Apoptosis signaling to mitochondria by death receptors and DNA damaging anti-cancer regimens
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Chapter 1

General Introduction
General Introduction

Studies in the nematode *Caenorhabditis elegans*, the fly *Drosophila melanogaster*, and mammalian cells have demonstrated that the molecular mechanisms of the execution machinery leading to apoptosis are highly conserved among metazoans. These mechanisms have been extensively reviewed\(^1\)\(^-\)\(^5\); therefore I will only describe some essential information in the following paragraphs of this chapter that is related to the work performed in this thesis.

**Cancer and apoptosis**

Tumorigenesis in mammals is a multistep process of about five to seven rate-limiting, stochastic events, leading to an unlimited growth advantage of the tumor cells\(^6\). Most cancers have acquired the same set of functional capabilities during their development, the so-called 'hallmarks of cancer'. In every case, alterations in defined pathways provide the tumorigenic phenotype, like self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis\(^7\). Normal tissues, however, rely on extracellular growth signals, growth inhibitory signals, survival signals generated by attachment to the extracellular matrix and soluble survival factors, as well as a control mechanism in the form of programmed cell suicide and limited life span. A great diversity of genetic alterations can lead to the same effect within one or more of the defined pathways that favor tumorigenesis. For instance, effector molecules can be amplified or mutated into constitutively active forms, or inhibitory molecules could be downregulated or mutated into inactive forms or even completely deleted from the genome. The cell type and the associated selection pressure will influence which kind of genetic alteration will take place.

Anticancer drugs can potentially kill cells in two fundamentally different ways; by interfering with cellular processes that are essential for maintenance of viability or by stressing the cell such that an endogenous physiological cell death mechanism is triggered, that indirectly prompts the cell to kill itself. Initially, the development of chemotherapeutic agents was based on the observation that tumor cells proliferate faster than normal cells. Thus, drugs that interfered with DNA replication or cellular metabolism were chosen. However, these drugs also affected rapidly dividing normal cells of the bone marrow and gut and thereby reducing the efficacy of the therapies. Besides this, drug resistance lowers the efficiency of treatments. Drug resistance can take place because proteins within the cell can interfere with either drug accumulation (drug efflux pumps like P-glycoprotein) or stability (detoxifiers like glutathione) thus
inhibiting drug-target interactions, or by mutations in proteins that are involved in various stress signaling pathways leading to cell death or cell cycle arrest.

Apoptosis, or programmed cell death, is a multistep cell death program that is present in a latent form in all cells of the body and is characterized by the active participation of the dying cell. In 1972, Kerr and coworkers were the first who described this kind of cell death, which is morphologically and biochemically different from necrosis. An apoptotic cell undergoes cell shrinkage, membrane blebbing and nuclear condensation and fragmentation, and subsequent disassembly into apoptotic bodies. Phagocytes engulf these apoptotic bodies thereby preventing harmful leakage of cell content of the dying cell into the surrounding tissue and avoiding an inflammatory response, which is a characteristic for necrosis. Apoptosis plays a crucial role in normal development, in maintenance of tissue homeostasis and in an effective immune system, by eliminating cells that are redundant, damaged or infected. It is therefore not surprising that dysregulation of this process is implicated in numerous pathological conditions, ranging from degenerative disorders to autoimmunity and cancer.

Alterations in apoptotic pathway components or their regulators have been detected in a variety of cancers, suggesting that loss of the ability of cells to undergo apoptosis might contribute to carcinogenesis. For instance, the p53 tumor suppressor gene is the most frequently mutated gene in human tumors, and loss of p53 function can both disable apoptosis and accelerate tumor development in transgenic mice. If there is DNA damage, this is sensed by kinases ATM and Chk2, which phosphorylate and stabilize p53. P53 can bring about a cell cycle arrest to repair the damage or activate the apoptotic pathway via transcription dependent and independent mechanisms. Also, functional mutations or altered expression of upstream regulators of p53 (like ATM, Chk2, Mdm2 and p19ARF) or downstream effectors (PTEN, Bax, Bak and Apaf-1) occur often in human tumors. Furthermore, dysregulation of Bcl-2 family members, like Bcl-2, Bcl-xL and Bad, is often observed in tumors. In fact, Bcl-2 was first identified based on its translocation in follicular lymphoma, and is overexpressed in a variety of cancers. Also post-mitochondrial mutations have been reported, like silencing of Apaf-1 in metastatic melanoma and leukemia cell lines or overexpression of inhibitor of apoptosis proteins (IAPs) or heat shock proteins. There is also a correlation between tumorigenicity and mutations in death receptors, like CD95 and TRAIL receptors, or with dysregulation of downstream signaling components, like caspase-8 and FLIP.

Thus, there is a relevant link between apoptosis and cancer. Since apoptosis resistance is one of the 'hallmarks of cancer' and conventional anti-cancer therapies kill cells by inducing apoptosis, it is important to develop novel anti-cancer therapies that can activate apoptotic pathways that are still operational in most of the cancers and that preferentially selectively induce apoptosis in tumor cells and leave healthy cells...
Untouched. It is therefore crucial to know what the fundamental mechanisms are of proteins that participate in apoptotic pathways.

**Caspases: the engine of cellular destruction**

A crucial event in apoptosis involves the activation of members of the caspase family of cysteine proteases, which coordinate destruction of the cell through limited proteolysis of an array of intracellular proteins\(^{31-33}\). To prevent unscheduled cell suicide, each caspase, essentially present in all cells, is synthesized as a dormant pro-enzyme that requires processing at caspase cleavage sites to generate the active enzyme\(^{2,34,35}\). Caspases, like Caspase-8,-9 and -10 that initiate apoptosis become activated by self-association and binding to activating adaptor or scaffold proteins via their long prodomain. This results in autocatalysis and the formation of the active tetrameric enzyme. Effector caspases like Caspase-3,-6 and -7 are activated by processing by initiator caspases or other proteases like Granzyme B. In turn, these activated effector caspases cleave an array of substrates resulting in the execution of the actual cell death. So, a chain reaction of caspase activation is the cell's death sentence.

At least 14 distinct mammalian caspases have been identified, with their orthologues present in species ranging from *C. elegans* to *D. melanogaster*. The involvement of the *C. elegans* CED-3 caspase homologue in apoptosis was first discovered by Yuan et al.\(^{36}\). Since then, compelling evidence has demonstrated that the mechanism of apoptosis is evolutionarily conserved and executed by this family of cysteine proteases. Caspases are named like that, since they all cleave after an aspartate residue in their substrates\(^{37}\). The function of caspases is either related to cytokine maturation or induction of apoptosis\(^{38}\). Caspases involved in apoptosis are generally divided into two categories based on their primary structure; the initiator caspases with their long amino-terminal prodomains include Caspase-2,-8,-9,-10 and the effector caspases, with short prodomains, include Caspase-3,-6,-7\(^{39}\). However, classification of the caspases based on their cleavage specificity gives a different view. Then Caspase-2,-3 and -7 are grouped together with a preference for Asp-Glu-X-Asp and the Caspases-6,-8,-9 and -10 have a preference for (Leu/Val)-Glu-X-Asp\(^{40}\).

**Caspases and their activation: extrinsic and intrinsic apoptosis pathways**

There are two major caspase-activating cascades that regulate apoptosis in mammals: one is initiated from the cell surface death receptors (extrinsic pathway) and the other is triggered by changes in mitochondrial membrane integrity (intrinsic pathway), see also Figure 1. The initiator caspases all contain a protein-protein interaction motif in their prodomain, either a caspase-recruitment domain (CARD) or two death effector domains
Figure 1 – Apoptosis pathways.

Some of the known components of the extrinsic (death receptor) and intrinsic (mitochondrial) apoptosis pathways are shown. Proteins in circles promote apoptosis, whereas proteins in squares have an inhibitory function. See text for explanation. Adapted from references 4 and 8.

(DED). These motifs interact with similar motifs present on oligomerized adaptorproteins, thus bringing multiple initiator caspases into close proximity with one another and facilitating their autoactivation.41

The extrinsic apoptosis pathway is induced upon activation of trimerized death receptors, belonging to the tumor necrosis factor (TNF) receptor superfamily, by the trimerized cognate ligands. Death receptors share the presence of a death domain (DD) in their cytoplasmic tail. The best characterized death ligand / receptor interactions include TNFα, CD95 ligand (CD95L, FasL) and TNF-related apoptosis-inducing ligand (TRAIL) with TNF receptor-1 (TNF-R1), CD95 (Fas, APO-1) and TRAIL-R1/-R2 (DR4/DR5), respectively. The initiator Caspases-8 and -10 are activated in response to ligation of a death receptor. Following stimulation by e.g. CD95L, the DD in the cytosolic tail of the trimerized death receptor CD95 associates with the DD of the adaptor molecule FADD (Fas-associated death domain). FADD then recruits procaspase-8 via a homophilic DED interaction. The protein complex thus formed at the tail of the death receptor, known as the death-inducing signaling complex (DISC)42,43 results in autocleavage and activation of Caspase-83,44. As illustrated in Figure 1, active Caspase-8 can initiate either directly the activity of a cascade of effector caspases or via the mitochondria, involving the pro-apoptotic Bcl-2 family member Bid. The importance of the mitochondrial contribution is dependent on the cell type.45
Initiator Caspase-9 is instead activated by assembly into a multiprotein caspase-activating complex, called the apoptosome, which contains besides Caspase-9, the Apaf-1 scaffold protein and Cytochrome c (Cyt c)\textsuperscript{46-48}. Diverse cellular stresses like radiation, chemotherapeutic drugs, glucocorticoids and cytokine deprivation, provoke the release of Cyt c from the mitochondria into the cytosol, in the intrinsic pathway, see also Figure 1\textsuperscript{49,50}. Cyt c associates in an ATP dependent manner to the carboxy-terminus of Apaf-1 and induces a conformational change in it\textsuperscript{51,52}. In this state Apaf-1 assembles into a heptameric structure that recruits procaspase-9 via a homophilic CARD interaction to the complex, where it undergoes auto-proteolytic activation\textsuperscript{53}. Then, active Caspase-9 activates the effector caspases, which cleave several substrates, leading to cellular and nuclear morphological changes and ultimately cell death.

Exactly by which mechanism initiator caspases achieve catalytic competence in their recruitment / activation complexes is still unresolved. Recently, it was suggested that within this proximity-induced activation model, dimerization of monomeric zymogens is the key event, rather than proteolysis. Various groups have shown that activation of Caspase-8 and -9 occurs via dimerization and that the internal proteolysis probably serves to partially stabilize the activated dimers or to release the dimers from the complexes\textsuperscript{54,57}. Dimerization causes a conformational change of the zymogen's catalytically active pocket, making it accessible to substrates\textsuperscript{56}. It is suggested that this activation by dimerization is the general mechanism to activate all initiator caspases. Recently, Kumar and colleagues have suggested something similar for Caspase-2 activation\textsuperscript{58}. They show that Caspase-2 may become recruited in a CARD-dependent manner to a novel Apaf-1-independent activation complex, where it becomes activated without the requirement of cleavage. Until now, the individual components of this complex have yet to be identified. Whether Caspase-2 can be really grouped among the initiator caspases is still not solved. Its activation mechanism, which is dependent on its prodomain, and various reports that demonstrate a link between Caspase-2 and release of mitochondrial pro-apoptotic factors upon DNA damage-induced death, make it likely\textsuperscript{59-62}. On the other hand, collective data on Caspase-2 deficient mice, its substrate specificity and reports (see also chapter 4 in this thesis) placing Caspase-2 downstream of the mitochondria in various apoptotic pathways, suggests that Caspase-2 could act as effector caspase\textsuperscript{40,63-65}.

In case of the effector Caspases-3,-6 and -7, which do not have a long prodomain and thereby can not oligomerize to an adaptor/scaffold complex, the activating event is proteolysis by an initiator caspase (-8,-9, or -10)\textsuperscript{66} or by other proteases like Granzyme B\textsuperscript{67}. In turn, these activated effector caspases cleave various protein substrates leading to the systematic and orderly disassembly of the dying cell. Up to now, more than 280 different caspase substrates are known. However, some substrates are just cleaved as bystanders, whereas others have a discrete function in propagation of the cell death process by turning off cell-protective mechanisms and
activating pathways that lead to cell destruction. These substrates include regulators of cell-cycle progression, DNA repair proteins and nuclear and cytoskeletal structural proteins, explaining the characteristic features of apoptosis.

It should be mentioned that also apoptosis-like programmed cell death can occur, which is caspase-independent. Both death receptors and DNA damage can trigger this caspase-independent cell death program that can involve organelles, including mitochondria, endoplasmic reticulum (ER) or lysosomes that activate other proteases.

**Bcl-2 family: regulators of the mitochondrial membrane integrity**
The mammalian Bcl-2 protein family consists of both pro- and anti-apoptotic proteins, which show sequence homology and structural similarity in their Bcl-2 homology (BH) regions. The anti-apoptotic members include besides Bcl-2, also Bcl-x, Bfl-1/A1, Mcl-1, Bcl-w and Boo/Diva/Bcl-B. They all share three to four Bcl-2 homology (BH) domains and contain a hydrophobic carboxy-terminal tail which localizes these members mainly to intracellular membranes like the outer mitochondrial membrane, the ER and the nuclear envelope. These members protect cells from a wide range of cytotoxic insults, like cytokine deprivation, UV and γ radiation and chemotherapeutic drugs. The pro-apoptotic Bcl-2 family members can be divided into two subgroups. The Bax type subgroup of pro-apoptotic members has two to three BH domains and is structurally very similar to the anti-apoptotic relatives. The group consists of Bax, Bak, Bok/Mtd, Bcl-x, Bcl-G and Bcl-Rambo. The second pro-apoptotic subgroup contains proteins that only share the BH3 region with the anti-apoptotic members and are therefore called 'the BH3-only's'. BH3-only members identified so far are Bid, Bad, Bik/Nbk, Bim/Bod, Bmf, Harakiri/DPS, Noxa, Puma/Bbc3, BNIP3/NIP3 and BNIP3L/Nix. Structural analysis of the Bcl-xL/Bak BH3 peptide complex has revealed that the BH3 domain of Bak binds to a hydrophobic cleft formed by the BH1, BH2 and BH3 domains of Bcl-xL. Anti-apoptotic and pro-apoptotic Bcl-2 family members may thus engage, by a BH3 domain-dependent mechanism, in the formation of heterodimers in which they mutually antagonize each other’s function.

The response of the mitochondrion to upstream stimuli is a critical control point in apoptosis, which is regulated by the pro- and anti-apoptotic Bcl-2 family members via a yet incompletely resolved mechanism. Several apoptotic stimuli, as described earlier, induce loss of mitochondrial membrane integrity, which allows the release of numerous mediators from the mitochondrial intermembrane space, like Cyt c, Smac/DIABLO, Omi/HtrA2, apoptosis inducing factor (AIF) and endonuclease G, see also Figure 1. These mediators all promote the apoptotic machinery via their own mechanism. Cyt c is a crucial component for the formation of
the apoptosome, which initiates the caspase cascade. As a further safeguard mechanism, Smac and Omi prevent the inhibitor of apoptosis proteins (IAPs) from inhibiting the activity of processed caspases. AIF and endonuclease G have been proposed to contribute to ‘caspase-independent’ nuclear changes and cell death. However, whether these released mediators are essential for the induction of apoptosis is not yet established. For instance, absence of Cyt c solely attenuates stress-induced apoptosis. However, microinjection of Cyt c into various cell types induces apoptosis in a caspase dependent manner. Loss of Smac does not influence the apoptotic process, although one could argue that Omi might still block IAP function. Nevertheless, inhibition of IAPs does not initiate apoptosis.

Recently, also the ER is thought to participate in stress-induced apoptosis that is imposed by disruption of calcium homeostasis or by excess of unfolded proteins. Bcl-2 can function on the ER and can protect against ER-stress induced apoptosis and against the cross talk between ER and mitochondria. Also Bax and Bak can localize to the ER and either one of the two is required for ER-stress induced apoptosis, and for the associated cleavage of ER-localized mouse Caspase-12. Whether Caspase-12 is required for ER-stress induced cell death is uncertain, since Caspase-12 deficient mice were partially resistant to ER-stress induced apoptosis. The human counterpart of Caspase-12 is a Caspase-12-like protein with a premature stop codon and several point mutations preventing the translated protein from having any caspase-like enzymatic activity.

In mammals, activation of BH3-only proteins integrates diverse apoptotic stimuli into one single pathway by triggering Bax/Bak-mediated mitochondrial dysfunction. The BH3-only proteins are considered to function as the cell death triggers, but pro-apoptotic Bax and/or Bak are also essential for the cell killing, which can be antagonized by anti-apoptotic Bcl-2 family members. Inactivation of Bax affected apoptosis only slightly and disruption of Bak had no evident effect, but inactivation of both genes dramatically impaired apoptosis in many tissues and prevented BH3-only proteins to kill. So, the presence of either Bax or Bak seems to be essential for apoptosis in many cell types and places the BH3-only proteins upstream in the pathway. Bax and Bak appear to permeabilize the outer mitochondrial membrane and allow the release of apoptogenic proteins, as mentioned above. In normal cells, Bax is predominantly a cytosolic monomer, but during apoptosis it undergoes a conformational change that exposes both the amino- and carboxy-termini, leading to mitochondrial translocation and oligomerization. Bak is constitutively localized to the mitochondrial membrane, but also changes conformation and forms larger aggregates upon apoptotic stimulation. In vitro, Bax and Bak homo-oligomers can permeabilize mitochondria and artificial protein-free liposomes and form pores through which Cyt c can pass. Although tBid can induce Bax and Bak oligomerization and insertion into the outer mitochondrial membrane, the exact mechanisms that cause
conformational changes and oligomerizations are still unknown\textsuperscript{135,136}. Incubation of tBid with mitochondria \textit{in vitro} does not allow for detectable Bid present in cross-linked Bak oligomers, which suggest a ‘hit-and-run’ activation mechanism\textsuperscript{131}, or perhaps Bid instead activates Bax/Bak indirectly. Though high concentrations of Bcl-2 proteins prevent Bax oligomerization and channel-forming activity\textsuperscript{124}, cross-linking studies reveal no Bcl-2 / Bax complexes\textsuperscript{137}. Alternatively, Bax might interact with components of the existing voltage-dependent anion channel to create a larger channel\textsuperscript{138,139}, but several studies have found no evidence for such complexes\textsuperscript{124,137,140}.

\begin{center}
\begin{tabular}{c c c}
\textbf{Model A} & \textbf{Model B} & \textbf{Model C} \\
\includegraphics[width=0.3\textwidth]{model_a.png} & \includegraphics[width=0.3\textwidth]{model_b.png} & \includegraphics[width=0.3\textwidth]{model_c.png} \\
\end{tabular}
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\textbf{Figure 2} – 	extit{Models for functional relationship of the different subgroups of the Bcl-2 family. (Model A)} The BH3-only proteins (BH3) directly engage and activate the pro-apoptotic Bax/Bak proteins, once they have overwhelmed the anti-apoptotic Bcl-2-type proteins (Bcl-2). \textit{(Model B)} The anti-apoptotic Bcl-2 type proteins prevent, either directly or indirectly, activation of pro-apoptotic Bax/Bak proteins. The BH3-only proteins (BH3) target only Bcl-2 type proteins and inactivate these members. \textit{(Model C)} Certain BH3-only proteins like Bad target only Bcl-2 type members (Bcl-2) and are thought to act as sensitizers, while other BH3-only proteins like tBid can engage both Bcl-2 type and Bax/Bak proteins and act as the activators. At least the class of activators has to be activated to activate Bax/Bak proteins.

Adapted from reference 1.

In principle, there are three main variations on the theme of the functional relationship between the different subgroups of the Bcl-2 family, as schematically represented in Figure 2, but collective data is still not conclusive. In model A, Bcl-2 type proteins sequester BH3-only proteins from Bax/Bak types, thereby preventing the BH3-only induced activation of Bax/Bak proteins. This model is in line with data on the capability of tBid to trigger Bax to permeabilize protein-free liposomes\textsuperscript{133}. However, so far only Bid and the BH3 peptide of Bid and Bim have been shown to bind Bax or Bak directly, and this model does therefore not explain how the other BH3-only proteins perform their activation on Bax/Bak\textsuperscript{91,131,141,142}. In model B, BH3-only proteins only
target Bcl-2-type members and relieve the inhibition of Bcl-2 on Bax/Bak-type members. In favor of this model all BH3-only proteins known so far are able to bind to the Bcl-2-type members. Although overexpression of Bcl-2 and Bcl-xL inhibit apoptosis induced by BH3-only proteins, which is in agreement with this model, mutants of Bcl-2 and Bcl-xL that could not bind to Bim, Bad or Bid are no longer able to protect cells from apoptosis induced by these BH3-only proteins\textsuperscript{123}. This suggests that there is an existing cooperation between BH3-only proteins and the pro-apoptotic Bax/Bak proteins which could no longer be prevented and partially supports model C. This model is a combination of the previous two. Bcl-2-type members have a neutralizing function by sequestering the BH3-only members and thereby prevent activation of Bax/Bak-type members. In addition, this model suggests a specialization within the BH3-only group; there are members, like Bad that predominantly have a sensitizing function and members like tBid that have an activating function. Both can by sequestered by Bcl-2-type members but, the tBid-like members (including Bim) have a lower binding affinity for Bcl-2 than the Bad-like members and are subsequently easily displaced by them from Bcl-2 and are free to directly activate Bax/Bak members. This model is based on data from \textit{in vitro} competition experiments with BH3 peptides\textsuperscript{141}. This is furthermore supported by data with a mutant of Bcl-xL that can no longer bind Bax or Bak, but still is able to prevent apoptosis. This suggests that Bcl-xL sequesters the BH3-only members rather than directly regulating the activity of Bax/Bak\textsuperscript{143}. A criticism on this model is that it would require the activation of the two proposed classes of BH3-only members, but single BH3-only proteins such as Bad are potent stimuli as well\textsuperscript{119,123}.

\textbf{Regulation of activity of the mitochondrial ‘cell death triggers’}

In order to prevent inappropriate cell death, BH3-only proteins are kept inactive by a number of different mechanisms until it is the right moment to come into action\textsuperscript{144}. Transcriptional control, induced by the tumor suppressor protein p53, has been described for Noxa and Puma in response to DNA damage\textsuperscript{98,100,101}. Puma expression is also regulated in a p53-independent manner, by glucocorticoids and serum deprivation\textsuperscript{99}. Mouse knockout studies for Puma show that Puma is required for the p53-dependent apoptotic responses to \(\gamma\) radiation and DNA-damaging drugs in thymocytes, as well as to c-Myc and E1A oncogenes in primary embryonic fibroblasts (MEFs)\textsuperscript{145,146}. Puma deficiency also protects lymphocytes from several p53-independent apoptotic stimuli. Characterization of Noxa knockout mice also indicate a role for Noxa in p53-dependent apoptosis, though less pronounced than found for Puma\textsuperscript{146,147}. Furthermore, the expression of Bid and Bim can be transcriptionally regulated by p53 and the forkhead transcription factor FKHR-L1, respectively\textsuperscript{148,149}. 
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In addition, post-transcriptional controls like sequestration or protein modification are ways to hold BH3-only proteins in check. For example, Bid requires cleavage to produce an active carboxy-terminal fragment (tBid) that translocates to the mitochondria to induce, among other factors, the release of Cyt c into the cytoplasm\textsuperscript{118,150-152}. Bid proteolysis is achieved by Caspase-8 upon ligation of death receptors or by the serine protease Granzyme B that is released from lytic granules into the target cell by cytotoxic T and NK cells\textsuperscript{153}. It is suggested that amino-terminal myristoylation on tBid facilitates the translocation to the mitochondria\textsuperscript{154}, where it is attracted to cardiolipin-enriched areas in the mitochondrial outer membrane\textsuperscript{155}. Bid-deficient mice appear normal, but are resistant to CD95-induced hepatocellular apoptosis\textsuperscript{156}, which reflects the requirement of the mitochondrial contribution to apoptosis induction in these cells. Furthermore, it is suggested that Bid serves as a chemosensitivity gene, since Bid-deficient MEFs were more resistant to adriamycin and 5-FU than wild type MEFs\textsuperscript{149}, though no differences were found in response to \(\gamma\) radiation, staurosporin or dexamethasone\textsuperscript{156}. In healthy cells, both Bim and Bmf are sequestered on cytoskeletal structures; the microtubular dynein motor or the actin-based myosin V motor complex, respectively\textsuperscript{95,157}. Stress signals, like cytokine deprivation and calcium flux for Bim, and anoikis and actin depolarizing drugs for Bmf, trigger their release, which is probably induced by their phosphorylation\textsuperscript{158}. Loss of Bim in mice causes extensive lymphoid and myeloid cell accumulation, indicating a role in deleting autoreactive lymphocytes \textit{in vivo}\textsuperscript{159,160} and its lymphocytes are resistant to cytokine withdrawal or taxol treatment, but remain sensitive to \(\gamma\) radiation, etoposide or dexamethasone\textsuperscript{161}. Another BH3-only protein, Bad, is a target for phosphorylation by protein kinase-A and -B/Akt (PKB). In healthy cells, survival factors induce phosphorylation of Bad and subsequently allow its sequestration by 14-3-3 proteins in the cytosol. Cytokine deprivation may induce apoptosis by permitting dephosphorylation of Bad and its subsequent release\textsuperscript{162-166}. Bad deficient mice, however, have no detectable abnormality and their neurons are normally sensitive to NGF deprivation in culture\textsuperscript{167}. This suggests that Bad just contributes in this pathway by perhaps supporting Bim, which is essential in this pathway\textsuperscript{161,168}.

Regulation of apoptosis pathways

Cells constantly receive signals from outside and inside the cell that can cause damage. To ensure that they can make the right decision, so either to die or to stay alive and repair the damage, the apoptotic process is strongly regulated. Both caspase activation and caspase activity are tightly controlled. Death receptor-induced caspase activation can be inhibited by cellular FLICE-inhibitory (c-Flip) proteins\textsuperscript{169}. There are two splice variants of c-Flip present within the cell. c-Flip-short (c-Flip\textsubscript{S}) resembles the viral variant of Flip. It contains two DED domains with which it can interact with FADD and
by that interfere with the recruitment and activation of procaspase-8 or -10 to the DISC from ligand-activated death receptors. c-Flip-long (c-FlipL) in addition contains a caspase-like domain and thus shares more structural similarity to Caspase-8/-10. However, c-FlipL is catalytically inactive due to mutations in the catalytic domain and can interfere thus with the processing of procaspase-8 and -10, but not with its recruitment to the DISC. c-FlipL is a different kind of caspase regulator than c-Flips. Low expression levels are required for the activation of Caspase-8, because it potently promotes procaspase-8 activation through heterodimerization\(^{248}\). Whereas at high expression levels, c-FlipL acts as an apoptosis inhibitor\(^{248}\). High levels of c-FlipL protein are detected in several malignant melanoma tumors and gastric, colon and bladder adenocarcinoma\(^{170-173}\).

Receptor-mediated cell death can also be inhibited by the expression of decoy receptors. These receptors look like death receptors but either completely lack the cytoplasmic death domain or contain truncated variants, and they therefore specifically sequester ligands away from their functional receptors. Decoy receptors can inhibit apoptosis induced by TRAIL (decoy receptors DcR1 and DcR2\(^{174,175}\)) and CD95L (decoy receptor DcR3\(^{176,177}\)). The TRAIL decoy receptors DcR1 and DcR2 are often downregulated in tumor cells but not in normal cells or differentially subcellularly localized\(^{178,179}\) and may thus protect normal cells from TRAIL-induced apoptosis. In contrast, the CD95 decoy receptor DcR3 is overexpressed in some lung and colon tumors, suggesting a possible role in tumor cell resistance to CD95L\(^{177}\).

As mentioned earlier, Bcl-2 family members regulate the mitochondrial membrane integrity and thereby the activation of inducer Caspase-9. Caspases can also be directly inhibited by members of the IAP family. XIAP, cIAP1 and cIAP2 for instance, directly inhibit Caspase-3, -7 and -9 by binding via one of their baculovirus IAP repeats (BIR) and masking the active site in the caspases\(^{180-184}\). Furthermore, the RING domains of XIAP, cIAP1 and cIAP2 have ubiquitin ligase (E3) activity and can act as adaptors to bind to the ubiquitin-conjugating enzymes to direct the transfer of ubiquitin to caspases and mediate their proteasomal degradation\(^{185-187}\). The activity of the IAP family of proteins is in their turn inhibited by the IAP-interacting regulatory proteins Smac in humans\(^{105}\) and Diablo in mice\(^{106}\) and HtrA2/Omi\(^{107,188,190}\) upon release of the mitochondria. And in the case of XIAP, data indicate that apoptotic stimuli like dexamethasone and etoposide lead to ubiquitylation and subsequent degradation of XIAP itself\(^{185}\). Aberrant expression or activity of IAPs has been implicated in human diseases, like t(11;18)(q21;q21) translocation of cIAP2 in mucosa-associated lymphoid tissue (MALT) lymphoma\(^{191}\), which results in a chimeric cIAP2-MALT1 protein that lacks the RING domain\(^{192}\). Overexpression of cIAP1 by amplification of the q22 region on chromosome 11 is found in oesophageal squamous-cell carcinoma\(^{193}\). High protein expression levels of XIAP in Hodgkin’s lymphoma-derived B cells play a crucial role in resistance to apoptosis\(^{194}\).
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Also PKB/Akt is a strong anti-apoptotic regulator. It phosphorylates a variety of proteins, including several associated with cell death pathways, leading to diminished apoptotic cell death. PKB-mediated phosphorylation of the pro-apoptotic Bcl-2 family member BAD leads to its sequestration by 14-3-3 proteins and its dissociation from the mitochondria. Likewise, phosphorylation of Caspase-9 inactivates this protease, thus blocking the propagation of death signals originating in the mitochondria. In addition, PKB promotes cell survival by phosphorylating and inhibiting the Forkhead transcription factor FKHR-L1 and thereby preventing the expression of the pro-apoptotic Bcl-2 family member Bim. Furthermore, it can upregulate Bcl-2 expression through the cAMP-response element-binding protein. Another regulator of cell survival, the NF-κB transcription factor, is also a target of PKB. PKB regulates NF-κB activation via accelerated degradation of the NF-κB inhibitory protein 1κBα, resulting in elevated levels of for instance Bcl-xL or c-Flip. Additionally, NF-κB regulates the expression of numerous other anti-apoptotic proteins, like Bfl-1/A1, Bcl-2, cIAP2 and XIAP or the serpin protease inhibitor 2A to protect against lysosome-mediated apoptosis.

The role of mitochondrial permeabilization: an evolutionary perspective

Concomitant studies in mammals and in lower eukaryotes, primarily C. elegans and D. melanogaster, have shown that the fundamental processes and protein families involved in apoptosis are evolutionarily conserved. Proteins from different species also activate or inhibit apoptotic processes within each other. However, the system in C. elegans is very simple and less diversified compared to the mammalian situation. D. melanogaster provides a system of intermediate complexity between nematodes and mammals.

In C. elegans, three proteins are required for programmed cell death: the pro-apoptotic BH3-only Bcl-2 family member EGL-1, the caspase-9 homologue CED-3 and the Apaf-1 adaptor homologue CED-4, whereas the anti-apoptotic Bcl-2 homologue CED-9 is essential for cell survival. In cells destined to survive, CED-9 binds directly to the adaptor CED-4, and co-localizes on mitochondria, and prevents it from activating the CED-3 caspase. However, when programmed cell death is initiated, the BH3-only protein EGL-1, which is the only initiator present in C. elegans, is transcriptionally upregulated and binds to and inactivates CED-9. This allows CED-4, which works autonomously in causing cell death, to move to the nuclear membrane and to multimerize with CED-3 and promote the autocatalytic activation of CED-3. Although Apaf-1 molecules have carboxy-terminal WD40 repeats, CED-4 lacks these repeats. Cyt c binds these WD40 repeats and changes the conformation of Apaf-1 in such a manner that it can recruit procaspase-9. Since CED-4 does not contain WD40 repeats it is perhaps not so surprising that C. elegans does not require Cyt c to activate CED-3. Thus, in the model system of C. elegans, the BH3-only protein neutralizes the Bcl-2-
type inhibition on Apaf-1/CED4 and Caspase-9/CED3 is activated without any mitochondrial involvement.

No Drosophila homologues of BH3-only proteins have been identified thus far. However, various apoptotic stimuli induce the upregulation of one or more of the cell death inducer proteins like Reaper, Hid, Grim and Sickle by the Hox transcription factor or by the Drosophila p53 orthologue. Genetic analysis indicates that the fly apical caspase Drone is essential for the pro-apoptotic activity of the Reaper, Hid, Grim (RHG) proteins. Drone competes with RHG proteins for binding to Drosophila inhibitor of apoptosis proteins (Diap1, and 2). Under normal conditions, Drone binds to Diap1 and is ubiquitinated by Diap1 and its associated ubiquitin-conjugating E2 enzyme (Morgue or Ubc1) and targeted for proteosomal degradation. However, following upregulation of RHG proteins e.g. Reaper displaces Drone from Diap1. Binding of Reaper to Diap1 leads to auto-ubiquitination and proteasome degradation of Diap1 instead, thus facilitating Drone stabilization and activation. There is no indication for a role of Cyt c release in Drosophila apoptosis, although the Drosophila Apaf-1 homologue Dark contains WD40 repeats that can bind Cyt c. Moreover, the IAP antagonists (e.g. Reaper) do not reside in mitochondria, like in mammalian cells arguing against a mitochondrial contribution in Drosophila. The Drosophila homologues of Bax-type proteins, Debcl, and of Bcl-2-type proteins, Buffy, both localize to mitochondria where they associate and counteract each other and function upstream of caspase activation. Whether they act downstream of RHG proteins or in a parallel pathway and are essential for caspase activation is still being resolved.

Anti-cancer therapy on modulators of apoptosis

As impaired apoptosis is one of the hallmarks of cancer, the search for therapeutic strategies to enhance apoptotic cell death is widely growing. These studies focus for instance on limiting the function of Bcl-2 and IAPs, or engaging the death receptor pathway. The oncogenic potential of anti-apoptotic Bcl-2 family members was first characterized for Bcl-2. The translocation of Bcl-2 in follicular lymphoma caused a reduction in cell death rather than enhanced proliferation. Mouse studies confirmed the oncogenic potential of elevated levels of Bcl-2, but since the chance of getting tumors in these mice is rare, it is suggested that additional mutations are required for transformation. When additional oncogenes are co-expressed, for instance Myc, which promotes proliferation, a dramatic synergism is observed in the formation of malignancy. Also indirect mutations in oncogenes induced by mitogens are found to increase the expression levels of anti-apoptotic Bcl-2 family members, like activation of the NF-κB pathway, which induces the expression of Bcl-xL and Bfl-1/A1 genes. To fully transform, cells e.g. have to bypass growth arrest as well as senescence, which can be achieved by elimination of p53 function. Most spontaneous B lymphoid tumors
that arise in \textit{myc} transgenic mice have in addition to elevated levels of Bcl-2 and/or Bcl-
\textit{x}_{1} mutations that eliminate p53 function\textsuperscript{239,240}. The pro-apoptotic Bcl-2 family members (BH3 only’s and Bax-like) probably all behave like tumor suppressors and thus need to be inactivated to allow transformation to occur. It is likely that these pro-apoptotic members have a substantial redundancy, so their tumor suppressor function may only arise in specific cell types. Downregulation of Bax is regularly found in haematopoietic malignancies\textsuperscript{241}, however \textit{bax-null} mice acquire few spontaneous tumors\textsuperscript{242}. Absence of both Bax and Bak greatly enhance the transformation compared to loss of either one of the two\textsuperscript{119}. Bim is a potent tumor suppressor at least in B cells. Although loss of Bim in itself does not enhance tumor incidence in mice within the first 12 months of their life, already loss of a single allele of Bim dramatically accelerates lymphoma in mice expressing a \textit{myc} transgene in the B lineage\textsuperscript{243}. Moreover, 50\% of Bid deficient mice develop chronic myelomonocytic leukaemia by two years of age\textsuperscript{244}. Since Puma and Noxa are both transcriptional targets of the tumor suppressor protein p53 and knock out studies of these genes indicate a role for them in p53-dependent apoptosis induction, these proteins are attractive candidates for being tumor suppressors as well. Nothing is known yet about tumor incidence in these recently made knock out mice.

Death receptor-induced apoptosis pathways can directly activate effector caspases, thus they can bypass certain inhibitions at the mitochondrial level that frequently occur in tumor cells and are not affected by loss of functional alleles of p53. In particular, the death ligand TRAIL harbors potential as anticancer agent. \textit{In vitro}, it killed the majority of malignantly transformed cell lines tested but not normal cells. And it shows no toxicity when systemically administered to animals in mouse and monkey preclinical models\textsuperscript{174,245}. Toxicity of TNF\textgreek{a} and CD95L precludes their use for systemic therapy.

\textbf{Scope of this thesis}

Conventional anti-cancer regimens like $\gamma$ radiation and chemotherapeutic drugs, kill cells by activating apoptotic signaling pathways, but apoptosis resistance is one of the ‘hallmarks of cancer’. This irony has led to the urge to design novel anti-cancer therapies that can activate apoptotic pathways in such a manner that the block(s), in resistant cancer cells, in these pathways can be circumvented. Insight in the molecular nature of apoptosis pathways can help to design novel types of therapies that activate apoptotic pathways that are still functional plus induce apoptosis as selectively as possible in tumor cells. Most apoptotic stimuli convey their signal on mitochondria, which is a critical control point in apoptosis signaling. The research presented in this thesis was therefore aimed at defining key mediators involved in communication to the mitochondria in apoptosis signaling induced by death receptors as well as DNA
damaging anti-cancer regimens. Jurkat T leukemic cells were mainly used as model system, because these cells are sensitive to multiple apoptotic stimuli, including death receptor stimulation and treatment with DNA damaging anti-cancer regimens like etoposide and γ radiation. Previously, our lab has generated CD95-resistant Jurkat variant clones, which fail to release Cyt c and as a consequence do not succeed to undergo apoptosis. These variant clones proved cross-resistant to apoptosis induction by etoposide and γ radiation as a consequence of lack of Cyt c release, indicating the presence of common aspects in apoptosis signaling by CD95, etoposide and γ radiation. Therefore, we also investigated at which level the mitochondrial activation was blocked and what the common and distinct aspects are in these pathways. Interestingly, TRAIL-induced Cyt c release and apoptosis signaling are still operational in the variant clones. In chapter 2, the communication to mitochondria is investigated in death receptor signaling and the important mediators for TRAIL-receptor and CD95 signaling are described. The pathways diverge downstream of tBid. Mitochondrial apoptosis signaling in the variant cells is blocked downstream of tBid but upstream of the mitochondria. Therefore, we subsequently looked for Bid interacting proteins to search for potential inhibitors (chapter 3). We identified Bfl-1 as a strongBid-interacting and inhibitory protein, but it appeared not to be the factor responsible for the common inhibition in the variant clones. However, its mechanism of action provided insight into a general mechanism of mitochondrial regulation by Bcl-2 family members. Next, we examined apoptosis signaling to the mitochondria in response to DNA damaging anti-cancer regimens and show the importance of aspartate-cleaved Bid as key mediator of the apoptotic response (chapter 4). As a result, this clarifies the cross-resistance in the variant clones. The work described in chapter 5 started with the observation that the p53 Drosophila target Reaper could induce Cyt c release in Xenopus egg extracts via an unknown mechanism. We investigated how Reaper induced apoptosis in mammalian cells and established that this occurs independently of the mitochondria via a novel described mechanism for Reaper. Finally, the results of all chapters are summarized and potential future perspectives are discussed in chapter 6.
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