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Combination of Systemic Chemotherapy with Local Stem Cell Delivered S-TRAIL in Resected Brain Tumors

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Abstract

Survival for glioblastoma multiforme (GBM) has failed to significantly improve despite success in pre-clinical models. Limitations to successful translation of new therapies include poor delivery of systemic therapies and use of simplified preclinical models which fail to reflect the clinical complexity of GBMs. TRAIL induces apoptosis specifically in tumor cells and we have tested its efficacy by on-site delivery via engineered stem cells (SC) in mouse models of glioblastoma multiforme (GBM) that mimic the clinical scenario of tumor aggressiveness and resection.

However, about half of tumor lines are resistant to TRAIL and overcoming TRAIL-resistance in GBM by combining therapeutic agents that are currently in clinical trials with SC-TRAIL and understanding the molecular dynamics of these combination therapies are critical to the broad use of TRAIL as a therapeutic agent in clinics. In this study we screened clinically relevant chemotherapeutic agents for their ability to sensitize resistant GBM cell lines to TRAIL induced apoptosis. We show that low dose cisplatin increases surface receptor expression of DR4/5 post G2 cycle arrest and sensitizes resistant GBM cells to TRAIL induced apoptosis. *In vivo*, using an intracranial resection model of resistant primary human-derived GBM and real time optical imaging, we show that a low dose of cisplatin in combination with synthetic extracellular matrix (sECM) encapsulated SC-TRAIL significantly decreases tumor re-growth and increases survival

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Author Contributions

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in mice bearing GBM. This study has the potential to help expedite effective translation of local stem cell-based delivery of TRAIL into the clinical setting to target a broad spectrum of GBMs.

Keywords

GBM; primary-human derived GBM; stem cell therapy; TRAIL; cisplatin; resection

Introduction

Survival for glioblastoma multiforme (GBM) has only minimally improved despite many successful therapeutic trials in pre-clinical GBM models. Unfortunately, the incidence of GBMs the most common malignant brain tumor is rising relative to other brain tumors with no dramatic improvement in median survival, in spite of maximal treatment with surgery, chemotherapy, and focal radiotherapy[1–3]. Since GBMs are characterized by microscopic tumor infiltration along the perivascular spaces and white matter fibers[4], local recurrences are very common despite wide surgical resection of GBMs with presumed clear tumor margins as defined with CT or MRI. Unfortunately, some of the targeted systemic therapies have yielded virtually ineffective given their inability to adequately cross the blood brain barrier at doses without increasing significant toxicity[5, 6].

Previously, we and others have shown that stem cells have unique tumor-specific homing properties and engineering of these stem cells to release anti-tumor proteins locally has shown to be effective in mouse models of malignant and invasive GBMs[7–12]. One particular tumor specific protein that has been characterized extensively is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) which induces death receptor (DR)4/5 specific apoptosis in a wide variety of glioma cell lines including intracranial human GBM xenografts in mice[10, 11, 13]. We have previously shown that the on-site delivery of TRAIL via stem cells(SC-TRAIL) has anti-tumor effects and survival benefits in solid and invasive GBMs[12]. The efficacy of SC-TRAIL is significantly higher as compared to systemically delivered TRAIL which is inefficient due to its short half life and potential increased toxicity to normal tissue[13, 14]. Recently, we have shown that therapeutic stem cells encapsulated in a biodegradable, synthetic extracellular matrix (sECM) within the resection cavity of a mouse GBM xenograft was more effective in stopping tumor growth and improving survival[12]. Unfortunately, it is well known that established GBM cell lines have varying resistance/sensitivity with greater than 50% of lines being resistant to TRAIL-induced apoptosis, therefore treatment strategies that promote sensitization to TRAIL induced killing are required for therapeutic efficacy[15, 16].

Moreover, when examining resistance to new treatment strategies in GBMs, translational relevance will need to include and highlight the importance in response of new therapies to primary patient-derived GBM tumors, which are developed via isolated glioblastoma stem cells (GSCs) from patients. These patient-derived GBMs provide for more accurate preclinical models which have been shown to recapitulate patient histopathology in intracranial xenografts and model the importance of intratumoral heterogeneity in facilitating resistance [17–20].

While previous preclinical models have shown efficacy and provide proof of concept for SC-TRAIL local therapy in TRAIL sensitive established glioma cell lines, in this study we explored overcoming TRAIL resistance in a patient-derived GBM intracranial xenograft resection model with the clinically approved and well established chemotherapy drug cisplatin [21–23]. We initially identify cisplatin among a number of clinically approved chemotherapeutic agents to best sensitize resistant GBM cells to TRAIL-induced death. We examine possible mechanisms for cisplatin induced TRAIL sensitization. Moreover, we show the efficacy of SC-TRAIL therapy combined with low dose cisplatin in an intracranial mouse xenograft of a TRAIL resistant primary human-derived GSC line after primary resection.

Results

Cisplatin sensitizes resistant GBM cell lines to TRAIL induced cell death

In order to examine TRAIL resistance in GBMs, we first screened a panel of established human glioma cell lines (U373, U251, LN229, LN308, U87, Gli79, Gli36EvIII, LN319) and primary patient derived GSC lines (GBM4, GBM8, BT74, GBM6, GBM23, GBM46, GBM64) for cell death after treatment with S-TRAIL at increasing doses (Fig. 1A). Only two out of the eight established GBM cell lines (U87, Gli36EvIII) and two out of seven primary human-derived GSC lines (GBM8, BT74) showed substantial (>50%) cytotoxicity at a high S-TRAIL dose (500 ng/ml). Three relatively TRAIL resistant established glioma cell lines U373, U251, and LN229 were subsequently assessed for the different effect of the clinically approved chemotherapies temozolomide, cisplatin, and etoposide in TRAIL sensitization [1, 21, 22](Fig. 1B). Temozolomide at 50 and 100 μ M concentration was not found to sensitize these GBM lines to S-TRAIL induced cytotoxicity, while cisplatin at 5 μ g/ml and 10 μ g/ml was found to have significant sensitizing effects ($P < 0.001$) for all three cell lines. Etoposide also showed effectiveness in sensitizing cells to S-TRAIL induced cell death, however not in all cell lines tested at lower doses. Given this result, we further analyzed the effects of cisplatin at different concentrations on three established TRAIL resistant glioma cell lines U373, U251, and LN229 and three TRAIL resistant primary patient-derived GSC lines GBM4, GBM23, and BT74 (Fig. 1C). The primary GSC lines were more sensitive to cisplatin (lethal concentration of 50% (LC50) GBM4 1.5 μ g/ml, GBM23 2.3 μ g/ml, BT74 1.4 μ g/ml), while the established glioma cell lines were less sensitive (LC50: LN229 23.1 μ g/ml, U251 18.9 μ g/ml, U373 7.6 μ g/ml).

Subsequently, the ability of low dose cisplatin (dose < LC25 (lethal concentration of 25%)) to sensitize to TRAIL induced apoptosis was examined in the TRAIL resistant established glioma cell lines LN229, U373, and the semi resistant U251. Western blotting revealed that the combination treatment of cisplatin and S-TRAIL in the LN229 and U373 cell lines resulted in increased levels of cleaved PARP (cPARP) protein compared with that from either treatment alone. For the U251 cell line, the TRAIL only treated group did have a significant difference in cPARP as compared to control which is consistent with the previous finding that this cell line is more sensitive to TRAIL (Figure 1A, U251 cell viability is 55% after 500ng/ml of S-TRAIL) (Fig. 1D). A significant cytotoxic effect was seen in cells treated with cisplatin and S-TRAIL as compared to all groups ($P < 0.05$) (Fig. 1D). Clear

differences in cell morphology were seen in photomicrographs of glioma cells in the different treatment groups (Fig. 1E). The enhanced S-TRAIL response with cisplatin treatment was also evident at the caspase 3/7 activation level (Supplementary Fig. S1). These results show that pre-treatment with low dose cisplatin facilitates TRAIL induced apoptosis in both TRAIL resistant and semi-resistant GBM cell lines.

Low dose cisplatin sensitizes TRAIL resistant primary patient derived GSC cell lines to S-TRAIL induced apoptosis and stem cell derived S-TRAIL in co-culture

In the relatively TRAIL resistant primary patient-derived GSC lines GBM4, GBM23, and BT74, the treatment of low dose cisplatin (< LC25 for all three cell lines) was found to facilitate TRAIL induced apoptosis. A significant cytotoxic effect was seen in cells treated with low dose cisplatin 0.1 µg/ml and S-TRAIL 200ng/ml as compared to all groups ($P<0.05$) (Fig. 2A). Given that the TRAIL resistant primary patient-derived GSC cell line GBM4 was sensitized to TRAIL induced killing via low dose cisplatin, we examined the use of stem cell-delivered TRAIL with low dose cisplatin on GBM4 cell viability in vitro. We engineered mouse adipose derived mesenchymal stem cells (MSC) to express a potent and secretable variant of TRAIL, S-TRAIL, designated as MSC-S-TRAIL and MSCs with only GFP as a control group (MSC-GFP). To observe the effect of MSC-S-TRAIL on GBM cells in vitro, we established co-cultures of glioma cells (GBM4-FmC) with either MSC-S-TRAIL or the control MSC-GFP after GBM4-FmC cells were either pretreated 3 days earlier with either low dose cisplatin (0.1 µg/mL) or vehicle (PBS). Pretreated cells with cisplatin facilitated MSC-S-TRAIL glioma induced killing as confirmed by a significant decrease in glioma cell viability as compared with all controls (MSC-GFP, MSC-GFP +cisplatin, MSC-S-TRAIL) (Fig. 2B). Fluorescent photomicrographs showed either encapsulated sECM MSC-S-TRAIL or MSC-GFP(green) and the floating neurospheres of GBM4-FmC(red) (Fig. 2C). Clearly, in the MSC-S-TRAIL treated with cisplatin group, there were smaller and less glioma neurospheres. These results reveal that MSCs engineered to secrete S-TRAIL can cause TRAIL induced killing in a resistant primary human derived GSC with low dose cisplatin pretreatment.

Low dose cisplatin effects in vitro and in vivo in primary patient derived GSCs

The TRAIL resistant primary GSC line GBM4 was examined for cell cycle effects of low dose cisplatin 0.1 µg/ml (a dose which was 10 times less than the LC50 concentration). After a single treatment of low dose cisplatin, there was a shift in the cell cycle at 48 hours from G1 to both S and G2 (Control->48hrs: G1 69% ->32%, S 25% ->40%, G2 6%->28%) with subsequent G2 cell cycle arrest at 72 hours (Control->72hrs: G1 69% -> 17%, S 25% -> 18%, G2 6%-> 66%) (Fig. 3A). There was no effect of low dose cisplatin on cell cycle at 24 hrs as compared to control (Supplementary Fig. S3).

To assess the effect of low dose cisplatin in an orthotopic model of GBM, we used the GBM4 cell line, engineered to express Fluc-mCherry, GBM4-FmC. Four weeks subsequent to intracranial injection of GBM4-FmC cells, mice were injected intraperitoneally (i.p.) for 3 days with low dose cisplatin 3mg/kg (human equivalent dose of 0.24 mg/kg or 9 mg/m²) or normal saline (control). A significant decrease in the rate of tumor growth (via measurements of bioluminescent signal after treatment) as compared to controls was

observed at 10 days (Fig. 3B). In-vivo low dose cisplatin was found to have anti-proliferative effects as indicated by immunohistochemistry staining showing that levels of the proliferation marker Ki67 was approximately five-fold less in the treatment group versus the control (Fig. 3C). These results reveal that low dose cisplatin causes G2 cell cycle arrest and reduces proliferation in the TRAIL resistant primary patient-derived GSC line, GBM4.

In order to examine the possible mechanism for S-TRAIL sensitization, GBM4 cells were treated with low dose cisplatin (0.1 µg/ml) and analyzed for DR4 and DR5 surface receptor expression via flow cytometry. Untreated GBM4 cells were found to have minimal to no DR4 surface receptor expression as indicated by an overlapping expression profile with the unstained group (Fig. 4A). Cells examined at 48 or 72 hours after cisplatin treatment showed marked increase in DR4 surface receptor expression. In contrast, untreated GBM4 cells were found to have some endogenous surface DR5 expression as compared to the unstained group (Fig. 4B). A clear increase in DR5 surface receptor expression levels was seen after cisplatin exposure. Interestingly, western blot analysis revealed similar total DR4 and DR5 protein expression among treated and untreated groups (Supplementary Fig. S2). Furthermore, DR4 and DR5 surface receptor expression was increased in treated GBM4 cells when examined at 72 hours as compared to 48 hours after low dose cisplatin treatment.

In order to evaluate the role of caspase mediated apoptosis, GBM4 cells were treated in the presence or absence of the pan-caspase inhibitor, Z-VAD-FMK (Fig. 4C, D). GBM4 cells treated with Z-VAD-FMK were noted to have relatively equivalent caspase 3/7 activity as compared to control, however, in the absence of Z-VAD-FMK the cisplatin and TRAIL combination group had significantly increased caspase 3/7 activity as compared to all groups (Fig. 4C). Moreover, the presence of Z-VAD-FMK abrogates the cytotoxic effect of TRAIL after pretreatment of cisplatin. Without Z-VAD-FMK, the combination of cisplatin and TRAIL causes a significant decrease in cell viability as compared to all other groups (Fig. 4D). These findings show that low dose cisplatin increases DR4 and DR5 surface receptor expression and that TRAIL mediated death after sensitization with cisplatin occurs largely via caspase mediated apoptosis.

Local stem cell derived TRAIL within intracranial resection cavity inhibits tumor growth and improves survival after low dose cisplatin treatment in a resistant primary human-derived GSC mouse resection model

A number of evidences suggest that MSC may influence tumor progression in several tumor types. In order to determine, the effect of engineered mouse adipose derived MSC on GBM growth, we implanted GBM4-FmC or a mix of GBM4-FmC and MSC-GFP and followed GBM growth in real time over-time. As shown in the summary graph and representative images there was no significant effect of MSC on tumor growth over-time (Supplmentray. Fig. S4). To establish a clinically relevant model which reflects the standard practice of GBM resection, we used our previously described mouse GBM resection model with local stem cell based therapy, incorporating systemic low dose chemotherapy to test for an effective treatment against TRAIL resistant primary derived GSC line [12]. Briefly, after creating a cranial window, GBM4-FmC cells were implanted and allowed to establish and grow for approximately 4 weeks, where mice subsequently were treated intraperitoneally

with low dose cisplatin (3 mg/kg) or normal saline (control) for 3 days and underwent subsequent resection with implantation of sECM encapsulated MSC. Fluorescent microscopy allowed for visualization of GBM4-FmC (red) tumor (Fig. 5A) and subsequent tumor resection with intracranial implantation of encapsulated sECM MSC-S-TRAIL or MSC-GFP (green) into resection cavity (Fig. 5B). Bioluminescent imaging showed that the group of mice treated with the combination of cisplatin and sECM encapsulated MSC-S-TRAIL had significantly less tumor growth after resection as compared to cisplatin in combination with MSC-GFP (stem cell therapy control) or solely MSC-S-TRAIL (systemic therapy control- normal saline) (Fig. 5D). Kaplan-Meier survival analysis revealed a significant benefit of low dose cisplatin therapy in combination with MSC-S-TRAIL as compared to the other control groups (Fig. 5E). Histopathological analysis of brain sections (Supplementary Fig. S5) where MSC-S-TRAIL or control MSC-GFP cells were directly injected intracranially into mice with GBM4-FmC tumors (treated either with cisplatin or normal saline) revealed a significantly higher number of cleaved caspase-3-positive cells in the combination group of cisplatin and MSC-S-TRAIL (Fig. 5F). These results show that sECM-encapsulated engineered MSC in combination with low dose systemic cisplatin has therapeutic benefits against primary patient-derived TRAIL resistant GBMs.

Discussion

In this study, we show that systemic treatment with low dose cisplatin in combination with local therapy within the tumor resection cavity using encapsulated stem cells engineered to secrete S-TRAIL has therapeutic benefits against a primary patient-derived TRAIL resistant GBM. Our model highlights an attempt to mimic the complex reality of treating GBMs.

Current clinical trials using systemic targeted TRAIL receptor agonists such as conatumumab or mapatumumab have largely been unsuccessful revealing the importance of TRAIL resistance, even when these targeted drugs are known to penetrate into tumor [24–27]. In addition to the obstacle of accessing the blood-brain barrier, these novel drugs, especially as monotherapies, are likely to fail in treating GBMs given the known problem of TRAIL resistance [28–31]. Moreover, recent data showing the remarkable intratumoral heterogeneity within GBMs sheds light on the reality that even if a certain population of cells are sensitive to a specific targeted therapy, there will likely be a group of resistant cells which can ultimately lead to failure of a specific monotherapy [17, 30, 32]. Combination treatment strategies based on improved molecular analysis and characterization of each patient's tumor tissue will allow for identifying multiple possible targets. Utilizing this information, combination treatment strategies using multimodal and multi-mechanistic therapy can provide for better overall outcomes.

Our laboratory has previously shown that TRAIL induces apoptosis specifically in sensitive tumor cells and we have tested its efficacy by on-site delivery via engineered stem cells in mouse models of TRAIL sensitive GBMs [12]. Initially, in this study we examine therapeutic agents that are currently clinically approved and have been used for GBM therapy to test for their ability to overcome TRAIL resistance [1, 21, 22]. We find that cisplatin, a platinum containing crosslinking DNA damaging agent, overcomes TRAIL resistance better than the other chemotherapeutic agents tested in established glioma cell

lines. We show the efficacy of low dose cisplatin alone or in combination with TRAIL in vitro in TRAIL resistant GBM cell lines and that this effect is mediated via caspase induced apoptosis. Although previous studies have examined TRAIL sensitization via use of chemotherapy agents [33, 34] in GBM, they do not examine and utilize models of primary patient-derived TRAIL resistant GSCs. The use of primary patient-derived GSCs has been shown to better recapitulate patient histopathology and the complexity of the intratumoral heterogeneity found in GBMs [17, 20, 32, 35, 36]. Therefore, in the present study, after identifying the potential therapeutic benefit of combining low dose cisplatin and TRAIL in established glioma cell lines, we specifically intended to perform all subsequent studies in the TRAIL resistant primary-patient derived GSC cell line, GBM4. Wakimoto et al. [20] have previously characterized GBM4 and have shown that it mimics patient histopathology and aggressiveness in a mouse intracranial xenograft [20]. In this study, the combination of low dose cisplatin and TRAIL was effective in decreasing tumor cell viability in the TRAIL resistant primary patient-derived GSC lines GBM4, GBM23, and BT74.

Given that systemically delivered TRAIL has previously been shown to be relatively ineffective given its relatively short half life, we engineered mouse adipose derived mesenchymal stem cells to secrete TRAIL (MSC-TRAIL) [13, 14]. We specifically use adipose derived stem cells given that autologous adipose tissue would be more easily attainable in the clinical setting as compared to neural or bone marrow derived stem cells facilitating more feasible translation of autologous non-immunogenic stem cell therapy. Also, adipose stem cells have been shown to have similar migratory capacity to gliomas as compared to neural or bone marrow derived stem cells [37]. In vitro, we show that encapsulated sECM MSC-TRAIL when cocultured with GBM4-FmC cells have anti-tumor effects only in tumor cells with prior low dose cisplatin treatment.

After consistent evidence showing the sensitization of TRAIL resistant GBM cells to low dose cisplatin, we examined the possible effects of low dose cisplatin in-vitro. We found that treatment lead to G2 cell cycle arrest at 72 hours, which also correlated with increased DR4/5 surface receptor expression. Interestingly, as the cells over time shift more from G1->S->G2, they also gradually increased DR4/DR5 surface receptor expression. We did not see differences in total DR4/5 protein with Western blotting (Supplementary. Fig 2), although this has been previously shown at higher treatment doses of cisplatin in established glioma cell lines [34]. From our results, low dose cisplatin likely sensitizes TRAIL resistant cells by increasing surface receptor expression of DR4/DR5 which also correlates with G2 cell cycle arrest. We realize that some cells remain resistant to TRAIL therapy after treatment with cisplatin and this might be accounted for by the concept of intratumoral heterogeneity with a subpopulation of cells resistance directly to cisplatin. Mechanisms of cisplatin resistance have been examined at the length in the literature which include prevention of interaction of cisplatin and DNA, inhibition of cisplatin effects on DNA, and obstruction of lethal downstream signals after DNA damage. [38]

In vivo, using an intracranial resection model of the resistant primary human-derived GBM, GBM4, low dose cisplatin in combination with encapsulated sECM MSC-TRAIL within resection cavity resulted in a significant decrease in tumor growth and increased survival in mice bearing GBM4 tumors. Again, the increased cell death was correlated with increased

cleaved-caspase 3 staining indicating the importance of TRAIL induced caspase mediated apoptosis after treatment with cisplatin.

Our results indicate that systemic low dose cisplatin treatment is able to sensitize resistant primary patient-derived GBMs to local stem cell based delivery of TRAIL within the resection cavity. Given that clinical trials for new therapies for GBMs are tested usually after recurrence with failure/resistance to standard therapy, our study highlights a preclinical model that attempts to mimic the reality of clinical GBM treatment including aspects of intracranial resection, use of resistant primary patient-derived cell line, and combination with a clinically accepted chemotherapeutic agent. This *in-vivo* model which incorporates a resistant GSC xenograft with tumor resection and combined local and systemic treatment attempts to reflect a more accurate depiction of the complexity and difficulty of treating GBMs, highlighting the reality that the efficacy of such new therapies will be examined in resistant recurrent GBMs in future clinical trials. In order to effectively combat this aggressive disease and facilitate future clinical trials with local stem based delivery of TRAIL, combination with clinically approved chemotherapeutic agents such as cisplatin at low doses will help for broader acceptance and more successful therapeutic results of this targeted novel treatment strategy.

Materials and Methods

Cell Lines and Reagents

Primary human-derived GSC lines GBM4, GBM8, BT74, GBM6, GBM23, GBM46, and GBM64 (previously isolated as described [20]) were grown in neurobasal medium (Invitrogen/GIBCO) supplemented with 3mmol/L of L-Glutamine (Mediatech), B27 (Invitrogen/ GIBCO), 2 mg/mL of heparin (Sigma), 20 ng/mL of human EGF (R&D Systems), and 20 ng/mL of human FGF-2 (fibroblast growth factor; PeproTech) as described [26]. Established human glioma cell lines U373, U251, LN229, LN308, U87, Gli79, LN319 and Gli36EvIII (Gli36 expressing a constitutively active variant of EGFR (EGFRvIII) [39]) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Mouse adipose derived mesenchymal stem cells (MSC; Cell Engineering Technologies, Coraville, IA) were cultured in low glucose DMEM supplemented with L-Glutamine (Mediatech), MEM non-essential amino acids (Mediatech), 15% FBS, and penicillin/streptomycin. Cisplatin used in both in-vivo and in-vitro studies was obtained in solution format at a concentration of 1mg/ml (Massachusetts General Hospital Pharmacy, Boston, MA). Dilutions were prepared in normal saline for in-vivo intraperitoneal (i.p.) injections and phosphate buffered saline (PBS) for in-vitro experiments. Temozolomide (TMZ, Sigma) used for in vitro studies was dissolved in DMSO at a 50 mM stock solution. Less than 0.5% DMSO was added to media for in-vitro experiments with corresponding controls. Etoposide used for in-vitro studies was obtained in solution format at a concentration of 20mg/ml (Massachusetts General Hospital Pharmacy, Boston, MA) and dilutions were prepared with PBS for in-vitro experiments. S-TRAIL was obtained from 293T cells transfected with LV-S-TRAIL and measured as previously described [7]. Encapsulation of cells occurred with

the following sECM components: Hystem and Extralink (Glycosan Hystem-C, Biotime Inc.); added together with cells per the manufacturer's protocol.

Viral vectors and Engineering Cell Lines

The following two retroviral (RV) vectors RV-S-TRAIL-IRES-GFP and RV-GFP, previously created and described [40], were used to transfect MSCs to create MSC-S-TRAIL and MSC-GFP. Briefly, MSCs were transduced with RV-S-TRAIL-IRES-GFP and RV-GFP, respectively, at a MOI of 8–10 and after 48 hours were sorted by GFP expression with a fluorescence-activated cell sorting (FACS Aria Cell-Sorting System, BD Biosciences, San Diego, <http://www.bdbiosciences.com>). A lentiviral vector Pico2-mCherry-Fluc (kindly provided by A. Kung, Dana-Farber Cancer Center) was used and packaged in 293T/17 cells as previously described [41]. GBM4 cells were transduced with LV-Pico2-Fluc.mCherry at a multiplicity of infection (MOI) of 2 in medium containing protamine sulfate (4 mg/mL) and selected with puromycin creating GBM4-FmC cell line. All cells were visualized by fluorescence microscopy for mCherry or GFP expression 36–48 hours after transduction.

Cell Viability and Caspase Assays

Initially, both established glioma cells and primary GSCs were screened for S-TRAIL sensitivity. Glioma cells were seeded on 96-well plates (1×10^4 per well for GSCs, 5×10^3 for established glioma cells) and treated with different doses of S-TRAIL (0, 50, 100, 500 ng/mL) and assayed for cell viability 48 hours after treatment. The effects of TMZ, etoposide, and cisplatin in sensitizing glioma cells was examined. 24 hours after seeding cells in a 96 well plate, cells were treated with either cisplatin (5 or 10 μ g/mL), etoposide (10 or 20 μ g/mL) or TMZ (10 or 100 μ M). 24 hours later after treatment with chemotherapeutic agent, S-TRAIL (200 ng/mL) was added and cells were subsequently assayed for cell viability 24 hours after treatment. Dose response curves for cisplatin were obtained after treatment with increasing doses after 48 hours. Lethal concentration doses were obtained via non-linear regression analysis. The effect of low dose cisplatin ($< LC_{25}$ for all cell lines) concentrations of 1 μ g/mL for established TRAIL resistant glioma cell lines and 0.1 μ g/mL for TRAIL resistant primary GSC line were examined in combination with TRAIL treatment. Cells were plated in 96 well plate (as described above) and treated with either low dose cisplatin or PBS. 48 hours after treatment, cells were treated with S-TRAIL (200 ng/mL) and viability was assayed 48 hours after treatment. Cell viability was measured using an ATP-dependent luminescent reagent (Cell-TiterGlo; Promega; Madison, WI). Caspase 3/7 activity was assessed similarly but the assay was performed 24 hours after S-TRAIL addition instead of 48 hours. Caspase activity was determined using DEVD-aminoluciferin (CaspaseGlo 3/7; Promega; Madison, WI) according to manufacturer's instructions. All experiments were performed in triplicates. Representative images of treated cells were processed with DP2-BSW Software (Olympus).

Co-culture experiments of primary GSCs and MSCs

TRAIL resistant primary GSCs, GBM4-FmC, were initially either treated with low dose cisplatin (0.1 μ g/mL) or PBS for 72 hours and subsequently co-cultured (1×10^4 per well for GSCs) in 96 well plate with encapsulated, either MSC-S-TRAIL or MSC-GFP cells (5×10^3

cells/4.5µl Hystem and Extralink, 2:1 ratio). Cells were visualized 48 hours post-GBM4-FmC addition to encapsulated MSCs using fluorescence microscope (Olympus IX51). Representative images were processed with DP2-BSW Software (Olympus). Glioma cell viability was measured by assessing the Fluc activity of cells with D-luciferin as described previously [42].

Cell-cycle analysis and detection of surface DR4 and DR5

For cell cycle analysis, GBM4 cells were treated with low dose cisplatin (0.1 µg/mL) for 48 or 72 hours. Control and treated cells were prepared per standard protocol and incubated with propidium iodide (10 mg/mL; Sigma) and RNase A (250 mg/mL) and subsequently analyzed by flow cytometry (BD FACSCalibur). For DR4 and DR5 surface receptor expression, GBM4 cells were treated with either PBS or low dose cisplatin (0.1 µg/mL) at 48 or 72 hours. Cells were prepared per standard protocol and then were stained with PE-conjugated anti-human DR4 (DJR1) or DR5 (DJR2-4) monoclonal antibodies (eBioscience, San Diego, CA, USA) in solution at 4 degrees for 30 min. Data was analyzed using FlowJo Software.

Western Blot analysis

Following treatment, cells were washed, then lysed with 20mM Tris-HCl pH8.0, 137mM NaCl, 10% glycerol, 1%NP-40, 2mM EDTA plus protease inhibitors(Roche) and phosphatase inhibitors (Sigma-Aldrich) at 4°C. Cell lysates were clarified by centrifugation at 16,000×g for 10 minutes, and the supernatant protein concentrations were determined by using a Bio-Rad protein assay kit. 6X SDS-sample buffer (Boston BioProducts) was added to protein samples and heated at 100°C for 3 minutes. 10–30µg of protein were resolved on SDS-PAGE gel and transferred to nitrocellulose membrane, and probed with primary antibodies DR4(ProSci), DR5(ProSci), poly(ADP-ribose) polymerase (PARP; Cell Signaling) or atubulin (Sigma), and detected by chemiluminescence after incubation with HRP (horse radish peroxidase)-conjugated secondary antibodies.

In vivo experiments in an intracranial xenograft model of primary human-derived GSCs

To examine the effects of low dose cisplatin in-vivo in a primary human-derived TRAIL resistant GSC mouse intracranial xenograft, athymic nude mice (4–6 weeks of age; Charles River Laboratories) were implanted with GBM4-FmC cells (3×10^5 cells per mouse) stereotactically (2.5-mm lateral from bregma, 2.5-mm deep, n=14). After 4 weeks of tumor growth as assessed by Fluc activity (tumor volumes) by bioluminescence imaging (BLI) described previously [43], each mouse was administered 3 mg/kg of cisplatin in a mixture of normal saline or control solution intraperitoneally (i.p.) for 3 days. Mice were imaged for Fluc activity(tumor volumes) by BLI on days 0(initial day of drug treatment), 5 and 10. To assess the efficacy of combination treatment of low dose cisplatin and MSC-S-TRAIL in a mouse model of tumor resection, a cranial window was created over the right cranium using a SZX10 stereo microscope system (Olympus) for subsequent implantation and tumor debulking for fluorescence guided surgery. Approximately 7 days later, GBM4-FmC cells (3×10^5 cells per mouse) were stereotactically implanted superficially into right hemisphere (2.5-mm lateral from bregma, 0.5-mm deep) into the brains of 21 mice. Again after 4 weeks of tumor growth as assessed by Fluc activity (tumor volumes), each mouse was administered

3 mg/kg of cisplatin in a mixture of normal saline or control solution intraperitoneally (i.p.) for 3 days (n=14 cisplatin treated, n=7 controls). 3 days after the last cisplatin treatment, mice underwent tumor resection and implantation of stem cells as previously described [12, 44]. This resulted in the following groups MSC-S-TRAIL+cisplatin (n=6), MSC-GFP+cisplatin (n=6), and solely MSC-S-TRAIL (n=7). For encapsulation prior to implantation into resection cavity, MSC-S-TRAIL or MSC-GFP cells (2×10^5) were resuspended in Hystem (6 μ l) and the matrix cross-linker Extralink (4 μ l) were added together and allowed gel for approximately 20 minutes before implantation. Mice were serially imaged for Fluc activity over 10 days. Mice were imaged for Fluc activity as well as followed for survival and sacrificed when neurological symptoms became apparent.

To examine the effects of MSCs alone on GBM4 tumor growth, nude mice (4–6 weeks of age; Charles River Laboratories) were implanted intracranial with GBM4-mCherryFluc cells (4×10^5) alone or GBM4-mCherryFluc cells (4×10^5) in a 2:1 mixture with MSC-GFP cells (n=4/ each group) and tumor cell fate were imaged for Fluc activity as described.

All in vivo procedures were approved by the Subcommittee on Research Animal Care at MGH.

Tissue processing and immunohistochemistry

Mice bearing tumors were perfused with formalin and brains were removed and sectioned. Brain sections were immunostained with antibodies against human Ki67 (DAKO, Carpinteria, CA), cleaved caspase-3 (Cell Signaling Technology) as described earlier [8]. Photomicrographs of immunohistochemistry and hematoxylin and eosin slides were processed using DP2-BSW Software (Olympus).

Statistical analysis

Results were analyzed by Student t-test when comparing 2 groups. Data were expressed as mean $\pm$ standard errors. Differences were considered significant at P values less than 0.05. Kaplan-Meier analysis was used for survival studies and comparisons were made using the log-rank test. Prism 6 software (GraphPad Software, Inc.) was used for statistical analysis including nonlinear regression analysis of dose response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TRAIL resistance and cisplatin sensitization of human gliomas to TRAIL induced cell death

TRAIL sensitivity/resistance of (A, upper panel) established human glioma cell lines (U373, U251, LN229, LN308, U87, Gli79, Gli36EvIII, LN319) (A, lower panel) and primary human-derived GSCs (GBM4, GBM8, BT74, GBM6, GBM23, GBM46, GBM64) to TRAIL mediated apoptosis. Viability of glioma cell lines in response to 48-hour treatment of different doses of S-TRAIL as measured by assay. Data are mean $\pm$ s.e.m. (B) Glioma cell viability showing the combined effect of temozolomide, cisplatin, or etoposide with S-TRAIL treatment on different TRAIL resistant glioma cell lines LN229, U251, U373. (**, $P < 0.01$; *, $P < 0.05$; N.S., not significant) (C) Cisplatin dose response curve in glioma cell lines. Increasing concentrations of cisplatin were assayed in the established cell lines LN229, U251, U373 and the primary derived GSC lines GBM4, GBM23 and BT74. (D, lower panel) Glioma cell viability showing the combined effect of low dose cisplatin and S-TRAIL treatment on different TRAIL resistant glioma cell lines (LN229, U251, U373) with cisplatin (1 μ g/ml) and S-TRAIL (200 ng/mL). (*, $P < 0.01$) (D, upper panel) Western blot analysis demonstrating changes in PARP cleavage with cisplatin (1 μ g/ml) alone or in combination with S-TRAIL (200 ng/mL). (E) Corresponding photomicrographs showing the changes in cell morphology after treatment.

Figure 2. Low dose cisplatin in combination with stem cell-derived TRAIL induces apoptosis of resistant primary derived GSCs

(A) Glioma cell viability showing the combined effect of low dose cisplatin and S-TRAIL treatment on the GBM4, GBM23, and BT74 TRAIL resistant GSC lines with cisplatin (0.1 $\mu\text{g/ml}$) and S-TRAIL (200 ng/mL). (B) Coculture of GBM4-FmC cells with stem cells producing TRAIL. Representative fluorescent co-culture photomicrographs with or without cisplatin treatment are shown (GBM4-FmC (red) and MSC-S-TRAIL or MSC-GFP; green). (C) GBM4-FmC cells were either pretreated with PBS (control) or pretreated with cisplatin

0.1µg/ml for 3 days. They were then co-cultured with encapsulated sECM-MSC (MSC-S-TRAIL or MSCGFP) for 2 days and assessed for viability by Fluc activity (*, $P < 0.01$).

Figure 3. Low dose cisplatin causes G2 cell cycle arrest and reduces proliferation of the TRAIL resistant primary derived GSC cell line, GBM4, in-vivo

(A) Cell cycle effects of 48 hr treatment of low dose cisplatin (1 $\mu\text{g/ml}$) on the primary human GSC line GBM4. (B) In-vivo effect of low dose cisplatin (3mg/kg) in the intracranial implanted GBM4-FmC tumor. Experimental timeline shown below. Plot showing the percentage BLI intensity relative to day 0 following treatment (n=7–8 per group). Representative pseudocolor BLI images on days 0 and 10. (* $P < 0.05$ at day 10). (C) Representative 40x images of tumors dissected from brains of control and cisplatin-treated mice 5 days after treatment. Upper panel: Fluorescent images showing GBM4-mCherry tumor. Lower panel: Ki67 immunohistochemistry with GFP secondary antibody used. (D) Quantification of Ki67 immuno-positivity (**, $P < 0.01$).

Figure 4. Low dose cisplatin increases DR4 and DR5 surface receptor expression and sensitizes the TRAIL resistant primary derived GSC cell line, GBM4, to caspase mediated apoptosis (A,B) Histograms showing cell surface levels of DR4 and DR5 in GBM4 cells treated with cisplatin (0.1 μ g/ml) and measured using PE-conjugated DR4 or DR5 antibodies. (C) Caspase-3/7 assays showing the combined effect of cisplatin and TRAIL treatment on GBM4 cells with cisplatin (0.1 μ g/ml) and S-TRAIL (200 ng/ml) in the presence or absence of pan-caspase inhibitor (Z-VAD-FMK) (*, $P < 0.05$) (D) GBM4 cell survival in the setting of pre-treatment with cisplatin 0.1 μ g/ml or vehicle followed by the addition of S-TRAIL (200ng/ml) in the presence or absence of Z-VAD-FMK (20 μ mol/l) (*, $P < 0.01$).

Figure 5. Encapsulated therapeutic MSCs inhibit tumor growth and improve survival in TRAIL resistant GBM4-FmC tumors after primary resection and treatment with low dose cisplatin therapy

(A) Light and fluorescence photomicrographs of an intracranial implanted GBM4-FmC tumor in a cranial window. (B) Light photomicrographs after resection of tumor and a fluorescence photomicrograph of the resection cavity containing representative encapsulated sECM MSC-GFP cells. (C) Timeline of in-vivo experiment (D). Encapsulated sECM MSC-S-TRAIL or MSC-GFP cells after cisplatin (3mg/kg) treatment and encapsulated MSC-S-TRAIL-GFP without cisplatin treatment were implanted intracranially after tumor resection and Fluc signal intensity was followed. Plot demonstrates the growth rate via relative change in Fluc intensity normalized to post-resection day 1. (* $P < 0.05$ MSC-S-TRAIL+cisplatin versus MSC-S-TRAIL and MSC-GFP+cisplatin at 10 days; * $P < 0.05$ MSC-S-TRAIL+cisplatin versus MSC-S-TRAIL at 6 days). (E) Kaplan-Meier survival curves. ($P < 0.05$, MSC-S-TRAIL+cisplatin versus MSC-S-TRAIL, and MSC-S-TRAIL+cisplatin versus MSC-GFP+cisplatin) (F, upper panel) Representative images of GBM4-FmC tumor sections after combined treatment (cisplatin or control, MSC-S-TRAIL or MSC-GFP) immunostained with an antibody to cleaved caspase 3 (blue; 20 $\times$ magnification); (F, lower panel) Quantification of cleaved caspase 3 immunopositivity. Data are mean $\pm$ s.e.m. (**, < 0.01).