A Serine Protease-Dependent Mechanism of Apoptosis after Caspase Arrest

Dissertation

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1. Introduction

1.1. Apoptosis

The term apoptosis derives from the Greek, originally meaning the dropping of leaves from a tree or petals from a flower. In modern biology, apoptosis is referred to as programmed cell death, a process by which a cell dies in a regulated and organized fashion. This form of cell death was classified as a morphological entity on its own in 1972 in a landmark paper by Kerr and coworkers and eventually termed apoptosis. However, the first reports of a form of cell death with distinct morphology were already published by the end of the 19th century. While in 1980 Willie complained about the fact that there was only little interest in this area of research, this has changed dramatically since. This is most impressively documented by the award of the 2002 Nobel Prize in Physiology or Medicine “for their discoveries concerning 'genetic regulation of organ development and programmed cell death’” to Sydney Brenner, H. Robert Horvitz and John E. Sulston. Therefore, the next section will describe the difference between controlled and uncontrolled cell death, characterize the role of apoptotic stimuli in the liver and finally introduce HepG2 cells as a model for liver research.

1.1.1. Apoptosis vs. necrosis

In contrast to apoptosis, necrosis – derived from the Greek expression for “deadness” or “dying” – describes a type of cell death which is characterized by edema and cell swelling, resulting in final rupture of the cell membrane. The typical features of a cell dying apoptotically comprise nuclear condensation with margination of chromatin, condensation of cytoplasm, rounding up, blebbing with maintenance of membrane integrity (zeiosis), and intranucleosomal DNA fragmentation. In contrast, necrosis mostly leads to disintegration and lysis of the nucleus, edematous swelling of organelles and cells, blebbing with rupture of the plasma membrane, and random DNA degradation. Apoptotic cells are phagocytosed by professional phagocytes or neighboring cells without inflammatory tissue reactions, whereas necrotic cell death often causes leukocyte infiltration and a general inflammatory response (for detailed review see reference). It is important to mention that, given the fact that many cells may display both apoptotic and necrotic features when dying, newer concepts of cell death do not only differentiate between apoptotic and necrotic cell death but also integrate mixed forms like “necrosis-like programmed cell death” and “apoptosis-like programmed cell death”.
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1.1.2. Death receptors in physiology and pathophysiology of the liver

The liver is a unique organ, also and especially to that regard that it has the capacity to regenerate after injury or partial resection of hepatic tissue, a fact that is already implied by the ancient Greek legend of Prometheus. The liver can compensate for resections of up to 70% of its mass by actuating a sequence of events that generally result in complete restitution of hepatic mass and function. Quiescent cells are primed to enter the cell cycle and then enter its different phases under the influence of various proteins, growth factors and cell cycle-dependent kinases. The cytokine tumor necrosis factor α (TNFα) plays a key role in the proliferation of the regenerating liver and its hepatic levels are rapidly increased after partial hepatectomy, TNF receptor 1 (TNF-R1) being absolutely essential for regeneration. The underlying mechanism is induction of c-Jun N-terminal kinase (JNK) and the transcription factors AP-1 and NF-κB, respectively. The important role of especially NF-κB has been impressively demonstrated by Rosenberg et al who showed that mice deficient in the NF-κB-transactivating gene RelA died at embryonic days 14-15 days due to massive liver apoptosis. This apoptosis was dependent on TNF-R1, as mice deficient of both RelA and TNF-R1 developed normally, a fact that shows that TNFα is a pleiotropic cytokine, i.e. it can induce different and even completely opposed processes like cell proliferation and cell death. In order to control these processes, both cytokine-dependent and -independent pathways act cooperatively. This requirement for multiple signals also protects the liver from undergoing hyperplasia by ensuring that cell growth and cell death are well balanced.

It is a characteristic of its pleiotropic nature that TNFα and another member of the TNF superfamily, the cytokine CD95L, also play an important role in the induction of hepatic apoptosis, as for example during viral hepatitis, inflammatory hepatitis, Wilson’s disease, alcoholic liver disease, endotoxemia-induced liver failure and ischemia/reperfusion-induced liver damage. The tight connection between TNFα and CD95L in controlled cell death of hepatocytes is exemplified by the finding that mice lacking both TNF-R1 and -2 are resistant to death and fulminant liver injury induced by an agonistic anti-CD95 antibody, whereas this is not the case for the TNF-R1 knockout. Moreover, not only TNFα but also CD95L has been shown to be both a mediator of hepatic apoptosis and regeneration. As a consequence, these two cytokines have been used to study the process and the molecular mechanisms of hepatocyte apoptosis for many years.

In summary, death receptors and their respective agonists exert pleiotropic functions as they couple to both cell proliferation and apoptosis, depending on the circumstances. This tightly
controlled balance is a prerequisite for organ homoeostasis and function and any serious imbalance will have pathophysiological or even lethal consequences.
1.1.3. HepG2 cells: a model for liver research

Cell lines derived from human hepatocellular carcinoma (HCC) are commonly used in order to study both physiology and pathophysiology of hepatocytes. Compared to primary hepatocytes they offer the unrivaled advantage of almost unlimited availability and easy handling. What is more, whereas the isolation of primary murine hepatocytes only requires certain skills and the appropriate instrumentation, primary hepatocytes from humans are not readily available, especially if cells from healthy donors are required. Also, experimental systems established with cell lines are usually relatively robust, i.e. results should be highly reproducible. In contrast, experiments using cells isolated from animals are often hampered by variations due to the use of cells derived from different animals or seasonal variations. Nevertheless, when working with cell lines, one should keep in mind that – as any cell or organism – also these cells acquire mutations. For this reason, a cell line that has been isolated thirty years ago might have lost certain features over the years and the thousands of passages it was eventually subjected to. For that reason, it is also possible that different laboratories, although using the same cell line, make different observations in the same experimental system. But for all that, due to their undisputed advantages cell lines still are the model of choice e.g. in the study of signaling processes or apoptosis.

The HCC lines most commonly used are Hep3B and HepG2 cells, respectively. As Hep3B cells harbor the integrated hepatitis B virus genome, this cell line has been assigned by the ZKBS (German Central Commission for Biological Safety) to risk category 2. Therefore, our study was conducted using HepG2 cells, a human hepatocellular carcinoma cell line that was established from the tumor tissue of a 15-year-old Argentine boy with hepatocellular carcinoma in 1975\textsuperscript{28}. HepG2 cells express a variety of liver functions\textsuperscript{29} as for example uptake of low density lipoproteins (LDL)\textsuperscript{30} and secretion of apolipoprotein B\textsuperscript{31}, fibrinogen\textsuperscript{32} or bile acids\textsuperscript{33,34}. As a consequence, they are in wide use as a model of hepatic physiology. However, HepG2 cells have also proven to represent an excellent model of hepatic apoptosis and many groups have studied the apoptosis of these cells induced by a variety of drugs or death receptor agonists\textsuperscript{35-44}. It is nevertheless of great importance to keep in mind that HepG2 are hyperdiploid, transformed cells and even though they share many features of primary hepatocytes they do not necessarily share any given pathway.
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1.2. Death receptors and their ligands

Up to the present, 29 members of the tumor necrosis factor (TNF) receptor family have been identified. They play important roles in many biological events and control e.g. innate and adaptive immune functions. Of these, the most thoroughly studied are cell death induction and regulation of the inflammatory process45. Moreover, death receptor ligands such as TNFα, CD95L and TRAIL have been explored as potential therapeutics in cancer therapy with limitations in the case of TNFα and CD95L due to systemic toxicity (for detailed review see references46,47).

Death receptors can be classified into three major groups, based on their cytoplasmatic sequences and signaling properties. The following section will introduce three members of the first group, namely TNF-R1, CD95/Fas and TRAIL-R1/2, which is characterized by a death domain (DD) in the cytoplasmatic tail. Activation of these receptors by their corresponding homo- or heterotrimeric ligands leads to recruitment of DD-containing adaptor molecules such as Fas associated death domain (FADD) or TNF-R associated death domain (TRADD) which – through interaction with their death effector domain (DED) – cause activation of the caspase cascade and induction of apoptosis. The second group of receptors contains one or more TRAF (TNF associated factor) –interacting motifs (TIMs) and comprises for example TNF-R2 or the lymphotoxin-β receptor (LTβR). Stimulation of these receptors activates multiple signal transduction pathways as nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK), p38, or phosphoinositide 3-kinase (PI3K). The third group includes the TRAIL receptors 3 and 4, decoy receptor 3 and osteoprotegerin, which do not contain functional intracellular domains or motifs. Even though this group cannot provide intracellular signaling, it can effectively compete with the other two groups of receptors for their corresponding ligands48.

1.2.1. TNF-R1 / TNFα

The TNF receptor 1 (TNF-R1) is a ubiquitous 55 kDa transmembrane protein which is trimerized upon binding of its ligand, TNFα. This cytokine is produced mainly by macrophages and in smaller amounts by several other cell types. It is involved in acute liver toxicity caused by xenobiotics22 or during systemic inflammatory response syndrome (SIRS)20. The 233 amino acid membrane-bound form is cleaved by TNFα converting enzyme (TACE) to yield the 157 amino acid, 17 kDa soluble cytokine. TNFα exerts pleiotropic effects on many different cell types and is implicated as an important mediator in various physiological and pathophysiological conditions16. Moreover, it has become clear that TNFα
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is an important mediator of cell death and its role in models of acute liver injury has been studied extensively\(^{20,22,24,49}\). The most recent concept of TNF-R1 mediated signal transduction involves the formation of sequential signaling complexes. The initial complex I is bound to the plasma membrane and consists of TNF-R1, the adaptor TNF-R associated death domain (TRADD), the kinase RIP1 and TNF-R associated factor 2 (TRAF-2) and rapidly leads to activation of NF-κB. It is only in a second step that TRADD and RIP1 associate with FADD and caspase-8, forming the cytoplasmatic complex II. Thus, in contrast to the classical model of TNF receptor signaling which proposed formation of a death-inducing signaling complex (DISC) consisting of TNF-R1, TRADD, FADD and caspase-8, TNF-R1-mediated signal transduction comprises a checkpoint which only allows for induction of apoptosis via complex II when the anti-apoptotic signal via NF-κB fails to be activated\(^5\).

1.2.2. CD95 / CD95L

The receptor CD95 (also known as Fas or Apo1) is ubiquitously expressed in various tissues with abundant expression in the thymus, liver, heart and kidney. Its ligand CD95L, however, is predominantly expressed in activated T lymphocytes or natural killer cells. It is also expressed constitutively in immune-privileged sites such as the testis and the eye, a fact that illustrates the importance of the CD95/CD95L system in T cell selection and immunity\(^{50}\). Binding of CD95L to its receptor induces formation of the DISC which – different from the TNF-R1 system – primarily consists of the trimerized receptor, FADD and caspase-8. It has been shown that death domain associated protein (DAXX), receptor interacting protein (RIP) kinase and RIP-associated ICH-1 homologous protein with a death domain (RAIDD) also associate with the CD95 receptor. However, the exact role of these proteins is unclear, as they are dispensable for CD95L-induced apoptosis in lymphoid cells\(^{51}\). Of interest, a variety of studies has shown that many chemotherapeutic drugs exert their mode of action via induction of the CD95 system\(^{52,53}\). Both p53- and AP-1-dependent mechanism for the upregulation of CD95 and CD95L, respectively, have been described\(^{40,54,55}\). Another interesting finding was that toxic bile salts induce apoptosis of mouse hepatocytes through ligand-independent oligomerization of CD95, the downstream signaling involving both caspase-8 and cathepsin-B\(^{56,57}\). Other publications show that, in contrast to TNFα, stimulation of CD95 causes apoptosis of murine hepatocytes which had not been sensitized by blocking transcription both \textit{in vitro} and \textit{in vivo}, in wild-type and as well as in TNF-R1 knock-out mice\(^{24}\). This shows that TNF-R1 and CD95 are at least in part independent and differentially regulated triggers of murine apoptotic liver failure.
1.2.3. TRAIL-R1/2 / TRAIL

Generally, TNF-related apoptosis-inducing ligand (TRAIL) is similar in a number of ways to CD95L, both in signaling and its effects. Being a point of controversy in the beginning, it has meanwhile been shown that the adaptor molecule FADD is required for both CD95- and TRAIL-mediated apoptosis. Moreover, in addition to caspase-8, also caspase-10 is recruited to and activated at the DISC of TRAIL and CD95 receptors, respectively. However, in contrast to CD95L, TRAIL – with one exception discussed below – is alleged to have selective antitumor activity because it is only causing apoptosis of transformed but not normal cells. It is believed that normal cells are protected from TRAIL by expression of the decoy receptors TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and osteoprotegerin, which do not possess an intracellular signaling domain. Indeed, resistance of tumor cells occasionally correlates with the expression of these decoy receptors. The selectivity of TRAIL for transformed cells made it a very promising agent for cancer therapy. However, the potential utility and safety of systemic administration of TRAIL has been questioned by the finding that – unlike monkey or mouse hepatocytes – human hepatocytes were susceptible to induction of human recombinant TRAIL in vitro. A way to avoid this serious problem is the use of anti-human TRAIL-R2 monoclonal antibody which exerts tumoricidal activity without inducing significant cell death in human hepatocytes. A recent report even discusses the possibility of viral transfer of the TRAIL gene to transformed cells by target techniques such as tumor specific promoters, showing that TRAIL gene transfer was able to kill liver cancer cells without toxicity to normal cells.
1.2.4. Downstream signaling

The current concept of caspase-dependent apoptosis differentiates between two separate albeit not exclusive apoptotic pathways, i.e. the extrinsic and the intrinsic pathway. The extrinsic pathway is initiated by binding of death receptor ligands to their respective receptors with subsequent activation of caspases-8 and -10 and is responsible for elimination of unwanted cells during development or immune system-mediated tumor removal. In contrast, the intrinsic or mitochondrial pathway is initiated by a variety of apoptotic stimuli such as ionizing radiation, heat shock, osmotic stress or chemotherapy, which lead to release of cytochrome c from the mitochondria and subsequent activation of caspase-9 (discussed in detail in section 1.3.1.1)\textsuperscript{67,68}. The two pathways are linked by the ability of caspase-3 to cleave the proapoptotic protein Bid, whose truncated form tBid causes release of cytochrome c by
mitochondrial outer membrane permeabilization (MOMP). In the last few years, many groups have studied the role of mitochondria in apoptosis which lead to identification of a number of pathways upstream of MOMP. The actual pore-forming effector molecules are proapoptotic Bcl-2 family members like Bax and Bak. They can be activated transcriptionally or by conformational change induced by cleavage or binding to an activated Bcl-2 -like protein such as Bad, tBid, Bim, Noxa or PUMA. The anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL oppose MOMP probably by heterodimerization with Bax-like proteins (e.g. Bax or Bak)\textsuperscript{11,69}. Thus, the ratio of anti- and proapoptotic molecules such as Bcl-2/Bax constitutes a rheostat that sets the threshold of susceptibility to apoptosis via the intrinsic pathway which utilizes the mitochondria to amplify death signals\textsuperscript{4}.

Besides cytochrome c, also other proapoptotic factors are released from the mitochondrial periplasm after MOMP. These include endonuclease G, apoptosis inducing factor (AIF), Smac/DIABLO and the serine protease Omi/HtrA2\textsuperscript{2}. Smac/DIABLO binds to the X-linked inhibitor of apoptosis protein (XIAP), thus preventing its binding to and inhibition of caspases-3, -7, and -9. Omi/HtrA2 (discussed in detail in section 1.3.3.3.2) utilizes the same binding motif as Smac/DIABLO to bind XIAP. However, through its serine protease activity it additionally cleaves and inactivates this anti-apoptotic protein. AIF and endonuclease G both translocate to the nucleus where they participate in DNA cleavage by mechanism which still remain to be clarified in detail\textsuperscript{70}.
Figure 1.2: Control of mitochondrial outer membrane permeabilization (MOMP) and downstream signaling after MOMP.
(Adapted from Wang).
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1.3. The role of proteases in apoptosis

As described in the previous sections, both the initiation and final execution of apoptosis are dependent on the activity of a various proteases. Therefore, the next section will present the major proteases that are, according to the current state of knowledge, involved in apoptotic cell death.

1.3.1. Caspases

Studies on one of the model organisms in developmental biology, the nematode worm *Caenorhabditis elegans*, revealed that the product of the *ced-3* gene was required for all developmental-required cell deaths in this worm. It was found that the CED-3 protein was closely related to the newfound human protease interleukin-1β converting enzyme (ICE), a finding that resulted in the discovery of several ICE-like proteases over the next few years which were subsequently renamed ‘caspases’\(^{71,72}\). The term ‘caspase’ is an abbreviation of ‘cysteine aspartate-specific protease’, indicating that caspases exhibit primary specificity for aspartic acid residues, which is a very uncommon among the known proteases, the only other enzyme with this primary specificity being the physiological caspase activator granzyme B\(^68\). Caspases exist within cells as zymogens, i.e. immature pro-enzymes that must undergo proteolytic cleavage at two aspartic acid sites in order to become activated. During this processing, the large and small subunits are separated and the pro-domain is released. Following cleavage, the large and small subunits join to form a heterodimer with an active site. Two heterodimers then form the active tetrameric enzyme with the active sites facing away from each other (Figure 1.3) As the zymogen itself already has a low level of intrinsic protease activity, pro-caspases can autocatalytically transactivate themselves when brought into close proximity\(^1\).
Caspases can be divided into two functional subgroups: those implicated in the processing of pro-inflammatory cytokines during immune response (caspases-1, -4, -5 and-11) and those that are activated during apoptosis (caspases-2, -3, -6, -7, -8, -9, -10 and -12)\(^7\) (Figure 1.4). The latter group is further subdivided into two groups, the first being the initiator caspases, i.e. caspases that are involved in the upstream signaling events during apoptosis and that tend to have long N-terminal pro-domains with interaction domains such as the death effector domain (DED) or caspase recruitment domains (CARD) that are also present in adaptor molecules at the DISC such as FADD, RAIDD or Apaf-1\(^7\). The second subgroup is the executioner caspases which are thought to be responsible for the actual demolition of the cell and tend to have short or absent pro-domains\(^3\). The next section will discuss the specific characteristics of the initiator and executioner caspases, respectively.

\[\text{Figure 1.3: The mechanism of caspase activation.} \]
(Adapted from Bleackley\(^1\).)
1.3.1.1. Initiator caspases

The initiator caspases that have been most intensively studied so far are caspase-8 and caspase-9. Caspase-8 is – together with caspase-10\textsuperscript{61} – implicated in the extrinsic pathway of apoptosis, as it has the ability to bind to adaptor proteins of the DISC via its two death effector domains (DED). Its potential to proteolytically generate tBid make caspase-8 a link between the extrinsic and intrinsic pathway of apoptosis. As for its activation, the induced
proximity model of Salvesen and Dixit\textsuperscript{75} proposed that the intrinsic enzymatic activity of the procaspase-8 zymogen was sufficient to autocatalytically produce active caspase-8 (or FLICE) after adaptor molecule-mediated clustering of many zymogen molecules at the DISC\textsuperscript{61,75,76}. This clustering is antagonized by the anti-apoptotic cellular FLICE inhibitory protein (cFLIP) which binds to FADD via its DED and thus prevents recruitment of caspase-8 to the DISC\textsuperscript{77}.

Caspase-9, together with apoptotic protease activating factor 1 (Apaf-1) and cytochrome c and dATP, forms the so called apoptosome, a protein complex that has the structure of a seven-spoked wheel, with a central hub that contains the CARD of Apaf-1, serving as the caspase-9 recruitment domain\textsuperscript{78}. Similarly to caspase-8, activity of caspase-9 is also controlled by an endogenous protein inhibitor, i.e. XIAP, which binds to caspase-9 via its Bir-3 domain\textsuperscript{79}. Of importance, whereas the concept that caspases are activated by cleavage of their interdomain linkers still is believed to apply for the executioner proteases, the most recent publications propose a different mechanism for the initiator caspases-8 and -9. The group of Guy Salvesen has shown recently that cleavage is neither required nor sufficient for activation of initiator caspases and that proximity-induced activation of these apical caspases is rather attributable to dimerization. Thus, internal proteolytic cleavage is only a secondary event which results in partial stabilization of the activated dimer\textsuperscript{80}.

There is no doubt that caspases-8, -9 and -10 are initiators of apoptosis. The classification of the caspases-2 and -12, respectively, still may differ according to the author. Caspase-2 has been shown to bind to RAIDD at the CD95 receptor via its CARD domain and thus might be regarded as an initiator caspase\textsuperscript{51}. Caspase-12 acts an initiator of stress-induced apoptosis of the endoplasmatic reticulum in a mechanism also involving calpain\textsuperscript{81,82}. However, our knowledge about the function and importance of these two enzymes is still far from being complete and further studies will be necessary in order to correctly classify them.

1.3.1.2. Executioner caspases

In stark contrast to the initiator caspase, the executioner caspases-3 and -7 exist in the cytosol as inactive dimers, being activated by limited proteolysis within their interdomain linkers. This cleavage is being carried out by an initiator caspase or occasionally also a non-caspase protease such as granzyme B. Caspase-6 has been much less intensively studied than caspases-3 and -7 but it is generally regarded as being an executioner caspase due to its lack of a long pro-domain and its cleavage downstream of the initiators.\textsuperscript{78} Studies have revealed an increasing number of substrates especially of caspase-3, which is generally being regarded as being the most prominent executioner caspase. Those substrates comprise structural
proteins like lamin A, vimentin and fodrin, the inhibitor of caspase-activated DNase (ICAD), the kinase RIP, its endogenous inhibitor XIAP or PARP. Lamin A and PARP are also cleaved by caspases-6 and -7, respectively. Even though there is redundancy in the substrates it has been shown that these three executioner caspases perform distinct, non-redundant roles during the demolition phase of apoptosis. Even though in vitro experiments have shown that caspases-6 and -7 play minor or highly specialized roles in the execution of apoptosis, they are unlikely to be functionally redundant. This is exemplified by the early embryonic lethal phenotype of the caspase-7 but not the caspase-3 knockout mouse.

1.3.2. Non-caspase proteases

In some cases, broad-range caspase inhibitors such as zVAD-fmk do not confer protection after induction of apoptosis because death signaling continues via caspase-independent processes. Thus, despite caspase inhibition, cells can still be phagocytosed and killed in an apoptosis-like fashion. This fact has initiated a fast-growing field of research, the study of caspase-independent apoptosis. Therefore, the next paragraphs will introduce the main enzymes involved in these processes, give an overview on the mechanisms that have been elucidated so far and point out the possible relevance of these findings for human pathophysiology.

1.3.2.1. Calpain

Calpain is a ubiquitous neutral cysteine protease with two major isoforms, m- and µ-calpain. Both are ubiquitously expressed and are composed of a large catalytic subunit of approximately 80 kDa and a smaller regulatory subunit of approximately 30 kDa. Although the catalytic subunits of the two isoforms are encoded by distinct genes and the two isoforms require different concentrations of cytosolic Ca²⁺ for their activation in vitro (i.e. milli- and micromolar concentrations, respectively), the 30 kDa regulatory subunit is common to both isoforms. A large variety of proteins have been shown to be calpain substrates, as for example actin, fodrin, gelsolin, c-fos, c-jun or p53. Moreover, calpain shares some common substrates with caspases, e.g. Bid, Bax and even pro-caspases-3 and -9 themselves. However, cleavage of the latter two neither activates nor inactivates these enzymes. It has also been reported that activated caspases cleave and inactivate calpastatin, the endogenous inhibitor of calpain. Participation of calpain has been demonstrated in apoptotic-like events during platelet-activation and experimental models of ethanol and microcystin-induced hepatotoxicity. Furthermore, activation of the calpain system has been found in the
pathophysiology of spinal cord injury\textsuperscript{92} and Alzheimer’s disease (AD)\textsuperscript{93}, making calpain inhibitors an interesting target of pharmacological research.

1.3.2.2. Cathepsins

All of the mammalian lysosomal cysteine proteases are known as cathepsins, however the converse is not true. Even though most known cathepsins belong to the papain family of cysteine protease (cathepsins B, H, L, S, C, K, O, F, V, X and W), there are also cathepsins that belong to the pepsin family of aspartate proteases (cathepsins D and E) or to the S10 and chymotrypsin families of serine proteases (cathepsins A and G, respectively)\textsuperscript{94}. For a long time, cathepsins were believed to be mainly involved in intracellular protein degradation\textsuperscript{95}. Meanwhile, it has become clear that they are also important in immunology and host defense\textsuperscript{96-99}. Interest in the role of cathepsins in apoptosis has arisen from the findings that they have been found to translocate out of lysosomes under special conditions and have the potential to induce apoptosis and activate caspases\textsuperscript{100-105}. This new interest was further boosted by the finding that the widely used caspase inhibitors zVAD-fmk, zDEVD-fmk and AcYVAD-cmk inhibited cathepsin B in an unspecific manner\textsuperscript{106,107}. Therefore, the next paragraphs will give a short overview on the current state of knowledge about the main cathepsins involved in apoptosis (for review see references\textsuperscript{95,108-111}).

1.3.2.2.1. Cathepsins B and L

Over the last few years, many studies have shown that the cysteine proteases cathepsin B and L play a pivotal role in a variety of apoptotic processes. It is known that cathepsin B can process and activate procaspases-1 and -11\textsuperscript{106,112}, while cathepsin L participates in activation of caspase-3\textsuperscript{113}. The significance of cathepsin B especially in apoptosis of hepatocytes has been clarified in detail by the group of Gregory J. Gores (for review see reference\textsuperscript{114}). In a model of bile salt-induced apoptosis of rat hepatocytes and hepatoma cells, contribution of cathepsin B\textsuperscript{115} and protection by overexpression of its endogenous inhibitor, cystatin A, have been demonstrated\textsuperscript{116}. Also, in TNF\textsubscript{α}-induced apoptosis of mouse hepatocytes, cathepsin B has been shown to mediate lysosomal permeabilization\textsuperscript{117} and promote release of cytochrome c via cleavage of Bid\textsuperscript{118}. Finally, the proof for the important role of cathepsin B especially in this model of apoptosis was brought forward by the fact that the cathepsin B knockout mouse turned out to be resistant to TNF\textsubscript{α}-mediated hepatocyte apoptosis and liver injury\textsuperscript{119}. Studies by other authors showed that cathepsin B also participates in TNF\textsubscript{α}-induced apoptosis of breast cancer cells\textsuperscript{120} and is the dominant execution protease in apoptosis of WEHI 164 fibroblasts\textsuperscript{121} where it mediates release of arachidonic acid\textsuperscript{122}. Compared to cathepsin B, there
1. Introduction

is much less evidence for an importance of cathepsin L in apoptosis. The most interesting data in this regard come from the cathepsin L knockout mouse, which looses its fur (with subsequent regrowth) at day 21 after birth as a result of altered hair follicle physiology. Of interest, whereas neither cathepsin B nor cathepsin L knockout mice develop a serious, i.e. life-threatening of lethal phenotype, the combined cathepsin B/L double knockout has turned out to be lethal within the first 30 days after birth, resulting in massive apoptosis of select neurons in the cerebral cortex and the cerebellar Purkinje and granule cells. This hints to an essential interplay of cathepsins B and L at least in the brain, which is further stressed by the finding that while cathepsin B expression is elevated in human glioma, selective suppression of cathepsin L by antisense cDNA significantly impaired glioma cell invasion and reduced their apoptotic threshold.

1.3.2.2.2. Cathepsin D

The role of the aspartate protease cathepsin D in apoptotic processes has been subject of extensive studies in the last few years. Apoptosis after translocation of cathepsin D to the cytosol with subsequent release of cytochrome c from mitochondria has been shown to occur in both neonatal rat cardiomyocytes and fibroblasts treated with the redox cycling quinone naphthazarin (5,8-dihydroxy-1,4-naphthoquinone). Activation of caspases and apoptosis was also induced after microinjection of cathepsin D into human fibroblasts. Moreover, translocation of cathepsin D and subsequent apoptosis has been demonstrated after lysosomal destabilization by lysosomotropic agents like α-tocopheryl succinate or the ceramide-related detergent sphingosine, whereas stabilization of lysosomes by imidazoline drugs protected astrocytes from cathepsin D-dependent oxidative cytotoxicity. In some cell types, cathepsin D also mediates TNFα- and CD95L-induced apoptosis. A possible explanation for the common activation of cathepsin D by such diverse apoptotic stimuli might be provided by the generation of ceramide by the lysosomal enzyme acid sphingomyelinase (ASM). Proapoptotic receptors or stimuli activating ASM include CD95, TNF-R1, interleukin-1 receptor, γ-irradiation, UV-light, ischemia, and infection of mammalian cells with pathogenic bacteria or viruses. Direct interaction of ceramide with cathepsin D results in autocatalytic proteolysis of the 52 kDa pre-pro-cathepsin D to form the enzymatically active 48/32 kDa isoforms of cathepsin D. Evidence for an important role of cathepsin D not only in pathophysiology but also in physiology is provided by the fact that a single nucleotide mutation in the cathepsin D gene leads to the neurodegenerative storage disease CONCL (congenital ovine neuronal ceroid lipofuscinoses) in sheep and that cathepsin D-deficient mice die by intestinal necrosis approximately 25 days after birth.
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Indeed, a recent report by Castino et al shows that inhibition of cathepsin D alone was sufficient to drive neuroblastoma cells into apoptosis and the authors suggest to include cathepsin inhibitors in therapeutic treatment of neuroblastomas\textsuperscript{142}.

1.3.2.2.3. Cathepsin G

In contrast to Cathepsins B, L and D, only very few publications have studied the role of cathepsin G in apoptotic processes. Cathepsin G is – together with cathepsin A – a serine protease member of the large cathepsin family and is abundantly expressed in neutrophils. It has been reported that cathepsin G can activate procaspase-7 by cleavage between the large and small subunits in the zymogen molecule \textit{in vitro}\textsuperscript{88}. It also cleaves the 113 kDa poly ADP-ribose polymerase (PARP) both \textit{in vitro} and during necrosis of Jurkat T cells. Interestingly, in contrast to PARP cleavage by caspases which yields fragments of 89 and 24 kDa, necrotic or \textit{in vitro} cleavage by cathepsin G only yields a major fragment of approximately 50 kDa\textsuperscript{143}. Translocation of cathepsin G from lysosomes to the cytosol has been observed during apoptosis of NB4 leukemic cells\textsuperscript{144} and the serine protease inhibitor squamous cell carcinoma antigen 2 (SCCA2) has been shown to inhibit cathepsin G and protect HeLa cells from TNF\textsubscript{α}-induced apoptosis\textsuperscript{145}. A more recent study has demonstrated that cathepsin G from neutrophils activates the kinases ERK, p\textsuperscript{38} MAPK and AKT in cardiomyocytes, resulting in decreased contractile function and apoptosis, a finding that the authors assume to have functional importance at sites of interstitial inflammation in the heart\textsuperscript{146}.

1.3.3.3. Serine proteases

There is growing evidence that serine proteases other than cathepsin G – together with caspases – take part in protein degradation during apoptosis, most of the evidence being based on the observation that particular apoptotic events can be prevented by broad-range inhibitors of serine proteases such as TLCK or TPCK\textsuperscript{147,148}. Hence, the following sections will present the most prominent serine proteases involved in apoptotic processes and discuss possible mechanisms of their action.

1.3.3.3.1. Granzyme B

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells utilize a granule exocytosis pathway for the elimination of pathogenic cells\textsuperscript{4}. The classical “lethal hit” model stated that following granule exocytosis, poly-perforin formed channels in the target cell membrane in a Ca\textsuperscript{2+}-dependent manner through which granzymes A and B gained entry to the target cell cytoplasm and substrates\textsuperscript{1}. Meanwhile it has been demonstrated that once released from the
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CTL, the differentially glycosylated active 29–33 kDa serine protease granzyme B binds to the mannose-6-phosphate receptor and is endocytosed but remains arrested in endocytic vesicles of the target cell, its release being mediated by perforin. Granzyme B, like caspases, has the unique feature of cleaving after aspartic acid residues and the fact that caspase require cleavage at such residues makes them an excellent target for granzyme B\textsuperscript{149}. Indeed, it has been shown that granzyme B is able to cleave and activate caspase-3 both in vivo and in vitro. Caspases-6, -7, -8, -9 and -10 are also substrates for granzyme B in vitro\textsuperscript{150}. Other substrates comprise the inhibitor of caspase-activated DNase (ICAD), poly ADP-ribose polymerase (PARP) and the proapoptotic BH3-only protein Bid. Cleavage of Bid leads to translocation of its truncated form tBid to the mitochondria, resulting in release of cytochrome c, which can lead to necrotic cell death in the absence of caspase activity. In case caspase-3 is active, Smac/DIABLO and HtrA2 which are also released from the mitochondrial periplasm neutralize and – in the case of HtrA2 – even proteolytically inactivate XIAP, thus leading to enhanced activity of caspases-3, -7 and -9 and subsequent apoptosis. The importance of granzyme B cleavage of Bid is further stressed by the finding that full caspase activity is only achieved in the presence of Smac/DIABLO in the cytoplasm\textsuperscript{151}. A recent report of Amsterdam et al shows that granzyme B is also expressed and activated in ovarian granulosa cells undergoing apoptosis, a finding that extends the role of this protease beyond CTL-mediated cell death\textsuperscript{152}.

1.3.3.3.2. Omi/HtrA2

Human Omi or HtrA2 (high temperature requirement of activation) is a recently described member of a novel family of mammalian serine proteases homologous to the \textit{Escherichia coli} chaperone HtrA\textsuperscript{153}. It is located in the mitochondrial periplasm and its physiological role in mammalian cells is not yet fully understood. It seems that HtrA2 is involved in cellular stress response\textsuperscript{154} as it is upregulated after heat shock and endoplasmatic reticulum stress induced by tunicamycin\textsuperscript{155}. Like cytochrome c, AIF, endonuclease G and Smac/DIABLO, HtrA2 is released from mitochondria during apoptosis and translocates to the cytosol and the nucleus. The mature 36 kDa form can both neutralize and inactivate the X-linked inhibitor of apoptosis protein XIAP by either binding it in a manner similar to Smac/DIABLO or by cleaving it, thus potentiating apoptosis\textsuperscript{70,156-161}. Moreover, HtrA2 is also able to induce apoptosis in a caspase-independent manner that exclusively relies on its protease function\textsuperscript{162-164}. The findings that HtrA2 might be involved in stomach cancer development\textsuperscript{165} and the pathogenesis of Alzheimer’s disease\textsuperscript{166} further demonstrate its importance in human pathology.
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1.3.3.3. AP24

The 24-kD apoptotic protease AP24 is a chymotrypsin-like serine protease that was isolated from the leukemia cell line U937 undergoing apoptosis induced by either TNFα, UV light or various DNA-damaging drugs like etoposide, camptothecin or chlorambucit. It causes internucleosomal DNA fragmentation in the nucleus, a process which is attenuated by metabolic depletion of nicotinamide adenine dinucleotide (NAD) or overexpression of the anti-apoptotic protein Bcl-2. In contrast, nutritional depletion of glutathione (GSH) leads to sensitization towards AP24-mediated apoptosis, a process which is inhibited by the addition of exogenous GSH. It has been shown that activity of caspase-3 is one of several upstream events required for the activation of AP24. Recent reports suggest that AP24 activates L-DNase II in TNFα-induced apoptosis, which could also account for the yet unresolved mechanism of how the ubiquitously expressed leukocyte elastase inhibitor (LEI) is transformed into the endonuclease L-DNase II during apoptosis. However, it is important to note that AP24 so far only has been described by its apparent molecular weight and sensitivity towards serine protease inhibitors but that its cDNA sequence still remains to be identified.
2. Aims of the study

Apoptosis is an essential process in development, organ homeostasis and disease, allowing for cell death in a controlled manner and without inflammatory tissue reactions. Death receptor agonists such as TNFα and CD95L have been implicated in all of these conditions and by their pleiotropic nature have the potential to elicit both cell growth and cell death, depending on the circumstances. In recent years, many studies have demonstrated that also a variety of non-caspase proteases play an important role in apoptosis induced by chemotherapeutic agents and death receptor agonists. Therefore, using the human hepatoma cell line HepG2, the aims of the present study were:

1) To investigate the participation of the lysosomal cysteine protease cathepsin B.
2) To characterize the role of caspases in death receptor agonist-induced apoptosis.
3) To test whether proteases other than caspases might be involved in this process.
### 3. Materials and methods

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
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<tbody>
<tr>
<td>Ac-DEVD-AFC</td>
<td>N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin</td>
</tr>
<tr>
<td>ActD</td>
<td>Actinomycin D</td>
</tr>
<tr>
<td>AEBSF</td>
<td>Pefabloc SC™/ 4-(2-Aminoethyl)-benzenesulfonyl fluoride</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumine</td>
</tr>
<tr>
<td>CA-074Me</td>
<td>[L-3-trans-(Propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E-64d</td>
<td>(2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>(Ethylenedinitrilo)tetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescence protein</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene-bis(oxyethylenenitrilo)tetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>Magnesium chloride</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>Sodium dihydrogenphosphate</td>
</tr>
<tr>
<td>NIW</td>
<td>1-Naphthalenesulfonyl-Ile-Trp-CHO</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TLCK</td>
<td>N₄p-tosyl-L-lysine chloromethyl ketone</td>
</tr>
</tbody>
</table>
### 3. Materials and methods

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<table>
<thead>
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<tr>
<td>TPCK</td>
<td>N,alpha-tosyl-L-phenylalanine chloromethyl ketone</td>
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<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
</tr>
<tr>
<td>Ucf-101</td>
<td>5-[5-(2-Nitrophenyl)furfurylidine]-1,3-diphenyl-2-thiobarbituric acid</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>z-RR-AMC</td>
<td>N-Benzylloxycarbonyl-Arg-Arg-7-amido-4-methylcoumarin</td>
</tr>
<tr>
<td>zDEVD-fmk</td>
<td>N-Benzylloxycarbonyl-Asp-Glu-Val-Asp(O-Me) fluoromethyl ketone</td>
</tr>
<tr>
<td>zVAD-fmk</td>
<td>N-Benzylloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone</td>
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</table>
3. Materials and methods

3.1. Substances

Human recombinant TNFα was generously provided by Prof. Dr. D. Männel (University of Regensburg, Germany), the activating anti-CD95 antibody CH11 and killerTRAIL were purchased from Biomol (Hamburg, Germany) and Alexis Biochemicals (Gruenberg, Germany), respectively. Mouse monoclonal anti-cytochrome c and anti-caspase-3 were purchased from BD Biosciences Pharmingen (Heidelberg, Germany), mouse monoclonal anti-caspase-8 and -9 from Oncogene Research Products (Boston, MA, USA) and rabbit polyclonal anti-Bid antibody from New England Biolabs (Frankfurt am Main, Germany). Mouse monoclonal anti-PARP was a kind gift of Prof. Dr. A. Bürkle (University of Konstanz, Germany). All antibodies were specific for human cells.

The caspase inhibitor zVAD-fmk, the caspase substrate Ac-DEVD-AFC and the cathepsin B substrate z-RR-AMC were purchased from Bachem (Bubendorf, Switzerland), the topoisomerase I inhibitor camptothecin and all protease and protein kinase inhibitors from Merck Biosciences (Schwalbach/Taunus, Germany).

PBS was obtained from PAA (Linz, Austria), FCS from Biochrom (Berlin, Germany) and RPMI 1640 from PAA, Cambrex Bio Science (Verviers, Belgium) or ATCC (Manassas, VA, USA). Alamar Blue™ was supplied by BioSource (Solingen, Germany).

Primers for PCR were purchased from Thermo Electron Corporation (Dreieich, Germany), restriction and DNA modifying enzymes from New England Biolabs (Frankfurt am Main, Germany).

All standard chemicals were purchased from established suppliers, in most cases Sigma-Aldrich (Taufkirchen, Germany).

3.2. Cell culture

HepG2 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in RPMI 1640 containing 10% FCS, 100 µg/ml penicillin and 100 U/ml streptomycin in a humidified incubator at 5% CO₂ / 95% air. FCS was inactivated by incubating in a water bath at a temperature of 55° C for 30 minutes prior to addition to the medium. Cells were split twice a week in a ratio of 1:5, using Accutase® (PAA, Linz, Austria) to detach adherent cells.

3.3. Isolation and culture of mouse hepatocytes

Isolation of hepatocytes from 8 weeks old mice was performed by the two-step collagenase
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perfusion method of Seglen\textsuperscript{174} as modified by Leist\textsuperscript{22}. After isolation, hepatocytes were plated in 200 µl RPMI 1640 medium containing 10% heat-inactivated FCS in collagen-coated 24 well plates at a number of 8 x 10\textsuperscript{4} hepatocytes per well. Cells were allowed to adhere to culture plates for 4 h before the medium was exchanged for RPMI 1640 without FCS. Adherence and incubations were carried out in a humidified atmosphere at 37°\textdegree C, 5% CO\textsubscript{2}, 40% O\textsubscript{2} and 55% N\textsubscript{2}.

3.4. Treatment with cytokines and inhibitors

HepG2 cells were sensitized with 1 µg/ml (400 ng/ml for primary murine hepatocytes) ActD 30 minutes before treatment with TNF\textalpha or 100 µM CHX 120 minutes before treatment with agonistic anti-CD95 antibody (clone CH-11) and killerTRAIL, respectively. ActD and CHX were dissolved in isotonic saline (DeltaSelect, Pfullingen, Germany), cytokines were diluted in saline with 0.1% HSA. Inhibitors were reconstituted in the appropriate solvent (in most cases DMSO) and diluted to working concentrations in isotonic saline. Inhibitors were generally added 30 minutes prior to challenge with cytokines. Final concentrations of DMSO never exceeded 1% and control cells were treated with vehicle in order to exclude any unspecific solvent effects.

3.5. Treatment with camptothecin

Camptothecin was reconstituted in DMSO and diluted to working concentrations in isotonic saline and added 30 minutes after addition of inhibitors. Control cells were treated with vehicle.

3.6. Caspase-3/-7 activity assay

Activity in cell lysates was assayed by cleavage of the synthetic substrate Ac-DEVD-AFC in a concentration of 50 µM in caspase assay buffer (50 mM HEPES, 50 mM NaCl, 10 mM EDTA, 10 mM DTT, 0.1% CHAPS, 5% glycerol, pH 7.20). Release of free AFC was monitored in intervals of 5 minutes for 30 minutes at 37º\textdegree C in a Wallac Victor\textsuperscript{2} multilabel counter (EG&G Wallac, Turku, Finland). One unit of specific activity was calculated as micromoles of substrate cleavage – as assessed by release of free AFC – per mg protein and minute [µmol * mg\textsuperscript{-1} * min\textsuperscript{-1}].

3.7. Cathepsin B activity assay

Activity in cell lysates was assayed by cleavage of the synthetic substrate z-RR-AMC in a concentration of 50 µM in cathepsin B assay buffer (50 mM sodium acetate, 4 mM EDTA, 10
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mM DTT, 1 mM Pefabloc® SC, pH 6.00). Release of free AMC was monitored in intervals of 5 minutes for 30 minutes at 37ºC in a Wallac Victor² multilabel counter. One unit of specific activity was calculated as micromoles of substrate cleavage – as assessed by release of free AMC – per mg protein and minute [µmol * mg⁻¹ * min⁻¹].

3.8. Cytotoxicity assay

Cytotoxicity was measured by the reduction of the tetrazolium dye Alamar Blue™ by viable cells. The assay was performed according to the manufacturer’s instructions. Vehicle-treated cells were used to set the basal level of cytotoxicity (i.e. 0% cytotoxicity), cells lysed with 0.1% Triton® X-100 were used to set its maximum level (i.e. 100% cytotoxicity). Also, release of the cytosolic marker enzyme lactate dehydrogenase was used as a parameter for cytotoxicity (→ section 3.9)

3.9 LDH activity assay

Lactate dehydrogenase was determined in culture supernatants (S), and in the remaining cell monolayer (C) after lysis with 0.1 % Triton X-100 according to Bergmeyer¹⁷⁵. The percentage of lactate dehydrogenase release was calculated from the ratio of S/(S+C).

3.10 Digitonin extraction of cytosol

Typically 2.5 x 10⁶ cells seeded in 6-well plates were used for this purpose. After medium was exaspirated, 350 ul of extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM Pefabloc® SC, pH 7.50) containing 40 µg/ml digitonin was added to each well. Extraction was performed for 10 minutes at room temperature on a orbital shaker at very low speed. Time of extraction and concentration of digitonin were optimized to get a maximal release of the cytosolic marker enzyme lactate dehydrogenase and a minimal release of the lysosomal marker enzyme β-hexosaminidase.

3.11. β-Hexosaminidase activity assay

Activity in cytosolic extracts was assayed by cleavage of the synthetic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in a concentration of 100 µM in hexosaminidase assay buffer (50 mM NaH₂PO₄, pH 5.00). Release of free 4-methylumbelliferyl was monitored in intervals of 5 minutes for 30 minutes at 37ºC in a Wallac Victor² multilabel counter. One unit of specific activity was calculated as micromoles of substrate cleavage – as assessed by release of free 4-methylumbelliferyl – per mg protein and minute [µmol * mg⁻¹ * min⁻¹].
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3.12. Preparation of cell lysates for enzyme activity assays

Cultured cells were lysed with PBS containing 0.1% Triton® X-100. Protein concentration was measured using an assay based on the bichinconinic acid method (Interchim, Montluçon, France) according to the manufacturer’s instructions. Standard curves of BSA were used as a reference.

3.13. Immunoblot (Western blot)

Cultured cells were lysed with lysis buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM imidazole, 2.5 mM EDTA, 2.5 mM DTT, 0.1% Triton® X-100, pH 7.40) and protein concentration was measured as described earlier. Briefly, an aliquot of each sample equivalent to 30 ug protein was boiled after addition of the appropriate amount of 5x sample buffer (5 mM EDTA, 162 mM DTT, 5% SDS, 50% glycerol, 0.5%o bromophenol blue, 188 mM Tris, pH 8.80). The samples were separated on 12% SDS-polyacrylamide gels (PAGE) and electrophoretically transferred to nitrocellulose filters using the Bio-Rad electrotransfer system (Bio-Rad Laboratories, Munich, Germany). Equal transfer was verified by Ponceau staining of the membranes. Caspases-8 and -9 were detected with the mouse monoclonal antibodies AM46T (clone 1-3) and AM47T (clone 1-2), respectively. Bid was detected with a rabbit polyclonal antiserum (#2002), for detection of PARP a mouse monoclonal antibody was used. Antigen-antibody complexes were visualized with HRP-coupled secondary antibodies (goat anti-mouse and goat anti-rabbit, Dianova, Hamburg, Germany) and a custom-made ECL detection system (2.5 mM luminol, 0.4 mM para-coumaric acid, 10 mM Tris base, 0.15l H2O2, pH 8.50).


Using a human XIAP construct (kind gift of Dr. Thomas Meergans, University of Konstanz) HA-tagged recombinant human XIAP(ABir-3) was generated by PCR using the primers ATC ATC GGA TCC ACC ATG GCA TAC CCA TAC GAT GTT CCA GAT TAC GCT ATG ACT TTT AAC AGT TTT GAA GGA T (sense) and ATC ATC AGG CCT TCA ACT TCG AAT ATT AAG ATT CCG GCC (antisense), respectively. The PCR product thus had an N-terminal HA-tag and BamHI and Stul restriction sites (5’ and 3’, respectively) which were used for ligation into the expression vector pcDNA5/FRT (BamHI / EcoRV digested). Integrity of the insert after ligation was confirmed by sequencing (Sequiserve, Vaterstetten, Germany).
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3.15. Cotransfection of EGFP and XIAP(ΔBir-3)

Cotransfections of EGFP and XIAP(ΔBir-3) were performed using the aforementioned HA-tagged XIAP(ΔBir-3) construct and the vector EGFP-pC1 in a ratio of 2.5:1. Transfection reagents used were Effectene® (Quiagen, Hilden, Germany) for HeLa cells and FuGene6® (Roche Biochemicals, Mannheim, Germany) for HepG2 cells, respectively. Expression of the recombinant proteins was checked by western blot.

3.16. Statistics

All data are given as means ± SEM. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnett’s or Bonferroni’s Multiple Comparison Test of controls vs treated groups. Statistical analysis that included all vs all comparisons was done using Tukey Multiple Comparison Test. All statistics were calculated using the program GraphPad Prism® 4.01 (GraphPad Software Inc.) and a p value <0.05 was considered as being significant.
4. Results

4.1. The role of cathepsin B in apoptosis of HepG2 cells

4.1.1. Induction of apoptosis by camptothecin

Since cathepsins have been shown to be effector proteases in hepatocyte apoptosis, we analyzed the role of cathepsin B (CatB) in apoptosis induced by the topoisomerase I inhibitor camptothecin, a chemotherapeutic and potent inducer of apoptosis in hepatoma cells. Figure 4.1 shows that treatment of HepG2 cells with camptothecin caused translocation of CatB from lysosomes to the cytosol in a time-dependent manner, which was not observed in control cells. Moreover, CatB activity in the cytosol could be completely inhibited by the highly specific CatB inhibitor CA-074Me.

We then tested whether inhibition of CatB had any effect on activation of caspases induced by camptothecin. As demonstrated in figure 4.2a, camptothecin caused time-dependent activation of effector caspases, which reached its peak after 20 hours and was completely inhibited by the pan-caspase inhibitor zVAD-fmk. Inhibition of CatB by CA-074Me resulted in a markedly decreased activation of effector caspases. In contrast, treatment with the nonspecific CatB/L/H inhibitor E-64d tended to even enhance caspase activation (data not shown).

However, the reduced caspase activity after inhibition of CatB did not result in a decreased cytotoxicity (Figure 4.2b). Yet, also complete inhibition of caspases by zVAD-fmk did not rescue HepG2 cells from cell death in this model.
4. Results

Figure 4.2: Time course of caspase activity (a) and cytotoxicity (b) after treatment with camptothecin. control (○): untreated cells. CPT (▲): 2.5 µM camptothecin. + CA-074Me (■): 2.5 µM camptothecin + 10 µM CA-074Me. + zVAD-fmk (▼): 2.5 µM camptothecin + 1 µM zVAD-fmk. Data represent mean ± SEM.

4.1.2. Induction of apoptosis by TNFα

We then assessed the role CatB in apoptosis of HepG2 cells after treatment with the death receptor agonist TNFα. Figure 4.3a shows that also in this model, inhibition of CatB significantly reduced the activation of effector caspases. But just like in the camptothecin model, inhibition of CatB and the subsequently diminished caspase activity did not confer any protection to HepG2 cells as compared to cells where CatB was not inhibited (Figure 4.3b).

Figure 4.3: Effect of CatB inhibition on concentration-dependent activation of caspases (a) and cytotoxicity (b) after ActD/TNFα. control (○): cells treated with 1 µg/ml ActD and TNFα up to 100 ng/ml. CA-074Me (▲): cells treated with 10 µM CA-074Me, 1 µg/ml ActD and TNFα up to 100 ng/ml. Data represent mean ± SEM. *: p < 0.05 for control vs CA-074Me. Two-tailed t-test.
4. Results

4.2. Death receptor agonist-induced apoptosis of HepG2 cells

Being slightly stunned by the fact that diminished caspase activity after inhibition of CatB did not result in decreased cytotoxicity we wanted to know to what extent caspases actually contribute to apoptosis in HepG2 cells. To test this, we used the well-established model of apoptosis induced by combined treatment of HepG2 cells with inhibitors of transcription/translation (ActD and CHX, respectively) and TNFα, agonistic αCD95 antibody (αCD95) or TRAIL.

4.2.1. Kinetics of caspase activity and cytotoxicity

Treatment of HepG2 cells with ActD/TNFα, CHX/αCD95 or CHX/TRAIL caused activation of effector caspases and subsequent cell death (Figure 4.4). Even though this activation was much more rapid in the case of TRAIL treatment, the peak in caspase activity was reached after approximately 8 hours in all three models. Similarly, cytotoxicity reached its plateau after approximately 20 hours in all three models, even though the induction was more rapid for cells treated with TRAIL.

**Figure 4.4:** Time course of cytotoxicity (●) and caspase activation (○) after treatment of HepG2 cells with the following combinations: 

- **a** 1 µg/ml ActD and 100 ng/ml TNFα.
- **b** 100 µM CHX and 1 µg/ml αCD95.
- **c** 100 µM CHX and 100 ng/ml TRAIL.
4.2.2. Correlation of caspase activity and cytotoxicity

We subsequently wanted to assess whether there was a correlation between caspase activity and cytotoxicity. To this end, we analyzed the concentration-dependent induction of caspase activity and cytotoxicity which is presented in figure 4.5. In all three models, increasing concentrations of death receptor agonist caused increasing caspase activity and cytotoxicity, respectively. Of importance, there was a significant correlation between caspase activity and cytotoxicity ($R^2 = 0.91$, 0.96 and 0.96 for TNF$\alpha$, $\alpha$CD95 and TRAIL, respectively), which suggested that activation of caspases was a causal event in this model.

\[ \text{Figure 4.5: Concentration curves for cytotoxicity (filled symbols) and caspase activation (open symbols) after addition of death receptor agonists to HepG2 cells that had (triangles) or had not (circles) undergone prior sensitization by ActD and CHX, respectively.} \]

a 0 - 100 ng/ml TNF$\alpha$ ± 1 µg/m ActD.

b 0 - 1 µg/ml $\alpha$CD95 ± 100 µM CHX.

c 0 - 100 ng/ml TRAIL ± 100 µM CHX.
4. Results

4.2.3. Effect of caspase inhibition on cytotoxicity

4.2.3.1. HepG2 cells

In the next experiments we wanted to test whether inhibition of caspases conferred protection to HepG2 cells treated with TNFα, αCD95 or TRAIL. As shown in figure 4.6, inhibition of caspases by the broadband inhibitor zVAD-fmk did not result in protection of HepG2 cells from cytotoxicity, which was only achieved at very high concentrations of the inhibitor.

Table 4.1 shows the corresponding IC₅₀ values of zVAD-fmk for half-maximal inhibition of caspase activity and cytotoxicity, respectively. The ratio of these values shows, that approximately 200 – 800-fold higher concentrations of zVAD-fmk were necessary for half-maximal inhibition of cytotoxicity than for half-maximal inhibition of caspase-activity, suggesting that protection through this inhibitor was only a secondary, unspecific effect and not attributable to its action on caspases.
4. Results

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (caspase activity) [nM zVAD-fmk]</th>
<th>IC$_{50}$ (cytotoxicity) [nM zVAD-fmk]</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActD/TNF$\alpha$</td>
<td>17</td>
<td>13800</td>
<td>1 : 812</td>
</tr>
<tr>
<td>CHX/$\alpha$CD95</td>
<td>57</td>
<td>11000</td>
<td>1 : 193</td>
</tr>
<tr>
<td>CHX/TRAIL</td>
<td>21</td>
<td>5500</td>
<td>1 : 262</td>
</tr>
</tbody>
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Table 4.1: Comparison of the IC$_{50}$ values for inhibition of caspase activity and cytotoxicity by zVAD-fmk in sensitized HepG2 cells treated with death receptor agonists.

4.2.3.2. Primary murine hepatocytes

This finding was in stark contrast to the situation in primary murine hepatocytes, where a significant correlation between decrease in caspase activity and decreased cytotoxicity could be observed (Figure 4.7), the IC$_{50}$ values being virtually identical (2 nM and 4 nM for inhibition of caspase activity and cytotoxicity, respectively).

4.2.4. Morphology of cell death after caspase arrest

In order to check whether cell death after inhibition of caspases was still apoptotic, we pre-treated HepG2 cells with 0, 1.5 $\mu$M – a concentration that completely inhibited caspase activity but had little to no protective effect regarding cytotoxicity – or 100 $\mu$M zVAD-fmk.

Figure 4.8 shows that cells that were treated with ActD/TNF$\alpha$ and 0 or 1.5 $\mu$M zVAD-fmk for 6 hours displayed the same apoptotic morphology (i.e. beginning zeiosis), showed nuclear condensation and exposed phosphatidylinerine on the outer membrane, another marker of...
apoptosis. Of note, the shape of nuclear condensation between these two treatments was not identical, the chromatin of HepG2 cells treated with 0 µM zVAD-fmk being more compacted at the periphery of the nucleus and less dispersed. In contrast, cells that were pretreated with 100 µM zVAD-fmk did not show any sign of apoptosis but were identical to untreated control cells in all parameters analyzed.

![Morphology (upper panel), nuclear condensation (middle panel) and phosphatidylserine exposure (lower panel) of HepG2 cells treated with either 1 µg/ml ActD (left column), 1 µg/ml ActD and 100 ng/ml TNFα (ActD/TNFα, second column), 1.5 µM zVAD-fmk + ActD/TNFα (third column) or 100 µM zVAD-fmk + ActD/TNFα (right column) for 6 hours.](image)

**Figure 4.8:** Morphology (upper panel), nuclear condensation (middle panel) and phosphatidylserine exposure (lower panel) of HepG2 cells treated with either 1 µg/ml ActD (left column), 1 µg/ml ActD and 100 ng/ml TNFα (ActD/TNFα, second column), 1.5 µM zVAD-fmk + ActD/TNFα (third column) or 100 µM zVAD-fmk + ActD/TNFα (right column) for 6 hours.

Interestingly, analysis of late morphology after 24 hours revealed that cells in which caspases had been inhibited by 1.5 µM zVAD-fmk displayed a distinct, round morphology, whereas cells in which caspases had not been inhibited had completely disintegrated into remnants, the so called ‘apoptotic bodies’ (Figure 4.9). Thus, inhibition of caspases had both an influence on early and late apoptotic morphology of HepG2 cells treated with death receptor agonists.
4. Results

4.2.5. Effect of overexpression of XIAP(ΔBir3)

In order to confirm the finding that inhibition of caspases was not sufficient to protect HepG2 cells from undergoing apoptosis, we transfecteded both HepG2 cells and HeLa cells (a cervix carcinoma cell line) with a plasmid encoding for an enhanced green fluorescent protein (EGFP)-coupled XIAP(ΔBir3), a protein that was different from wild-type XIAP inasmuch that it had the potential to inhibit caspases-3 and -7 but not caspase-9. As can be seen in figure 4.10, inhibition of caspases-3/-7 by XIAP(ΔBir3) significantly reduced ActD/TNFα-induced cell death in HeLa but not in HepG2 cells, confirming our previous findings from the experiments using zVAD-fmk.

Figure 4.9: Morphology of HepG2 cells treated with either a 1 µg/ml ActD and 100 ng/ml TNFα (ActD/TNFα) or b 1.5 µM zVAD-fmk + ActD/TNFα for 24 hours. Arrows indicate cells with the characteristic round shape found only in the late stage of apoptosis after caspase arrest.

Figure 4.10: Protective effect inhibition of caspases-3/-7 by overexpressed XIAP(ΔBir3) in HeLa (a) but not in HepG2 cells (b) sensitized with either 100 µM CHX (HeLa) or 1 µg/ml ActD (HepG2) and subsequently challenged with 100 ng/ml TNFα. Data represent mean ± SEM. *: p < 0.05 for EGFP control vs EGFP + XIAP(ΔBir3). T-test.
4. Results

4.3. Death receptor agonist-induced apoptosis of HepG2 cells after caspase arrest

The finding that inhibition of caspases was not sufficient to protect HepG2 cells from undergoing apoptosis induced by either camptothecin or death receptor agonists TNFα, αCD95 and TRAIL, respectively, implicated that caspases were dispensable for HepG2 cells in order to undergo apoptosis. Therefore, further investigations were aimed at analyzing this newfound mechanism of apoptosis after caspase arrest in detail.

4.3.1. Cleavage of PARP, Bid and release of cytochrome c

Release of cytochrome c from the mitochondria to the cytosol and cleavage of PARP and Bid are typical parameters of death receptor agonist-induced apoptosis. As shown in Figure 4.11a, inhibition of caspases by 1.5 µM zVAD-fmk in HepG2 cells treated with ActD/TNFα resulted in little to no cleavage of PARP and Bid, whereas cytochrome c was still being released. In contrast, when cells were treated with CHX/αCD95 (Figure 4.11b) or CHX/TRAIL (Figure 4.11c), cleavage of PARP and – to a lesser extent – also Bid could still be observed, even though for these two cytokines, HepG2 cells had been pretreated with 10 µM zVAD-fmk to completely exclude any residual caspase activity. Similarly to ActD/TNFα, cytochrome c was still released when caspases were inhibited, albeit this release was slightly delayed.

**Figure 4.11**: Time course of cleavage of PARP and Bid and release of cytochrome c, respectively, in HepG2 cells treated with: a 1 µg/ml ActD and 100 ng/ml TNFα ± 1.5 µM zVAD-fmk. b 100 µM CHX and 1 µg/ml αCD95 ± 10 µM zVAD-fmk. c 100 µM CHX and 100 ng/ml TRAIL ± 10 µM zVAD-fmk.
4. Results

4.3.2. Cleavage of initiator caspases

As illustrated in the introduction, the initiator caspases-8 and -9 have been shown to be prone to degradation by non-caspase proteases. For this reason, we analyzed cleavage of caspase-8 and -9 after caspase arrest in contrast to control treatment. Again, little to no cleavage was observed in cells treated with ActD/TNFα and 1.5 µM zVAD-fmk (Figure 4.12a), whereas in cells that had been treated with CHX/αCD95 or CHX/TRAIL, inhibitor concentrations as high as 10 µM did not prevent cleavage of initiator caspases (Figures 4.12b and c, respectively).

**Figure 4.12:** Time course of cleavage of caspase-8 and caspase-9, respectively, in HepG2 cells treated with:

- a 1 µg/ml ActD and 100 ng/ml TNFα ± 1.5 µM zVAD-fmk.
- b 100 µM CHX and 1 µg/ml αCD95 ± 10 µM zVAD-fmk.
- c 100 µM CHX and 100 ng/ml TRAIL ± 10 µM zVAD-fmk.
4. Results

4.3.3. Protection by the plant compound glycyrrhizin

Glycyrrhizin is a natural compound isolated from the liquorice root. Beyond its industrial use as a sweetener, extracts from the liquorice root play an important role in traditional Chinese medicine. Studies have shown that glycyrrhizin was able to prevent TNFα-induced apoptosis of HepG2 cells\textsuperscript{178}, αCD95-induced fulminant hepatitis in mice\textsuperscript{179} and also is a promising agent for chemoprevention of hepatocellular carcinoma associated with chronic hepatitis B and C, respectively\textsuperscript{180}. Therefore, we wanted to test whether it also conferred protection to death receptor agonist-induced apoptosis after caspase arrest by 1.5 µM zVAD-fmk.

Figure 4.13 demonstrates that glycyrrhizin protected HepG2 cells from apoptosis induced by CHX/αCD95 in a concentration-dependent manner. Moreover, this protection was conferred independently of caspase activity, i.e. in both apoptosis with or without caspase arrest.

![Figure 4.13](image)

Of note, similar effects as assessed by cytotoxicity assays could be observed for HepG2 cells treated with ActD/TNFα and CHX/TRAIL ± 1.5 µM zVAD-fmk (data not shown). However, whereas in the case of αCD95, treatment with high concentrations of glycyrrhizin resulted in a cell morphology that was identical to untreated control cells, this was not the case for TNFα and TRAIL, where the morphology could neither be assigned to untreated control nor apoptotic cells.
4. Results

4.3.4. Increased cytotoxicity after inhibition of c-Jun N-terminal kinase (JNK)

As illustrated in the introduction (section 1.2), death receptors also couple to signaling via protein kinases such as JNK or p38. For example, Liedtke et al have shown in HuH7 human hepatoma cells that JNK transduces antiapoptotic signals, which modulate the strength and time course of FADD-dependent cell death involving mitochondrial permeability transfer\[^{181}\]. For this reason, we analyzed the effect of the selective JNK inhibitor SP600125 on apoptosis of HepG2 cells induced by ActD/TNF\(\alpha\) in the absence or presence of caspase activity. Figure 4.14 shows that – while inhibition of protein kinase p38 did not have any effect – inhibition of JNK significantly augmented cytotoxicity in both models, i.e. whether caspasess where active or not. In contrast, no effect was observed for untreated control cells. A similar effect was observed for apoptosis induced by CHX/\(\alpha\)CD95 or CHX/TRAIL (data not shown).

![Figure 4.14: Inhibition of JNK by SP600125 (4 \(\mu\)M, shaded bars) significantly augments cytotoxicity in both HepG2 cells treated with 1 \(\mu\)g ActD and 100 ng/ml TNF\(\alpha\) (ActD/TNF\(\alpha\)) or ActD/TNF\(\alpha\) + 1.5 \(\mu\)M zVAD-fmk (+ zVAD-fmk). Data represent mean ± SEM. ***: p < 0.001 for control vs SP600125. One-way ANOVA, Bonferroni’s Multiple Comparison Test.](image-url)
4. Results

4.3.5. Protective effect of serine protease inhibitors on cytotoxicity

In order to further characterize caspase-independent cell death of HepG2 cells we screened inhibitors of a variety of different proteases. Those were added to sensitized HepG2 treated with either TNFα, αCD95 or TRAIL and their effect on apoptosis both with and without caspase arrest was assessed. As shown in figure 4.15a, the unspecific serine protease inhibitors TLCK and TPCK and the Omi/HtrA2 inhibitor Ucf-101 significantly augmented ActD/TNFα-induced cytotoxicity in cells without caspase arrest, whereas they conferred protection to cells where caspases were inhibited. In contrast, the broadband serine protease inhibitor AEBSF augmented cytotoxicity in both models.

![Figure 4.15: Effect of serine protease inhibitors on death receptor agonist-induced apoptosis of HepG2 cells treated with either saline (control), AEBSF (375 µM), Ucf-101 (50 µM), TLCK (100 µM) or TPCK (25 µM) and: a 1 µg/ml ActD and 100 ng/ml TNFα ± 1.5 µM zVAD-fmk. b 100 µM CHX and 1 µg/ml αCD95 ± 1.5 µM zVAD-fmk. c 100 µM CHX and 100 ng/ml TRAIL ± 1.5 µM zVAD-fmk. Data represent mean ± SEM. */**/***: p < 0.05/0.01/0.001 for saline vs zVAD-fmk. One-way ANOVA, Dunnett’s Multiple Comparison Test.]

This protective effect of serine protease inhibitors only after caspase arrest could be confirmed for cells treated with CHX/αCD95 (Figure 4.15b) and CHX/TRAIL (Figure 4.15c), respectively, where caspase arrest combined with either TLCK, TPCK or Ucf-101 resulted in a significant reduction of cytotoxicity. On the contrary, inhibition of cathepsins B, L, H, or D with their respective inhibitors did not have any effect on cytotoxicity in all three models (data not shown).
4. Results

4.3. Prevention of cytochrome c release by TLCK

The results from the previous experiment suggested a switch to a serine protease-dependent mechanism of apoptosis after caspase arrest. To further confirm the important role of serine proteases in this specific setting, we analyzed the effect of combined inhibition of both caspases and serine proteases on the release of cytochrome c. As can be seen in figures 4.16a, b and c, combined addition of both zVAD-fmk and TLCK markedly reduced release of cytochrome c as compared to zVAD-fmk alone. Of note, this effect was most pronounced for cells treated with αCD95 (Figure 4.18b) and TRAIL (Figure 4.18c).

Figure 4.16: Combined inhibition of caspases by zVAD-fmk (1.5 µM for panel a, 10 µM for panels b and c) and serine proteases by TLCK (100 µM) reduces time-dependent release of cytochrome c from the mitochondria in HepG2 cells treated with:

- a 1 µg/ml ActD and 100 ng/ml TNFα ± TLCK
- b 100 µM CHX and 1 µg/ml αCD95 ± TLCK
- c 100 µM CHX and 100 ng/ml TRAIL ± TLCK.
4. Results

4.4. Selective sensitization of HepG2 cells CD95L and TRAIL but not TNFα

With respect to the augmentation of death receptor agonist-induced cytotoxicity observed after inhibition of JNK (section 4.3.4), we were curious to know whether inhibition of JNK without prior inhibition of translation/transcription by ActD and CHX, respectively, had the potential to sensitize HepG2 cells to death receptor agonists. This was of special interest because development of resistance to death receptor agonists like TRAIL is often found in transformed cells of cancer patients.

4.4.1. Selective sensitization by c-Jun N-terminal kinase (JNK) inhibition

Figure 4.17 demonstrates that inhibition of JNK by SP600125 selectively sensitized HepG2 cells to apoptosis induced by αCD95 and TRAIL but not TNFα. In addition, also in this model, inhibition of caspases by 1.5 µM zVAD-fmk did not confer any protection.

![Figure 4.17: Inhibition of JNK by SP600125 (4 µM, ▲) selectively sensitizes HepG2 cells to cytotoxicity induced by αCD95 and TRAIL (panels b and c, respectively) but not TNFα (c), without any protective effect by additional inhibition of caspases by zVAD-fmk (1.5 µM, ■). Data represent mean ± SEM.](image-url)
4. Results

4.4.2. Selective sensitization by histone deacetylase (HDAC) inhibition

Another inhibitor we tested was M344, which selectively inhibits histone deacetylase. This enzyme regulates histone acetylation, thereby modulating the transcriptional activity of certain genes and has recently been shown to sensitize melanoma cells to TRAIL\textsuperscript{182}. Similarly to SP600125, M344 selectively sensitized HepG2 cells to αCD95 and TRAIL but not TNFα (Figure 4.18). Conversely to the result obtained with the JNK inhibitor, additional inhibition of caspases markedly decreased cytotoxicity, a fact that points to differences in the underlying mechanisms of sensitization.

![Figure 4.18: Inhibition of HDAC by M344 (1 µM, ▲) selectively sensitizes HepG2 cells to cytotoxicity induced by αCD95 and TRAIL (panels b and c, respectively) but not TNFα (c). Additional inhibition of caspases by 1.5 µM zVAD-fmk (■) markedly decreased cytotoxicity. Data represent mean ± SEM.](image-url)
5. Discussion

In the present study we wanted to characterize the role of the lysosomal cysteine protease cathepsin B and participation of caspases and other proteases in different models of apoptosis in HepG2 cells. As a result, we could show that inhibition of caspases in death receptor agonist-induced apoptosis causes a switch to a novel serine protease-dependent mechanism in this model.

5.1. The role of cathepsin B

By analysis of cytosolic CatB activity (Fig. 4.1) in HepG2 cells treated with camptothecin (CPT) we could clearly show that this cysteine protease is released from the lysosomes during apoptosis. Even though its inhibition by the specific, cell-permeable compound CA-074Me markedly attenuated caspase activity (Fig. 4.2a), it did not have any effect on CPT-mediated cytotoxicity (Fig. 4.2b). This finding contrasts with data from Roberts et al\(^{114}\), who have – in a similar model of CPT-induced apoptosis in Hep3B cells, another human hepatoma cell line – demonstrated that inhibition of CatB by CA-074Me markedly attenuated apoptosis. However, also complete inhibition of caspases by the broadband inhibitor zVAD-fmk failed to confer even the slightest protection. Of importance, the morphology of the cells as assessed by light microscopy was still apoptotic when either CatB or caspases were inhibited. This was quite contrary to our expectations, because the current concept of apoptosis induced by chemotherapeutics is that it is mediated by caspases. Indeed, we could show that caspases were activated in a both time- and concentration-dependent manner in this model (data not shown). Yet, this finding delivered an explanation for the absent effect of attenuated caspase activity after CatB inhibition, suggesting that caspases were dispensable for HepG2 cells in order to undergo apoptosis in this model.

Congruently, the results from the ActD/TNF\(\alpha\) model pointed to the same direction, showing that although inhibition of CatB significantly attenuated activation of caspases (Fig. 4.3a), it failed to protect HepG2 cells from apoptosis (Fig. 4.3b). Again, this was quite unexpected, because in this death receptor-mediated model of apoptosis, inhibition of CatB had a very profound effect on caspase activation, which was attenuated to levels as low as 55% of controls.

Taken together, we could demonstrate that inhibition of cathepsin B attenuated activation of caspases in both CPT- and ActD/TNF\(\alpha\)-induced apoptosis of HepG2 cells. This might be explained by the ability of CatB to cleave Bid and thus generate tBid, leading to release of
cytochrome c from the mitochondria and activation of caspase-9\textsuperscript{108}. Inhibition of CatB therefore is likely to result in suppression or at least delay of the mitochondrial amplification loop of caspase activation.

Most interestingly, the results of both the CPT and ActD/TNF\textgreek{a} model strongly suggested that activity of caspases was not essential for HepG2 cells in order to undergo apoptosis. As this was quite against our expectations and had – to the best of our knowledge – not been described before, the following experiments concentrated on the study of the role of caspases in death receptor agonist-induced apoptosis of these cells.

5.2. Caspases in death receptor agonist-induced apoptosis

In accordance with the results from many other groups, HepG2 cells sensitized with ActD or CHX responded to treatment with either TNF\textgreek{a}, \textgreek{a}CD95 or TRAIL with activation of caspases and subsequent cell death (Fig. 4.4). Thereby, increasing concentrations of death receptor agonist resulted in increasing caspase activity and cytotoxicity, respectively, the latter two being significantly correlated (Fig. 4.5). These results fit well in the current concept of death receptor signaling, as stimulation of death receptors should result in activation of caspase-8 at the DISC and subsequent activation of the effector caspases. Moreover, this effect is expected to be concentration-dependent under non-saturating conditions, i.e. unto the point where every single receptor is occupied by its respective ligand.

The finding that inhibition of caspases by zVAD-fmk was not able to prevent cytotoxicity in this setting (Fig. 4.6), however, strongly suggested that caspases are activated in but are dispensable for death receptor agonist-induced apoptosis in HepG2 cells. What is more, the big differences between the IC\textsubscript{50} values for caspase inhibition and protection from cell death, respectively (Table 4.1) implies nonspecific inhibition of another target. Indeed, a publication that was one of the major triggers of studies of the role of cathepsin B in apoptosis had demonstrated that zVAD-fmk and other classical caspase inhibitors do inhibit cathepsin B at high concentrations\textsuperscript{106}. Also, studies conducted in our lab had shown that concentrations of the caspase inhibitor zDEVD-fmk as low as 1 \textmu M were sufficient to completely inhibit both cathepsins B and L \textit{in vitro}, even though those enzymes did not cleave the synthetic caspase substrate DEVD-AFC. In contrast, the cathepsin inhibitors E-64, CA-074 (i.e. the analogues of the cell-permeable prodrugs E64d and CA-074Me) and NIW in concentrations up to 50 \textmu M had no inhibitory effect on recombinant human caspases-2, -3, -6, -7, -8, -9 and -10 (data not shown). This demonstrates that neither zVAD-fmk nor zDEVD-fmk – which allegedly is
specific for the executioner caspases – exert specific action only on caspases, at least in high concentrations.

The presumption of an unspecific effect of zVAD-fmk was further backed by the results obtained with primary murine hepatocytes, where inhibition of caspases was associated with a reduction in cytotoxicity. In fact, many studies have shown that death receptor agonist induced apoptosis in these cells is strictly dependent on caspases and that inhibition of caspases confers protection\textsuperscript{183-185}. In this regard, one of the early studies has shown that zVAD-fmk inhibited ActD/TNF\textsubscript{α}-induced apoptosis in both primary murine hepatocytes and HepG2 cells\textsuperscript{21}. Interestingly, as caspase activity assays were not readily available at this time, the authors did not correlate protection and caspase activity, but calculated the IC\textsubscript{50} values for protection of primary murine hepatocytes and HepG2 cells conferred by zVAD-fmk. In analogy to our findings, a more than 200-fold higher concentration was necessary for half-maximal inhibition of cell death in HepG2 cells as compared to primary murine hepatocytes. However, as caspase activity had not been determined in this study, the existence of a caspase-independent apoptotic mechanism in HepG2 cells was far from being self-evident at this time.

Our finding that caspase inhibition correlates with protection in primary murine hepatocytes but not in hepatoma cells also rises the question whether this is a general feature of hepatoma or even cancer cells in general. If this indeed would be the case and given that human hepatocytes behave like their murine counterpart, the use of low concentrations of zVAD-fmk might allow to selectively kill transformed cells while protecting normal hepatocytes from e.g. death receptor agonist-induced apoptosis \textit{in vivo}. Of course, studies in either nude mice with transplanted tumors or mice with induced hepatocellular carcinoma will be necessary in order to decide whether there really is a common principle which might suggest a therapeutic interest of our findings.

As to the morphology of HepG2 cells in our model, whereas inhibition of caspases did not abrogate exposure of phosphatidylserine, it caused differences in the shape of chromatin condensation (Fig. 4.8) and conspicuously in the late stage of apoptosis (Fig. 4.9). Whereas the latter does not seem to have been described so far, a type of chromatin condensation similar to what we observed after caspase arrest has been described for caspase-independent, AIF-driven apoptosis\textsuperscript{11}. Moreover, if released from the mitochondria, both AIF and endonuclease G might account for degradation of nuclear DNA \textsuperscript{70,160}. Having also shown that, in contrast to HeLa cells, inhibition of caspases-3 and -7 by XIAP(ΔBir3) did not confer any protection to TNF\textsubscript{α}-induced apoptosis of HepG2 cells (Fig. 4.10), it was quite evident that
these should have a caspase-independent backup mechanism in order die decently. Therefore, our following experiments were aimed at investigating this newfound mechanism in broader detail.
5.3. Death receptor agonist-induced apoptosis after caspase arrest

5.3.1. Shared and unique features

In order to further characterize apoptosis of HepG2 cells after caspase arrest we chose to compare two settings, i.e. apoptosis without and with caspase inhibition by zVAD-fmk. Strikingly, inhibition of caspases did not prevent release of cytochrome c from the mitochondria (Fig. 4.11), indicating that caspase activity was not necessary for mitochondria outer membrane permeabilization. Moreover, also cleavage of PARP and – to a lesser extent – Bid was not abrogated by caspase inhibition, especially in HepG2 cells treated with αCD95 or TRAIL. This finding is well in accordance with literature, as many publications have shown that these two proteins are prone to degradation by various non-caspase proteases such as calpain, cathepsins B and G or granzyme B. In particular, a role of especially calpain seems quite feasible as this enzyme is expressed ubiquitously and activated by elevated levels of cytosolic calcium. In this matter, recent publications have shown that TNFα-mediated apoptosis of both mouse fibroblasts and rat hepatoma cells is associated with an increase in cytosolic calcium levels.

The fact that – in the case of αCD95 and TRAIL – even concentrations of up to 10 µM zVAD-fmk did not prevent cleavage of procaspases 8- and -9 (Fig 4.12) also suggests that a different, non-caspase protease is involved. Indeed, both calpain and granzyme B have been shown to be able to cleave either procaspases-8 or both procaspases-8 and -9, albeit without activating them. Thus, cleavage of these two procaspases does not necessarily result in their activation, which anyway would have been prevented by zVAD-fmk in our setting. As a consequence, it follows that our findings rather represent a secondary, downstream event due to the action of a non-caspase protease.

The observation that glycyrrhizin protected HepG2 cells in both models (Fig. 4.13) implies that this compound acts on a target that is shared by both the caspase-dependent and -independent apoptotic pathway. While the exact mechanism by which glycyrrhizin mediates hepatoprotection still remains elusive, the fact that we could observe protection in both models and for all three death receptor agonists could hint at a very upstream target that is common among the different signaling pathways. Of note, our finding that glycyrrhizin completely prevented CHX/αCD95-induced apoptosis of HepG2 cells contradicts the observations of another group that described protection of the same cell line from ActD/TNFα but not ActD/αCD95. It seems unprobable that these obvious differences are solely due to sensitization of with ActD instead of CHX, even though we have to take this possibility into
5. Discussion

account. However, *in vivo*-studies have revealed that glycyrrhizin protects from αCD95-induced hepatitis in mice[^178], which clearly demonstrates that its action is not limited to the TNFα/TNF-R1 signaling pathway.

Another feature that we found to be shared by both pathways was the sensitizing effect of JNK inhibition (Fig. 4.14). This effect had been described for HuH7 cells by Liedtke et al, showing that inhibition of JNK augmented apoptosis induced by ActD/TNFα[^181]. Interestingly, the authors could show that overexpression of dominant negative forms of TRAF-2 and TAK-1, two upstream kinases of JNK, resulted in an earlier and stronger induction of apoptosis in this model. Of importance, neither activation of ERK nor NF-κB was influenced. Quite unexpectedly, dominant negative c-Jun, a downstream target of JNK that together with c-Fos forms the transcription factor AP-1, had no effect. This shows that the effect of JNK inhibition was not attributable to a block of AP-1, but rather a different target of JNK. It follows that anti-apoptotic signaling by JNK is another shared feature of caspase-dependent as well as -independent apoptosis of HepG2 cells as inhibition of this kinase by SP600125 augmented cytotoxicity in both settings. It is worth mentioning that – depending on the cell type – JNK inhibition also might protect from apoptosis (reviewed by Varfolomeev and Ashkenazi[^189]).

In summary, we could show that death receptor agonist-induced apoptosis after caspase arrest shares a variety of features with the classical caspase-dependent pathway as for example release of cytochrome c. Most importantly, our results show that disappearance of procaspases does not necessarily mean that these are activated but might as well merely represent their proteolytic cleavage by non-caspase proteases. Future experiments that have to be done in order to characterize downstream signaling in HepG2 cells after binding of death receptor agonists are overexpression of dominant negative FADD (dnFADD) or cFLIP. Overexpression of dnFADD – which consists only of the death domain (DD) – abrogates recruitment of procaspase-8 to the DISC. However, signaling via molecules that require only the as an adaptor DD in order to bind to the DISC should still be possible. Similarly, cFLIP – which is homologous to caspase-8 but lacks protease activity and hence is a competitive negative regulator of this protease – should specifically block caspase signaling.

In order to further characterize the protease that was responsible for caspase-independent apoptosis of HepG2 cells, we screened the effect of a variety of protease inhibitors on both apoptotic pathways, a strategy that eventually proved to be successful.
5. Discussion

5.3.2. A switch to serine protease-dependent apoptosis

The fact that TNFα had been shown to elevate cytosolic calcium levels and the known potential of the calcium-dependent cysteine protease calpain to degrade a variety of classical caspase substrates (discussed in section 5.3.1) naturally raised our suspicion. However, the unspecific calpain/cathepsin inhibitor E-64d, as well a variety of other inhibitors of cathepsins B, L and D (CA-074Me, NIW and pepstatin A, respectively) did not have any protective effect in either setting. In stark contrast, unspecific inhibitors of serine proteases like TLCK and TPCK protected HepG2 cells from apoptosis induced by either TNFα, αCD95 and TRAIL, but only when caspases were inhibited by 1.5 µM zVAD-fmk (Fig 4.15). In the settings without caspase arrest, these inhibitors had no effect or in the case of ActD/TNFα even proved to significantly augment cytotoxicity. Protection from cytotoxicity after caspase arrest was also conferred by the inhibitor of Omi/HtrA2, which is another serine protease. The fact that inhibition of serine proteases conferred protection only when caspases were inactive inevitably leads to the conclusion that HepG2 cells have the potential to switch to a caspase-independent and serine protease-dependent apoptotic mechanism. During the last few years, quite a number of publications have proven the existence of apoptosis that depends on serine proteases. Still, the potential of a cell line to switch to an alternative pathway of apoptosis if the other is blocked has – to the best of our knowledge – not been described so far. This does not necessarily mean that it is restricted to only HepG2 cells. As a matter of fact, we could demonstrate the existence of a similar mechanism in the mouse hepatocyte cell line AML-12 (unpublished data). A possible explanation for the fact that this alternative apoptotic pathway in HepG2 and maybe also other cells has been overlooked so far might be unspecific inhibition of this protease by zVAD-fmk. Indeed, a closer look at literature reveals that concentrations of more than 100 µM zVAD-fmk are commonly used to protect cells from – as the authors argue – caspase-dependent apoptosis. However, our results show that this protection might not be due to inhibition of caspases alone (which are completely inhibited by concentrations below 1 µM zVAD-fmk in most cells) but another protease. This is most impressively exemplified by a publication of Nakayama et al. in which the authors studied ethanol-induced apoptosis of HepG2 cells. They showed that concentrations of up to 400 µM zVAD-fmk were necessary in order to protect cells from apoptosis, whereas a combination of 50 µM zVAD-fmk and 100 µM TLCK had the same effect. As the authors did not correlate inhibition of caspases and cytotoxicity, it is tempting to speculate that they have simply overlooked a switch from one apoptotic pathway to another.
5. Discussion

In any case, their observations are well in accordance with our own findings, although different models were used and different conclusions were drawn from the results obtained. Another question that remained to be answered in our model was whether the putative serine protease is activated upstream or downstream of the mitochondria. The fact that the Omi/HtrA2 inhibitor Ucf-101 conferred significant protection in apoptosis after caspase arrest indicated that this protease might be released from the mitochondria after mitochondrial outer membrane permeabilization. The latter event is not inevitably dependent on proteolytic events like generation of tBid but might as well be initiated by a displacement in the ratio of Bcl-2 and Bax (section 1.2.4). Yet, our results clearly showed that inhibition of both caspases (by 1.5 µM zVAD-fmk) and serine proteases (by 100 µM TLCK) significantly reduced the release of cytochrome c from the mitochondria as compared to controls. This demonstrates that the serine protease acts upstream of the mitochondria and that Omi/HtrA2, even though it might play a role in execution of apoptosis, is only of minor importance. What is more, using human recombinant Omi/HtrA2 we could also show that this enzyme is inhibited by neither TLCK nor TPCK (unpublished observations). This indicates that the protective effect of Ucf-101 might be attributable to inhibition of a serine protease other than HtrA2, but as we did not test the effect of Ucf-101 on cytochrome c release, this remains speculative at this moment.
5.3.3. Proposed mechanism

Putting all our results together, we propose a new, refined mechanism of death receptor-induced apoptosis in HepG2 cells which is represented by Figure 5.1. As discussed in the previous sections, treatment of sensitized HepG2 cells leads to extrinsic activation of caspase-8 which in turn activates downstream caspases-3 and -7. Moreover, caspase-8 also launches the intrinsic pathway by proteolytically generating tBid, leading to mitochondrial outer membrane permeabilization, release of cytochrome c and subsequent activation of caspase-9 in the apoptosome, thereby amplifying activation of downstream caspases. Inhibition of caspases by low concentrations of the unspecific caspase inhibitor zVAD-fmk (Fig. 5.1 a) causes a switch to an alternative, serine protease-dependent mechanism of apoptosis (Fig. 5.1 b). This serine protease, whose mechanism of activation remains elusive so far, acts upstream of mitochondria, leading to release and cytochrome c and subsequent apoptosis. Treatment of cells with the unspecific serine protease inhibitors TLCK and TPCK, the Omi/HtrA2 inhibitor Ucf-101 or very high concentrations of zVAD-fmk eventually results in protection from cell death, but only after caspase arrest. It is important to stress the fact that neither exclusive caspase arrest nor exclusive inhibition of serine proteases alone has any protective effect in this model.

Figure 5.1: Inhibition of caspases in death receptor agonist-induced apoptosis of HepG2 cells by low but effectively inhibiting concentrations of zVAD-fmk (a) causes a switch to another, serine protease-dependent apoptotic mechanism (b). This protease acts upstream of mitochondria and can be inhibited by the unspecific serine protease inhibitors TLCK and TPCK, the Omi/HtrA2 inhibitor Ucf-101 and high concentrations of zVAD-fmk.
5. Discussion

In summary, our results clearly demonstrate that HepG2 cells have the potential to switch from a caspase-dependent apoptotic pathway to another, serine protease-dependent if the former is inhibited. What is more, it poses the question whether this might be a mechanism that is, if not commonly shared by all transformed cells, featured by cells other than just HepG2 cells. To this regard we could indeed show that also in another transformed cell line, caspase arrest causes a switch to serine protease-dependent mechanism. Again, this alternative pathway could be inhibited by either TLCK, Ucf-101 or very high concentrations of zVAD-fmk (unpublished data from Petra Richl). What is more, preliminary studies showed that also in AML-12 cells, a nontransformed cell line from derived from hepatocytes of mice transgenic for transforming growth factor α (TGFα)190, inhibition of caspases did not correlate with protection from cell death. This observation is of special interest, because these cells were suggested to be a model in which to study hepatocyte growth and differentiation, implying that they are more similar to primary hepatocytes than for example HepG2 cells. Still, further studies will be necessary in order to elucidate the role the described switch to apoptosis dependent on serine proteases in a variety of cell lines. Also, studies in different types of primary cells will reveal whether this switch only exists in some cell lines or is a much more common principle. Finally, current work concentrates on the identification of the serine protease(s) involved by an affinity site labeling approach, a method by which we hope to further elucidate the details of this novel, caspase-independent apoptotic pathway.
5. Discussion

5.4. Selective sensitization to αCD95 and TRAIL but not TNFα

Our studies on the potential of certain drugs to sensitize HepG2 cells to death receptor agonist without prior inhibition of translation / transcription was none of the original aims of the present study. In fact, the observation that inhibition of c-Jun N-terminal kinase (JNK) as well as histone deacetylase (HDAC) caused selective sensitization of HepG2 cells to αCD95 and TRAIL but not TNFα was quite surprising, as it had not been reported so far. The fact that there was – in contrast to models that sensitize by ActD or CHX – no sensitization to TNFα excludes the possibility that the underlying mechanism might be inhibition of transcription or translation, because both would also sensitize to TNFα. In addition, JNK inhibition is very likely to sensitize by a different mechanism than does inhibition of HDAC. This is illustrated by the fact that caspase arrest had no effect in the model of sensitization by SP600125 (the JNK inhibitor) but caused a marked decrease of cell death in cells sensitized by the HDAC inhibitor M344 (Fig. 4.17 and 4.18, respectively). An explanation is offered by reports that have shown that inhibition of HDAC in melanoma cells leads to upregulation of proapoptotic proteins like Bid, Bax and procaspases-8 and -3, whereas the anti-apoptotic proteins Bcl-XL and XIAP are downregulated\(^{182}\). In accordance with this finding, overexpression of Smac/DIABLO\(^{191}\) or downregulation of Bcl-2, FLIP or XIAP\(^{192}\) has been shown to sensitize otherwise resistant hepatocellular carcinoma and melanoma cells, respectively, to TRAIL. In addition, sensitization of HCC cells by chemotherapeutic drugs has been shown to be dependent on caspase-8 recruitment to the DISC\(^{193}\). These findings clearly show that HDAC inhibition interferes with both negative and positive regulators of the classical caspase-dependent apoptosis. Therefore, the observed reduction/delay in cell death of HepG2 cells sensitized with by HDAC inhibition was not unexpected.

Sensitization to TRAIL by HDAC inhibition in otherwise resistant cell lines is a recent, although not completely new finding\(^{194}\) and inhibitors of HDAC therefore are regarded as promising adjuvants in therapy of TRAIL-resistant tumors. However, no reports on a selective sensitization to αCD95 and TRAIL but not TNFα exist, most likely because only the use of TRAIL is of broader medical relevance, as the two other cytokines exert unacceptable side effects. Most importantly, our results show that CD95 and DR4/5 share common signaling pathways that seem to be only of minor importance in TNF-R1 signaling. As there are still a lot of open questions regarding death receptor signaling especially via CD95 and DR4/5, our findings are – if not only interesting – also of a modest importance.
6. Summary

Apoptosis is an essential process in development, organ homeostasis and disease, allowing for cell death in a controlled manner. Death receptor agonists like TNFα, CD95L and TRAIL are important mediators of apoptosis as binding to their respective receptors on susceptible cells leads to a series of proteolytical events that finally results in apoptotic death of susceptible cells.

The present study investigated the role of non-caspase proteases in apoptosis of the human hepatoma cell line HepG2. In particular, the role of the lysosomal cysteine protease cathepsin B in DNA damage- and death receptor agonist-induced apoptosis was analyzed. In addition, the role of caspases and non-caspase proteases in cell death induced by the death receptor agonists TNFα, agonistic αCD95 antibody or TRAIL was studied in detail. Using this model, we also assessed the potential of various drugs to either modulate apoptosis of sensitized cells or sensitize cells to apoptosis induced by death receptor agonists. In summary, the following results were obtained:

1. Camptothecin treatment caused translocation of cathepsin B from the lysosomes to the cytosol in a time-dependent manner.
2. Inhibition of cathepsin B in camptothecin-induced apoptosis lead to a markedly decreased activation of caspases. In accordance, cathepsin B inhibition in apoptosis induced by ActD/TNFα caused a significant reduction in caspase activation. However, neither inhibition of cathepsin B nor caspases was able to confer protection from apoptosis.
3. Treatment of sensitized (ActD and CHX, respectively) cells with TNFα, agonistic αCD95 antibody or TRAIL resulted in concentration- and time-dependent activation of caspases and subsequent cytotoxicity. Moreover, caspase activity and cytotoxicity were significantly correlated ($R^2 = 0.91$, 0.96 and 0.96 for TNFα, αCD95 and TRAIL, respectively), suggesting a causal role of caspases.
4. Inhibition of caspases by zVAD-fmk was sufficient to protect from death receptor agonist-induced apoptosis in primary murine hepatocytes but not in HepG2 cells. In these, the ratio of IC$_{50}$ values for inhibition of caspase activity and cytotoxicity (1:812, 1:193 and 1:262 for TNFα, αCD95 and TRAIL, respectively) suggested that protection by zVAD-fmk was attributable to unspecific inhibition of a non-caspase protease.
5. Cell death after caspase arrest was characterized by classical apoptotic features like \textit{z}eiosis, chromatin condensation and phosphatidylserine exposure. Differences could be observed for the shape of chromatin condensation which was rather disperse than compacted and the late morphology of cells, which had not disintegrated into apoptotic bodies but rather displayed a distinct round shape.

6. Inhibition of effector caspases-3 and -7 by overexpression of their endogenous inhibitor XIAP(\text{\textcopyright}Bir3) did not confer protection in HepG2 cells, whereas it conferred significant protection in HeLa cells.

7. Caspase arrest did not prevent release of cytochrome c from the mitochondria. Also, cleavage of PARP and pro-caspases-8 and -9 could be observed in the absence of caspase activity.

8. Protection conferred by the plant compound glycyrrhizin as well as augmented apoptosis by the c-Jun N-terminal kinase inhibitor SP600125 was observed in apoptosis both with and without caspase arrest.

9. Caspase arrest lead to a switch to a serine protease-dependent mechanism of apoptosis which acts upstream of mitochondria. Only combined inhibition of both caspases and serine proteases was able to rescue cells from death receptor agonist-induced apoptosis.

10. Both inhibition of c-Jun N-terminal kinase and histone deacetylase selectively sensitized HepG2 cells to apoptosis induced by \textit{\alpha}CD95 and TRAIL but not TNF\textit{\alpha}, the underlying mechanisms remaining to be elucidated.

In summary, the results of this thesis demonstrate that caspase arrest does not protect HepG2 cells from undergoing cell death but rather causes a switch to a novel, serine-protease dependent mechanism of apoptosis.
7. Deutsche Zusammenfassung


3. Die Behandlung sensitivierter Zellen mit an TNFα, αCD95 oder TRAIL führte zu einer konzentrations- und zeitabhängigen Caspase-Aktivierung und nachfolgendem Zelltod. Zudem waren Caspase-Aktivität und Zelltod signifikant korreliert ($R^2 = 0.91$, $0.96$ und $0.96$ für TNFα, αCD95 bzw. TRAIL), was eine kausale Beteiligung der Caspasen nahe legt.
7. Deutsche Zusammenfassung

4. Eine Hemmung der Caspasen durch den Breitband-Inhibitor zVAD-fmk schützte primäre murine Hepatozyten aber nicht HepG2-Zellen vor Todesrezeptor-induzierter Apoptose. In letzteren lässt der Quotient der IC\textsubscript{50}-Werten für die Hemmung der Caspase-Aktivität bzw. Zytotoxizität (1:812, 1:193 and 1:262 für TNF\textsubscript{α}, αCD95 bzw. TRAIL) darauf schliessen, dass der durch zVAD-fmk vermittelte Schutz auf die unspezifische Hemmung eines anderen Faktors oder einer anderen Protease zurück zu führen ist.


6. Eine Hemmung der Effektor-Caspasen -3 und -7 durch Überexpression ihres endogenen Inhibitors XIAP(ΔBir3) vermittelte keinen Schutz in HepG2 Zellen, während hingegen sie in HeLa-Zellen einen signifikanten Schutz vermittelte.


8. Der durch den pflanzlichen Wirkstoff Glyzerrhizin vermittelte Schutz vor Apoptose bzw. die durch den JNK-Inhibitor SP600125 vermittelte verstärkte Apoptose wurde unabhängig davon beobachtet, ob die Caspasen aktiv waren oder gehemmt wurden.


10. Sowohl die Hemmung der c-Jun N-terminalen Kinase (JNK) als auch der Histondeacetylase (HDAC) bewirkte in HepG2-Zellen ohne vorherige Hemmung der Transkription bzw. Translation eine selektive Sensitivierung gegen die αCD95- und TRAIL-vermittelte, nicht aber die TNFα-vermittelte Apoptose.

Zusammenfassend zeigen die Ergebnisse der vorliegenden Arbeit, dass eine Hemmung der Caspasen in HepG2-Zellen diese nicht vor dem Zelltod schützt, sondern vielmehr ein Umschalten zu einem neuartigen, Serinprotease-abhängigen apoptotischen Mechanismus bewirkt.
8. References

8. Foundation N. Nobel Prize in Physiology or Medicine, 2002.


27 Budd RC. Death receptors couple to both cell proliferation and apoptosis. \textit{J Clin Invest} 2002; 109: 437-441.


34 Cantz T \textit{et al.} MRP2, a human conjugate export pump, is present and transports fluo 3 into apical vacuoles of Hep G2 cells. \textit{Am J Physiol Gastrointest Liver Physiol} 2000; 278: G522-531.


40 Muller M \textit{et al.} Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. \textit{J Clin Invest} 1997; 99: 403-413.


8. References


51 Villunger A *et al.* Fas ligand-induced c-Jun kinase activation in lymphoid cells requires extensive receptor aggregation but is independent of DAXX, and Fas-mediated cell death does not involve DAXX, RIP, or RAIDD. *J Immunol* 2000; **165**: 1337-1343.

52 Watson A. The role of Fas in apoptosis induced by anticancer drugs. *Hepatology* 1999; **29**: 280-281.


57 Higuchi H *et al.* Bid antisense attenuates bile acid-induced apoptosis and cholestatic liver injury. *J Pharmacol Exp Ther* 2001; **299**: 866-873.


61 Sprick MR *et al.* Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *Embo J* 2002; **21**: 4520-4530.


8. References

86 Aoki K et al. Complete amino acid sequence of the large subunit of the low-Ca2+-requiring form of human Ca2+-activated neutral protease (muCANP) deduced from its cDNA sequence. FEBS Lett 1986; 205: 313-317.
8. References


8. References


120 Mathiasen IS, Hansen CM, Foghsgaard L, Jaattela M. Sensitization to TNF-induced apoptosis by 1,25-dihydroxy vitamin D(3) involves up-regulation of the TNF receptor 1 and cathepsin B. Int J Cancer 2001; 93: 224-231.


127 Roberg K. Relocalization of cathepsin D and cytochrome c early in apoptosis revealed by immunoelectron microscopy. Lab Invest 2001; 81: 149-158.


8. References


145 McGettrick AF, Barnes RC, Worrall DM. SCCA2 inhibits TNF-mediated apoptosis in transfected HeLa cells. The reactive centre loop sequence is essential for this function and TNF-induced cathepsin G is a candidate target. Eur J Biochem 2001; 268: 5868-5875.


8. References

8. References


187 Draper DW, Harris VG, Culver CA, Laster SM. Calcium and its role in the nuclear translocation and activation of cytosolic phospholipase A(2) in cells rendered sensitive to TNF-induced apoptosis by cycloheximide. *J Immunol* 2004; 172: 2416-2423.

188 Kim BC *et al.* Tumor necrosis factor induces apoptosis in hepatoma cells by increasing Ca(2+) release from the endoplasmic reticulum and suppressing Bcl-2 expression. *J Biol Chem* 2002; 277: 31381-31389.


192 Chawla-Sarkar M *et al.* Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. *Cell Death Differ* 2004.
8. References
