Effect of Irradiation on stem cell based delivery of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) in gliomas

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Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive form of brain tumor and accounts for more than half of these cases. Despite clinical advances over the last few years, the median survival for patients with this malignancy has not been significantly improved. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) that has been proven to posses cancer cell specific killing potential, has drawn considerable attention for anti-tumor therapy. However, studies have shown that a significant subpopulation of the heterogeneous GBMs are TRAIL resistant. In this study, we aim to explore the effect of irradiation on sensitizing TRAIL resistant GBM cells to stem cell delivered TRAIL therapy and to mechanistically evaluate the pathways contributing to modulation of TRAIL sensitivity. Our studies demonstrate that irradiation specifically increases the surface expression levels of death receptors (DR) 4/5 on GBM cells and sensitizes GBM cells to TRAIL mediated apoptosis. We expect that these studies will have implications in translating TRAIL based therapies into clinics for treating GBMs.

Samenvatting

Glioblastoma multiforme (GBM) is de meest voorkomende en meest agressieve hersentumor die we kennen, en vertegenwoordigt de helft van alle primaire hersentumoren. Ondanks de klinische vooruitgang de afgelopen jaren, is er geen significante verbetering in de gemiddelde overleving van patienten met deze aandoening. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) heeft bewezen kanker specifieke cel dodende eigenschappen en is een mogelijke strategie voor anti-tumor therapie. Echter, uit onderzoek blijkt een significante subpopulatie van de heterogene GBMs TRAIL resistent zijn. In deze studie, hebben we onderzocht wat het effect van radiotherapie is op de gevoeligheid van GBM cellen voor TRAIL therapie toegediend middels mesenchymale stam cellen. Ook zijn de pathways verantwoordelijk voor veranderingen in TRAIL sensitiviteit bestudeerd. Onze studies laten zien dat radiotherapie GBM cellen gevoeliger maakt voor TRAIL geïnduceerde apoptose. Dit wordt mogelijk veroorzaakt door een toename van de expressie van de death receptors (DR) 4/5 op het oppervlak van de GBM cellen. Wij verwachten dat deze studie gevolgen zal hebben voor het vertalen van TRAIL gebaseerde therapie naar kliniek als behandeling voor GBMs.
# Table of contents

1. Introduction and Problem definition ................................................. 4
2. Materials and Methods ........................................................................ 7
3. Results ................................................................................................... 10
4. Discussion ............................................................................................... 14
5. Conclusion ............................................................................................... 16
6. Acknowledgements ................................................................................. 17
7. References ................................................................................................ 18
8. Supplements ............................................................................................. 23
Introduction

**GBM**

Glioblastoma Multiforme (GBM) is the most common and aggressive primary brain tumor in humans and is known for its very poor prognosis (1, 2). Without therapy, patients with GBM uniformly die within 3 months. Patients treated with optimal therapy, including surgical resection, radiation therapy, and chemotherapy, have a median survival of approximately 15 months, with less than 25% of patients surviving up to 2 years and less than 5% of GBM patients surviving 5 years post diagnosis (3-5). No underlying cause has been identified for the majority of malignant GBMs. The only established risk factor is exposure to ionizing radiation (4). Evidence for an association with head injury, occupational risk factors, and exposure to electromagnetic fields is inconclusive (4, 6, 7). When the tumors grow, they cause mass effect on important structures in the brain and this can interfere with thought, memory, emotion, movement, vision, hearing, touch, and other brain functions (8). Tumor associated edema can also affect brain function. Common symptoms include seizures, nausea and vomiting, headache, personality changes, and hemiparesis (9). However, the kind of symptoms produced depends highly on the location the tumor, more so than on its pathological properties (10). So, although uncommon, a tumor can remain asymptomatic until it reaches an enormous size. The classic headaches that are suggestive of increased intracranial pressure are most severe in the morning and may wake the patient from their sleep, but many patients experience headaches that are indistinguishable from tension headaches (3, 9).

Current treatment for GBM consists of maximal surgical tumor resection, radiation and chemotherapy (11). Even patients who undergo a gross total resection of their malignant glioma have a high recurrence rate because of the invasiveness and infiltration into the adjacent functional brain parenchyma (12). Therefore additional radiation therapy and chemotherapy is necessary. Temozolomide is currently the most commonly used chemotherapy treatment (13-15). Despite in depth knowledge of GBM pathogenic lesions and decades of advances in neurosurgery, radiation therapy and clinical trials, little improvement in the medium 15-month patient survival has been achieved. This is mostly the result of resistance to the conventional therapies (16, 17). Another problem in the treatment of GBMs is efficient delivery of therapeutic agents to the tumor. The blood brain barrier and vascular dysfunction in the tumor prevent many drugs from reaching the tumor cells (18). One of the approaches to overcome this problem is drug delivery directly to the brain.

**Irradiation**

Irradiation has been a successful cancer treatment for decades. In GBM tumors, it has been used next to and in combination with surgical tumor resection and chemotherapy. The addition of radiotherapy increases the survival among GBM patients with 3 to 4 months (14, 19). Conventional radiotherapy consists of 60 Gy of partial-field external-beam irradiation delivered 5 days per week in fractions of 1.8 to 2.0 Gy (3). After standard radiotherapy 90% of the tumors recur at the original site (20). Radiation induces apoptotic cell death by manifesting DNA damage, leading to p53-dependent cell cycle arrest (21), and inducing the intrinsic pathway leading to apoptosis (Figure 1).
TRAIL
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an attractive anti-tumor agent as it induces apoptosis in tumor cells while sparing normal cells (22, 23). TRAIL binds to its death domain-containing receptors (DR) TRAIL-Receptor1/DR4 and TRAIL-Receptor2/DR5 on the cell surface and this leads to the formation of the death-inducing signaling complex (DISC). This complex consists of the receptor, the adaptor protein Fas-associated death domain (FADD), and caspase-8. Formation of the DISC, leads to activation of pro-caspase-8. Activated caspase-8 then cleaves downstream substrates resulting in, ultimately, the activation of caspase-3 and subsequently apoptosis. Active caspase-8 also causes cleavage of Bid into tBid, which translocates to the mitochondria leading to the release of Cytochrome c. Cytochrome c can activate caspase-9, which then activates caspase-3 (24)(Figure 1). TRAIL treatment has shown impressive tumor reducing results for GBM tumors (22, 25, 26). The Shah laboratory has previously engineered a tumor specific secretable form of recombinant TRAIL, S-TRAIL. When S-TRAIL is delivered through viral vectors or different stem cell types it has shown anti-tumor effects in glioblastomas (27, 28). Because of its selective induction of apoptosis, without damaging normal cells and tissues, TRAIL is one of the most promising candidates for cancer therapy (29). However, almost 50% of the tumor lines are resistant to TRAIL mediated apoptosis. That is why there is urgent need to find new ways/mechanisms to sensitize these lines to TRAIL mediated apoptosis. Recent reports show that chemotherapeutic agents or radiotherapy can enhance TRAIL sensitivity by increasing the expression of TRAIL receptor 1 (DR4) and/or TRAIL receptor 2 (DR5) in a range of tumors (30). These findings suggest that a synergistic antitumor effect might be achieved using combination therapies.

Figure 1: Schematic representation of the intrinsic and extrinsic apoptotic pathway.
Apoptosis induction via the death receptors (DR4/DR5) can result in activation of the extrinsic and intrinsic pathways. The TRAIL ligand binds to functional receptors DR4 and DR5. Binding of TRAIL ligand or receptor-specific agonistic antibodies to DR4 and DR5 induces trimerization of the receptors. The cytoplasmic part of the DR4 and DR5 receptors contain death domains that enable recruitment of Fas-associated protein with death domain (FADD) and pro-caspase 8, enabling activation of pro-caspase 8 to its active form; caspase 8. Caspase-8 activates downstream effector caspases both directly and through the activation of the mitochondrial apoptosis pathway through BID cleavage. Once activated, effector caspases cleave downstream substrates and induce DNA fragmentation, ultimately leading to apoptosis.
Stem cell based therapies for tumors

Stem cells have the ability to home to intracranial pathologies, such as GBM tumors (31, 32). Also, they do not trigger an immense immune reaction in unrelated donor transplantation (33). These characteristics suggest that stem cells have potential use as effective delivery vehicles for therapeutic genes in GBM treatment (31). In prior research it has been established that mesenchymal stem cells (MSCs) migrate effectively to the primary tumor mass and also infiltrate in the micro-invasive tumor deposits in the brain (34). When MSCs are engineered to secrete tumor specific therapeutic protein, such as TRAIL, they have anti-tumor effects in mouse models of GBM (34, 35). Recently, the Shah laboratory has shown that these therapeutic MSCs, when encapsulated in synthetic extracellular matrices (sECM), are able to eradicate recurrent GBMs in a novel clinically relevant mouse model of GBM resection (36). This strategy is emerging as one of the most promising cell based therapeutic approaches to eradicate GBMs.

However, it is still desirable to increase the migratory capacity of MSCs to tumors even more, because this would thereby increase the delivery of the therapeutic genes to the tumor. Enhancing the MSC localization to tumors has been investigated by genetically manipulating MSCs to over-express target receptors against epidermal growth factor-producing gliomas (37) and the overexpression of any receptor on MSCs related to tumor tropism may improve their migration capacity to specific tumor cells. Another potential method to enhance engraftment is based on the observation that MSCs migrate to damaged tissue after local tumor irradiation. Several studies have shown greater MSC migration to irradiated tumors compared with un-irradiated tumors (38-40). The mechanism responsible for this increased migration and the factors related to the targeted tropism of MSCs remain to be elucidated; however, it is thought that irradiation increases inflammatory signaling, which involves secretion of chemokines or growth factors and may attract MSCs to the tumor microenvironment (38).

In this study, we aim to sensitize TRAIL resistant glioma lines to stem cell delivered TRAIL therapy, with the use of irradiation and study any possible effects and mechanistically evaluate the possible effect of radiation on TRAIL pathway to induce tumor selective apoptosis.
Material and Methods

Cell Culture
Human glioma line U373 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) media supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA). The primary cell lines GBM4 and GBM64 were grown in Neurobasal media (Invitrogen, Carlsbad, CA) supplemented with rhEGF (R&D Biosciences), rhFGF(R&D Biosystems), L-Glutamine (Invitrogen) and Heparin (Stem Cell Technologies). The mouse Mesenchymal Stem Cells (mMSC) were cultured in DMEM supplemented with FBS, Horse Serum (Invitrogen) and other supplements.

All the cells were supplemented with antibiotics (1% pen strep) and incubated at 37 °C in a humidified atmosphere.

Irradiation
For the viability assays, cells were dissociated and seeded at 1x10^4 or 5x10^3 cells per well in a 96-well culture plate, and 6 hours later exposed to γ-radiation using a gamma-cell irradiator at various doses as indicated. For the Western Blot analysis, co-culture assays and Flow Cytometry experiments, cells were dissociated and plated in a T75-flask. After 6 hours, the cells were irradiated as described above. For irradiation of the tumors in vivo the mice were anesthetized with 80 µl ketamine/xylazine peritoneally and their bodies were covered with a lead shield. Their heads were exposed to 10 Gy.

Cell Viability Assay
GBM cells where plated in a 96-well format at 1x10^4 cells per well, irradiated and treated with increasing doses of TRAIL (200ng/ml, 400 ng/mL) according to an experimental setup described later, and analyzed after 48 hours after TRAIL addition. Cell viability was measured by a quantitative luminescence assays using an ATP-dependent luminescent reagent (CellTiter-Glo, promega). Assays were performed according to the manufacturer’s instructions, and plates were read in a luminometer at 0.5 s per well. For the studies with stem cells, mMSCs were encapsulated in a biodegradable, synthetic extracellular matrix (sECM). The sECM components, Hystem and Extralink (Glycosan Hystem-C, Biotime Inc.) were reconstituted according to the manufacturer’s protocol. mMSCs (1x10^4) expressing GFP-Fluc, or S-TRAIL, were re-suspended in Hystem (60 µl) and the matrix was cross-linked by adding Extralink (30 µL). One drop (4.5 µl) of the sECM-mMSC hydrogel was placed in the middle of each glass-bottomed well of a 96-well plate. Once the gel sets in, 50 µL GBM cell media was added. GBM cells were dissociated 6h prior to irradiation. Cells were then irradiated at 10 Gy. The GBM cells (1x10^4 cells) were added to the 96-well plate containing the encapsulated mMSCs 48 Hour after radiation. Cell viability was measured 48 hours after the addition of the GBM cells by a quantitative luminescence assays using 10µg of D-luciferin (Perkin-Elmer) per well and plates were read in a luminometer with an integration time of 0.5 s.
Flow Cytometry to analyze TRAIL Death Receptors

GBM cells were treated with control (PBS) or irradiation (10 Gy) for 48h and were collected and stained with PE-conjugated anti-human DR4 (DJR1) or DR5 (DJR2-4) monoclonal antibodies (eBioscience, San Diego, CA, USA) in 0.5% BSA/PBS solution at 4°C for 30 min. Rinses were performed with 0.5% BSA/PBS at 4°C. Flow cytometry was performed using FACSaria IIu cells sorter and results were analyzed by FlowJo software.

Western Blotting

Whole cell lysates were extracted from gamma-irradiated GBM4 cells, TRAIL or the combination of Irradiation + TRAIL. Protein samples were loaded (20-30 ug) in a precast 10% gel (Ready Gels, Bio-Rad) and run at 110V for 90 min. After running the gel was transferred to PVDF membrane (Millipore Immobilon-P x IPVH 000 01) for 1 hour at 90V. After transfer, the membrane was blocked in suitable blocking buffer for 30-60 min and incubated in primary antibody overnight at 4°C. The membrane was then washed with 1xTBST solution thrice and incubated in secondary antibody for 1-2 hours. After secondary antibody the membrane was washed again with 1xTBST for 3 times and developed by using chemiluminescence.

Primary antibodies used:
- Cleaved-PARP (1:1000) (Cell Signaling Technology, Danvers, MA)
- pChk1 (1:500) (Cell Signaling Technology, Danvers, MA)
- pChk2 (1:500) (Cell Signaling Technology, Danvers, MA)
- α-Tubulin (1: 10,000) (Sigma-Aldrich, St. Louis, MO)

Secondary antibodies used:
- Goat anti Mouse-HRP (1:1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA)
- Goat anti Rabbit-HRP (1:1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA)

In-vivo Mouse Xenograft tumor models

Athymic female nude mice (6-8 weeks of age; Charles River Laboratories) 25-30 g in weight were used for the intracranial xenograft GBM model. The mice were anesthetized peritoneally with Ketamine/Xylazine anesthesia (90 mg/kg of Ketamine and 9 mg/kg of Xylazine per mouse) and immobilized in a stereotactic frame. The skin was opened and a hole was drilled 2 mm right from the sagittal suture. (Figure 4A) 2*10^5 5 GBM4-expressing Fluc-mCherry (FMC) cells were implanted stereotactically in the right frontal lobe using a Hamilton syringe (Hamilton Company, Reno, NV) using an automatic injector. After the injection was complete, the needle was slowly retracted and the hole in the skull was closed with bonewax, and the skin was closed using 4-0 Vicryl suture (Ethilon, NJ). The subcommittee and Research Animal Care at Massachusetts General Hospital approved all in vivo procedures.

Non-invasive Bioluminescence Imaging

Tumor growth was followed by using Bioluminescence imaging (BLI). Mice were anesthetized using the Ketamine regimen and also injected intraperitoneally with 1mg of D-luciferin per mouse and imaged for 5 min. Mice were imaged on days 3, 10, 19, 27, and 34 after implantations.
**Immunohistochemistry**

Mouse brains were perfused, fixed in 40% Formalin solution under deep anesthesia. The excised brains were stored in formalin at 4 °C for 24 hours after which they were transferred to 30% sucrose and stored at 4 °C until further use. Tissues were sectioned (25 micron thickness) and washed with PBS (3x10 min.) in a 24 well plate. After washes the specimens were blocked in blocking buffer (0.3% BSA, 8% serum, 0.3% Triton x-100) for 1 hour and later incubated in primary antibodies, Ki67 (1:50) (DakoCytomation A/S, Glostrup, Denmark) and pChk2 (1:200) (Cell Signaling Technology, Danvers, MA) at 4°C overnight. The next morning sections were washed with PBS (3x10 min) and then incubated in secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2 hours at 4°C. After secondary antibody the sections were washed once more with PBS (3x10 min.) and then mounted onto glass slides. The sections were sealed using Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA) and analyzed.

**Statistical Analysis**

Data was analyzed by student’s t-test when comparing two groups and by One-Way ANOVA, followed by Dunnett’s post-hoc test, for multiple comparisons. Data were expressed as mean SD, and differences were considered significant at p < 0.05.
Results

Irradiation significantly induces damage in GBM cells and also activates TRAIL-mediated apoptosis

A panel of patient derived primary GBM cell lines were screened for their sensitivity for TRAIL. (Figure 2A) From this panel GBM4 and GBM64 were selected for further studies. U373, an established glioma cell line resistant to TRAIL was used as a positive control, because of its known response to irradiation therapy (41).

Figure 2: Cytotoxic effects of TRAIL, Irradiation and Combined therapy on different GBM cell lines. (A) Viability analysis on different GBM cell lines treated with increasing doses of TRAIL. (B) GBM4 cells were exposed to radiation (40 Gy, 80 Gy) for 48hrs, and then total cell extracts were analyzed by Western Blot with phospho-Chk1 (pChk1) and phospho-Chk2 (pChk2). α-Tubulin was used as a loading control. (C) U373, GBM4, GBM64 cells were plate in 96-well plates. 48 hours after plating they were treated with TRAIL. The viability of the cells was analyzed after another 48, or 72 hours. For the effect of irradiation (10 Gy) and the effect of combined irradiation and TRAIL therapy the cells were dissociated and plated in a 96-well plate 6 hours before radiation. 48 Hours after irradiation the cells were treated with TRAIL. The viability of the cells was assayed after another 48 (GBM4) or 72 hours (U373, GBM64). Statistical comparison was done using One-Way ANOVA with post-hoc Dunnett’s test. *p < 0.05 compared to IR, p < 0.05 compared to TRAIL.
To ascertain the influence of irradiation on the induction of DNA damage, Western Blot analysis was carried out on GBM4 cells exposed to different doses of irradiation (40 Gy and 80Gy). A dose dependent increase in expression in pChk1 and pChk2 was observed following irradiation as compared to control cells (Figure 2B). The effects of irradiation alone, TRAIL, and combination were studied on GBM4, GBM64, and U373 cells. Significant reduction in cell viability of GBM4 cells as measured by changes in luminescence signal intensity was observed 48 hours after TRAIL addition, while a similar effect was observed in GBM64 and U737 72 hours after TRAIL treatment (Figure 2C).

**Radiation induces DNA damage and inhibits glioma cell proliferation in vivo**

To study the effect of radiation on the growth of GBM4 glioma cells in vivo, we employed an intracranial xenograft mouse model where in GBM4 glioma cells were stereotactically

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**Figure 3: In vivo experiments**

(A) Tumor injection side; GBM4-FMC cells were injected 2 mm right from the sagittal suture, 2.2 mm deep. (B) A representative image of one mouse with implanted GBM4-FMC cells, followed over 34 days. (C) Mice were implanted with GBM4-FMC (red) tumor cells and irradiated on day 34. 14 Days after radiation (10 Gy) one irradiated and one unirradiated (control) were sacrificed and then stained with Ki67 (green) antibody, and pChk2 (green) antibody.

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11
implanted, followed for tumor growth by bioluminescence imaging (Figure. 3B) and then radiated at 10Gy on day 35 following tumor implantation. Control and radiated mice were harvested 14 days following radiotherapy. Ki67 staining on brain sections revealed a marked reduction in proliferation of GBM4 cells as compared to control. A significant increase in pChk2 expression was observed in radiated GBM4 mice as compared to control (Figure 3C).

**In vitro cell viability assays with encapsulated stem cells**

To determine the effect of TRAIL on irradiated GBM cells a cell viability assay was carried out using sECM encapsulated Mesenchymal stem cells. Irradiation, or mMSC-TRAIL alone showed a marginal effect on glioma cells. Whereas the combined treatment of mMSC-TRAIL and irradiation did have a significant cytotoxic effect as measured by changes in luminescence signal intensity 72 hours after combining the glioma cells with encapsulated stem cells (Figure 4A).

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**Figure 4:** Therapeutic potential of mMSC secreting TRAIL in glioma therapy. (A) The effect of mMSC-TRAIL in combination with irradiation treatment was analyzed by a co-culture experiment. mMSC-GFP and mMSC-TR-GFP were encapsulated in a biodegradable synthetic extracellular matrix (sECM) and drops of the gel were plated in a 96-well plate. GBM4-FMC cells were exposed to 10 Gy at the same time. 48 hours after irradiation glioma cells were added to the 96-well plate. The viability of the glioma cells was analyzed after 72 hours. Statistic comparison was done using One-Way ANOVA with post-hoc Dunnett’s test.* p < 0.05 compared to IR, # p < 0.05 compared to TRAIL (B) Pictures of the co-culture experiment described at 3A. GBM4-FMC cells (red) and mMSC-GFP, mMSC-TR-GFP (green)

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**Radiation enhances surface expression of death receptors on glioma cells**

To explore the underlying mechanisms by which irradiation enhances TRAIL-mediated cell death, we performed a Flow cytometry analysis on GBM4 cells and determine whether irradiation had an influence on the death receptor expression on glioma cells. Our results showed an increase in the surface expression of DR4 and DR5 in radiated GBM4 (10Gy) cells as compared to control (Figure 5C).
Figure 5: **Enhanced TRAIL induced apoptosis via up-regulation of DR4 and DR5 by irradiation**

(A) GBM4 cells were exposed to irradiation (10 Gy) for 48hrs, and then total cell extracts were analyzed by Western Blot with CL-PARP. α-Tubulin was used as a loading control. (B) CL-PARP Band intensity of the Western Blot data showed in Figure 5A (C) Flow cytometry analysis was performed on irradiated (10 Gy) GBM4 cells that were stained for DR4 and DR5 expression. The shift to the right indicates an increased expression of DR4 and DR5 on the cell membrane of the GBM4 cells after treatment with irradiation.
Discussion

In this study we explored the effect of irradiation on stem cell delivery of TRAIL. We tested the sensitivity of several patient derived - primary glioma lines, and chose two TRAIL resistant lines (GBM4, GBM64) for our further experiments. We also used U373, an established glioma cell line reported to be effective in a combination therapy of radiation and TRAIL treatment as a positive control (41). The use of patient derived primary glioma lines in a resection mouse model makes the findings of the current study more clinically relevant as compared to the use of an established glioma cell line in an intact mouse model.

Radiation has been reported to have a marked effect on cell proliferation both in-vitro and in-vivo. However, different cell lines respond differently to various doses of radiation. Our findings indicate that a radiation of 10 Gy was the most effective dose and which was established following treatment of GBM4 cells at various doses of radiation (Suppl. Fig. 1). Establishing an optimal radiation dose is essential for the success of the subsequent combination therapy with stem cell – delivered TRAIL. Irradiation of GBM4 cells markedly increased the expression of p-Chk1 and p-Chk2 as compared to control. Also, the increase in expression was found to be dose dependent. Phospho-Chk1 and Phospho-Chk2 play an important role in DNA damage checkpoint control and tumor suppression (42). Activation of Chk1 and Chk2 occurs in response to blocked DNA replication and certain forms of genotoxic stress, like irradiation (43, 44). The increase in expression of p-Chk1 and 2 in the current study clearly indicates that DNA damage is induced in GBM 4 cells when subjected to irradiation.

After following the growth of GBM4 cells in vivo, we irradiated (10Gy) the mice after they had developed significant sized tumors as determined via bioluminescence. We sectioned (both control and irradiated brains) and stained the sections with Ki67 and pChk2. Ki67 is a marker of cell proliferation, (45). We found a decrease in Ki67 in the irradiated sections. This indicates that irradiation decreases the rate of tumor cell proliferation. The irradiated sections stained for pChk2 show an increased expression. This shows that irradiation also induces DNA damage in vivo.

Further to explore the effect of radiation on TRAIL mediated cell viability response, assays were performed with GBM4, GBM64 and U373 cells wherein they were either irradiated or treated with purified s-TRAIL or both and we found that the combination therapy significantly lowered the cell viability. However, the effect was observed at different time points following s-TRAIL addition in different cell lines. GBM4 cells showed a 60% reduction in cell viability 48h post TRAIL treatment while GBM64 and U373 also showed a marked reduction in cell viability 72h following TRAIL treatment (Fig 1B). This suggests that the sensitization of different TRAIL-resistant glioma cell lines is variable and needs to be examined further.

To further validate our findings and to confirm that the TRAIL mediated apoptotic pathway is activated following irradiation in TRAIL resistant glioma cell lines, western blot analysis was performed. Irradiated GBM4 cells showed a marked increase in the expression of cleaved PARP as compared to control, radiation, or TRAIL treatment alone. Poly ADP-ribose polymerase (PARP), found in the cell nucleus, is involved in DNA repair. Activation of PARP occurs in
response to environmental stress, like irradiation (37). Cleavage of PARP indicates that the cell is undergoing apoptosis. Our Western Blot analyses show a significant increase in cleaved PARP expression when irradiation was combined with TRAIL therapy as compared to control or irradiation and TRAIL therapy alone. This finding suggests that irradiation sensitizes GBM4 cells to TRAIL therapy.

In order to understand the mechanism by which irradiations sensitizes glioma cells to TRAIL therapy, we examined the surface receptor expression levels for death receptors (DR) 4 and 5 by flow cytometry analysis. DR4 and DR5 play an important role mediating apoptosis in the cells. TRAIL induces apoptosis by binding to the death receptors on the cell surface (46, 47). Results show a shift in the curve to the right indicating an increased DR4 and DR5 surface level expression as compared to control, further strengthening our hypothesis that radiation causes an increase in recruitment of the death receptors. However, further mechanistic evaluation is warranted to confirm the findings.

Furthermore, to explore the therapeutic efficacy of the combination therapy, in-vitro assays were performed using stem cell – delivered TRAIL. Mouse mesenchymal stem cells were encapsulated in a synthetic hyaluronic acid – based biodegradable extracellular matrix were plated with irradiated GBM4 cells along with appropriate controls. Results showed that irradiated GBM4 cells had a significant reduction in cell viability when cultured with mMSCs secreting TRAIL as compared to control mMSCs. Both mMSC lines expressed GFP for better visualization. These findings strengthen our rationale of using encapsulated stem cells secreting TRAIL to treat gliomas.

However, further in vivo experiments in intracranial resection mouse model are needed to validate these findings. A thorough in-vivo demonstration of the hypothesized effects are warranted and a further mechanistic evaluation for strengthening different findings. Given the preliminary evaluation irradiation does sensitize TRAIL resistant cells to stem cell delivered TRAIL therapy.
Stem cell based therapies have evolved the past few years and have been successfully demonstrated in GBMs. TRAIL has been found to specifically induce cell death in tumor cells. However, because of the heterogeneity of high-grade gliomas, resistance to TRAIL is common. Hence, novel approaches that sensitize GBM cells to stem cell mediated TRAIL therapy are much needed. In this current study we found that irradiation sensitizes TRAIL resistant patient derived gliomas to stem cell based TRAIL therapy.
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References


Supplementary Figure 1: **The effect of various irradiation and TRAIL doses on GBM4 cells.**

(A) Viability analysis on GBM4 glioma cell line treated with increasing doses of IR (0.2, 0.5 and 10 Gy) and two different doses of TRAIL (200ng/mL, 400ng/ml).