Molecular regulation of death receptor- and DNA damage-induced apoptosis

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Chapter 7

Summarizing Discussion

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Promoting apoptosis is an effective strategy to kill tumor cells. Because of different aberrations acquired during oncogenesis, tumor cells are often more prone to die via apoptosis than normal untransformed cells [1]. Defects in apoptosis pathways can however limit the therapeutic efficacy of anti-cancer regimens [2]. The research presented in this thesis focused on elucidating different aspects of the molecular regulation of the apoptotic responses to death receptor ligands and conventional anti-cancer regimens, with the aim to 1) identify novel biomarkers of resistance and 2) aid the development of new cancer therapeutics that can break resistance and enhance their effects.

Molecular regulation of death receptor- and DNA damage-induced apoptosis

Conventional chemotherapeutics induce apoptosis via the intrinsic pathway, which entirely relies on the mitochondria. They activate specific BH3-only proteins, which promote mitochondrial outer membrane permeabilization and subsequent activation of the effector Caspases [3]. Death receptor ligands activate the extrinsic pathway of apoptosis by engaging their pro-apoptotic receptors and activating Caspase-8/-10. Upon activation, Caspase-8/-10 can directly activate the effector Caspases, but also activate BH3-only protein Bid to engage the mitochondrial pathway for Caspase activation [4]. Currently, cells are distinguished into two types on basis of their dependence on the mitochondrial apoptosis pathway for the response to death receptor ligands: Type II cells rely on the mitochondria, whereas Type I cells are believed die entirely independently of them [5]. Based on our findings in **chapter 2**, we propose that cells should not simply be categorized as either Type I or Type II cells as in fact all cell types, to varying extent, require the mitochondria for elimination by death receptor agonists. Importantly, the distinction between Type I and Type II cells has been made on basis of short-term apoptosis assays. Cells were shown to be either resistant to Fas ligand upon Bcl-2 overexpression (Type II), or did not appear to be affected (Type I) [5]. We show that, when clonogenicity is taken into account, Type I cells also require the mitochondria for an optimal response to death receptor agonists. A significant proportion of cells of different classical Type I cell types was found to be clonogenically resistant to TRAIL upon Bcl-2 overexpression or Bid RNAi. This partial resistance to TRAIL was reflected in a reduced efficiency to activate effector Caspases.

Qualitative differences between Type I and Type II cells have been suggested to underlie their differential dependence on the mitochondria. Whereas Type II cells were unable to robustly activate Caspase-8/-10 and depended on the mitochondrial pathway of effector Caspase activation, Type I cells could activate sufficient amounts of Caspase-8/-10 to directly activate the effector Caspases and bypass the mitochondria [5]. Differences in gene expression profiles have also been reported, with Type I cells expressing mesenchymal-like genes and Type II cells expressing epithelium-like markers [6]. There are several indications however that the cellular differences in mitochondrial reliance for death receptor-induced apoptosis are not qualitative in nature, but rather quantitative, depending on the level of expression of pro- and anti-apoptotic proteins. Protein levels of XIAP, which inhibits partially activated effector Caspases, appear to be important in this respect. Recently, Jost et al. have shown that deletion of XIAP converts the typical Type II hepatocytes into Type I cells [7]. In support, another study showed that XIAP strongly regulates effector Caspase activation in Type II cells. Corresponding with their Type II nature, Hela cells overexpressing Bcl-2 were resistant to TRAIL-induced apoptosis. Downregulation of XIAP by RNAi rendered these cells independent of the mitochondria, allowing efficient killing by TRAIL [8]. We found that XIAP is also a bottleneck in Type I cells for death receptor-induced apoptosis. Mitochondrial release of Smac/DIABLO and XIAP inhibition were key for optimal killing of Type I MCF-7 cells by

TRAIL. RNAi-mediated XIAP downregulation re-sensitized resistant Bcl-2-expressing Type I cells to TRAIL. Expression of a cytosolic variant of Smac/DIABLO (ΔSmac) and treatment with the mimetic LBW242 restored TRAIL sensitivity of resistant Bid RNAi MCF-7 cells more efficiently, with LBW242 restoring TRAIL-induced apoptosis to the extent observed in control cells. This suggests that targeting of other IAP family members than XIAP by Smac/DIABLO is essential. CIAP1 and cIAP2, which regulate NF-κB activation at the DISC, are likely targets. Indeed, Smac/DIABLO mimetic Compound A has been shown to target cIAP-1 and hereby induce NF-κB activation and TNFα-dependent apoptosis [9].

Our findings prove that individual cells within a population differ with respect to their dependence of the mitochondria for the response to death receptor ligands. In support, Spencer et al. have recently demonstrated that the heterogeneity that exists in cancer cell populations also causes great cell-to-cell variability in the timing and probability of apoptotic execution by TRAIL [10]. We found that, within a Type I cell population, mitochondria-dependent cells exhibited very low Caspase-3 expression, which translated in a high XIAP:Caspase-3 ratio. This is in agreement with XIAP being an important regulator of death receptor-induced apoptosis and arrest of clonogenicity.

Interestingly, we found that Caspase-9 is not required for TRAIL-induced apoptosis. Unlike Bcl-2 overexpression and Bid RNAi, expression of dominant-negative Caspase-9 did not mediate any clonogenic resistance to TRAIL in Type I MCF-7 cells. In support, Shawgo et al. [11] have recently shown that Caspase-9 is dispensable for the response to Fas ligand in Type II Jurkat cells. Thus, although Caspase-9 is absolutely essential for the initial Caspase-3 cleavage via the intrinsic apoptosis pathway (e.g. mediated by DNA damage), it is irrelevant for this purpose during death receptor-induced apoptosis. Caspase-8/-10 is/are apparently the only inducer Caspase(s) required for Caspase-3 cleavage in response to death receptor ligands.

Our findings have clinical implications for treatment of both Type I and Type II tumor cells with death receptor agonists such as TRAIL. Cells with a block in the mitochondrial pathway, e.g. through elevated expression of Bcl-2 and/or XIAP, are expected to evade treatment regardless of their Type I or Type II classification. Our study shows that combined treatment with Smac/DIABLO mimetic is required to kill these cells.

Bid plays an important role in death receptor-induced apoptosis, as it connects the extrinsic pathway with the mitochondrial route for effector Caspase activation. Bid activation and activity can be regulated through different post-translational modifications, which affects the response to death receptor agonists particularly in Type II-like cells. Phosphorylation of full-length Bid by Casein Kinases inhibits cleavage by Caspase-8 in the unstructured loop and prevents the formation of an N-terminal fragment (tBid-N) and the active fragment tBid-C [12]. When tBid-C is generated, a Glycine is exposed that can be myristoylated, facilitating targeting to the mitochondrial membrane [13]. In **chapter 3**, we reveal an additional mechanism of regulation of tBid-C activity [14]. We show that, following Bid cleavage by Caspase-8, tBid-N needs to be ubiquitinated and degraded by the proteasome to allow tBid-C to expose its pro-apoptotic BH3 domain and mediate mitochondrial permeabilization. We found that the ubiquitination of tBid-N is unconventional, as the acceptor sites are neither lysine nor the N-terminus. Instead, we found that serines, cysteines and threonines in the first 30 amino acids of tBid-N were the main acceptor residues. Conjugation to cysteine residues is not unusual, as Ubiquitin is transferred to ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzymes this way. However, cysteine ubiquitination of non-E2 or E3 proteins has only been reported once before. The cytoplasmic tail of major histocompatibility complex I (MHC-I) was shown to be ubiquitinated on cysteines by the herpes-virus E3 ligase MIR1

[15]. Ubiquitination of serines and threonines is less common but has also been reported. The cytoplasmic tail of MHC-I has also been found to be ubiquitinated on serines and threonines by the herpes-virus E3 ligase mK3 [16]. Our findings are the first to implicate a mammalian (human) machinery in such unconventional ubiquitination and the search for the responsible ligase, associated ubiquitin conjugation enzymes as well as possible deubiquitination enzymes is ongoing in our laboratory. Several other Bcl-2 proteins are also regulated by ubiquitination and proteasomal degradation, such as Bcl-2 [17], Mcl-1 [18] and Bfl-1 [19], Bax [20], Bim [21], Bik [22] and tBid-C [23]. However, as is generally the case, ubiquitination and proteasomal degradation negatively regulate their activity. In contrast, degradation of tBid-N represents a form of positive regulation; it promotes the pro-apoptotic activity of Bid.

In **chapter 4**, we unambiguously demonstrate that Bid can also be an important mediator of the apoptotic response to DNA damage. Participation of Bid in this response has been heavily debated. Whereas several studies showed Bid to be required for DNA damage-induced apoptosis [24-26], others found Bid to be irrelevant [27,28]. We believe our study can reconcile these discrepant findings, as we found that the cellular p53 status dictates whether Bid is required for DNA damage-induced apoptosis. Whereas p53-proficient cells required Bid for IR- but not etoposide-induced apoptosis, p53-deficient cells required Bid for both responses. The differences observed in previous studies in dependence on Bid for DNA damage-induced apoptosis might be caused by the use of different immortalization procedures for MEF transformation and their differential effects on p53 activity. Bid was found to be important for DNA damage-induced apoptosis in both SV40- and hTERT-immortalized MEF [24], but not in E1a/Ras-immortalized MEF [27]. Immortalization with the SV40 genome is known to be mediated by the direct inhibitory effect of large T antigen on p53 activity [29]. Whereas hTERT mainly promotes cell survival by stabilizing chromosomal ends through extension of the telomeric repeat sequences, it has also been shown to inhibit p53-dependent apoptosis [30]. A decreased ability to activate p53 and its target BH3-only proteins Puma and Noxa might render SV40- and hTERT-immortalized MEF more dependent on Bid for DNA-damage induced apoptosis than otherwise transformed cells. This is however speculation and should be properly tested. To further implicate Bid in the apoptotic response to DNA damage in p53-deficient cells, $Bid^{-/-}$ and $p53^{-/-}$ mice should be crossed and their cells should be examined for their responses to etoposide and IR. We expect that Bid;$p53^{-/-}$ cells are more resistant to these agents than $p53^{-/-}$ cells.

As a mediator of DNA damage-induced apoptosis, Bid might also function as a tumor suppressor. In support, $Bid^{-/-}$ mice have been shown to be predisposed to development of a disorder resembling myeloid leukemia, manifesting from the age of 12 months. This finding has however been challenged by a recent study, in which no abnormalities were observed in $Bid^{-/-}$ mice in the 24-month period studied [27]. Additional studies should reveal whether loss of Bid can contribute to tumorigenesis. Based on our findings, we believe that $Bid^{-/-}p53^{-/-}$ mice might be particularly prone to tumor development, even more so than $p53^{-/-}$ mice.

During death receptor-induced apoptosis, Bid is activated through cleavage by Caspase-8/-10 in its unstructured loop and, as shown in **chapter 3**, subsequent ubiquitination and proteasomal degradation of the generated tBid-N fragment. We found that Bid is activated differently during DNA damage-induced apoptosis (**chapter 4**). Mutation of all known cleavage sites in the unstructured loop and even complete deletion of the loop did not affect Bid activity, implying that full-length Bid might induce apoptosis. Mutational analysis and Bcl-2 over-expression experiments indicated that Bid needs to expose its BH3 domain to mediate DNA damage-induced apoptosis. How full-length Bid would be able to do so is currently unclear. Post-translational modification of Bid might be required to induce a conformational

change, which frees the BH3 domain from the structural constraint of the N-terminal domain. Within the N-terminal domain, helix 1 or 2 might be targeted. Interestingly, a single mutation of leucine 35 within helix 2 of mouse Bid has been shown to be sufficient to break the interaction between the N-terminus and the BH3 domain and to activate full-length Bid to the same extent as tBid-C [31]. Phosphorylation or ubiquitination of a residue within helix 1 or 2 might have the same effect. Possibly, Serine 27 (S27) in helix 1 in mouse Bid (S29 in human Bid) could be a target of phosphorylation for this purpose. It would be interesting to test whether mutation of S27 to a non-phosphorylatable residue can render Bid inactive in DNA damage-induced apoptosis. Vice versa, alteration of S27 into an aspartate residue, which can mimic phosphorylation, might activate full-length Bid.

Combination treatments with death receptor agonists

Blockades in the intrinsic apoptosis pathway in tumor cells, e.g. imposed by defective p53 or elevated Bcl-2 expression, cause resistance to different anti-cancer regimens. Combining these regimens with death receptor ligands appears to be an effective approach to break resistance, as both the intrinsic and extrinsic apoptosis pathways are hereby activated. Blockades in the intrinsic apoptosis pathway might hereby be bypassed and additive or even synergistic responses might be obtained. Indeed, we show in **chapter 5** that a new formulation of Fas ligand, APO010, synergizes with different conventional anti-cancer regimens in killing p53-mutant Bcl-2 overexpressing cells, which are insensitive to the individual treatments [32]. Wherever tested, the combined effects observed did not involve breakage of mitochondrial resistance imposed by Bcl-2. Instead, in Jurkat cells we found that etoposide, ionizing radiation, UV radiation, HDAC inhibitors trichostatin A (TSA) and valproic acid (VPA) and proteasome inhibitors MG132 and lactacystin commonly downmodulated both c-FLIP$_S$ and c-FLIP$_L$ levels and hereby mediated synergy with APO010. C-FLIP$_L$ can both promote and inhibit Caspase-8 activation, depending on the levels recruited to the DISC [33,34]. However, as supported by RNAi, we show that downregulation of both c-FLIP$_S$ and c-FLIP$_L$ strongly sensitizes to Fas ligand-induced apoptosis. As observed with etoposide, c-FLIP downregulation most likely allowed enhanced activation of Caspase-8 at the DISC. All regimens hereby enabled direct activation of the effector Caspases in response to APO010, allowing a bypass of the mitochondria and thus mediating a switch from a Type II to a Type I phenotype. In HCT-15 cells, both UV and VPA mediated downmodulation of c-FLIP$_S$ and c-FLIP$_L$, which correlated with enhanced sensitivity to APO010. Etoposide also enhanced the response to APO010 but did not downregulate c-FLIP levels, indicating that it can also sensitize by a different mechanism. Thus, c-FLIP downregulation is an important, but not the only, mechanism through which various conventional anti-cancer regimens can sensitize to APO010 and mediate a conversion from a Type II to a Type I phenotype. In line with our results, different other studies have shown that downregulation of c-FLIP can sensitize tumor cells to death receptor ligands; it has been documented for e.g. neuroblastoma cells [35], prostate cancer cells [36] and Hodgkin/Reed-Sternberg cells [37]. Also, 5-FU has been shown to downregulate c-FLIP levels in the DISC and enhance the response to TRAIL [38,39].

In etoposide-treated cells, inhibition of JNK restored c-FLIP levels and blocked sensitization to APO010. This is in line with the finding that JNK mediates c-FLIP downregulation by activating its E3 ubiquitin ligase Itch [40] and with an earlier report that showed that inhibition of JNK also reverses etoposide-mediated sensitization to TRAIL [41]. JNK inhibition could not restore c-FLIP levels in cells exposed to ionizing radiation, UV radiation, TSA, VPA, MG132 and lactacystin, indicating that these stimuli mediate c-FLIP downregulation in a JNK-independent manner.

Although we see that pre-treatment with the

different conventional anti-cancer therapeutics tested allows for effective killing of Bcl-2-overexpressing Type II cells by APO010, we find that we do not reach the apoptosis levels obtained with APO010 alone in control cells. In view of our results presented in **chapter 2**, we therefore predict that a mitochondrial block imposed by Bcl-2 will enhance clonogenicity of the treated cells. If so, additional treatment with Smac/DIABLO mimetic might be required to obtain therapeutic responses.

We have recently tested the potential of APO010 as an anti-cancer therapeutic *in vivo* in combination with radiotherapy. Unfortunately, although *in vitro* results were promising, APO010 could not enhance the anti-tumor effect of radiotherapy *in vivo* at doses approximating maximal tolerable levels [42]. Very recently, Chen et al. have published that, at physiological levels, Fas ligand does not induce apoptosis but activates pro-survival pathways and promotes rather than antagonizes tumor growth [43]. Complete loss of Fas receptor in mouse models of ovarian and liver cancer reduced cancer incidence and tumor size *in vivo*. Moreover, different types of cancer cells were shown to be dependent on constitutive Fas activity for optimal growth *in vitro*. This means that Fas ligand and formulations based on it might not be suitable for anti-cancer therapy.

Discovery of SRP72 as a regulator of TRAIL-induced apoptosis

Death receptor ligands TNFα, Fas ligand and TRAIL can commonly induce apoptosis independently of the p53 status of cells, which makes them interesting candidates for cancer therapy. However, TRAIL has the unique ability to selectively kill tumor cells and leave normal untransformed cells unharmed. This feature has particularly sparked the interest in TRAIL as an anti-cancer therapeutic. Promisingly, we previously found that TRAIL, unlike APO010, strongly synergizes with radiotherapy in killing tumor cells both *in vitro* and *in vivo*, without causing any toxicity [44,45]. Several clinical trials are currently being performed with TRAIL and agonistic antibodies to its receptors DR4 and DR5, alone and in combination with different conventional therapeutics [46].

We are beginning to understand why tumor cells are more sensitive to TRAIL than untransformed cells. A recent study showed that the enhanced expression of O-glycosyltransferase GALNT14 correlates with TRAIL sensitivity of different tumor cell types. O-glycosylation of DR4 and DR5 in the Golgi apparatus was found to be required for their clustering at the plasma membrane and for induction of TRAIL-induced apoptosis [47]. To search for additional molecules regulating TRAIL sensitivity, we performed a large loss-of-function RNAi screen in breast carcinoma MCF-7 cells. Hereby, we identified signal recognition particle 72 (SRP72) as a critical factor for TRAIL-induced apoptosis (**chapter 6**). SRP72 is one of 6 protein subunits that, together with single RNA subunit, constitute the signal recognition particle (SRP). As a complex, the SRP binds to the signal peptide of newly synthesized secretory and membrane proteins and regulates their import into the endoplasmic reticulum, hereby controlling their secretion and membrane transport, respectively [48]. Interestingly, SRP72 has been previously identified with a siRNA-based RNAi screen as a specific regulator of DR4-mediated apoptosis in both cervical carcinoma Hela cells and colon carcinoma HCT15 cells. SRP72 depletion by RNAi was shown to cause resistance to an agonistic antibody to DR4 by mediating a strong reduction of DR4 cell surface levels. In contrast, DR5 cell surface levels were unaffected, as was the response to a DR5-specific antibody [49]. In agreement, we observed that SRP72 RNAi renders breast carcinoma MCF-7 cells resistant to TRAIL-induced apoptosis and clonogenic elimination. Although we also observed selective downregulation of DR4 cell surface levels in SRP72 RNAi MCF-7 cells, we found that this was not the cause of TRAIL resistance. By individually silencing DR4 and DR5 expression by RNAi, we found that MCF-7 cells died almost exclusively via DR5 in response to TRAIL. This indicated that SRP72 RNAi mediates resistance to TRAIL in MCF-7 cells by inhibiting DR5-mediated apoptosis. Further analysis suggested

that DR5 DISC formation was impeded in SRP72 deficient cells. A molecular explanation for this observation has not yet been found. Perhaps, transport of O-glycosyltranferases to the Golgi apparatus is also disturbed in SRP72 deficient cells. This would indirectly mediate a reduction in DR5 O-glycosylation and hamper its ability to cluster and form the DISC. Why SRP72 deficiency specifically abrogates membrane transport of DR4 is not clear either. A higher affinity of the SRP for the DR5 signal peptide than for that of DR4 might be an explanation. If so, switching the signal peptides of DR4 and DR5 should restore DR4 membrane transport and impede that of DR5. Interestingly, we found that SRP72 specifically regulates TRAIL-induced apoptosis; SRP72 deficiency blocked the response to TRAIL but not to TNFα and Fas ligand. SRP72 might thus be a specific biomarker for TRAIL sensitivity. In addition, we noticed that many different tumor cell types display elevated SRP72 expression. This might imply that enhanced SRP72 expression in tumor cells is a cause of their TRAIL sensitivity.

Outlook

The research presented in this thesis has focused on inducing apoptosis in tumor cells to limit their clonogenicity. Conventional anti-cancer therapeutics are often very successful in limiting clonogenicity of tumor cells by inducing cell death, (irreversible) cell cycle arrest or mitotic catastrophe. A question that therefore often arises is: "Why try to kill tumor cells via apoptosis when existing therapeutics can be very effective in other ways?" Many new discoveries in the field of apoptosis research in the past two decades have helped to answer this question and it is now clear that there are several advantages of killing tumor cells via apoptosis [2]. First, induction of cell death is preferable to mediation of cell cycle arrest, as cells are eliminated and are unable to contribute to tumor relapse. Second, apoptosis is preferable to necrosis, as it prevents the release of toxic cellular content into surrounding tissue and hereby avoids induction of a damaging inflammatory response. Third, apoptosis-inducing agents, such as TRAIL, can be very tumor cell specific and mediate much less toxicity to normal tissue than conventional therapeutics. In addition, TRAIL can kill tumor cells independently of the p53 status, which strongly affects the response to chemotherapeutics. Finally, unlike chemotherapeutics, many apoptosis inducing agents are not mutagenic and will thus not contribute to potential malignant transformation of healthy cells.

Understandably, resistance to apoptosis will limit the potential of apoptosis-inducing therapeutics. In this thesis, we have shown that defects in Bid activation [14] and mitochondrial permeabilization [32,50] can cause resistance to death receptor ligands and DNA damaging regimens. However, we also showed that such blockades can be circumvented by combining these agents with conventional or new anti-cancer therapeutics [32,50]. We found that tumor cells with a mitochondrial block, regardless of the Type I or Type II classification, develop clonogenic resistance to TRAIL but can be effectively re-sensitized through co-treatment with Smac/DIABLO mimetic. In addition, we showed that different anti-cancer regimens sensitized Bcl-2 overexpressing Type II Jurkat cells to APO010, by mediating c-FLIP downregulation, and hereby converted these cells into 'Type I like' cells. Because Fas has recently been discovered to promote tumor growth at physiological levels and because it mediates dose-limiting liver toxicity [43,51,52], APO010 will probably not be suitable for anti-cancer therapy. Rather, combining these stimuli with TRAIL has greater potential. However, to obtain therapeutic levels of cell death, such treatments should in addition be combined with a Smac/DIABLO mimetic.

A combination of TRAIL with BH3 mimetic ABT-737, which neutralizes anti-apoptotic Bcl-2 proteins and hereby lowers the threshold for mitochondrial pemeabilization, might also be an effective approach to sensitize tumor cells to TRAIL. However, ABT-737 has a limited binding profile similar to that of Bad, targeting only Bcl-2, Bcl-x_L and Bcl-w and not A1 and

Mcl-1 [53]. Cells overexpressing A1 or Mcl-1 have been shown to be resistant to ABT-737 [54]. Hence, the challenge is now to develop a BH3 mimetic that mimics the BH3 domain of e.g. Bid and targets all 5 anti-apoptotic Bcl-2 proteins. Such a BH3 mimetic might be even more effective than Smac/DIABLO mimetics, as it allows for the release of all mitochondrial factors, not just of Smac/DIABLO.

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