Chapter 7

Effect of γ-radiation on rhTRAIL efficacy in glioblastoma multiforme cells

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Abstract

Objectives: It has been suggested that radiation can enhance the apoptosis-inducing efficacy of the TNF Related Apoptosis Inducing Ligand (TRAIL). Whether or not this is a general effect seen in all TRAIL receptor-positive cell lines and whether or not this also enhances the ultimate loss of clonogenicity of tumor cells remains to be elucidated. In this study we evaluated the effect of combined $\gamma$-radiation-TRAIL therapy in a glioblastoma cell line, measuring both early apoptotic cell death and clonogenic ability as endpoints.

Methods: Glioblastoma A172 cells were treated by TRAIL, radiation or a combination of both treatments and cell death was analyzed by short term assays (Crystal Violet; FACS analysis) and a long term assay (clonogenic). Cell surface expression of the TRAIL receptor before and after radiation was evaluated by flow cytometry.

Results: Glioblastoma A172 cells expressed both the TRAIL R1 and –R2 receptor on the cell surface, but radiation did not increase the cell surface expression of these 2 receptors. Yet, the combination of radiation and TRAIL lead to a small synergy in apoptosis induction. However, this did not translate to synergy in ultimate loss of clonogenicity where radiation and TRAIL merely showed additive effects.

Conclusion: Up-regulation of TRAIL receptors by radiation does not seem a generic feature that is seen in all cell lines. Synergistic induction of apoptosis can occur, either via upregulation of TRAIL receptors (literature studies) or via dual triggering of synergizing signaling pathways that induce apoptosis (as suggestive from this study). This, however, does not (always) results in synergy at the level of clonogenic ability. Therefore when combining TRAIL treatment as an adjuvant to radiotherapy in the treatment of patients with malignant brain tumors, a synergistic interaction between the modalities is not to be expected.
Introduction

Glioblastoma multiforme tumors (GBM) tumors have been shown to be intrinsically resistant to radiation and although radiation has effect on overall survival, cures are not obtained. The development of new treatment modalities for patients with a GBM with the primary goal to enhance survival is an ongoing process. Such a new treatment modality could be the death inducing ligand TRAIL in combination with radiation. The subject of this paper is the death inducing effect of TRAIL in combination with radiation in a glioblastoma cell line.

Recombinant human TNF Related Apoptosis Inducing Ligand (rhTRAIL), a soluble death inducing ligand, induces cell death via binding to the transmembranous TRAIL-R1 and/or TRAIL-R2 receptors which triggers apoptosis. In a previous study we detected the presence of TRAIL-R1 and TRAIL-R2 receptors on primary GBM tumor tissue and showed the association of these receptors with tumor cell survival [1]. Various in vitro and animal in vivo experiments have shown the death inducing potential of TRAIL, without obvious toxicity for normal cells [2,3]. These results may implicate that rhTRAIL can be used as a therapeutic modality in the treatment of patients with various tumors, including those patients with a GBM. Unfortunately some GBM cell lines show also resistance to TRAIL [4,5].

An option may be to combine radiation and rhTRAIL to overcome resistance to either therapy. In several in vitro and ex vivo studies on non-GBM cells this was shown to be an effective combination therapy [6-11]. In fact, it has been suggested for leukemic and breast cancer cells that radiation upregulates TRAIL-receptors and enhances its cell surface expression, hereby enhancing the cellular sensitivity to TRAIL-mediated cell killing [10,11,8]. In contrast to this idea, Ciusani et al. [12] found no detectable effect of radiation (2Gy) on TRAIL receptor expression in two brain tumor cell lines (U373 and the SW1783). They also reported (data not shown) that apoptosis measurement (Annexin-V) after combinational treatment showed a modest (SW1783) to no (U373) increase in the number of apoptotic cells.

Most papers, addressing the combinational effect of radiation and TRAIL agonists, evaluate apoptosis as a short term end-point. Therefore, these studies do not take into account other modes of cell death that influence ultimate loss of clonogenicity of tumor cells. In other words, the same cells that would normally die late after radiation due to mitotic catastrophe may now die earlier from rhTRAIL induced (early) apoptosis due to radiation-induced upregulation of the TRAIL receptors, meaning that tumor cell death would be accelerated but ultimately not increased. This could lead to false conclusions regarding the potential enhancing effect of radiation on TRAIL and vice versa. In this study we therefore re-evaluated the effect of γ-radiation in combination with TRAIL therapy in a glioma cell line, evaluating both early apoptotic cell death and clonogenic ability as endpoints.
Materials and Methods

Cell lines

The glioblastoma cell line A172 was purchased from ATCC (Manassas, USA). A172 cells were cultured in DMEM, supplemented with 10% fetal calf serum (FCS) and 4 mM L-glutamine (Cambrex Bio Science) at 37°C in humidified 5% CO2 atmosphere.

TRAIL, antibodies and DiOC6

Recombinant human soluble TRAIL (rhTRAIL) was kindly provided by S. de Jong (University Medical Center Groningen, University of Groningen, Department of Internal Medicine, section Medical Oncology). Monoclonal (human) antibodies against TRAIL receptor TRAIL-R1 and TRAIL-R2 were purchased from Alexis Biochemicals (Benelux). Phycoerythrin-labeled goat anti-mouse IgG (GAM-PE) was used as a secondary antibody (DakoCytomation, Denmark). The cell-permeant green-fluorescent lipophylic dye DiOC6 was purchased from Molecular Probes, Eugene, OR.

Radiation

Cells were washed twice with phosphate buffered saline (PBS) and trypsinized, followed by neutralisation of trypsin with DMEM medium. After centrifugation (1500 rpm for 8 minutes) cells were resuspended in fresh medium at a concentration of 5 * 10^5 cells/ml (clonogenic assay) or 1 * 10^6 cells/ml (crystal violet assay, flow cytometry). In all experiments (unless mentioned otherwise), radiation was performed in suspension, using a 137-Cs gamma ray unit (IBL 637, CIS Biointernational, GIF sur Yvette, France) at a dose rate of 0.7895 Gy/min.

Flow cytometry

Flow cytometric analysis was performed at various intervals after radiation (8, 24 and 48 hrs). Cells were irradiated while plated (and attached) in T75 flasks. After radiation with 2 or 5 Gy, flasks were put back in a culture stove (37°C, 5% CO2). At the day of analysis, cells were washed twice with PBS and trypsinized. Cells were resuspended in fresh medium at a concentration of 5 * 10^5 cells/ml. After centrifugation (1600 rpm for 5 minutes) cells were resuspended in medium containing the various antibodies (aTRAIL-R1, aTRAIL-R2). Cells were incubated for 40 minutes on ice. After washing the cells with fresh medium, cells were incubated with the secondary antibody, GAM-PE, for 40 minutes (on ice). Cells were then washed twice with fresh medium and FACS analysis was performed using the ELITE flow cytometer.
Apoptosis was also measured using flow cytometry. Cells were harvested and radiated with 2 or 5 Gy. After radiation, cells were plated in 12-well plates (0,5 * 10^6 cells/well). Eight hours after radiation, rhTRAIL or medium was added to the different wells. The next day, all cells (adherent and non-adherent) were harvested. After centrifugation, cells were resuspended and DiOC6 was added. After incubation for 30 minutes at 37°C, cells were washed and analyzed by the Calibur flow cytometer.

**Crystal violet viability assay**

Tumor cell viability was assessed by a crystal violet assay. Cells were washed twice with PBS and trypsinized, followed by neutralisation of the trypsin with DMEM medium. After centrifugation (1500 rpm for 8 minutes) cells were resuspended in fresh medium and radiated. After radiation, cells were seeded in flat-bottom 96-well microculture plates at a concentration of 3*10^4 cells/well in 200 μl medium. Eight hours after radiation, 100 μl medium was removed and replaced by 100 μl fresh medium containing the various concentrations of rhTRAIL. Cells were reincubated overnight cells followed by a washing with PBS, staining with crystal violet solution and solubilized in 1% SDS. Absorbance was read at 575 nm by an ELISA-plate reader.

**Clonogenic assay**

Cells were washed twice with PBS and trypsinized. Cells were radiated and plated in 60 mm petri-dishes. Appropriate dilutions were made to yield 50 – 100 colonies per petri-dish. After plating the sample cells, 105 feeder cells (cells lethally radiated with 100 Gy) were added to each plate. Eight hours after radiation, rhTRAIL was added. Cells were stored in an incubator at 37°C and 5% CO2, for 12 – 14 days. After this period the medium was removed and cells were washed once with PBS. After fixating with 70% ethanol, cells were stained with 1% crystal violet solution and washed twice in tap water. Colonies containing more than 50 cells were counted. Survival was calculated with the formula: (Number of colonies counted/number of cells plated) x (1/plating efficiency) The plating efficiency (PE) is the colony count resulting from 100 untreated cells. In this study, the PE of A172 cells was 65%.

**Data analysis and statistics**

Interactions between TRAIL and radiation were analyzed by the fractional inhibition method. When expressed as the fractional inhibition cell viability, additive inhibition produced by both inhibitors (Ia,b) occurs when Ia,b =Ia + Ib; synergism when Ia,b >Ia + Ib and antagonism when Ia,b< Ia + Ib. Differences between two groups were tested for
Significance using a two-tailed non-parametric Mann-Whitney test. Differences between multiple groups (>2) were tested using one-way analysis of variance (ANOVA).

Results

Early cell death induced by rhTRAIL and radiation

First, we tested if combined treatment of radiation and recombinant human (rh) TRAIL would lead to enhancement of cell death in glioblastoma A172 cells using the crystal violet assay (Figure 1A). Rapid cell death after radiation alone was only 4%. Recombinant human (rh)TRAIL resulted in respectively 45% (10 ng/ml) and 65% (100 ng/ml) apoptosis. The combined treatment of radiation and rhTRAIL induced a significantly higher fraction of cell death than rhTRAIL alone (55% respectively 77% for 10 ng/ml and 100 ng/ml (p=0.01)). In Figure 1B it can be seen that the extent of rapid cell death after the combined treatment was significantly synergistic, albeit that the magnitude of the synergistic effect was small (rhTRAIL 10 ng/ml: p = 0.04; rhTRAIL 100 ng/ml: p = 0.02).

To test whether the rapid cell death was indeed due to increased apoptosis induction, we used flow cytometry. Figure 2 (pg 192) shows the FACS results of A172 cells after treatment with rhTRAIL, radiation or a combination of rhTRAIL and radiation. The fraction of apoptotic cells (cell under the pre G1 peak) increased from 5% (no treatment) to 40% and 77% after 10 or 100 ng/ml rhTRAIL respectively (Figure 2A, pg 192). After irradiation (2Gy) alone 11% of cells are apoptotic by these criteria (Figure 2B, pg 192). The combined treatment of radiation and rhTRAIL (10 ng/ml and 100 ng/ml) resulted respectively in 54.93% and 90.91% apoptosis. So, these data are consistent with the crystal violet data (Figure 1) and with the idea that this assay predominantly registers apoptotic cell death.

Clonogenic assay shows additive killing after combination treatment

To test whether the combined treatments not only accelerate cell death but also increase the ultimate level of cell death, we evaluated the ability of cells to form colonies after the single and combined treatments (Figure 3). Remarkably, whereas the early cell death assays only revealed 4% (crystal violet) respectively 11 % (FACS) cell death after 2Gy alone, only 25% of the cells were able to form colonies after this dose of radiation. This indicates that early apoptotic cell death only plays a minor role in radiation-induced cell death of A172 cells.

Cell survival was reduced in the non-irradiated group to 84.3%, 23.0% and 0.009% after 1, 5 and 10 ng/ml rhTRAIL respectively (Figure 3). Compared to the 40-45% apoptotic cell death after 10 ng/ml rhTRAIL in the short term assays (Figure 1 and 2 (pg 192)) this means that even TRAIL induces additional (delayed) cell death through non-classical pathways.
besides via rapid apoptosis alone. Treatment with 100 ng/ml rhTRAIL could not be analyzed due to the massive toxicity in the clonogenic assay (no colonies were detected).

As shown in Figure 3, the combined treatment with 2 Gy radiation and variable rhTRAIL concentrations (1, 5, 10 ng/ml) had merely additive effects on the clonogenicity of glioma cells. After treatment with radiation and 10 ng/ml TRAIL, there were no colonies found in plates where 40,000 cells were plated. Therefore, cell survival after this combined treatment was indicated as less than 0.0025% (shown by the arrow in Figure 3). So, although the combined treatments led to slightly more than additive rapid apoptosis, no enhancement of ultimate cell death was seen after the combination treatment.

**TRAIL receptor expression is not increased after radiation**

To test the idea that radiation may up-regulate TRAIL receptors at the cell surface and as such increase rapid apoptosis, we evaluated the cell surface expression of the TRAIL receptors TRAIL-R1 and TRAIL–R2 in A172 cells, by flow cytometry. Only 2.1% of the A172 cells expressed TRAIL-R1, whereas TRAIL-R2 was present on 99.2% of the cells (data not shown). After radiation (2 or 5 Gy), no significant radiation dependent changes were detectable in the expression of these two TRAIL receptors (Figure 4 and 5). The overall expression of TRAIL-R1 receptors did increase with time within all experimental groups, however, no statistical difference could be found in this increase between the control group and the radiation groups (Figure 4A). Also the number of TRAIL-R1 receptor positive cells remains extremely low over time and there was no significant increase after radiation (Figure 4B). Although upregulation of TRAIL-R2 receptor expression was seen after radiation, this effect was not significant and independent of time after radiation (Figure 5A). Also, the percentage of TRAIL-R2 positive cells was high and remained high with time without being affected by radiation (Figure 5B).
Rapid cell death induced as determined by the Crystal Violet assay after radiation, rhTRAIL (10 and 100 ng/ml) or a sequential treatment of radiation and rhTRAIL.

Panel A: Fraction of cell death after the respective treatments (* = rhTRAIL).

Panel B: comparison between the “observed” cell death and the calculated “additive” effect of both treatments (TRAIL 10= TRAIL 10 ng/ml). Data points represent the means and 95% confidence interval of 3 independent Crystal Violet assays.
Clonogenic survival of A172 cells after treatment with graded concentrations of rhTRAIL either given alone (control, squares) or in combination with 2Gy irradiation (circles). The parallel lines indicate an additive effect of the combined treatment with radiation and rhTRAIL. The plating efficiency of the untreated A172 cells was 65 and set as 100%. ↓: Less than 0.0025% survival. This figure is a representative of 2 independent experiments performed in triplicate.
Effect of radiation on TRAIL-R1 cell surface expression (panel A) and the percentage of TRAIL-R1 positive cells as measured by FACS analyses.

Panel A shows the mean fluorescence intensity as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares). The mean fluorescent intensity immediately after (sham) treatment of control cells was set as 100%. Bars represent SD of two independent experiments.

Panel B shows the fraction of cells with cell surface expression of the TRAIL-R1 receptor as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares).
Figure 5

**Panel A** shows the mean fluorescence intensity as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares). The mean fluorescent intensity immediately after (sham) treatment of control cells was set as 100%. Bars express SD of two independent experiments.

**Panel B** shows the fraction of cells with cell surface expression of the TRAIL-R2 receptor as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares).

Effect of radiation on TRAIL-R2 cell surface expression (panel A) and the percentage of TRAIL-R2 positive cells as measured by FACS analyses.

Panel A shows the mean fluorescence intensity as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares). The mean fluorescent intensity immediately after (sham) treatment of control cells was set as 100%. Bars express SD of two independent experiments. Panel B shows the fraction of cells with cell surface expression of the TRAIL-R2 receptor as a function of time in culture of untreated cells (triangles) or after irradiation of the cells with 2 Gy (diamonds) or 5 Gy (squares).
Discussion

It had been suggested by several investigators that radiation may lead to an enhancement of TRAIL-toxicity and as such this combined modality may have therapeutic potential. [6-12,14] In most cases, the effect of radiation and TRAIL on cell survival had been only investigated using short term viability assays (crystal violet, MTT). [6-11,14] However, these assays do not take into account (later) cell death e.g. due to mitotic catastrophy of cells that did not undergo apoptosis initially. As we show here for radiation, such can lead to a dramatic underestimation of ultimate loss of reproductive capacity of cells (the ultimate goal in cancer therapy) as it is detected by clonogenic assay. Similar data were reported in a recent study by Nagane et al. [14]. In their study, a synergistic effect on apoptosis (TUNEL assay) by the combination therapy was found in 2 cell lines (T98G and U251). However, in only one cell line (T98G) this translated into some synergy when evaluated by the clonogenic assay. In the other cell line (U251) only additivity was found for this endpoint, like what we show here for the A172 cells. Therefore short term assays may be mis-interpreted in terms of synergy if certain treatments lead to accelerated (apoptotic) cell death without having an effect of the extent of ultimate cell death.

In our current study, we tried to test whether radiation would also synergistically interact with TRAIL in inducing cell death in a glioblastoma cell line A172 in order to bypass the typical radio-resistance of GBM tumor cells. Although, we found borderline significant synergy for early cell death induction (apoptosis, MTT), as was found by many others [6-12,14] no evidence was found to support the idea that the combination treatment enhanced the extent of ultimate killing (clonogenic assay) of radio-resistant A172 cells. Rather, the treatments were additive.

A possible explanation of synergistic apoptosis induction after combination treatment, which is frequently stated in the literature, could be radiation-induced upregulation of TRAIL receptors, thereby enhancing receptor modulated rhTRAIL killing [7,14,15]. In this study, no radiation-induced upregulation of membrane bound TRAIL-R1/R2 receptors in A172 cells was detected. Nor was a detectable increase found in the fraction of cells that expressed these receptors at their surface. This may explain why we only found borderline synergy for early death induction. The fact that still some synergy is found may indicate that radiation triggers some signaling pathways that facilitate TRAIL-induced apoptosis or vice versa. In any case, the effect we found in glioma cells was minor and consistent with Ciusani et al. who also found no up-regulation of TRAIL receptors (FACS analysis) after ionizing radiation (2 Gy) on glioma cell lines [12]. Yet, Nagane et al. did find up-regulation of TRAIL-R2 protein levels after radiation in some of the glioma cell lines investigated [14]. However, they did not evaluate whether this also lead to increased membrane receptor expression. So unlike other cell lines such as acute T lymphoblastic leukemia (MOLT-4) cells [11] and colorectal carcinoma (Colo205; HCT-15), lung (NCI-H460)[16] and breast carcinoma cells (MCF7)[10], several glioma cells may be radiation
resistant in terms of up-regulating these receptors and this may in fact contribute to a reduced in situ tumor radio-resistance on top of their intrinsic cellular radio-resistance.

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 [6-12]. In none of these studies the efficacy of TRAIL and radiation was determined used a clonogenic assay. 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.

Finally, our findings demonstrate that glioma cells do express TRAIL receptors at their cell surface and are sensitive to TRAIL-induced cell death. This implies that glioma radio-resistance may not be associated with a cross-resistance to TRAIL. Hence, our study does not exclude rhTRAIL treatment as an adjuvant therapy in the treatment of patients with a malignant brain tumor. However, although only one glioma cell line was studied here, our data combined with Nagane et al. [14] suggest that a synergistic interaction between the modalities is not to be expected for several gliomas.
References

