Chapter 8

General discussion and future perspectives
The GBM is a highly aggressive tumor and shows major radio and chemo-resistance.

As radio-and chemotherapy exert their cell killing effect through the intrinsic “mitochondrial” apoptosis pathway of the GBM it seems logically that defects in the mitochondrial pathway may contribute to the intrinsic resistance of these conventional therapies. To bypass intrinsic resistance it is mandatory to evaluate the apoptosis inducing effect of drugs which can activate the “extrinsic” pathway of GBM cells, for example TRAIL [1, 8, 9, 13, 16, 18, 23]. The organized way of getting rid of malignant cells by apoptosis in combination with the lack of neuro- or systemic toxicity makes TRAIL an interesting molecule to treat the GBM acting through the extrinsic apoptosis inducing pathway [2, 28]. The applicability of TRAIL as apoptosis inducing agent has been extensively evaluated in several publications describing both in vitro and in vivo results (see chapter 2). On the other hand various tumor cell lines, including GBM cell lines, also show variable sensitivity to TRAIL [19, 30]. Thus both extrinsic as intrinsic resistance occurs in GBM cells. To overcome resistance TRAIL therapy could be combined with a variety of conventional and/or novel targeted therapies (chapter 2). In addition, to evaluate and develop TRAIL therapies insight must be given in receptor expression of the death inducing receptors and pathway defects occurring in GBM cells which contribute to the resistance of the GBM cell to TRAIL [22, 28].

Chapter 2 discusses the biology of TRAIL/TRAIL receptor signaling, focuses on the promises and pitfalls of recombinant TRAIL, as a therapeutic agent alone and in combinational therapeutic approaches, for GBM. Chapter 2 sums up various defects within the extrinsic pathway which can be “targeted” to partially restore sensitivity for TRAIL. The conclusion from this chapter is that the vast body of evidence from preclinical data indicates that the rational design of combinational TRAIL-based approaches with conventional as well as novel therapeutics may ultimately help to combat GBM. As TRAIL alone shows no major systemic toxicity it remains the question if after combinational treatment no systemic toxicity will occur [14]. Although animal in vivo studies with TRAIL en Temozolomide showed no systemic toxicity, we know from patients treated with radiotherapy and Temozolomide that approximately 7% of these patients show grade 3 to 4 hematological disturbances leading to cessation of the therapy [30]. Other novel drugs such as Bortezomib and rapamycine show in vitro favorable death inducing effects on tumor cells but might exert toxic effects in the human in vivo situation. It has been shown that bortezomib in combination with rhTRAIL induced hepatotoxicity in in vitro experiments [20]. A therapeutic window could be detected for cell lines other than GBM lines. In contrast to other tumors it might be difficult for intracranial gliomas, which are relatively surrounded by an intact blood brain barrier, to find the right therapeutic window.

The aim of the study in Chapter 3 was to quantify the expression of the death receptors TRAIL-R1, TRAIL-R2 on primary GBM tumor specimens, with immunohistochemistry and RT-PCR. Immunohistochemistry showed that TRAIL-R2 was predominantly expressed on GBM cells, however the percentage of GBM cells expressing TRAIL-R2 was relatively
low, around 30%. In comparison TRAIL-R1 was only expressed around 19%. Interestingly, the fact that TRAIL-R1 expression on the mRNA level has an inverse relation with the grading of gliomas, this was not seen with TRAL-R2. Through down regulation of TRAIL-R1 on both the protein and receptor level high grade glioma tumor cells might escape apoptosis induction from TRAIL-TRAIL receptor interaction. As a GBM tumor expresses more TRAIL-R2 it seems logically that “targeted” therapy directed against TRAIL receptors is focused on the TRAIL-R2 receptor. A recent study confirms our findings that the TRAIL-R2 receptor is the predominant TRAIL receptor in glioma cells and that TRAIL-R1 is of minor importance [27]. It has been shown that TRAIL receptors 1 and 2 have quite distinct crosslinking requirements for the initiation of apoptosis [25, 31]. TRAIL-R2 appears to signal apoptosis only after efficient receptor crosslinking by either native membranous TRAIL, aggregated sTRAIL variants, or by TRAIL preparations secondarily crosslinked by antibodies. Apoptosis signaling by TRAIL-R1 appears to be relatively independent of the receptor crosslinking characteristics of a particular form of TRAIL. Furthermore, it was shown that TRAIL-R2 had superior binding affinity for TRAIL, resulting in predominant binding of TRAIL to TRAIL-R2 over TRAIL-R1. These findings emphasize the rationale of “targeting” TRAIL to GBM cells [3, 5]. Therefore to fully exploit the therapeutic potential of TRAIL, a number of characteristics of both the TRAIL receptor system and TRAIL should be taken into account. For example the fact of widespread expression of the various TRAIL receptors throughout the human body; the differential binding affinities and crosslinking requirements of the agonistic receptors TRAIL-R1 and TRAILR2 and the solution behavior of particular TRAIL preparations are all important factors to deal with. On basis of the above mentioned essentials a novel fusion protein (scFvC54:sTRAIL) was developed (Chapter 4) with enhanced tumor selective apoptosis induction toward EGP2-positive tumor cells [4]. The characteristics of scFvC54:sTRAIL showed enhanced apoptosis inducing capacity by activating the TRAIL-R2 receptor through crosslinking. Furthermore, the fusion protein showed the potential to induce apoptosis in a monocellular way but more interesting also in a bicellular way in which specific binding to one cell results in the crosslinking of TRAIL receptors on a neighbouring tumor cell. After the development of the scFvC54:sTRAIL and the demonstration that the concept of “targeting” TRAIL to membranous bound antigens resulted in enhanced apoptosis inducing effects, various other fusion proteins were developed. Another fusion protein which was developed (scFv425:sTRAIL) had specificity for the EGF receptor. As GBM cells over express the EGF receptor, the scFv425:sTRAIL fusion protein can be used in the treatment of GBM. This fusion protein simultaneously blocks EGFR signaling; thereby sensitizing tumour cells to apoptosis, and induces apoptosis via TRAIL receptor signaling. This fusion protein efficiently activates apoptosis and shows promising in vivo activity [6]. This novel scFv425:sTRAIL fusion protein was subject of research in Chapter 5 and 6. First we evaluated the in vitro apoptotic inducing effects of scFv425:sTRAIL on various cell lines including GBM cells. A positive linear correlation between scFv425:sTRAIL binding and EGFR expression was found (Chapter 6). Not surprisingly, no correlation was found between the amount of EGFR, TRAIL-R1 or TRAIL-R2 expression and toxicity.
This is in concordance with the literature, and underscores the theory that the amount of apoptosis inducing effects of TRAIL is not limited by the numeric expression of membranous receptors but is more likely dependent on intracellular resistance effects.

The focus of Chapters 5 and 6 was on the delivery of the scFv425:sTRAIL fusion protein, since delivery might hamper the use of TRAIL or TRAIL fusion proteins in clinical use. It has been shown that only marginal amounts of TRAIL bypass the blood brain barrier (BBB) after systemic application. The question arises whether other delivery methods might be more efficient in delivering TRAIL to the tumor. In Chapter 5 a microencapsulation technique was evaluated for continuously delivery of TRAIL. The result of this study showed that CHO-K1 cells transfected with the gene encoding for the scFv425:sTRAIL protein can, after encapsulation in alginate capsules, produce stable scFv425:sTRAIL proteins. Also biocompatibility of the alginate capsules in mice brains was found suggesting no major immunological reaction towards these capsules. This study also demonstrated that producer cells could maintain normal growth capacity in capsules made of both intermediate and high-G alginate. Also the death inducing capacity of the produced fusion proteins was preserved. A potential drawback of the microencapsulation technique is the development of necrosis of the TRAIL producing cells between 16 and 30 days after encapsulation, due to growth expansion of the producer cells and the subsequent shortage of nutrients.

One major question remains to be answered. Can the microencapsulated TRAIL producer cells construct enough fusion proteins to deliver a toxic concentration of TRAIL in the vicinity of the tumor? As we know from diffusion techniques in general that the penetration of drugs into the brain will be only a few millimeters. As the microencapsulation delivery method depends on diffusion this might hinder its clinical applicability, unless many capsules can be implanted in and around the tumor remnants.

In Chapter 6 we therefore evaluated if the CED technique which is based on convection of drugs could be of interest for delivery of TRAIL to an intracerebral tumor. The aim of this study was to evaluate the efficacy of a single chain (scFv425):sTRAIL fusion protein with specificity for the EGF receptor in a variety of human cell lines and in an animal brain tumor model. Although the binding of the scFv425:sTRAIL fusion protein to the SW948 was relatively low (in comparison to some of the other cell lines), the sensitivity of SW948 cells to scFv425:sTRAIL proved to be extremely high. SW948 colon cancer cells were selected to be stereotactically xenografted in the cerebrum of SCID mice. Using this procedure over 90% of the injected mice developed SW948 xenografts within 14 days and seemed to be a excellent brain tumor model to investigate the scFv425:sTRAIL antitumor activity. We chose to evaluate xenograft acceptance and antitumor activity by performing pre and post treatment MRI’s. By performing a pre-treatment scan we could actually demonstrate uniform tumor growth with low variance and therefore we needed fewer animals for this study in comparison with studies evaluating the effect
of anticancer treatment by measuring the survival period of the animal. Disappointing was the conclusion that under the experimental conditions described in this manuscript, convection enhanced delivery of scFv425:sTRAIL by osmotic micro pumps appears to be insufficient to inhibit tumor growth. The limiting factor was the concentration of the scFv425:sTRAIL used in this study. Other methods of delivery of TRAIL, for GBM, are under investigation such as delivery of TRAIL by TRAIL producing stem cells [15, 17, 21, 24, 35]. Interestingly, systematic delivery of mesenchymal stem cells which produced TRAIL could prolong the survival of brainstem glioma-bearing mice, therefore mesenchymal stem cells may be an effective vehicle for the targeted delivery of therapeutic agents to gliomas [35].

As mentioned previously GBM cells exhibit intrinsic and extrinsic resistance for (non) conventional therapies. Numerous in vitro studies have demonstrated a variance in sensitivity of GBM cell lines for TRAIL or chemo/radiotherapy. It is known from human in vivo studies that various chemotherapeutics, used as monotherapy or in combination with radiotherapy do not lengthen overall survival [10, 12, 32, 33]. The only exception is Temozolomide in combination with radiotherapy [30]. In general the treatment strategy for GBM is surgery with radiotherapy. Temozolomide is only given when the patient meets certain criteria, such as a good performance score. Therefore it seems logical to evaluate possible synergistic apoptosis inducing effects of combination therapy with RT and TRAIL. In chapter 7 the combined death inducing effects of conventional radiotherapy and recombinant human TRAIL (rhTRAIL) were evaluated. We tried to test whether radiation would synergistically interact with TRAIL in inducing cell death in a glioblastoma cell line A172 in order to bypass the typical radioresistance of GBM tumor cells. Although, we found borderline significant synergy for early cell death induction (MTT), as was found by many others [8, 9, 11, 16, 18, 26, 29, 36] no evidence was found to support the idea that the combination treatment enhanced the extent of ultimate killing (clonogenic assay) of radioresistant A172 cells. Rather, the treatments were additive. Besides the conclusion that glioma cells may not show (much) synergy between radiation and TRAIL, our data also argue for re-evaluation of the observed synergy between these modalities seen in rapid death endpoint assays in other cell lines. In fact, our finding that the extent of TRAIL-induced clonogenic death exceeded the extent of rapid apoptotic cell death underscores the need for such re-evaluation. However, although only one glioma cell line was studied here, our data combined with Nagane et al. [26] suggest that a synergistic interaction between the rhTRAIL and radiotherapy is not to be expected for the GBM.
Future perspectives

Targeting aspects

In this thesis a novel targeted TRAIL fusion protein was tested with specificity for the EGP2 receptor which is present in colorectal cancer and lung tumors. It has been shown in the literature that the production of such targeted TRAIL fusion proteins is possible, and results in a drug with superior characteristics concerning its apoptosis inducing capacity compared to TRAIL alone. So, in parallel to the above, for GBM treatment with TRAIL, high effectiveness might be found in the use of a TRAIL fusion protein directed against a GBM specific membrane antibody like the EGF receptor (scFv425:sTRAIL) or its variants (mutants) that are even more specific for GBM (the EGFvIII receptor).

Both fusion proteins have been recombinantly manufactured and are available for in vitro research and could be used to evaluate their apoptosis effect on glioblastoma cells. Besides targeting specific receptors like EGP2 and EGFR, one might also raise effectiveness of TRAIL fusion products by trying to address the most specific TRAIL receptors. Recently a novel fusion protein scFv425:sTRAILmR1-5 was designed with specificity for the TRAIL-R1 receptor. In vitro, it showed superior apoptosis inducing activity in comparison with scFv425:sTRAIL in various cell lines including glioma cell lines. Since TRAIL-R1 has a different activation profile in respect to TRAIL-R2 and TRAIL-R1 is present on GBM cells (chapter 3) it would be interesting to evaluate the effect of this novel fusion protein in a broader GBM cell panel with and without adjuvant therapies such as RT and Temozolomide.

Other targets for the treatment of GBM with TRAIL and its possible fusion products might be the VEGF receptor. Although this receptor is not unique for GBM vasculature it is abundantly present there, as well as on most of the tumour cells themselves. Apoptosis induction by a anti-VEGFR-TRAIL fusion protein could be the result of a direct effect on the tumour cells or an indirect effect on the tumour vasculature. The toxicity profile of such anti-VEGFR-TRAIL fusion protein has to be established first, in vitro and in animal studies. This approach is different from toxicity of a product directly related with tumor cell kill, since endothelium within the heterogeneity of a GBM might behave differently from isolated endothelial cell lines. Mixed cell cultures and/or GBM spheroids may be the best models for such tests but are difficult to handle. Even better might be a genetically engineered GBM mouse model. As tumors are heterogeneous in nature and represent a mixture of multiple cell types (eg endothelium) changes in the tumor microenvironment have been shown to have a critical influence in the initiation and progression of tumors. These important characteristics are ultimately lost when cellular signalling is studied in vitro. Some of these limitations can be overcome by growing cancer cells in 3D cell culture systems. It is thus important to extend analysis to the study of tumors, both from murine xenograft models and genetically engineered mouse models of GBM.
Resistance aspects

It has been discussed previously that resistance against TRAIL is an topic which needs to be explored in order to find biological solutions to overcome this resistance and thereby optimizing TRAIL therapy. One way to overcome or by-pass resistance is to design multimodality treatment paradigms, since monotherapies will fail too easily by the activation of resistance pathways. Another way is to look more in detail to what is known about the resistance mechanisms. In GBM, EGFR and other tyrosine kinase receptor signalling pathways are well known aberrant pathways that contribute to this resistance against TRAIL induced apoptosis. Maybe that today’s popular tyrosine kinase inhibitors (TKIs) can have a role in a multimodality attack against the tumor, circumventing the resistance pathways. Some of these kinase inhibitors, even used as monotherapy, have shown promising results in preclinical trials. It remains to be seen whether they will perform well in the clinic. Phase II clinical trials with EGFR-TKIs (ZD-1839, gefitinib; OSI-774, erlotinib) demonstrated some responses, however it is much more interesting, in the light of the above reasoning, to see whether a combination treatment of a TKI with (targeted) TRAIL shows more therapeutic effect.

Another interesting pathway to attack in combination with TRAIL inducing apoptosis is the so called cell survival pathway, the PKB/mTOR pathway. By hitting PI3K from the PKB/mTOR-pathway GBM cell survival can be limited, supporting the effect of other anti GBM drugs. A recent study argues for combining PI3K inhibitors (LY294002) with TRAIL receptor agonists or conventional chemotherapeutic agents in order to “prime” glioblastoma cells for death receptor- or chemotherapy-induced cell death.

In the field of combinations with “pathway inhibitors” the possible value of mTOR inhibitors should be mentioned as well. One of these, Everolimus®, is already on the market as second line chemotherapy for patients with renal cell cancer, and seems worthwhile to explore for GBM treatment in combination with a TRAIL fusion protein.

Radiation

Although Radiation therapy has been proven effective against GBM since many years – and has become “standard therapy” after or instead of surgery – the effectiveness is limited, because of “radioresistance”. Such resistance might be overcome by a multimodality attack combining radiation therapy with (targeted) therapies. In chapter 7 the effect of combined γ-radiation-TRAIL therapy in a glioblastoma cell line, measuring both early apoptotic cell death and clonogenic ability as endpoints was explored. The clonogenic results did not show a synergistic effect in contrast to the early apoptotic death assay which suggested synergistic activity. It should be mentioned here again that when short term assays are used to detect possible enhancing effects of combination therapies, they
may lead to inappropriate conclusions about synergy. Only clonogenic assays or isobolographic calculations give trustful clues about the existence of synergy, only additional effects or none of these.

Addressing the issue of the combination therapy of irradiation and TRAIL it might be concluded so far that combining both therapies will lead to more cell kill on the short term; long term prognosis, however, seems not to be influenced. To lengthen overall survival the addition of chemotherapy and/or pathway inhibitors, mentioned above, on top of radiation and TRAIL must be investigated.

Delivery

The ability to bypass tumor cell resistance for TRAIL or other therapies is one hurdle to take but the delivery of large drugs in general to the site of the tumor is another one. Targeted TRAIL therapy can only be successful if the TRAIL fusion protein is delivered to the site of the tumor and especially its infiltrating zone. The Convection Enhanced Delivery (CED) technique (chapter 6) and the alginate microencapsulation method (chapter 5) of TRAIL fusion proteins and other novel therapeutics could be helpful in achieving this goal. The next step in analyzing the feasibility of alginate encapsulated (scFv:sTRAIL protein) producer cells in treating brain tumors is to implant encapsulated producer cells in the brain of mice with and without an intracerebral tumor. Diffusion of the protein within the tumor, mass effect of the intracerebral lesion, edema surrounding the tumor, brain compliance and cerebrospinal fluid flow will be factors influencing the in vivo efficacy of the microencapsulation method and must be analyzed before any prediction can be made if the microencapsulation method will be a future therapeutic modality in the treatment GBM. Also the efficacy of CED of scFv425:sTRAIL in the brain of mice that were stereotactically xenografted with EGFR-positive tumor cells was investigated. Under the experimental conditions described in this manuscript (chapter 6), convection enhanced delivery of scFv425:sTRAIL by osmotic micro pumps appeared to be insufficient to inhibit tumor growth. In our current model loading of the osmotic pump with higher concentrations of scFv425:sTRAIL, using pumps with higher output capacity, as well as starting the treatment at lower initial tumor burden may be necessary to evaluate the anti-tumour efficacy of this novel approach.

Stem cells

Several studies have demonstrated the existence of cancer stem cell (CSC)s in solid tumors, including GBM. It is now generally assumed that these CSC in particular have the ability to reinitiate tumor cell proliferation and that they form the basis for chemoresistance and radioresistance. CSC’s today form the new major target for experiments concerning
the eradication of cancer and this holds true for GBM as well. Therefore, it is extremely
worthwhile to test the effectiveness of TRAIL and its variants (fusion proteins) against these
CSC’s. This can be done by engineering human mesenchymal stem cells (MSC) to express
secretory-TRAIL. It was demonstrated that MSC could be used as delivery vehicles
for sTRAIL targeting glioblastoma stem cells (GBSC) in vivo resulting in in vivo antitumor
activity. The most prominent membrane “receptor” in GBM CSC’s seems to be CD133.
Therefore, the first step should be to produce an antiCD133 fusion TRAIL protein and look
for its antitumor effect.

Conclusion

We can conclude that ample in vitro and in vivo studies have shown that TRAIL is efficient
in killing tumor cells with no or minimal systemic toxicity. Furthermore, the design of TRAIL-
based therapy combined with other modalities, thereby augmenting apoptosis induction
or overcoming resistance is a well proven approach and should also be applied in future
therapeutic strategies for patients with GBM.

Although in vitro and in vivo studies show promising results the only way to find out if sys-
temic application of TRAIL, its variants or TRAIL receptor blocking antibodies, is feasible
and safe, is through the initiation of a phase I/II study for GBM patients. According to a
large number of studies and the stimulating results, Genentech and Amgen have launched
phase II clinical trials in non-Hodgkin’s lymphoma and non-small-cell lung carcinomas with
rhTRAIL [34]. Also phase I trials with anti-DR5/anti-DR4 antibodies have been initiated and
show safe toxicity profiles [7, 28, 34]. It’s time to make full use of the TRAIL-TRAIL receptor
interaction in GBM tumors, we cannot stay behind.
References


