATGCGTGATGTTTCTAAAGAAGATCAC L591 (271)
CTTACACGTGAAGAAATTGTGGAATTGATGCGTGATGTTTCTAAAGAAGATCAC L428 (271)
CTTACACGTGAAGAAATTGTGGAATTGATGCGTGATGTTTCTAAAGAAGATCAC KMH2 (271)
CTTACACGTGAAGAAATTGTGGAATTGATGCGTGATGTTTCTAAAGAAGATCAC XM_003524 (271)
CTTACACGTGAAGAAATTGTGGAATTGATGCGTGATGTTTCTAAAGAAGATCAC Consensus (271)
```

Caspase-3 Alignment

Section 7

```
AGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTGAGCCATGGTGAAGAAGGAATA L1309 (325)
AGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTGAGCCATGGTGAAGAAGGAATA L1236 (325)
AGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTGAGCCATGGTGAAGAAGGAATA L591 (325)
AGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTGAGCCATGGTGAAGAAGGAATA L428 (325)
AGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTGAGCCATGGTGAAGAAGGAATA KMH2 (325)
AGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTGAGCCATGGTGAAGAAGGAATA XM_003524 (325)
AGCAAAAGGAGCAGTTTTGTTTGTGTGCTTCTGAGCCATGGTGAAGAAGGAATA Consensus (325)
```

Section 8

```
ATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAAATAACAAACTTTTTCAGA L1309 (379)
ATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAAATAACAAACTTTTTCAGA L1236 (379)
ATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAAATAACAAACTTTTTCAGA L591 (379)
ATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAAATAACAAACTTTTTCAGA L428 (379)
ATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAAATAACAAACTTTTTCAGA KMH2 (379)
ATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAAATAACAAACTTTTTCAGA XM_003524 (379)
ATTTTTGGAACAAATGGACCTGTTGACCTGAAAAAAATAACAAACTTTTTCAGA Consensus (379)
```

Section 9

```
GGGGATCGTTGTAGAAGTCTAACTGGAAAACCCAAACTTTTCATTATTCAGGCC L1309 (433)
GGGGATCGTTGTAGAAGTCTAACTGGAAAACCCAAACTTTTCATTATTCAGGCC L1236 (433)
GGGGATCGTTGTAGAAGTCTAACTGGAAAACCCAAACTTTTCATTATTCAGGCC L591 (433)
GGGGATCGTTGTAGAAGTCTAACTGGAAAACCCAAACTTTTCATTATTCAGGCC L428 (433)
GGGGATCGTTGTAGAAGTCTAACTGGAAAACCCAAACTTTTCATTATTCAGGCC KMH2 (433)
GGGGATCGTTGTAGAAGTCTAACTGGAAAACCCAAACTTTTCATTATTCAGGCC XM_003524 (433)
GGGGATCGTTGTAGAAGTCTAACTGGAAAACCCAAACTTTTCATTATTCAGGCC Consensus (433)
```

Section 10

```
TGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTGGTGTTGATGAT L1309 (487)
TGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTGGTGTTGATGAT L1236 (487)
TGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTGGTGTTGATGAT L591 (487)
TGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTGGTGTTGATGAT L428 (487)
TGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTGGTGTTGATGAT KMH2 (487)
TGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTGGTGTTGATGAT XM_003524 (487)
TGCCGTGGTACAGAACTGGACTGTGGCATTGAGACAGACAGTGGTGTTGATGAT Consensus (487)
```

Section 11

```
GACATGGCGTGTCATAAAATACCAGTGGAGGCCGACTTCTTGTATGCATACTCC  L1309 (541)
GACATGGCGTGTCATAAAATACCAGTGGAGGCCGACTTCTTGTATGCATACTCC  L1236 (541)
GACATGGCGTGTCATAAAATACCAGTGGAGGCCGACTTCTTGTATGCATACTCC  L591 (541)
GACATGGCGTGTCATAAAATACCAGTGGAGGCCGACTTCTTGTATGCATACTCC  L428 (541)
GACATGGCGTGTCATAAAATACCAGTGGAGGCCGACTTCTTGTATGCATACTCC  KMH2 (541)
GACATGGCGTGTCATAAAATACCAGTGGAGGCCGACTTCTTGTATGCATACTCC  XM_003524 (541)
GACATGGCGTGTCATAAAATACCAGTGGAGGCCGACTTCTTGTATGCATACTCC  Consensus (541)
```

Section 12

```
ACAGCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATC  L1309 (595)
ACAGCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATC  L1236 (595)
ACAGCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATC  L591 (595)
ACAGCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATC  L428 (595)
ACAGCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATC  KMH2 (595)
ACAGCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATC  XM_003524 (595)
ACAGCACCTGGTTATTATTCTTGGCGAAATTCAAAGGATGGCTCCTGGTTCATC  Consensus (595)
```

Caspase-3 Alignment

Section 13

```
CAGTCGCTTTGTGCCATGCTGAAACAGTATGCCGACAAGCTTGAATTTATGCAC  L1309 (649)
CAGTCGCTTTGTGCCATGCTGAAACAGTATGCCGACAAGCTTGAATTTATGCAC  L1236 (649)
CAGTCGCTTTGTGCCATGCTGAAACAGTATGCCGACAAGCTTGAATTTATGCAC  L591 (649)
CAGTCGCTTTGTGCCATGCTGAAACAGTATGCCGACAAGCTTGAATTTATGCAC  L428 (649)
CAGTCGCTTTGTGCCATGCTGAAACAGTATGCCGACAAGCTTGAATTTATGCAC  KMH2 (649)
CAGTCGCTTTGTGCCATGCTGAAACAGTATGCCGACAAGCTTGAATTTATGCAC  XM_003524 (649)
CAGTCGCTTTGTGCCATGCTGAAACAGTATGCCGACAAGCTTGAATTTATGCAC  Consensus (649)
```

Section 14

```
ATTCTTACCCGGGTTAACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTT  L1309 (703)
ATTCTTACCCGGGTTAACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTT  L1236 (703)
ATTCTTACCCGGGTTAACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTT  L591 (703)
ATTCTTACCCGGGTTAACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTT  L428 (703)
ATTCTTACCCGGGTTAACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTT  KMH2 (703)
ATTCTTACCCGGGTTAACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTT  XM_003524 (703)
ATTCTTACCCGGGTTAACCGAAAGGTGGCAACAGAATTTGAGTCCTTTTCCTTT  Consensus (703)
```

Section 15

```
GACGCTACTTTTCATGCAAAGAAACAGATTCCATGTATTGTTTCCATGCTCACA  L1309 (757)
GACGCTACTTTTCATGCAAAGAAACAGATTCCATGTATTGTTTCCATGCTCACA  L1236 (757)
GACGCTACTTTTCATGCAAAGAAACAGATTCCATGTATTGTTTCCATGCTCACA  L591 (757)
GACGCTACTTTTCATGCAAAGAAACAGATTCCATGTATTGTTTCCATGCTCACA  L428 (757)
GACGCTACTTTTCATGCAAAGAAACAGATTCCATGTATTGTTTCCATGCTCACA  KMH2 (757)
GACGCTACTTTTCATGCAAAGAAACAGATTCCATGTATTGTTTCCATGCTCACA  XM_003524 (757)
GACGCTACTTTTCATGCAAAGAAACAGATTCCATGTATTGTTTCCATGCTCACA  Consensus (757)
```

Section 16

```
AAAGAACTCTATTTTTATCACTAA  L1309 (811)
AAAGAACTCTATTTTTATCACTAA  L1236 (811)
AAAGAACTCTATTTTTATCACTAA  L591 (811)
AAAGAACTCTATTTTTATCACTAA  L428 (811)
AAAGAACTCTATTTTTATCACTAA  KMH2 (811)
AAAGAACTCTATTTTTATCACTAA  XM_003524 (811)
AAAGAACTCTATTTTTATCACTAA  Consensus (811)
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Summary

Apoptosis represents an efficient mechanism to eliminate unwanted cells and is carried out by a family of cysteine containing aspartate-specific proteases, termed capases. Suppression of caspases leads to defective apoptosis and contributes to the pathogenesis of cancer by several mechanisms. The Hodgkin and Reed-Sternberg cell (H-RS) is a malignant germinal center B cell with rearranged but nonproductive immunoglobulin genes. However, through yet unknown mechanisms, H-RS cells resist the apoptotic fate normally suffered by defective B cells with crippled immunoglobulin genes. The H-RS cells were previously shown to be resistant to CD95-induced apoptosis. In this work, the intracellular events after induction of the mitochondrial apoptosis pathway have been scrutinized. The data provided show that Hodgkin's disease derived (HD) B cell lines are uniformly resistant to staurosporine, a protein kinase C inhibitor that preferentially stimulates the mitochondrial apoptotic pathway. Staurosporine failed to induce cytochrome c release from mitochondria in HD B cells and correspondingly, activation of caspases was not observed. In staurosporine-treated Hodgkin cells, Bax, a pro-apoptotic Bcl-2 family member, remained in its inactive state, indicating that these cell lines have a defect in this crucial step in apoptotic signaling upstream of the mitochondria. Further analysis of the caspase cascade downstream of mitochondria, showed that addition of cytochrome c failed to stimulate caspase-3 activation in cytosolic extracts of HD B cell lines. This appeared to be due to the overexpression of the X-linked inhibitor of apoptosis (XIAP) in these cell lines. Co-immunoprecipitation studies revealed that XIAP constitutively bound to caspase-3 and prevents the proteolytic processing of caspase-3 required for its activation. Removal of XIAP by either, addition of a XIAP-inhibitor Smac, or by immunodepletion, restored cytochrome c-triggered processing and activation of caspase-3. Finally, XIAP also inhibits caspase-9 in HD B cell lines,

suggesting a dual inhibitory role of XIAP in these cell lines, first by acting as a caspase-9 inhibitor and blocking of the initial cleavage of caspase-3, and then by inhibiting caspase-3 enzymatic activity and its maturation. In summary, defects in two crucial steps of the mitochondrial apoptotic pathway have been identified in H-RS cells that result in the complete abrogation of this pathway. Many of the drugs used for chemotherapy converge at the mitochondrial site of apoptosis and induce apoptosis by promoting the efflux of mitochondrial pro-apoptotic factors into the cytosol followed by activation of caspase cascades downstream of mitochondria. Defective mitochondrial apoptotic pathways contribute to resistance against multiple apoptotic stimuli, which may explain the resistance of H-RS cells against immune surveillance and chemotherapy at late stage of disease.

Zusammenfassung

Apoptose, auch als programmierter Zelltod bezeichnet, ist ein essentieller Mechanismus zur Aufrechterhaltung der Gewebehomöastasis und steht damit der Zellteilung als negativ regulierender Mechanismus gegenüber. Während der Apoptose aktiviert die Zelle ein internes Zerstörungsprogramm, dessen charakteristische Merkmale Veränderungen an der Plasmamembran (*membrane blebbing*), die Kondensierung des Zellkernes, der Abbau der Kern-DNA, sowie die darauf folgende Phagozytose der Zellreste durch Nachbarzellen ohne Entzündungsreaktion, sind. Im vielzelligen Organismus ist die Apoptose ein natürlicher Bestandteil der Ontogenese und unter anderem an der Entwicklung der Organe beteiligt, außerdem an der Beseitigung von gealterten, sowie infizierten oder mutierten Zellen. Somit wird durch die Apoptose eine effiziente Elimination von unerwünschten Zellen erreicht, und ihre Inhibition trägt zur Pathogenese verschiedener Erkrankungen bei, unter anderem zur Karzinogenese. Die Haupteffektoren der Apoptose sind Aspartat-spezifische Proteasen, die sogenannten Caspasen. Die Aktivierung von Caspasen kann durch mindestens zwei Apoptosesignalwege stattfinden: Zum einen kann ein Signalweg durch die Aktivierung der TNF- (*Tumor necrosis factor*) Rezeptorfamilie durch ihre spezifischen Liganden eingeleitet werden. Dies führt zur Rekrutierung und Aktivierung von Caspase-8, welche ihrerseits Caspase-3 aktiviert. Aktivierte Caspase-3 degradiert Apoptose-spezifische Substrate in der Zelle und führt damit zum Abbau der Zelle in den späten Schritten der Apoptose. Im zweiten Signalweg spielen Mitochondrien eine zentrale Rolle. Die Aktivierung des mitochondrialen Apoptosesignalweges wird durch Proteine der Bcl-2-Familie reguliert. Diese Proteinfamilie besteht aus anti-apoptotischen sowie pro-apoptotischen Proteinen, von denen Bax das bislang

bestuntersuchte pro-apoptotische Protein darstellt. Im Falle eines apoptotischen Stimulus findet eine allosterische Konformationsänderung des Bax-Proteins statt, welche zur Verankerung des Proteins in der mitochondrialen Außenmembran und seiner Oligomerisierung führt. Durch diese Oligomere werden pro-apoptotischen Moleküle aus Mitochondrien ins Zytosol freigesetzt, zum Beispiel Cytochrom c und Smac (*second mitochondria-derived activator of caspases*). Das zytosolische Cytochrom c initiiert die Formierung des Apoptosoms, das aus Apaf-1, Caspase-9 und Cytochrom c besteht. Die rekrutierte Caspase-9 wird im Apoptosom aktiviert und kann anschließend Caspase-3 aktivieren. Aktive Caspase-3 degradiert gezielt spezifische zelluläre Substrate und führt damit zum Tod der Zelle.

Die maligne Hodgkin-Reed-Sternberg (H-RS)-Zelle ist ein Keimzentrums-B-Lymphozyt mit rearrangierten aber nicht produktiven Immunglobulin-Gensegmenten. Durch einen bislang unbekannten Mechanismus gelingt es der H-RS-Zelle, der Apoptose, die normalerweise eine B-Zelle mit nicht-produktiven Immunglobulingenen eliminiert, zu entkommen. Vier Hodgkin-Zelllinien, aus Geweben von Hodgkin-Patienten etabliert, wurden in der vorliegenden Arbeit im Hinblick auf Defekte von apoptotischen Signalwegen untersucht. Bereits publizierte Untersuchungen zur Apoptose in diesen Zelllinien zeigten eine allgemeine Apoptoseresistenz. Auf Grund der Resistenz gegenüber der Apoptose-Induktion durch Fas-Ligand wurde ein Defekt des Rezeptor-vermittelten Signalweges der Apoptose postuliert.

In der vorliegenden Arbeit wurden die intrazellulären Signalwege der Apoptose nach Induktion mit Staurosporin, einem Induktor des mitochondrialen Apoptosesignalweges, untersucht. Diese Untersuchungen zeigten eine generelle Resistenz der H-RS-Zelllinien gegen Staurosporin-induzierten Zelltod. Die detailierte Analyse des mitochondrialen Apoptosesignalweges führte zur Entdeckung von zwei

Defekten. Zum einen wurde ein Defekt im Signalweg oberhalb von Mitochondrien charakterisiert, der zur Inhibition der Konformationsänderung von Bax führte. Nicht aktiviertes Bax kann jedoch die Freisetzung von Cytochrom c aus Mitochondrien nicht induzieren. Folglich wird auch die anschließende Caspasekaskade und damit die Apoptose nicht initiiert. Zur weiteren Analyse der Caspasekaskade wurden zytosolische Fraktionen von Zellen isoliert und die Caspaseaktivität nach der Zugabe von Cytochrom c *in vitro* untersucht. Diese Untersuchungen ergaben, dass die Hodgkin-Zellen in einem zweiten Signalschritt defekt sind, die Cytochrom c-vermittelte Caspaseaktivierung ist vollständig inhibiert. Die Prozessierung und Aktivierung von Caspase-9 und Caspase-3 sind blockiert und als Folge davon wurde keine Caspaseaktivität detektiert. Diesem zweiten Defekt im apoptotischen Signalweg in Hodgkin-Zelllinien liegt die Überexpression von XIAP (*X-linked Inhibitor of Apoptosis)*, einem Protein der IAP-Familie (*Inhibitors of Apoptosis*), zu Grunde. Die IAP-Proteine sind Inhibitoren der Apoptose, die ihre Funktion durch direkte Interaktion mit Caspasen ausüben. Die vorliegenden Daten zeigen, dass XIAP mit Caspase-3 assoziiert ist und damit die Aktivierung und Aktivität von Caspase-3 inhibiert. Die Depletion von XIAP hingegen resultierte in der Wiederherstellung der Caspaseaktivität. Auch in Gegenwart des Proteins Smac, das an XIAP bindet und seine Funktion blockiert, war die Caspaseaktivität wieder hergestellt. Diese Ergebnisse weisen auf eine zentrale Rolle von XIAP in der Inhibition der Caspasekaskade in H-RS-Zelllinien hin.

Zusammenfassend zeigt die vorliegende Arbeit, dass in H-RS-Zelllinien zwei Defekte in intrazellulären Signalwegen zur vollständigen Inhibition der Apoptose führen. Während sich der eine Defekt in der Signalkette oberhalb von Mitochondrien, im Bax-Aktivierungsschritt, befindet, liegt der zweite Defekt im Signalweg unterhalb der Mitochondrien in der Inhibition der Caspaseaktivierungskaskade durch die

Überexpression von XIAP. Das Vorliegen mehrerer Defekte in Apoptosesignalwegen von Hodgkin-Zellen erklärt ihre Resistenz gegen Immunabwehr und Chemotherapie.

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Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Jonathan Howard betreut worden.

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