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Targeted induction of apoptosis for cancer therapy

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Summary and perspectives

It has become increasingly clear that aberrancies in the cellular apoptotic machinery play an important role in the process of malignant transformation and tumor progression. Intriguingly, cancer cells are often reliant on these aberrancies for continued survival. Therefore, tumor-selective re-activation of apoptosis represents a promising therapeutic strategy, which in this thesis was pursued by tumor-selective delivery and activation of the Death Inducing Ligands TRAIL and FASL.

Both TRAIL and FASL are essential effector molecules that are active on the cell surface of various immune effector cells. Interestingly, TRAIL and FASL, in particular the soluble derivatives thereof, also possess promising tumor-selective activity in their own right. Therefore, both sTRAIL and sFASL are of considerable interest as anti-cancer agents. However, for their clinical use one can anticipate a number of fundamental problems that need to be addressed first. This includes the differential affinities and crosslinking requirements of sTRAIL for the various TRAIL receptors.

Previously, it has been reported that sTRAIL preferentially binds to TRAIL-R2 over TRAIL-R1 due to the respective affinities of ≤ 2 nM versus 70nM for these receptors. From these differential affinities it can be predicted that in a therapeutic setting conventional sTRAIL preferentially binds to TRAIL-R2, whereas TRAIL-R2 is actually rather unresponsive to conventional sTRAIL. Consequently, relatively high doses of conventional sTRAIL are needed to reach the therapeutic threshold for TRAIL-R2 signaling at the site of tumor.

In contrast, the specific binding of scFv:sTRAIL fusion proteins to a pre-selected target antigen results in selective accretion at the cell surface of targeted tumor cells. Consequently, the concentration of fusion protein is locally increased and the respective fusion protein is converted into a membrane-bound molecule. As a result, apoptotic signaling by both TRAIL-R1 and -R2 is efficiently activated.

In addition, it has recently been uncovered that different tumor types preferentially signal apoptosis via either TRAIL-R1 or TRAIL-R2. Solid tumors such as colon and breast carcinoma are predominantly sensitive to activation of apoptosis via TRAIL-R2 signaling. In contrast, hematological malignancies, such as B-cell chronic lymphocytic leukemia, are predominantly sensitive to activation of apoptosis via TRAIL-R1 signaling. Several research groups have generated TRAIL-R1 and/or TRAIL-R2 selective mutants of sTRAIL that show enhanced and selective pro-apoptotic activity towards tumor cells expressing the relevant TRAIL-receptor. Therefore, we have exchanged the wild-type sTRAIL domain for either a TRAIL-R1 or a TRAIL-R2 selective mutant in some of our fusion proteins. Research is currently ongoing to determine their potential increase in selectivity and efficacy on selected tumor types.

An important feature of both scFv:sTRAIL and scFv:sFASL fusion proteins is their so-called anti-tumor bystander activity as discussed in **chapters 4 and 7**. The robust anti-

tumor bystander activity of both scFv:sTRAIL and scFv:sFASL fusion proteins may be of clinical relevance to attack target antigen-negative tumor cells that would otherwise escape from targeted therapy.

Obviously, the choice of the targeted antigen is of the utmost importance to maximize the tumor-selective activity of our fusion proteins. Unfortunately, fully tumor-specific cell surface antigens are very rare and perhaps even non-existent or may be unpractical for clinical use. As a second best option, target antigens should be highly (over)expressed in neoplastic tissues and should preferentially play fundamental roles in the pathogenesis of cancer. Ideally, target antigen activation (e.g. phosphorylation) correlates with its function and signaling can be pharmacologically inhibited. Merely binding of the antibody fragment to this target should already result in an anti-tumor effect and/or should sensitize the targeted cell to TRAIL or FASL-mediated apoptosis.

These notions led us to select human EGFR as a highly promising candidate target antigen for scFv:sTRAIL fusion proteins, as described in **chapter 5**. Indeed, targeted delivery of sTRAIL to the EGFR, using EGFR-blocking antibody fragment scFv425, simultaneously inhibited EGFR-mitogenic signaling, sensitized tumor cells to apoptosis, and activated TRAIL receptor apoptotic signaling. Interestingly, co-treatment of EGFR-positive tumor cells with scFv425:sTRAIL and the EGFR-tyrosine kinase inhibitor Iressa synergistically induced apoptosis, indicating the potential clinical benefit of simultaneous extra- and intra-cellular inhibition of EGFR-signaling.

Of particular interest is EGFRvIII, a mutant form of EGFR that is expressed on the cell surface of glioma and in lung and ovarian carcinoma¹⁻³. Importantly, EGFRvIII is not detectably expressed by normal tissues, including those tissues with high levels of wild-type EGFR expression. We have constructed fusion proteins containing an scFv with high affinity specificity for EGFRvIII. Unfortunately, production of the anti-EGFRvIII:sTRAIL fusion protein in CHO cells yielded high-levels of aggregated fusion protein, unsuitable for EGFRvIII-restricted induction of apoptosis (unpublished data). We are currently trying to resolve this technical problem that is most likely related to improper protein folding of this fusion protein during protein synthesis.

Taken together, when compared to conventional sTRAIL and sFASL both scFv:sTRAIL and scFv:sFASL fusion proteins have strongly improved therapeutic characteristics. The choice for therapeutic application of either molecule may depend on the characteristics and requirements of the particular disease to be treated. These characteristics and requirements need to be investigated in greater detail in order to further tailor and optimize the respective fusion protein for a particular disease.

The pre-clinical data in this thesis provide clear proof of principle for the therapeutic potential

of targeted delivery and local activation of sTRAIL and sFASL for cancer therapy. Obviously, several aspects still need to be addressed in order to determine the clinical feasibility of this approach. An essential step is to determine the safety and efficacy of scFv:sTRAIL and scFv:sFASL fusion proteins in relevant animal models. In this respect, *in vivo* studies are currently being conducted in our lab in which human cell lines are xenografted into nude mice. The cell lines used have been lentivirally equipped with the luciferase gene to allow for *in vivo* real-time monitoring of tumor growth and response to therapy using bioluminescent imaging⁴. Recently, novel fluorescent probes have been developed for real-time *in vivo* imaging of caspase-8 and caspase-3 activation⁵. These probes appear to be ideal to accurately image and measure therapeutic apoptosis induction during treatment with our fusion proteins. Simultaneously, these probes can also be used to monitor the development of unwanted collateral apoptosis induction in normal cells and tissues. Additionally, the tissue distribution and half-life of the respective scFv:sTRAIL/scFv:sFASL fusion proteins needs to be accurately assessed.

Alternative target antigens and targeting domains

As indicated above the choice of the particular target antigen for our fusion proteins may be of eminent importance for tumor cell-restricted delivery and efficient activation of apoptosis. This is exemplified by the specific inhibition of the mitotic signaling activity of EGFR by scFv425:sTRAIL, as delineated in **chapter 5**. This strategy can be extended to other target antigens that are involved in complementary or alternative apoptotic signaling routes. An interesting example of such a target antigen is CD20, which upon cross-linking by the monoclonal antibody Rituximab potently activates apoptosis⁶. Our preliminary experiments indicate that scFvRit:sFASL, a fusion protein comprising a Rituximab-derived scFv antibody fragment genetically fused to sFASL, simultaneously activates CD20- and FAS-apoptotic signaling pathways. The preliminary data indicate that scFvRit:sFASL shows superior apoptotic activity compared to the parental Rituximab antibody.

Sometimes it may be necessary or even beneficial to deliver sTRAIL and sFASL using targeting domains other than those derived from antibodies. This is for instance the case when the encoding cDNAs for the VH and VL domains of a given anti-tumor antibody are not (yet) available or obtainable.

In addition, when the target antigen of interest is a receptor of some sort, it may be useful to deliver sTRAIL or sFASL by exploiting the receptor's natural ligand. An interesting example that we are currently working on is the recently identified ligand of CD7, designated K12⁷. We selected K12 because of its low nanomolar affinity for the CD7 antigen. Furthermore, it is known that CD7 is critically involved in activation of apoptosis

after cross-linking by another potential CD7 ligand, the lectin Galectin-1. We anticipate that our K12:sFASL and K12:sTRAIL fusion proteins display enhanced targeting and pro-apoptotic activity towards CD7-positive tumor cells.

Mammalian antibodies and (recombinant) derivatives thereof have long been the paradigm for targeted delivery of therapeutics. Recently, several alternative and artificial targeting domains coming from (non-)mammalian species have been identified. Repeat proteins such as ankyrin repeat (AR), leucine-rich repeat (LRR), or tetratricopeptide repeat (TPR) proteins are abundant specific binding molecules in nature. They are composed of small structurally homologous units (repeats) that stack to form a repeat domain. These non-globular repeat proteins have been subjected to protein engineering to serve as binding molecules with strongly improved thermostability⁸. Combinatorial libraries of designed ankyrin repeat proteins (DARPin) have been constructed from which candidate (e.g. tumor-selective) DARPins can be selected at unparalleled speed⁹. Importantly, DARPins can be cheaply produced at very high yields in simple prokaryotic organisms.

Overcoming apoptosis resistance

Literature reports that ~50% of tumor cell lines are resistant to TRAIL. Moreover, TRAIL-resistant cells often show cross-resistance to e.g. chemotherapeutics. The concept outlined here for the targeted delivery of sTRAIL and sFASL will obviously fail when the targeted tumor cells are resistant to apoptosis due to one or more defects in Death Receptor-mediated apoptotic signaling. Such resistance can be due to down-regulation or mutational inactivation of initiator caspase-8 or alternatively by epigenetic silencing of agonistic TRAIL-receptors.

To overcome Death Receptor-related resistance issues, the targeted delivery and activation of other effector moieties that possess divergent pro-apoptotic activity is warranted. Of particular interest are those proteins that play an important role in the elimination of aberrant cells by the immune system. In analogy to TRAIL, the intrinsic selectivity of such physiological effector molecules for diseased or aberrant cells can be exploited.

Worth mentioning in this respect are members of the protein family of Galectins¹⁰. Galectins are highly conserved animal lectins with beta-galactoside-binding activity for glycosylated proteins. Galectins play key roles in innate and adaptive immune responses through sugar-dependent and -independent mechanisms. The first identified member, Galectin-1, is expressed in a number of different tumor types and was shown to modulate the tumor immune response by eliminating infiltrating T cells. Reversely, several T-ALL leukemia cell lines are sensitive to apoptosis induction by recombinant Galectin-1. Very recently it was shown that multidrug resistant tumor cells of various origins are sensitive to apoptosis induction by Galectin-1.

Of note, for most activities the physiologically active form of Galectin-1 is a homodimer. Unfortunately, the *in vivo* efficacy of Galectin-1 is limited because at lower concentrations the equilibrium is rapidly shifted towards the inactive monomeric form.

However, this apparently unfavorable feature of Galectin-1 may well be exploited by constructing scFv:Galectin-1 fusion proteins. We hypothesize that for scFv:Galectin-1 sufficient tumor cell accretion will be feasible, whereby the therapeutic apoptotic activity of Galectin-1 will be locally (re)activated.

Concluding remarks

An important 'universal' issue in oncology is the available therapeutic window for selective activation of apoptosis in cancer cells. Although the approach outlined in this thesis shows marked selectivity for cancer cells, single agent therapy might prove not selective enough. Seemingly apoptosis-resistant tumor cells may have highly elevated apoptotic thresholds that can only be lowered by a combinatorial use of various pro-apoptotic agents as discussed in **chapter 2**. Some of the prominent examples are agents that are able to re-activate p53 and those that inhibit up-regulated anti-apoptotic proteins such as BCL-2 and XIAP.

Integration of the various anti-cancer concepts may help to rationally design combinatorial treatment strategies that enhance or restore the sensitivity of tumor cells to apoptosis induction. The most promising combinations will probably involve those drugs that work along different or complementary apoptotic signaling routes with non-overlapping toxicities towards normal cells.

The clinical success of such combinatorial strategies will rely heavily on patient-tailored identification of the respective cancer-related aberrancies. This will require the development of reliable, cost-effective and high-throughput diagnostic tools.

Laser-capture microscopy and DNA-micro array technology make it possible to evaluate large quantities of gene-expression data from individual cancer cells. However, currently it is still difficult to extract meaningful information from these data and to relate them to tumor-specific phenomena. Nevertheless, further improvements in this field are anticipated that might help to identify hitherto unknown routes and mediators of tumor-specific apoptosis induction. In turn, these findings might help to identify new targets for cancer cell-restricted activation of apoptosis.

Finally, the field of glycobiology, an as yet under-investigated area, is starting to yield a wealth of information that can be exploited for specific targeting of cancer cells. Glycosylation is a highly diverse and non-template driven process that can generate enormous informational content. Consequently, glycosylation represents one of the most diverse families of recognition patterns. It has since long been recognized that

cancer cells display aberrant glycosylation. Because of the highly specific interactions of these aberrantly glycosylated proteins with physiological receptors, such as members of the Galectin family, cancer specific glycosylation is a promising target for intervention. Indeed, it can be anticipated that as more insight is gained into the glycobiology of cancer, a plethora of specific targets, targeting moieties, and effector moieties will become available for future therapy.

Taken together, as the molecular aberrations in apoptosis regulation in cancer cells are elucidated, the rational design of combinatorial approaches paves the way towards enhanced and tumor-selective apoptosis induction that will help fight cancer in a clinical setting.

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