Targeted induction of apoptosis for cancer therapy
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Introduction to the thesis
Programmed cell death, known as apoptosis, is an essential cellular homeostasis mechanism that ensures correct development and function of multi-cellular organisms. The pivotal importance of the correct execution of apoptosis is apparent from the many human diseases with aberrancies in apoptosis, including cancer. During cancer development, various imbalances can arise in the apoptotic machinery. Consequently, sensitivity towards apoptosis is progressively reduced, which ultimately leads to inappropriate cell survival and malignant progression. However, it has become clear that cancer cells are often reliant on these aberrancies for continued survival. Perhaps counter intuitively, cancer cells can in fact be more prone to apoptosis than normal cells. The apoptosis-prone phenotype of cancer cells is masked and counterbalanced by upregulation of one or more anti-apoptotic mechanisms. Therefore, it is of enormous therapeutic interest to selectively tip the balance of the cellular fate of cancer cells towards apoptosis.

Indeed, the rational design of novel agents that can selectively induce apoptosis in cancer cells is a rapidly developing field, as exemplified by the plethora of such agents reported in contemporary literature. In chapter 2, a selection of novel rationally designed anti-cancer approaches that target apoptosis-related aberrancies is discussed. The aim of this chapter is to integrate new insights in apoptosis biology with recent progress in the different fields of targeted strategies. This integration of concepts might add to the development of a new therapeutic approach perhaps best described as “Targeted apoptosis induction in cancer”, which is in fact the subject of this thesis.

The next five chapters of this thesis are published reports on our novel therapeutic strategy designed to selectively activate apoptosis in cancer cells by targeted delivery of two prominent members of the TNF-superfamily of Death Inducing Ligands, namely TRAIL and FASL. TRAIL and FASL are essential effector molecules that are involved in the targeted elimination of cancerous and virus-infected cells by T- and NK-cells. T-cells selectively eliminate target cells by MHC-restricted activation of apoptosis, which is commenced only after specific recognition, binding, and co-stimulatory immune activation. Unfortunately, the passive transfer of tumour-specific T-cells in cancer therapy has proven to be extremely difficult. However, soluble forms of the effector molecules involved, c.q. soluble-TRAIL (sTRAIL) and soluble FASL (sFASL) have retained residual and promising intrinsic tumour-selective activity in their own right.

However, several potential limitations for the therapeutic application of soluble recombinant forms of TRAIL and FASL have been described. One fundamental problem to be addressed is the widespread expression of the various cognate death receptors throughout the human body. This unfavorable surplus of potential binding sites will hamper tumour-selective accretion of both TRAIL and FASL. In addition, the recombinant soluble forms
of TRAIL and FASL appear less efficacious at inducing apoptosis than the corresponding membrane-bound versions.

The aim of the research described in this thesis was to develop a general strategy that augments the therapeutic value of apoptosis inducing ligands, such as TRAIL and FASL. In chapter 3 we started this endeavor by designing a recombinant fusion protein, designated scFvC54:sTRAIL, in which sTRAIL was genetically linked to the scFvC54 antibody fragment that specifically targets the Epithelial Glycoprotein-2 (EGP2). EGP2, also known as EpCAM, is a well-established tumour-associated target antigen frequently overexpressed on the cell surface of various human carcinomas. EGP2 has been extensively studied as a target antigen for antibody-mediated imaging and immunotherapy.

Incubation with scFvC54:sTRAIL resulted in strong and selective binding to the cell surface of EGP2-positive cells only. Consequently, the soluble scFvC54:sTRAIL fusion protein was converted into a membrane-bound form, which enabled the efficient crosslinking of agonistic TRAIL receptors 1 and 2. Moreover, the pro-apoptotic signaling observed for scFvC54:sTRAIL was particularly strong as a result of reciprocal apoptosis induction between EGP2-positive target cells. The latter situation closely resembles the way in which the immune response is resolved by fratricide apoptosis induction of activated T-cells.

Of note, it has become evident that selective crosslinking and activation of not only TRAIL-R1 but also of TRAIL-R2 can be of considerable importance for TRAIL-based therapy. Recently, it was demonstrated that apoptosis in certain tumours is predominantly engaged via either TRAIL-R1 or TRAIL-R2. Unlike conventional recombinant sTRAIL preparations, scFvC54:sTRAIL can efficiently induce apoptosis in target cells not only via TRAIL-R1 but also via TRAIL-R2 signaling.

To accelerate clinical application of our fusion proteins we developed a rapid and versatile plasmid-based expression platform that is suitable for eukaryotic expression in industrial-grade production cell lines. Using this platform, stable homogenous scFvC54:sTRAIL trimers were produced in the absence of high-molecular-weight protein aggregates. This absence of aggregates is an important feature of our production system since sTRAIL (and sFASL) aggregates have been implicated in toxicity towards normal human cell types.

A serious limitation of many current antibody-based approaches is the escape of target antigen-negative tumour cells from therapy. Target antigen-negative tumour cells may be pre-existent in the lesion or can arise during or after therapy due to target antigen shedding, masking, or therapy-induced down-regulation. In chapter 4, we assessed the so-called bystander effect, predicted as being an essential part of the concept of targeted
apoptosis induction by scFv:sTRAIL fusion proteins (see chapter 4, Fig.1, page 62). The bystander effect is based on the principle that targeted tumour cells are not only eliminated, but are also exploited to convey a therapeutic effect towards neighboring tumour cells that lack expression of the target antigen. For scFvC54:sTRAIL, selective binding to EGP2-positive tumour cells allows for the crosslinking of agonistic TRAIL receptors on neighboring tumour cells, even when they lack EGP2 expression.

To determine the anti-tumour bystander activity of fusion protein scFvC54:sTRAIL, a panel of cell lines was selected representing three major human malignancies, namely acute lymphoblastic T-cell leukemia, B-cell lymphoma, and glioblastoma multiforme, all of which are normally EGP2-negative. By retroviral-transduction, variants of these cell lines that ectopically express EGP2 at the cell surface were generated. Using mixed cultures of parental EGP2-negative bystander cells and EGP2-positive target cells, we showed that, as predicted, selective binding of scFvC54:sTRAIL to EGP2-positive target cells conveyed an exceptionally potent anti-tumour bystander effect in EGP2-negative tumour bystander cells. The anti-tumour bystander activity of scFvC54:sTRAIL was detectable at target-to-bystander cell ratios as low as 1:100 and did not occur in the absence of target cells. Importantly, scFvC54:sTRAIL showed no detectable signs of “innocent” bystander activity towards freshly isolated blood cells. We concluded that the anti-tumour bystander activity of scFv:sTRAIL fusion proteins is a robust feature that may be important in cases where cancer cells escape from targeted therapy due to (partial) loss of target antigen expression.

In chapters 3 and 4 we exploited EGP2 as a model target antigen, since EGP2 has no intrinsic signaling activity that might interfere or obscure the evaluation of target-cell restricted apoptosis as induced by scFvC54:sTRAIL. In chapter 5, we extended our investigations by targeting an antigen of which signaling activity is important in carcinogenesis. As a clinically relevant prototype of such a signaling target antigen we selected the epidermal growth factor receptor (EGFR). Aberrant EGFR signaling has since long been recognized as an important contributor to malignant progression by e.g. enhancing cancer cell growth and increasing resistance of cancer cells to apoptosis. Several strategies designed to inhibit aberrant EGFR-signaling have been developed, including monoclonal antibodies (MAb C225 (Cetuximab) and MAb 425) and small molecule tyrosine kinase inhibitors such as Iressa (also known as ZD1839 or Gefitinib). The clinical efficacy of both classes of EGFR-signaling antagonists relies on multiple anti-cancer mechanisms, including inhibition of cell cycle progression, inhibition of metastatic potential, and an increased susceptibility to apoptosis. Importantly, synergistic tumoricidal effects have been reported upon combination of EGFR-signaling antagonists with recombinant sTRAIL. Therefore, we constructed a novel scFv:sTRAIL fusion protein, designated scFv425:sTRAIL,
designed to selectively target EGFR and thereby block EGFR mitogenic signaling, while simultaneously triggering TRAIL-receptor-mediated apoptotic signaling. As expected, \textit{in vitro} treatment with scFv425:sTRAIL resulted in its specific accretion at the cell surface of EGFR-positive cells only and in rapid inactivation of EGFR and downstream mitogenic signaling. As a result, EGFR-positive cells were sensitized to apoptosis. Simultaneously, the TRAIL domain of the scFv425:sTRAIL fusion protein selectively induced apoptosis by crosslinking of agonistic TRAIL-receptors. Similar to scFvC54:sTRAIL, scFv425:sTRAIL conveyed a potent anti-tumour bystander effect towards EGFR-negative bystander tumour cells in mixed culture experiments. Of note, co-treatment of EGFR-positive tumour cells with the EGFR-tyrosine kinase inhibitor Iressa and scFv425:sTRAIL resulted in a potent synergistic pro-apoptotic effect. Taken together, the favorable characteristics of scFv425:sTRAIL, alone and in combination with Iressa, indicate the potential value of scFv425:sTRAIL for the treatment of EGFR-expressing malignancies.

In \textit{chapter 6}, the feasibility of targeted induction of apoptosis by scFv:sTRAIL fusion proteins in hematological malignancies was assessed. To this end we constructed scFvCD7:sTRAIL, which harbors specificity for the T-cell leukemia-associated antigen CD7. Treatment with scFvCD7:sTRAIL induced CD7-restricted apoptosis in a series of malignant T-cell lines. Importantly, normal resting leukocytes, activated T-cells, and vascular endothelial cells (human umbilical vein endothelial cells) were fully resistant to apoptosis induction by scFvCD7:sTRAIL. Compared to scFvCD7:ETA, a CD7-specific pseudomonas Exotoxin-A immunotoxin, scFvCD7:sTRAIL possessed superior pro-apoptotic activity towards tumour cells. Moreover, scFvCD7:ETA did show toxicity towards normal CD7-positive peripheral blood lymphocytes. \textit{In vitro} treatment of blood cells freshly-derived from T-acute lymphoblastic leukemia patients resulted in marked apoptosis of the malignant T cells that was strongly augmented by the chemotherapeutic agent vincristin. These pre-clinical data clearly indicate that scFvCD7:sTRAIL is a potent and leukemia-specific therapeutic agent that might be useful in the treatment of CD7-positive acute T-cell leukemia and lymphoma. This novel strategy might help broaden the therapeutic spectrum for T-cell leukemia, since current treatment is predominantly limited to conventional cytotoxic therapy with only limited therapeutic response and significant morbidity.

In \textit{chapter 7}, the concept of targeted delivery of sTRAIL as described and discussed in \textit{chapters 3 - 6} was extended to the targeted delivery of sFASL. Previously, it was reported that recombinant sFASL induced severe liver toxicity in mice, a finding that
precluded its therapeutic application in humans. However, recent evidence indicates that this toxicity is due to contaminating high molecular-weight aggregates present in the respective sFASL preparations. In fact, it was demonstrated that homogeneous trimeric sFASL itself is non-toxic but also lacks tumoricidal activity. Consequently, new sFASL-based anti-cancer strategies have to meet the criterion of strictly localized activation of sFASL at the tumour cell surface. To test the feasibility of meeting these criteria, the sTRAIL domain from scFvCD7:sTRAIL (see chapter 6) was exchanged for sFASL, yielding fusion protein scFvCD7:sFASL. Fusion protein scFvCD7:sFASL proved to be biologically inactive as a soluble homotrimer and acquired tumoricidal activity only after specific binding to tumour cell surface-expressed CD7, as evidenced by its potent CD7-restricted activity on T-ALL cell lines and patient-derived T-ALL, PTCL, and CD7-positive AML cells. In addition, apoptosis induction by scFvCD7:sFASL was augmented by various conventional and experimental drugs, including proteasome inhibitor Velcade. Importantly, normal human lymphocytes and endothelial cells were resistant to treatment, while in activated T-cells only moderate induction of apoptosis was detected. Together, these results establish the feasibility of localized activation of sFASL at the tumour cell surface, which may rekindle the interest in application of sFASL in tumour therapy.

In chapter 8, a comprehensive summary of the results and conclusions is provided along with perspectives for further development of the deliberate and selective induction of apoptosis in the treatment of human cancer.