On TRAIL for glioma therapy?
Kuijlen, Josephus Marie André

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TRAIL-receptor expression is an independent prognostic factor for survival in patients with a primary glioblastoma multiforme

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Abstract

Purpose:
In order to improve the survival of patients with a glioblastoma multiforme tumor (GBM), new therapeutic strategies must be developed. The use of a death inducing ligand such as TRAIL (TNF Related Apoptosis Inducing Ligand) seems a promising innovative therapy. The aim of this study was to quantify the expression of the death regulating receptors TRAIL-R1, TRAIL-R2 and TRAIL on primary GBM specimens and to correlate this expression with survival.

Experimental design:
Expression of TRAIL and TRAIL-receptors was assessed by immunohistochemistry, both quantitatively (% of positive tumor cells) and semi-quantitatively (staining intensity) within both the perinecrotic and intermediate tumor zones of primary GBM specimens. RT-PCR of GBM tissue was performed to show expression of TRAIL receptor mRNA.

Results:
Immunohistochemistry showed a slight diffuse intracytoplasmic and a stronger membranous staining for TRAIL and TRAIL receptors in tumor cells. Semi-quantitative expression of TRAIL showed a significantly higher expression of TRAIL in the perinecrotic zone than in the intermediate zone of the tumor (p=0.0001). TRAIL-R2 expression was significantly higher expressed than TRAIL-R1 (p=0.005). The antigenic load of TRAIL-R2 was positively correlated with survival (p=0.02). Multivariate analysis of TRAIL-R1 within the study group (n=62) showed that age, gender, staining intensity, antigenic load, % of TRAIL-R1 expression, were not statistically correlated with survival however radiotherapy was significantly correlated with survival however radiotherapy (p=0.0001). Subgroup analysis of patients who had received radiotherapy (n=47) showed a significant association of % of TRAIL-R1 expression and the antigenic load of TRAIL-R1 with survival (multivariate analysis: p=0.036 respectively p=0.023).

Multivariate analysis of TRAIL-R2 staining intensity and antigenic load, within the study group (p=0.004 resp. p=0.03) and the subgroup (p=0.002 resp. p=0.004), showed a significant association with survival. RT-PCR analysis detected a negative relation between the amount of TRAIL-R1 mRNA and the WHO grade of astrocytic tumors (p=0.03).

Conclusions:
TRAIL-R1 and TRAIL-R2 expression on tumor cells are independent prognostic factors for survival in patients with a glioblastoma multiforme. Both receptors could be targets for TRAIL therapy. As TRAIL-R2 is more expressed, in comparison with TRAIL-R1, on GBM tumor cells, TRAIL-R2 seems to be of more importance as a target for future TRAIL therapy than TRAIL-R1.
**Introduction**

Neurosurgical debulking of a glioblastoma multiforme tumor followed by localized radiation of the cranium up to 60 Gy is still the standard therapeutic regimen for patients with this highly malignant primary brain tumor. The overall median survival after this conventional treatment lies between 9 and 12 months [1]. Radiotherapy has proven to prolong overall median survival for several months. Adjuvant chemotherapy has been investigated in various trials since the 1970’s with variable outcome. Although the recently presented Phase III study on Temozolomide (EORTC 26981) showed a statistically significant (2-3 months) prolonged survival for patients with a newly diagnosed GBM [2], this is still a minor gain. Therefore it is worthwhile to develop new therapeutic strategies for patients with a GBM.

One such strategy could be the use of apoptosis inducing ligands to eliminate tumor cells. TRAIL (TNF Related Apoptosis Inducing Ligand) is a promising death inducing ligand as this ligand induces apoptosis by ligation to its cognate cell surface receptors (TRAIL-R1 and R2) [3]. TRAIL can be present as membranous bound (memTRAIL) but can also be proteolytically cleaved from the membrane to form a soluble ligand (sTRAIL) [4]. The soluble form of TRAIL can be used for therapeutic options. Interestingly, soluble TRAIL induces apoptosis in tumor cells, and not in normal tissue [3]. Other data available showing possible side effect of sTRAIL on normal tissues although these might be related to dose or the modified form of sTRAIL used in these experiments [5-7].

TRAIL receptor expression on glioma cell lines has been frequently studied [8, 9-11], however only one study [12] addressed the expression of TRAIL receptors in primary tumor tissue.

The aim of this study was to quantify the expression of the death receptors TRAIL-R1, TRAIL-R2 and the membranous TRAIL, on primary GBM tumor specimens, with immunohistochemistry and RT-PCR. Receptor expression results were correlated with survival.
Material and methods

Patients

A total of 132 patients were diagnosed with a primary glioblastoma multiforme, according to the WHO criteria [13], in our hospital between January 1998 and January 2003. Exclusion criteria for this study were; stereotactic biopsy (57 patients), secondary GBM (10) and mixed tumors containing zones with sarcomatous or oligodendroglial differentiation (3). Finally, tumor tissue of 62 patients was included in this study.

Baseline characteristics

Several baseline patient characteristics were evaluated: gender, age at diagnosis, interval between start of symptoms and operation, postoperative radiotherapy and survival period. “Age at diagnosis” is defined as the age of the patient at the date of the first MRI/CT on which the tumor was diagnosed. The “period between start of symptoms and operation” is defined as the time between the manifestation of the first clinical symptoms, as registered by the referring neurologist, and the operation day. The survival period was calculated as the period from date of surgery until death.

Radiographic parameters

Pre-operative tumor size was measured on a gadolineum enhanced T1 weighted MRI, or if MRI scans were not available, on a contrast enhancing CT scan. Because we did not apply volumetric computer assisted measurements on the pre-operative scans we calculated the tumor volume by assuming that it was a sphere. The length and width of the tumor on the slice with the largest defined mass lesion was summed up and divided by 4 and this outcome was used as the radius.

Immunohistochemistry (IHC)

Paraffin embedded tumor tissue samples from patients with a GBM after debulking, were used. All the specimens were reviewed by the authors WFAdD and HH. Only patient samples with sufficient tissue that met all four WHO criteria [13] of a glioblastoma were used in this study. Tumor tissue from other types of primary brain tumors and normal brain tissue obtained from autopsies were used as control. Formalin-fixed, paraffin-embedded sections (4 μm) were cleared in xylene and rehydrated in a graded alcohol series. For TRAIL-R2 staining, antigen retrieval was performed by adding slides to a 10 mM citric acid monohydrate (Merck, Darmstadt, Germany) solu-
tion in demineralized water, pH=6.0, and subsequent microwave treatment at 100°C for 8 min at 700 W. Endogeneous peroxidase activity was blocked by treatment of slides with 1% peroxide in phosphate buffered saline solution (PBS, 0.14 M NaCl, 2.7 mM KCl, 6.4 mM Na₂HPO₄·2H₂O, 0.15 mM KH₂PO₄, pH 7.8) for 30 min. Slides were then washed twice with PBS and pre-incubated for 15 min with avidin and biotin blocking reagent (Vector Laboratories, Burlingame, CA). Slides were then stained for 60 min with TRAIL antibody (TRAIL (K-18), cat nr: sc-6079 Santa Cruz Biotechnology Inc, Santa Cruz, CA) diluted 1: 25, TRAIL-R2 antibody (TRAIL-R2 (DR5) (Ab-1); cat nr: PC392 Oncogene, Cambridge, MA) diluted 1:100, TRAIL-R1 antibody (DR4 (C-20), cat nr: sc-6823, Santa Cruz Biotechnology Inc, Santa Cruz, CA) diluted 1:100. All antibodies were diluted in PBS, 1% BSA. For the negative controls, no antibody was added to the PBS or the primary antibody was replaced by normal goat IgG (TRAIL, TRAIL-R1) or normal rabbit IgG (TRAIL-R2). Positive controls were tumor tissue samples, found on previous occasions to stain positive and first trimester placenta tissue for TRAIL and kidney tissue for TRAIL-R1 and TRAIL-R2.

After washes in PBS, the sections were exposed to the secondary biotinylated rabbit anti goat IgG diluted 1:300 (TRAIL, TRAIL-R1) or swine anti rabbit diluted 1:300 (TRAIL-R2) for 30 min, followed by the amplification system streptavidin-biotin complex. Secondary antibodies were diluted in PBS with 1% BSA and 1%AB serum. The slides were then treated with DAB and H₂O₂ for 10 min and counterstained with hematoxylin.

Preparation of RNA

Snap-frozen sections (10×10μm), of 19 primary GBM tissue samples, were cut and placed in a tube containing 300 μl lysisbuffer with 2.1 μl Beta-ME. For the RNA-isolation a RNA RT-PCR Miniprep kit (Stratagene) was used. RNA was stored at minus 20 degrees Celsius. Synthesis of cDNA was performed by using random primers Hexamers. In a PCR-tube 10 μl RNA solution, 1 μl random primers Hexamers (300 ng) and 1 μl 10 mM dNTP-mix were incubated by 65°C for 5 min. Directly after this the mix was put on ice. Then 4 μl 5× First-Strand buffer, 2 μl 0,1 M DTT and 1 μl RNaseOUT Recombinant Ribonuclease Inhibitor (40 units/μl, Invitrogen) were added. The mixer was incubated at 25 °C for 10 min and thereafter for 2 min at 42 °C before adding 1 μl Superscript II (200 units, Invitrogen). Then the mixer was incubated for 50 min. by 42 °C and for inactivating of the reaction incubated by 70 °C for 15 min. The cDNA was stored at -20°C.

RT-PCR

For the RT-PCR 2 primer sets were designed by using the sequence from PubMed (NM-003842, NM-147187 and NM-003844) and Primer design 3. Polymerase chain reaction for TRAIL R1 was performed using the forward primer 5’-AGAGAGAAGTCCCTGCACCA-3’
and reversed 5’-GTCCTCAGGGGCTACAT-3’ and for TRAIL R2 forward 5’-GATGGTCAAGGTCGGTATT-3’ and reversed 5’-TACGGCTGCAACTGTGACTC-3’. One μl of each cDNA was amplified using PCR Master Mix (Amersham Biosciences) under the following PCR conditions; 5 min. 94 °C, 35 cycles of 94 °C for 45 s, 59 °C for 45 s and 72 °C for 90 s, followed by 72°C for 7 min. The PCR-products were detected on a 1% agarose gel with Ethidium bromide and analyzed with the gelpdoc 1000 (Biorad) with the household gene GAPDH as reference. The RT-PCR data from the GBM samples (n=19) was compared to RT-PCR data from other primary astrocytic brain tumors ( pilo- cytic astrocytoma (WHO 1) n=3; diffuse astrocytoma (WHO 2) n=6; anaplastic astrocy- toma (WHO 3) n=3; secondary GBM (WHO 4)(n=3) and normal brain tissue (n=4). Splice variants of TRAIL-R2 were also analyzed (TRICK 2a and TRICK 2b).

Method of histological evaluation

All specimens were evaluated by the investigators (WFdD and HH) who were blinded for the patient data. Firstly, a semi-quantitative score was used to define the staining pattern. Staining patterns were recorded in the perinecrotic zone and intermediate zone. The perinecrotic zone was defined as the zone immediately surrounding a zone of necrosis. This zone was confirmed with VEGF-A and HIF-1-alpha expression (data not shown) [14-16]. In this relatively hypoxic zone, intracellular processes in tumor cells will differ from tumor cells in the intermediate zone, which is less hypoxic. The intermediate zone was defined as the region between the perinecrotic zone and the zone of invasion of cells into the normal brain tissue. As infiltrating tumor cells originate from the intermediate zone, this zone is the most important zone to evaluate histochemically. The semi-quantitative staining score is defined as a four point scale. No staining was scored as 0, low staining as 1, moderate as 2 and high intensity staining as 3. Secondly, within the intermediate zone, 1 high power field with 400 to 1200 cells was counted for staining of TRAIL or its TRAIL receptors. The percentage of positive cells (% TRAIL-R1 or R2) was calculated. Thirdly, within the intermediate zone the “antigenic load” (AGL) of the tumor was calculated. The antigenic load is the product of the percentage of positive cells and the semi-quantitative staining score. In this way both parameters are incorporated in a single value and this value, as we think, presents a more balanced outcome regarding TRAIL receptor presence in tumor tissue. The antigenic load varies from 0 to 300.

Statistical methods

Statistical comparisons were made with a Student’s t-test if data had a Gaussian distribution and a Mann-Whitney U test was used when data was not normally distributed. The non-parametric Spearman correlation test was used to compute correlation. A Kruskal-Wallis test was used to analyze multiple group comparisons. Kaplan-Meier
estimates were used to compute survival. Semiquantitative expression of TRAIL-R1 and TRAIL-R2 (staining intensity and AGL) was dichotomized in order to create larger groups with more power for analyses. No staining (0) and low staining (1) were grouped together into a new category called “staining 0/1” and moderate (2) and high staining (3) were grouped into the category “staining 2/3”. The cut-off point for the antigenic load was set a 20 for TRAIL-R1 and at 40 for TRAIL-R2. These cut-off points were chosen because they represent the median expression for both receptors.

Univariate and multivariate Hazard Ratios and the 95% CI’s were computed using the Cox proportional hazard analyses (SPSS 12). Proportional hazard analyses were done in the total study group and in a group of patients consisting of patients who had received radiotherapy. Within the text this group is called the subgroup. Differences found were significant at the p-level smaller than 0.05. All p-values reported are two-tailed.
Results

Patient characteristics

All patients had a Karnofsky Performance Score (KPS) of 70 or above, before operation. The mean age at diagnosis of the overall group was 59 years (95% CI: 56-62) with a male preponderance (69%). Mean pre-operative tumor volume was 43 cm³ (95% CI 36-49 cm³). Seventy-six percent of the patients received radiotherapy. The remaining patients (24%) did not receive radiotherapy because of rapid deterioration after surgery, which consequently led to a KPS beneath 70 and radiotherapy was abrogated. None of the patients within this study received any form of chemotherapy because in the study period, in the Netherlands, chemotherapy did not belong to the standard treatment of patients with a GBM. The mean survival of the total group (Figure 1) was 259 days (95% CI: 214-304). Age and gender were not associated with survival (Table 3) in contrast to radiotherapy, which was an independent factor for survival (Table 3).

Immunohistochemical evaluation of GBM tissue samples

In general, immunohistochemistry (IHC) for TRAIL, TRAIL-R1 and TRAIL-R2 displayed diffuse but slight background staining despite avidin-biotin blocking. However, staining of the astrocytes could be clearly distinguished from the background staining. The IHC for TRAIL (Figure 2A, pg 187), TRAIL-R1 (Figure 2B, pg 187) and TRAIL-R2 (Figure 2C, pg 187) showed a diffuse cytoplasmic staining together with a more intense membranous staining.

TRAIL expression

The semi-quantitative assessment showed a significantly stronger TRAIL expression in the perinecrotic zone when compared with the intermediate zone (1.53 vs. 0.90; p < 0.0001).

Quantitative measurement showed that 13% (95% CI: 8-17) of the tumor cells in the intermediate zone, were positive for TRAIL expression. The mean antigenic load for TRAIL in the intermediate zone was 22 (95% CI: 11-34) (Figure 3). No correlation could be found between survival and the antigenic load for TRAIL within the tumor specimens.

TRAIL-R1 expression

No difference in staining intensity of TRAIL-R1 was observed between the perinecrotic and intermediate zone. Forty-seven of the 62 tumors showed TRAIL-R1 expression within the intermediate zone (Table 1). Table 1 shows the mean antigenic load (AGL) of TRAIL-R1 within the intermediate area in relation to the different staining categories.
Quantitative measurement of the mean percentage of TRAIL-R1 expression on tumor cells in the intermediate zone was 19 % (95% CI: 12-26) (Figure 3A). A positive correlation was found between survival and the percentage of positive tumor cells expressing TRAIL-R1 (Spearman r: 0.25; 95% CI: -0.006 to 0.48; p=0.049 (Table 2)). The antigenic load of TRAIL-R1 expression was 39 (95% CI: 26-53) (Figure 3B). The antigenic load of TRAIL-R1 (Spearman r: 0.28; 95% CI: 0.030 to 0.50; p=0.024) (Figure 4 A; Table 2) was positively correlated with survival.

Univariate and multivariate analysis (Table 3 and Fig 5) were performed to evaluate if TRAIL-R1 and R2 expression were independent factors for survival. Radiotherapy was significantly associated with survival in univariate and multivariate analyses. Therefore we excluded the patients who did not receive radiotherapy after surgery and also performed a separate univariate and multivariate subgroup analyses on those patients who had received radiotherapy (n=47).

Analysis in the total study group (n=62) showed that the dichotomized staining intensity for TRAIL-R1 (0/1 vs. 2/3) (p=0.04), AGL TRAIL-R1 (p=0.016) and % of TRAIL-R1 expression on tumor cells (p=0.03) were all significantly associated with survival in univariate analysis (Table 3). In the multivariate analysis the TRAIL-R1 factors were not shown to be separable prognostic markers for survival (Table 3).

However, multivariate analysis in the subgroup (patients who had received radiotherapy (n=47)) showed significance for two of the three parameters (staining TRAIL-R1 p=0.12; AGL TRAIL-R1 p=0.023; % TRAIL-R1 p=0.036).

**TRAIL-R2 expression**

In general, the semi-quantitative assessment showed a more intense staining pattern for TRAIL-R2 than for TRAIL-R1, although statistical significance was not reached. TRAIL-R2 expression (Table 1) was present in 57 of the sixty-two tumor specimens. The mean quantitative expression of TRAIL-R2 on tumor cells within the intermediate zone of the tumor was 30% (95% CI: 25-36) (Figure 3A). The percentage of tumor cells expressing TRAIL-R2 was significantly higher than TRAIL-R1 (p=0.005) (Figure 3A). The difference in antigenic load between TRAIL-R1 and TRAIL-R2 was statistically significant (p=0.01) (Figure 3B). The antigenic load for TRAIL-R2 was 56 (95% CI: 42-70). A positive correlation was found between survival and the antigenic load of TRAIL-R2 (Spearman r: 0.29; 95% CI: 0.030 to 0.51; p=0.022) (Figure 4 B).

Survival curves of the staining intensity or the antigenic load (AGL) within the subgroup are expressed in Figure 6.

Analysis in the total study group (n=62) showed that the dichotomized staining intensity for TRAIL-R2 (0/1 vs. 2/3) (p=0.009) and AGL TRAIL-R2 (p=0.003) were significantly
associated with survival in univariate analysis (Table 3). The % of TRAIL-R2 expression on tumor cells (p=0.07) did not reach significance. In multivariate analysis, both dichotomized staining intensity and AGL TRAIL-R2 maintained significance (p=0.004 resp. p=0.03) and the % TRAIL-R2 expression was almost significant (p=0.059)(Table 3).

Multivariate analysis within the subgroup showed that the parameters dichotomized staining TRAIL-R2 (p=0.002) and AGL TRAIL-R2 (p=0.004) were independent factors associated with survival. The % TRAIL-R2 expression (p=0.08) was not associated with survival.

RT-PCR analysis of TRAIL receptors in astrocytic tumors and normal brain

**TRAIL-R1**
TRAIL-R1 mRNA could be detected in all tissue samples tested for mRNA expression (Figure 7). RT-PCR showed a significant difference in mRNA expression of TRAIL-R1 within the five groups of primary brain tumors (Kruskal-Wallis-test; p=0.03) (Figure 8A). The primary GBM group showed a relative downregulation of TRAIL-R1 mRNA in compared to the other primary tumors and normal brain tissue. There seems to be an inverse relation between the mRNA expression and the WHO grade.

**TRAIL-R2**
The splice variants of TRAIL-R2 were detected in all tumors (Figure 7), upregulation of either of the variants was not detected. The normalized level of TRAIL-R2 was a fraction higher in compared to TRAIL-R1 (Figure 8B). No significant difference in TRAIL-R2 mRNA could be detected among the various astrocytic tumors (p=0.07).
Kaplan Meier curve of overall survival of 62 patients with a glioblastoma multiforme. Mean survival is at 259 days.
The percentage of positive tumor cells (a) of TRAIL-R1, TRAIL-R2 and TRAIL within the intermediate zone of the tumor tissue of patients with a GBM. The “antigenic load” (b) is the product of the staining intensity and the percentage of TRAIL-receptor or TRAIL positive tumor cells. The antigenic load is scaled from 0 to 300. For both parameters a significant difference between TRAIL-R1 and TRAIL-R2 expression could be found. Bars represent 95% CI.
Correlation between survival and the antigenic load of TRAIL-R1 (Spearman r: 0.28; 95% CI: 0.030 to 0.50; p=0.024) and TRAIL-R2 (Spearman r: 0.29; 95% CI: 0.030 to 0.51; p=0.022).
Cox proportional hazard survival curves based on multivariate analysis of patients with a GBM with regard to TRAIL-R1 expression. (a) survival curves related to staining intensity for the total group. (b) dichotomized analysis of AGL ≤ 20 vs. > 20 within the subgroup (N=47) (p=0.023).
Cox proportional survival curves based on multivariate analysis of patients with a primary GBM with regard to TRAIL-R2 expression. (a) survival curves related to staining intensity for the total group (N=62). (b) survival curves (p=0.002) of low (0/1) versus high (2/3) staining intensity of TRAIL-R2 within the subgroup (N=47). (c) dichotomized analysis of AGL ≤ 40 versus > 40 within the subgroup (p=0.004).
Figure 7

Representative samples of mRNA expression of TRAIL-R1 (154 bp) and TRAIL-R2 splice variants (TRICK2a (93 bp) and TRICK-2b (181 bp)) of primary astrocytic tumors and normal brain tissue. All samples showed TRAIL-R1 and TRAIL-R2 variant expression. The negative control (not shown) showed no expression. GAPDH (500 bp) was used as reference. Pilocytic astrocytoma: lane 8; diffuse astrocytoma: lane 5, 10, 12; anaplastic astrocytoma: lane 3 and 7; primary GBM: lane 4, 6, 13; secondary GBM: lane 11 and 14; normal brain tissue: lane 1, 2, and 9. Lane 15 is a 100 bp ladder.
RT-PCR expression of TRAIL-R1 and TRAIL-R2 in various astrocytic brain tumors and normal brain. A Kruskal-Wallis test between the various astrocytic groups showed a statistical significant difference for TRAIL-R1 (p=0.03). TRAIL-R2 mRNA is higher expressed in comparison to TRAIL-R1. No significant difference was found between the various grades of astrocytic tumors for TRAIL-R2 (p=0.07). GBM n=19; pilocytic astrocytoma n=3; diffuse astrocytoma n=6; anaplastic astrocytoma n=3; secondary GBM n=3 and normal brain tissue n=4. GAPDH was used as reference.
### Table 1: Expression pattern of TRAIL receptors in glioblastoma multiforme tumors within the intermediate zone

<table>
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<tr>
<th>Staining category</th>
<th>TRAIL-R1 (n=62)</th>
<th>TRAIL-R1 (n=62) mean antigenic load</th>
<th>TRAIL-R2 (n=62)</th>
<th>TRAIL-R2 (n=62) mean antigenic load</th>
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<td>0</td>
<td>15 (24.2%)</td>
<td>0</td>
<td>5 (8.1%)</td>
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<tr>
<td>1</td>
<td>12 (19.4%)</td>
<td>12.9</td>
<td>23 (37.1%)</td>
<td>26.3</td>
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<td>2</td>
<td>28 (45.1%)</td>
<td>54.5</td>
<td>19 (30.6%)</td>
<td>68.5</td>
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<td>3</td>
<td>7 (11.3%)</td>
<td>106.5</td>
<td>15 (24.2%)</td>
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### Table 2: Correlation between survival and TRAIL receptor expression within the intermediate zone

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<th>Survival</th>
<th>TRAIL-R1 (% pos. cells in tumor)</th>
<th>TRAIL-R2 (% pos. cells in tumor)</th>
<th>TRAIL-R1 (antigenic load)</th>
<th>TRAIL-R2 (antigenic load)</th>
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Table 3 Univariate and multivariate analyses of survival in patients with a GBM

<table>
<thead>
<tr>
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<th>Total study group (n=62)</th>
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<th>Multivariate analysis</th>
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Subgroup (n=47)

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</tr>
<tr>
<td>% TRAIL-R1 expression</td>
<td>1</td>
<td>1.00-1.02</td>
<td>0.054</td>
</tr>
<tr>
<td>% TRAIL-R2 expression</td>
<td>1.01</td>
<td>1.00-1.03</td>
<td>0.056</td>
</tr>
</tbody>
</table>
Discussion

Multivariate analysis in the total study group did not associate TRAIL-R1 (staining intensity, AGL and % TRAIL-R1 expression) as an independent variable for survival.

Fifteen patients (24%) of the total study group, rapidly deteriorated after neurosurgical debulking and for this reason these patients did not receive radiotherapy. Most of these patients had a KPS between 70 and 80 and in our hospital patients with a KPS between 70 and 80 are eligible for operation. The consequence of operating this patient group was the fact that a minor discomfort during hospital stay or after discharge lead to deterioration of the patient with dropping of the KPS under 70 and subsequently these patients were not eligible for further postoperative radiotherapy. The rapid deterioration preceded the start of the radiotherapy and with this in mind, not having had radiotherapy was not the reason for their rapid deterioration but other influences were. To eliminate the radiotherapy bias on the patient group, these patients were excluded in a multivariate subgroup analysis.

In the subgroup analysis TRAIL-R1 (AGL and % TRAIL-R1 expression) were independent variables for survival. The staining intensity was also in the subgroup analysis not associated with survival. Therefore the staining intensity alone cannot be used to categorize patients in a certain prognostic survival group. However measurement of the staining intensity can be of importance as an adjuvant parameter in relation to quantitative measurements such as the percentage positive tumor cells for TRAIL-R1 expression. The study results show that the antigenic load, used as a variable, in evaluation of TRAIL receptor expression, is more significant associated with survival than the individual semi-quantitative or quantitative parameters.

RT-PCR analysis detected an inverse correlation between the amount of TRAIL-R1 mRNA and the WHO grade of astrocytic tumors. Low grade astrocytic tumors showed a higher expression of TRAIL-R1 mRNA than high grade astrocytic tumors. The results of both the multivariate analysis and the RT-PCR indicate that TRAIL-R1 expression is a prognostic factor for survival in patients with a primary GBM. Sträter et al. found similar results in patients with a colon carcinoma [17].

Multivariate analysis of TRAIL-R2 (staining intensity and AGL) shows that these variables are independently associated with survival both in the total study group and in the subgroup.

As shown in Table 3, age was not independently associated with survival. Within this study only primary GBM’s were subject of investigation. Secondary GBM were not included. Secondary GBM’s tend to have a peak incidence at a younger age than primary GBM. Therefore our study population is older and because of the fact that they are all
primary GBM’s, the standard deviation of the parameter age within the study group is small with the consequence that age is not associated with survival.

TRAIL-R1 is a death inducing receptor which is present on normal human tissue [18] and also on various tumors including brain neoplasms [12]. Soluble TRAIL can bind to the TRAIL-R1 receptor, thereby inducing an intracytoplasmic cascade of effector proteins, which eventually leads to apoptosis [8,19]. Interestingly, apoptosis can be induced even at low concentrations of sTRAIL binding to TRAIL-R1. In that respect TRAIL-R1 could be of importance as a target for human anticancer TRAIL therapy.

Frank et al. [12] described TRAIL receptor expression in the cytoplasm of various tumors, including 5 glioblastoma specimens as well as in normal brain. They found TRAIL receptor expression on the mRNA level within every tumor specimen they investigated. We also detected TRAIL-R1 receptor expression on the mRNA level in 100% of the glioblastomas, other glial tumors and normal cerebral tissue. TRAIL-R1 mRNA was higher expressed in the low grade astrocytomas when compared to high grade tumors.

Through downregulation of TRAIL-R1 on both the protein and receptor level high grade glioma tumor cells might escape apoptosis induction from TRAIL-TRAIL receptor interaction.

In order to be informed about the actual receptor status of GBM tumor tissue, TRAIL receptor expression on the cell membrane should be assessed on the protein level. The IHC data in this study showed both cytoplasmic and, a more intense, membranous staining. The staining intensity in the intermediate zone was stronger than in the perinecrotic zone, although this difference was not significant. On the basis of the semi-quantitative IHC analysis, TRAIL-R1 was present in more than 75% of the GBM tumors. Morphometrical analysis showed a mean TRAIL-R1 expression in 19% of the tumor cells (95% CI: 13-25%) which was significantly correlated with survival. The antigenic load was also significantly positively correlated with survival.

TRAIL-R2 is like TRAIL-R1 a death inducing receptor. However, apoptosis induction through this receptor differs from that of TRAIL-R1. TRAIL-R2 can only be activated by very high levels of monomeric soluble TRAIL or crosslinked soluble TRAIL [20], while TRAIL-R1 can be activated by monomeric sTRAIL. These distinct apoptosis activating mechanisms between TRAIL-R1 and TRAIL-R2 may have consequences for treatment of GBM patients with soluble human TRAIL.

In general, semi-quantitative expression of TRAIL-R2 was more intense than TRAIL-R1, although statistical significance was not reached. TRAIL-R2 was expressed in more than 90% of the tumors. Furthermore, a positive correlation was found between tumor cell expression of TRAIL-R1 and TRAIL-R2. The quantitative and semi-quantitative analysis
of TRAIL-R2 did not correlate with survival. Surprisingly the antigenic load for TRAIL-R2, like TRAIL-R1, was significantly correlated with survival and seems of more importance as a prognostic parameter than the separate quantitative and semi-quantitative results.

A mean of 27% of the tumor cells was positive for TRAIL-R2 expression. The difference in percentage of tumor cells expressing TRAIL-R2 compared to TRAIL-R1 was statistically significant. The data presented in this study confirm the results of in vitro studies regarding the higher expression of TRAIL-R2 in GBM cell lines, compared with TRAIL-R1 [8]. The difference however is not as distinct as it is in the in vitro data. The percentage of TRAIL receptor expression in tumor tissue in this study is much lower compared with the in vitro data from tumor cell lines. In GBM cell lines, 58 to 99% TRAIL-R2 positive cells were observed using FACS analysis [8]. No relevant TRAIL-R1 expression in these GBM cell lines was found. One could argue the relevancy of the amount of TRAIL receptor expression in cell lines that are homogenous tumor cells and therefore not representative for the heterogeneous phenotype of primary GBM tissue. Another argument explaining the difference, in expression level of both receptors, between the in vitro and our data may be due to the method of detection. Flow cytometric evaluation is more sensitive to detect TRAIL receptor expression on tumor cell membranes than immunohistochemistry. In that respect FACS analysis of cell suspensions of primary tumor tissue could give much more insight in the death inducing receptor expression on primary GBM tumor tissue.

Like Frank et al. [12] we also found in all of the investigated tissue specimens (glioma tumor tissue and normal brain tissue) TRAIL-R2 mRNA expression and the TRAIL-R2 transcript variants TRICK-2a and TRICK-2b. In contrast with TRAIL-R1 mRNA expression, no correlation of TRAIL-R2 mRNA expression, with survival could be found.

The relatively low percentage of TRAIL-R1 and TRAIL-R2 positive tumor cells raises the question, whether treatment of glioblastomas with TRAIL alone will be sufficient, or that combination therapies might be more effective. From the study of Stupp et al. [2] we can conclude that, Temozolomide as an adjuvant to radiotherapy, prolongs overall survival with 2 to 3 months. As with Temozolomide we might suspect that TRAIL therapy also prolongs survival particularly in glioblastomas with a high percentage of TRAIL receptors and therefore TRAIL can be of importance as an adjuvant therapy besides radiotherapy and chemotherapy.

Finally we analyzed TRAIL expression within GBM tumor tissue. TRAIL is also endogenously expressed within various tumors. The presence of TRAIL in tumors and its absence in normal brain tissue is not fully understood [21, 22]. Rieger et al. [23] stated that TRAIL expression on tumor cells may be a part of the defense system of glioma cells to mediate tumor immune escape, as has been suggested for the CD95 ligand. Membranous TRAIL expression could be used as a protective shield against activated
human T cells that want to attack the immunogenic tumor cell. TRAIL expressed on tumor cells could bind to TRAIL receptors on T cells and subsequently induce apoptosis in these T cells and thereby inactivating the immune defense system. In this study TRAIL expression was shown in 85% of the tumors and a mean of 13% TRAIL positive tumor cells was found. There was a stronger staining intensity of TRAIL within the perinecrotic zone in comparison with the intermediate zone, and this difference was found to be statistically significant (p=0.0001). This result differs from data showing that FasL, like TRAIL a member of the TNF family, was present on glioma cells but without preferential perinecrotic distribution [24].

In two publications [21,23] semi-quantitative TRAIL expression on human brain tumors using immunohistochemistry is described. In the astrocytoma group grade 1 to 4, 100% of these tumors were positive for TRAIL.

Soluble TRAIL is an apoptosis inducing ligand and one of the TNF related ligands. Other ligands belonging to this family are TNF-alpha and FasL. All three ligands can induce apoptosis. However therapeutic systemic levels of FasL and TNF-alpha were found to be lethally toxic in humans and in animals [25,26]. TRAIL was studied in rodents and in non-human primates and showed no toxicity [3]. Because of its favorable toxicity profile and its apoptosis inducing potential in various glioblastoma cell lines it seems to be a suitable antineoplastic agent.

However, the widespread expression of TRAIL receptors throughout the human body and the uncertainty about TRAIL-related toxicity towards certain normal cells and tissues [5,6] and also the relative low expression of TRAIL-receptors on glioblastoma tissue might hamper its clinical development. To prevent toxicity towards normal tissue it is of great importance to deliver TRAIL as selectively as possible to tumor tissue with a maximal mean tolerable dose (MTD). It could well be that the MTD for TRAIL is high enough to induce an apoptotic signal through the TRAIL-R1 receptor but too low for activation of the TRAIL-R2 receptor. A possible solution to bypass systemic toxicity of TRAIL and to optimize its apoptosis inducing potential for both receptors is target cell restricted enhanced apoptosis induction through a scFv:sTRAIL protein with specificity for a preselected tumor cell surface target antigen. Recently it was demonstrated that augmentation of the therapeutic value of TRAIL can be achieved by increasing its tumor selective binding properties through genetic fusion to a tumor-selective antibody fragment (scFv) [20,27]. Primary glioblastomas selectively overexpress the EGFR and the concept of targeting TRAIL to this receptor could be applicable in glioblastoma treatment [28]. Through this concept TRAIL can be targeted in a specific way to the tumor and thereby bypassing normal tissues expressing also TRAIL receptors. In this way systemic toxicity can be minimized. Another advantage of the target cell restricted apoptosis induction concept is the fact that both TRAIL-R1 and TRAIL-R2 are activated at the same time although both receptors have a distinct apoptosis induction mechanism. This
study shows that TRAIL-R2 is more expressed within GBM tumor tissue than TRAIL-R1. Therefore TRAIL-R2 is an important target for TRAIL therapy. Because of its dependence on crosslinked TRAIL in order to induce apoptosis, the target cell restricted apoptosis induction through a scFv:sTRAIL protein might be of great importance in future TRAIL therapy.

Since we do not know how many tumor cells must be positive for TRAIL receptors to induce enough cell kill for tumor reduction, future clinical trials with TRAIL or scFv:sTRAIL proteins in GBM patients should include assessment of the TRAIL receptor status and cell surface target antigen levels on tumor tissue in order to optimize the therapeutic use of TRAIL in the individual patient.
References
