Combining radiotherapy with death ligands in cancer treatment: feasibility and molecular mechanisms
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General Discussion
Combined modality treatment with radiotherapy and death ligands: clinical prospects

Improving the therapeutic efficacy of radiotherapy with death receptor agonists such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) or APO010 (MegaFas Ligand, [1]) is an elegant and rational strategy, since these regimens induce (partially) different routes to cell death. Depending on the type of malignancy, radiotherapy mainly reduces clonogenicity of tumor cells by inducing (irreversible) cell cycle arrest and non-apoptotic cell death and only partially by inducing (mitochondrion-mediated) apoptotic cell death. Therefore, a therapeutic window to reduce clonogenic survival of tumor cells exists by combining radiotherapy with death receptor agonists that specifically induce apoptosis - via both mitochondrion-dependent and -independent routes. The data presented in this thesis suggest that the combined treatment with radiotherapy and TRAIL (Chapter 2) holds clinical promise. We observed a strong combined effect, both in vitro and in vivo of the treatment in a p53 mutant, Bcl-2 overexpressing lymphoid malignancy (Jurkat-Bcl-2), which represented a clinically challenging situation. For APO010 (Chapter 4), we observed a strong combined effect of the combination in vitro in a wide range of tumor cell lines tested (from both solid and hematolical origin). However, we encountered dose-limiting systemic toxicity of APO010 in the absence of a therapeutic effect of the combined treatment in vivo.

Radiotherapy and TRAIL receptor agonists: prospects

The selective capacity of TRAIL to induce apoptosis in tumor cells, while sparing cancer cells [2, 3] has motivated extensive pre-clinical research efforts. At present, TRAIL and agonistic antibodies targeting TRAIL-R1 and TRAIL-R2 have advanced to phase II clinical trials, and therefore toxicity of the monotherapy is now well established (reviewed in [4]). In addition, a number of phase I and II trials combining conventional chemotherapeutics and TRAIL receptor agonists have been completed, showing promising anti-tumor activity in a range of hematological and solid tumors, but reaching dose-limiting toxicity (reviewed in [4]). Phase I clinical studies combining radiotherapy and TRAIL receptor agonists have not yet been reported. Due to the local effect of radiotherapy on the tumor, the combined effect of radiotherapy and systemically administered death receptor agonists will only be reached at the tumor site. This potentially increases the therapeutic window when compared to systemically administered chemotherapeutics.

The results presented in this thesis, using a p53-mutant, Bcl-2 overexpressing hematological malignancy - as a prototype of challenging clinical situation - demonstrated a clear combined effect in vitro, in the absence of systemic toxicity (Chapter 2). In addition, in xenograft models of (solid) breast tumors, combined treatment with radiotherapy and TRAIL [5, 6] or TRAIL receptor mAbs [7] also demonstrated anti-tumor efficacy, with differential importance for p53 in the observed combined effects. Collectively, these results indicate that combined treatment with TRAIL and radiotherapy is a promising strategy to pursue in a phase I clinical trial in the near future.

Radiotherapy and TRAIL receptor agonists: which agonist to select in terms of efficacy and toxicity?

Prior to a phase I clinical trial using radiotherapy and TRAIL receptor agonists, a careful decision must be made as to which TRAIL receptor agonist will be used. There are both advantages and disadvantages associated with the use different recombinant human (rh) TRAIL formulations or agonistic monoclonal antibodies (mAbs) targeting TRAIL-R1 or TRAIL-R2. Compared to TRAIL-R1 or TRAIL-R2 agonistic mAbs, untagged rhTRAIL (Genentech/Amgen) has a relatively short half-life (~32 min) in cynomolgus monkeys [2], requiring repeated administration to maintain a therapeutic plasma concentration. Native TRAIL has been evaluated in a phase II clinical trial as monotherapy as well as in combination with rituximab (reviewed in [4]), but this TRAIL formulation may not achieve the highest therapeutic effects. Preclinical data (including our own, not shown) indicate that TRAIL cross-linking increases agonistic activity to many tumor cell lines, presumably by the ability to cross-link multiple receptors by the same molecule. Therefore, multimerized TRAIL formulations (e.g. isoleucine-zippered TRAIL [8]) may prove to be more effective in a clinical situation and their (pre-)clinical development should be further evaluated.

As TRAIL targets both TRAIL-R1 and TRAIL-R2, one functional death receptor on tumor cells may suffice to respond to TRAIL treatment. The choice to use either TRAIL-R1 or TRAIL-R2 antibodies in patients should be carefully considered and to be used only to treat those tumors that have functional expression of the relevant death receptor. However, expression of TRAIL receptors per se does not imply tumor sensitivity to TRAIL, which makes it difficult to predict how tumor cells will respond to (combined) treatment.

As there has not (yet) been a method reported that identifies whether tumor cells will be sensitive to TRAIL or to combined treatment with radiotherapy, we emphasize it would be most informative to test primary tumor material (both of hematological and solid origin) for treatment sensitivity ex vivo as an indication for tumor sensitivity (and patient selection) to combined treatment regimens.

Normal tissue toxicity has been investigated intensively for rhTRAIL and much less for the agonistic TRAIL receptor mAbs. TRAIL was shown to selectively kill tumor cells and leave normal cells unharmed. Therefore
toxicity accompanying TRAIL receptor antibody therapy should be carefully monitored in ongoing and future clinical trials. The mechanism, by which normal cells are protected from TRAIL-induced apoptosis, is not entirely understood, since TRAIL death receptors are expressed on a wide range of tumor types, but also normal tissue [9, 10]. It was postulated that TRAIL decoy receptors present on normal cells could prevent TRAIL-induced toxicity; however no correlation was found between the expression of decoy receptors and TRAIL sensitivity [11, 12].

Toxicity data of combined treatment with (conventional) chemotherapeutics and TRAIL receptor agonists from in vivo tumor models are mostly obtained by application of human TRAIL receptor agonists in mice. Since TRAIL shows a certain degree of species specificity (e.g. Figure 2B Introduction, [3, 13]), these experimental approaches will be of limited predictive value for toxicity induced by the combined treatment in clinical trials. Therefore, we wanted to evaluate the feasibility and toxicity of combined treatment with radiotherapy and mouse TRAIL in mouse models of spontaneous tumorigenesis. However, almost all of the primary cell lines derived from these tumors (including breast, mesothelioma and lung) were resistant to apoptosis induction by either human or mouse TRAIL, even in combination with radiotherapy in vitro. Although the primary tumor may respond differently than its cell line derivatives, the lack of a batch of mouse TRAIL to be used in vivo at the time prevented us from initiating these studies.

Toxicity data from TRAIL receptor agonist monotherapy (as well as combined treatment with a variety of (conventional) chemotherapeutics) is accumulating from ongoing and completed clinical trials. Although toxicity should be monitored carefully and continuously, additional TRAIL receptor agonist-mediated toxicity is expected to be limited (but can at present not be rationally predicted) in a combined setting with locally applied radiotherapy.

Radiotherapy and TRAIL receptor agonists: potential limitations and solutions

One potential limitation of the combined treatment with radiotherapy and death ligands in the treatment of cancer is the elimination of metastases. Although an enhanced combined effect of the treatment is expected at the primary tumor site, this effect will not be manifest in metastasized tumors.

Engaging the immune system in combination treatment with TRAIL and conventional therapy presents itself as an interesting option. The idea is that antigens derived from tumor cells killed by TRAIL can be presented to the immune system. Pre-clinical studies using anti-TRAIL receptor antibodies in combination with immunostimulatory antibodies in mice showed promising therapeutic responses [14]. Here, professional antigen presenting cells (APCs) were boosted by anti-CD40 to endocytose and present antigens derived from tumor cells that had undergone apoptosis in response to anti-TRAIL receptor antibodies. Cytotoxic T-cells, in turn recognized tumor antigens, and (also aided by mAb to the costimulatory receptor CD137) eliminated tumor masses. In addition, immunological memory was formed and upon subsequent rechallenge, tumors were eliminated. As many tumors are resistant to TRAIL-induced apoptosis, or present only self-antigens, this strategy may not always be successful. It has recently been documented that radiotherapy modulates the peptide repertoire of targeted (tumor) cells [15]. Therefore, radiotherapy could potentially induce a tumor-specific immune response by (1) specifically killing tumor cells, allowing for enhanced presentation of tumor-antigens, leading to an anti-tumor immune response that also eliminates metastases and (2) by inducing presentation of alternative peptides in irradiated tumor cells, which could evoke a stronger immune response to the primary tumor. This concept should be addressed in appropriate tumor models and be accompanied by careful monitoring for any signs of auto-immunity.

Radiotherapy and CD95/Fas agonists: concluding remarks

Combined effects using radiotherapy and TRAIL in pre-clinical animal models are present in a range of hematological and solid tumor cell lines that also include a mutant p53 status and Bcl-2 overexpression. In addition, the first therapy responses of patients treated with TRAIL receptor agonists are also encouraging, with acceptable normal tissue toxicity. It will therefore be of interest to initiate a clinical trial combining TRAIL (or TRAIL receptor agonistic antibodies) with radiotherapy in the near future. These studies will also shed light on the tumor types that respond best to combined treatment that could in turn be (further) exploited to identify the molecular mechanisms underlying combined treatment responses.

Radiotherapy and CD95/Fas agonists: efficacy, limitations and future prospects

Although CD95/Fas agonists have not been pursued for clinical development due to lethal hepatotoxicity in mice treated with anti-CD95 antibodies [16], APO010 has now entered a phase I clinical trial to test safety (ClinicalTrials.gov identifier: NCT00437736). We reasoned that there was a window of opportunity for us to test the combination of radiotherapy and APO010. As radiotherapy and APO010 had a strong combined effect in vitro, we expected to sensitize tumor cells by radiotherapy to such low doses of APO010 that normal tissue toxicity would be acceptable. However, in the model systems used, APO010 reached dose-limiting systemic toxicity in the absence of a therapeutic effect (Chapter 4). In an intraperitoneal (i.p.) ovarian tumor mouse xenotransplant model, a combined therapeutic effect with APO010 and cisplatin was observed, which
correlated with synergistic apoptosis induction in vitro [17]. In this study, APO010 was injected i.p. at the site of the tumor. Although the half-life of APO010 in plasma was short, it reached a maximum of ~80 ng/ml within 2 h after i.p. injection [17]. It is therefore conceivable that the effective dose at the tumor site in that case was many fold higher than the IC_{50} for the same cells, as determined by in vitro apoptosis assays (5 ng/ml). In our case (Chapter 4), the tumors were implanted subcutaneously or intra-thoracically, while APO010 was applied i.p.. Possibly therefore, the APO010 concentrations reached within the tumor were lower than the concentrations that were effective in apoptosis-induction in the combined setting in vitro. Alternatively, CD95-triggering can induce a variety of cellular responses, as also indicated in the introduction, such as inflammation and NF-kB activation in both tumor cells and in cells in the (tumor) microenvironment. These alternative signaling pathways and cross-talk of tumor cells with stromal cells may greatly influence the anti-tumor effect of APO010.

As the maximal tolerable dose of APO010 decreased from 15 µg/kg in rats to 5 µg/kg in monkeys (http://www.nccr-oncology.ch/htdocs/Files/Newsletter_5%20page%20par%20page-FINALE.pdf ), it could be seen that the dose-limiting toxicity in humans using systemically administered APO010 is even lower. Therefore, a combination with systemically administered APO010 is not likely to become a very promising therapeutic strategy. In soft tissue sarcomas, TNFa - which induces severe toxicity when applied systemically - is effective when applied regionally [18]. Therefore future studies should aim at assessing therapeutic efficacy of locoregionally applied APO010, either alone or in combination with radio-/chemotherapy.

Mechanisms underlying responses to combined modality treatment

In order to predict which patients would benefit from (and hence, be selected for) combined treatment with radiotherapy and death receptor agonists, it is essential to understand the molecular mechanisms underlying combined treatment responses. The data presented in this thesis indicate that downregulation of the anti-apoptotic molecule c-FLIP is an important determinant in sensitization to death receptor-induced apoptosis, at least for CD95 (Chapter 5). Not only did (high doses) radiotherapy induce c-FLIP downregulation, a wide range of stimuli that were found to sensitize cells to CD95-mediated apoptosis also downregulated c-FLIP protein levels. Deliberate downregulation of c-FLIP sensitized tumor cells to CD95-mediated apoptosis and (consequently), the sensitizing effect by the different stimuli was largely abrogated. We do not imply that c-FLIP downregulation is the only mechanism by which tumor cells can be sensitized to death receptor agonists, but we and others have shown that it is clearly an important contributor.

Patients with tumors that express high c-FLIP levels may not benefit from death receptor monotherapy in future clinical studies. This suggests that these patients specifically could be assigned to a treatment schedule in which death receptor agonists are combined with radio- and/or chemotherapy for optimal treatment responses.

Similarities and differences for the role of c-FLIP in sensitization to either TRAIL receptor or CD95-mediated apoptosis

In a number of studies c-FLIP downregulation has been implicated in enhanced sensitivity to TRAIL-induced apoptosis (e.g. colon carcinoma cells [19] or hepatocellular carcinoma [20]). However, as we demonstrated in Chapter 3 for an intermediate single dose of ionizing radiation (10 Gy) and published by others for cisplatin and doxorubicin [20], tumor cells were sensitized to TRAIL-induced apoptosis in the absence of c-FLIP downregulation. Common in all studies was the altered ratio of inducer caspases versus c-FLIP in the DISC, which could explain improved inducer caspase activation and subsequent apoptotic execution. We have also observed in Chapter 3 that etoposide could strongly sensitize Jurkat-Bcl-2 cells to TRAIL-induced apoptosis. As tested in Chapter 5, etoposide downregulated c-FLIP levels, and could have contributed to TRAIL sensitization to a large extent. The nature of the mechanisms by which low dose ionizing radiation sensitized Jurkat-Bcl-2 cells to TRAIL can only be speculated upon and should be addressed in future studies. These mechanisms may include altered receptor distribution at the plasma membrane (perhaps into ‘lipid rafts’), allowing for enhanced pro-apoptotic signalling. We have attempted to address this point by disturbing lipid rafts using β-methyl cyclodextrin in Jurkat-Bcl-2 cells (data not shown), but these results are difficult to interpret and still inconclusive, as β-methyl cyclodextrin induces a variety of cellular responses and showed toxicity to our cells in the concentration range used. Other potential mechanisms of radiation-induced TRAIL sensitization may include post-translational modifications of TRAIL-R1 [21], or TRAIL-R2 [11].

We are not the first to show that c-FLIP downregulation sensitized cells to CD95-mediated apoptosis; it has previously been documented for e.g. neuroblasma cells [22], prostate carcinoma cells [23] and Hodgkin/Reed-Sternberg cells [24]. Although c-FLIP can also augment Caspase-8 activation in the DISC [25, 26], we identified c-FLIP downregulation as a common mechanism by which a wide range of stimuli sensitized Jurkat-Bcl-2 cells to CD95-mediated apoptosis (Chapter 5). This downregulation most likely allowed for enhanced activation of inducer caspases by an altered ratio of inducer caspases versus c-FLIP levels in the CD95
death inducing signaling complex (DISC). This in turn, allowed for the activation of effector caspases, without breaking mitochondrial resistance imposed by Bcl-2. The mechanisms of c-FLIP downregulation induced by the different stimuli are probably very different. For HDAC inhibitors and etoposide there is evidence that post-translational mechanisms affect c-FLIP levels (Chapter 5, [27]), whereas c-FLIP downregulation induced by proteasome inhibitors is most likely affected at the level of translation by impaired NF-κB signaling [28, 29].

In a pre-sensitization set-up, a dose of 10 Gy could efficiently sensitize Jurkat-Bcl-2 cells to TRAIL-induced apoptosis, in the absence of c-FLIP downregulation (Chapters 2 and 3). In the same set-up, a single dose of 10 Gy was not sufficient to sensitize Jurkat-Bcl-2 cells to APO010-induced apoptosis (data not shown). However, a single high radiation dose of 30 Gy efficiently sensitized Jurkat-Bcl-2 cells to APO010-induced apoptosis, which was strongly correlated with reduced c-FLIP levels, indicating that in Jurkat-Bcl-2 cells, sensitization to CD95-mediated apoptosis relied stronger on c-FLIP downregulation than did sensitization to TRAIL-induced apoptosis. Collectively, these data suggest that c-FLIP downregulation can greatly facilitate both TRAIL receptor and CD95-mediated apoptosis. However, other mechanisms - that have to be better defined – can also contribute to sensitization to (particularly TRAIL) death receptor agonists.

Regulation of TRAIL receptor membrane expression
A requirement for tumor sensitivity to death receptor agonists is the expression of a functional death receptor on the tumor cell surface. During death receptor agonist therapy, it can be envisioned that tumor cells acquire therapy resistance by downregulating or inactivating death receptors. Therefore, understanding the regulation of death receptor expression at the cell surface may aid to design optimal treatment regimens for cancer patients receiving death receptor-based therapy. In Chapter 6, we have focused on the regulation of TRAIL death receptors and identified a mechanism by which TRAIL-R1 membrane levels are differentially regulated from those of TRAIL-R2. A number of E3 ubiquitin ligases of the MARCH protein family targeted TRAIL-R1 at lysine 273, thereby reducing cell surface levels of TRAIL-R1. A number of MARCH proteins are ubiquitously expressed [30], but in particular MARCH 1 transcript was found to be overexpressed in breast cancer (www.oncomine.org). Apoptosis signaling, death receptor trafficking and cell surface expression appear to be intimately linked. It is conceivable that MARCH overexpression (by mediating TRAIL-R1 downregulation) may confer TRAIL resistance. Future studies will have to elucidate whether (and how) overexpression of MARCH proteins impact on TRAIL sensitivity. Ideally this should be tested in a system which lacks TRAIL-R2 or by using agonistic antibodies to TRAIL-R1, since TRAIL can signal for apoptosis through both TRAIL-R1 and TRAIL-R2 and the impact of the MARCH proteins on TRAIL-R2 downregulation were much less pronounced.

If a correlation can be found between MARCH overexpression and sensitivity to TRAIL receptor agonists, this could potentially be used either to rationally assign patients to death receptor therapy. Alternatively, studies could aim at improving the efficacy of death receptor therapy by interfering with MARCH protein expression.

Concluding remarks
The research presented in this thesis provides new insights in the therapeutic potential and the underlying molecular mechanisms of improving the efficacy of radiotherapy with death receptor agonists. Radiotherapy and APO010 showed a strong combined effect on apoptosis induction and clonogenic survival in vitro, but APO010 could not enhance the anti-tumor effect of radiotherapy in vivo at doses approximating maximal tolerable levels. Radiotherapy and TRAIL however, showed good anti-tumor responses both in vitro and in vivo, without causing toxicity in the model system that we employed. We have identified c-FLIP downregulation as an important mechanism by which tumor cells are sensitized to CD95-mediated apoptosis, not only by ionizing radiation, but by a wide range of (stress) stimuli. In addition, we have identified a novel mechanism by which TRAIL-R1 membrane levels can be regulated.

The results obtained in this thesis imply that the most promising clinical development in combining radiotherapy with death receptor agonists is the combination with radiotherapy and TRAIL (or possibly TRAIL-receptor agonists). These agents have entered phase II clinical trials as monotherapy and therefore toxicity in humans has well been assessed, and could therefore relatively safely be combined with radiotherapy. The underlying molecular mechanisms combined treatment efficacy could (1) provide a new basis for research in aiming to further improving the therapeutic efficacy of death receptor agonists and (2) aid in selecting those patients that are most likely to benefit from combined treatment in a therapeutic setting.
References