Silencing IL-13Rα2 Promotes Glioblastoma Cell Death via Endogenous Signaling

Linda C. Hsi¹,³,⁴, Suman Kundu¹, Juan Palomo², Bo Xu¹, Ryan Ficco¹, Michael A. Vogelbaum², and Martha K. Cathcart¹,³

Department of Cell Biology¹, Brain Tumor and NeuroOncology Center², Cleveland Clinic, and Department of Molecular Medicine³, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, Ohio 44195

Running title: IL-13Rα2 inhibition promotes glioblastoma apoptosis via 15-LOX-1

Abbreviations: GBM, glioblastoma multiforme; IL-13, interleukin-13; IL13Rα2, interleukin-13 receptor alpha 2; 15-LOX-1, 15-lipoxygenase-1; 13-S-HODE, 13-S-hydroxyoctadecadienoic acid; 13-S-HpODE, 13-S-hydroperoxyoctadecadienoic acid; PPARγ, peroxisome proliferators activated-receptor gamma; LA, linoleic acid; AA, arachidonic acid; siRNA, small interfering RNA.

Grant support: Supported in part by grant ACSODSR2009-2 from the American Cancer Society (LCH) and PO1-HL087018 from the NIH (MKC).

¹To whom requests for reprints should be addressed: Linda Hsi, Department of Cell Biology NC/10, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave., Cleveland, Ohio 44195. Phone: (216)444-0472; Fax: (216)444-9404; E-mail: hsil@ccf.org.
Disclosure of Potential Conflict of Interests: None of the authors report any conflicts of interest.

Manuscript: Word count: 4997; 7 Figures total
Abstract

Glioblastoma multiforme (GBM) is one of the most lethal forms of cancer, with a survival rate of only 13-27% within 2 years of diagnosis despite optimal medical treatment. We hypothesize that the presence of a unique IL-13Rα2 decoy receptor prevents GBM apoptosis. This receptor has a high affinity for IL-13 (Interleukin-13), binds the cytokine, and competitively inhibits the intracellular signaling cascade initiated by IL-13. In cells lacking the IL-13Rα2 decoy receptor, IL-13 initiates the production of 15-lipoxygenase-1 (15-LOX-1), which has been implicated in cellular apoptosis. Our group and others have shown that induction of 15-LOX-1 correlates with tumor cell death in colorectal, pancreatic, and prostate cancer. How 15-LOX-1 induces apoptosis remains unclear. Preliminary evidence in GBM cells implicates an apoptotic process mediated by peroxisome proliferator-activated receptor gamma (PPARγ). 15-LOX-1 metabolites can modulate PPARγ and activation of PPARγ can suppress tumor growth. We hypothesize that in GBM, IL-13 can induce 15-LOX-1, which regulates cell apoptosis via signaling through PPARγ and that expression of IL-13Rα2 prevents apoptosis and contributes to tumor growth. Our in vitro and in vivo data support this. Knocking down IL-13Rα2 with siRNA dramatically induces 15-LOX-1 expression, promotes apoptosis and reduces GBM tumor growth in vivo. These findings identify a mechanism for eliminating the blockade of endogenous IL-13 signaling and promotion of apoptosis and characterizes a role for 15-LOX-1 in GBM apoptosis. Identifying a mechanistic pathway that can be targeted for pharmacological intervention will have applied implications to developing novel and effective treatments for GBM.
Introduction

Glioblastoma multiforme (GBM) is the most common malignant brain tumor in adults and one of the most lethal forms of cancer. Studies report that the overall survival of patients with newly diagnosed GBM is 13-27% within 2 years of diagnosis, despite optimal medical treatment, i.e., surgery, radiotherapy and temozolomide (1-2). Poor long-term survival is due to local infiltrative growth within the brain that makes complete surgical resection virtually impossible, the intrinsic radio- and chemotherapeutic resistance of glioma cells, and their high rate of mutation (2-3). It has been suggested that reducing various signaling pathways such as PI3K, Akt, mTOR, or NF-B may not only reduce tumor growth, but also reduce the migratory abilities of glioma cells to restore proapoptotic drug sensitivity to current chemotherapies (4). Within the last few years the introduction of the oral methylator temozolomide (TMZ) has greatly enhanced the treatment of high grade gliomas when used in combination with surgery and radiotherapy (2). Nevertheless, less than ten percent of patients remain alive five years post-diagnosis. This dismal prognosis compares unfavorably with other cancers such as breast, colon, lung, and prostate cancer. New treatments for GBM are urgently needed. Many alternative approaches such as gene therapies, vaccination/immunotherapies, and direct toxin injections are in various stages of preclinical and clinical development (5-7). There is great need for investigations into novel treatment options for this deadly disease.

Recently, several different approaches have been explored in an attempt to increase survival from GBM. Pro-apoptotic gene therapy approaches, with targets such as TNFα, FasL, and TRAIL have been proposed; however, these approaches have shown increased neurotoxicity due to non-specific killing of non-tumoral brain cells (6). Other approaches, such as Ad-p53, which target a majority of all tumor cells have not been successful because of lack of efficacious transduction of a large percentage of cells (8). Several vaccination approaches have been tested in preclinical and early phase clinical trials (9-10). Vaccination approaches will
require testing in large Phase III trials before conclusive evidence of their clinical effectiveness can be provided (10). Another approach has been the targeting of receptors exclusively expressed on glioma cells with cytotoxic proteins. This approach has had clinical toxicity that is likely due to binding of the toxin to immune cells bearing the physiological receptor (11-12).

GBM expresses IL-13 receptors (13-14). IL-13 is a pro-inflammatory, Th-2-derived cytokine that is known to induce apoptosis in many different cell types, including GBM cells (15-16); however, even in the presence of endogenous or artificially elevated IL-13, GBM cells maintain a very low level of apoptosis. One emerging explanation for this phenomenon is the presence of an IL-13Rα2 receptor subunit by GBM. IL-13Rα2 is overexpressed in a variety of human tumors and previous studies have shown that it can serve as a biomarker of disease and a target for cancer therapy (17). IL-13 plays a central role in inflammation and immune responses and binds to two receptor subunits, IL-13Rα1 and IL-13Rα2 (18). IL-13Rα1 is a ubiquitously expressed low-affinity IL-13R, but after binding to IL-13, it recruits IL-4Rα and forms a high-affinity IL-13R complex (type II IL-13R) and mediates signal transduction through the JAK-STAT pathway (19-20). IL-13Rα2, on the other hand, binds IL-13 with extremely high affinity and internalizes, but it does not mediate signal transduction (21). It has been hypothesized that the extracellular domain of IL-13Rα2 may serve as a decoy receptor for the type II IL-13R complex (22-23).

GBM has high levels of IL-13Rα2 receptor present compared to normal tissue. For example, ~80% glioblastoma tumors express high levels of IL-13Rα2, whereas corresponding normal brain tissues do not show detectable levels of this receptor (14). IL-13Rα1 is a normal receptor for IL-13 and once bound, initiates a signaling cascade which culminates in the transcription of several different genes. One of the resulting proteins is 15-lipoxygenase-1 (15-LOX-1), an enzyme that can oxidize esterfied or free fatty acids and that can promote apoptosis (24-26). For example, 15-LOX-1 has been shown to play important roles in colorectal
cancerous growth (27-29). Restoring 15-LOX-1 expression by various means has been shown to restore and be mechanistically linked to apoptosis in colorectal cancer cells (24-25, 29). We hypothesized that induction of 15-LOX-1 may also promote apoptosis in GBM; however, in GBM, the presence of IL-13Rα2 acts like a decoy receptor and does not allow normal IL-13 signaling to occur.

15-LOX-1 catalyzes the oxidization of arachidonic acid (AA) and linoleic acid (LA) into 15-(S)-hydroperoxyeicosatetraenoic acid (15-HPETE) and 13-(S)-hydroperoxide octadecadienoic acid (13-HPODE), respectively. Interestingly, these are the metabolic precursors to 15-(S)-hydroxyeicosatetraenoic acid (15-HETE) and 13-(S)-hydroxy-octadecadienoic acid (13-HODE), both of which are known to activate the ligand-dependent nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ). Recently, PPARγ has emerged as a potential player in the IL-13-induced apoptosis pathway (30). The mechanism by which PPARγ inhibits proliferation varies greatly among cell types, and is not fully understood in GBM cells (31-32); however, it does appear that some ligands for PPARγ can induce apoptosis in GBM cells (32-33).

Toxins have been designed to target the IL-13 receptor, which generally would bind to all IL-13 receptors and are meant to be toxic to the cells bearing IL-13 receptors. One such agent is IL13-PE38QQR, the recombinant cytotoxin composed of IL-13 and a truncated form of Pseudomonas aeruginosa exotoxin A. This approach was tested preclinically and in Phase I-III clinical trials (7). Although there were high expectations for this approach, the Phase III trial failed to achieve clinical endpoints. Reasons for failure are likely to have been the short half life of IL13-PEQQR, clinical toxicity which limited the doses that could safely be delivered to patients, and little control over toxin administration. The clinical toxicity was likely due to binding of the toxin to the physiological IL-13Rα1/IL-4α type II receptor. This toxin would be predicted to be harmful for normal immune cells bearing the IL-13Rα1 receptor.
Rather than pursuing the targeting of the IL-13Rα2 receptor with a toxin or blocking IL-13 interaction with its receptors, we have taken an alternative approach to specifically inhibit expression of IL-13Rα2 in glioma cells using siRNA. The siRNA does not inhibit the expression of the physiological IL-13Rα1/IL-4α receptor. With this novel approach, by specifically targeting and silencing the IL-13Rα2 receptor only, we now allow normal endogenous IL-13 signaling cascades to proceed which can then initiate apoptotic pathways leading to GBM tumor cell death. We hypothesize that an siRNA targeted to IL-13Rα2 will effectively and specifically eliminate IL-13Rα2 expressing GBM cells with reduced neurotoxicity and off-target effects.

**Materials And Methods**

**Cell Culture**

U87 and A172 cells (obtained from the American Type Culture Collection) were grown at 37°C in a humidified 5% CO₂/95% air atmosphere. Cells were passaged within 6 months of receiving the cell lines from the established cell bank. The cancer cells were authenticated by their ability to form cancer tumors in athymic nude mice. The cells were tested for mycoplasma-free by PCR methods before this study. The culture medium for U87 cells was Eagle’s minimal essential medium (EMEM) (1X) (Life Technologies) with Earles salts supplemented with 10% fetal bovine serum, sodium pyruvate, non-essential amino acids (Invitrogen), penicillin, and streptomycin. The culture medium for A172 cells was DMEM supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin. Trypsin (Life Technologies) was used to subculture cells. Unless otherwise specified, all chemicals and reagents were from Sigma.

**Treatment with IL-13**

Treatment groups were compared with vehicle controls. Treatment with IL-13 (Biosource) was
done at the dose and for the times indicated. Dose and time ranges were selected based on previous reports utilizing this compound (34).

RNA Interference

Smartpool siGENOME siRNA reagents for IL-13Rα2 siRNA were obtained from Dharmacon (Lafayette, La) and handled according to the manufacturer’s instructions. Transfection of siRNA was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s specifications. Control cells were similarly transfected without siRNAs (i.e. vehicle only). Untreated cells, mock transfected cells, and transfection of non-target siRNA were used as controls. Non-target siRNA’s were obtained from Dharmacon. After transfection, cells were replenished with regular medium for various time periods. Cells were assayed for silencing 24, 48, and 72 hours after transfection. Treatment groups were compared with vehicle controls.

Western Blot Analysis of 15-LOX-1, PPARγ, IL-13Rα2 Protein

Following appropriate treatment protocols, we analyzed cell lysates for 15-LOX-1, PPARγ, and IL-13Rα2 levels. Cells were lysed in lysis buffer containing protease and phosphatase inhibitors. Protein concentration was measured by Bradford’s method. Western blot analysis was performed using standard Western blotting techniques with specific antibodies directed against human cellular proteins. 15-LOX-1, PPARγ, and IL-13Rα2 Western blot analysis was performed using a 15-LOX-1 primary antibody 1:1000 (antibody supplied by J. Cornicelli), a PPARγ primary antibody (Santa Cruz Biotechnology SC-7273) at a dilution of 1:2000, and an IL-13Rα2 primary antibody (R&D Systems MAB614) at a dilution of 1:2000 respectively. In general, SDS-PAGE and Western blotting techniques were carried out as described previously (28). β-tubulin was used as a loading control.

Briefly, treated and control cells groups were washed twice with ice-cold PBS and lysed
in protein lysis buffer containing protease and phosphatase inhibitors. Aliquots of the protein preparation were heated to 70°C in protein sample buffer (Invitrogen) and separated by a 4%-12% gradient gel (Novex) according to the manufacturer’s instructions. Proteins were transferred onto nitrocellulose membranes (Invitrogen). Blots were blocked with 10% non-fat dry milk in 20mM Tris-buffered saline (pH7.4) containing 0.05% Tween 20 (TBS-T) and washed. The blots were then incubated in 1% milk in TBS-T with an appropriate primary antibody. After washing, blots were incubated with an appropriate horseradish peroxidase-linked secondary antibody (Amersham). After reaction with chemiluminescence reagents (Amersham ECL system), bands were detected by exposure to film (Amersham). All other reagents were from Sigma.

**RT-PCR Analysis**

The RT-PCR conditions, primers, and sequences for 15-LOX-1 were performed as described previously (35). PCR products were resolved on 2% agarose gels.

**Measurement of IL-13**

IL-13 was measured using the Quantikine Human IL-13 kit (R&D Systems) according to the manufacturer’s specifications. All samples were assayed in duplicate. Each assay was carried out in at least two independent experiments.

**Cellular Apoptosis**

Independent methods were used to measure apoptosis. Apoptosis was measured by Elisa cell death detection kit (Roche), which detects mono- and oligonucleosomes in the cytoplasmic fraction of cell lystates, according to the manufacturer’s specifications. Each assay was carried out in at least three independent experiments. AnnexinV/propidium iodide staining was performed followed by FACScan flow cytometry. Equal numbers of cells (1x10^5) were plated in
6-well dishes and then following treatment of interest, cells were washed twice in PBS and then
resuspended in Annexin V binding buffer containing 2 μl Annexin V-fluorescein isothiocyanate
(FITC) and 10 μl of propidium iodide (PI). After incubation in the dark at room temperature for
15 minutes, the cells were immediately analyzed by FACScan flow cytometry. For statistical
analysis, the Student’s t test was used to determine the statistical differences between various
experimental and control groups. Data were reported as the mean ± standard deviation. A
P<0.05 was considered significant.

Treatment with IL-13, PPARγ agonists, or PPARγ antagonists

Treatment groups were compared with vehicle controls. IL-13 (50 μg/ml) (Biosource),
rosiglitizone (5μM) (Cayman Chemical), or troglitizone (5μM) (Cayman Chemical) were used for
treatment. Cells were treated from 0 (control) to 96 hours as noted. Cells were pretreated with
the PPARγ antagonist GW9662 (5μM) (Cayman) for 30 min prior to any additional treatment.
Dose and time ranges were selected based on previous reports utilizing these compounds (36).
Each assay was carried out in at least three independent experiments.

Tumorigenesis in nude mice

U87 cells were harvested, counted, and suspended in an equal volume of Matrigel. 2.5 x 10^6
cells in 500μL matrigel were injected under the dorsal skin of nude mice. The animals were left
for 2 weeks without any treatment for uniform development of visible tumors. Ten mice were
used in each group. Tumor volume was measured beginning from week 2 using a digital
vernier caliper. Tumor volume was calculated using the formula [(smallest diameter^2 x widest
diameter) /2], and the growth curves were plotted for each group. Starting tumor volume
ranges for each group were as follows: Control, 143-380 mm^3; non-target siRNA, 198-353
mm³; IL-13Rα2 5μM siRNA, 151-309 mm³; IL-13Rα2 10μM siRNA, 158-341 mm³. The mice were then injected at the tumor site with IL-13Rα2 siRNA (5 or 10μM), non-target siRNA (10μM), or vehicle alone twice per week for four weeks. The siRNA plasmid vector was mixed with *in vivo*-jetPEI (Ployplus-transfection) in 5% glucose solution (100 μl per injection). SiRNA was infused at a rate of 20 μl/min and the needle was left in place for 1 min post injection before removal to prevent backflow. Animals were assessed visually every day thereafter. Tumor volume measurements were taken prior to each injection. At the end of the fourth week, the tumors were measured and the animals were sacrificed and photographed. The tumors were surgically removed, weighed, and photographed. All animal experiments were approved by and in compliance with our Institutional Animal Care and Use Committee. Animals exhibiting abnormal behavior (e.g. hair loss, jerking movements, hemiparesis, loss of grooming behavior) or evidence of pain or distress were euthanized according to approved protocols. Animals with ulcerated tumors were also euthanized according to protocol. The results were statistically evaluated using ANOVA. A value of P<0.05 was considered statistically significant.

**Results**

**IL-13Rα2 siRNA inhibits IL-13Rα2 expression in GBM cells.**

The GBM cell line A172 was treated with IL-13Rα2 siRNA. The siRNA inhibited the levels of IL-13Rα2 mRNA in a dose-dependent manner. By real-time PCR (RT-PCR), 5nM and 10nM IL-13Rα2 siRNA inhibited approximately 65% and 80% of the IL-13Rα2 mRNA respectively (Figure 1A). As a control, the effect of IL-13Rα2 siRNA on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was examined. The IL-13Rα2 siRNA did not have an effect on GAPDH mRNA production. The IL-13Rα2 protein expression was also significantly down-regulated by treatment with IL-13Rα2 siRNA at both 48 and 72 hours (Figure 1B).
IL-13Rα2 siRNA treatment induces apoptosis in GBM cells.

To determine whether IL-13Rα2 blocks GBM apoptosis, we treated A172 cells with IL-13Rα2 siRNA (5nM) for 48 and 72 hours. Apoptosis was assessed by Annexin V/PI staining followed by FACScan flow cytometry. Treatment with IL-13Rα2 siRNA (5nM) induced apoptosis in a time dependent manner with 25% and 65% cell death at 48 and 72 hours respectively (Figure 2A). Treatment with the PPARγ antagonist GW9662 (5μM) reduced this effect, suggesting the involvement of PPARγ in GBM apoptosis. Control cells, cells treated with GW9662 alone, oligofectamine, or non-target siRNA controls did not have increased apoptosis.

Treatment with IL-13Rα2 siRNA promotes 15-LOX-1 expression in GBM cells.

The production of 15-LOX-1, an enzyme whose production has been previously linked to the IL-13 signaling cascade through the type II receptor (34), and is known to catalyze the production of PPARγ specific ligands and apoptosis was examined next. To explore this, the IL-13Rα2 decoy receptor was inhibited using IL-13Rα2 siRNA. RT-PCR was used to determine the effects of the siRNA on the production of 15-LOX-1 mRNA levels. Inhibition of IL-13Rα2 by 5nM and 10nM IL-13Rα2 siRNA increased the production of 15-LOX-1 20 and 30 fold, respectively (Figure 2B). This is particularly interesting because the cells were not exposed to any exogenous IL-13, the usual inducer of 15-LOX-1 expression. The up-regulation of 15-LOX-1 suggests that the cells are releasing IL-13 endogenously. We have confirmed the presence of IL-13 in the GBM cell supernates by Elisa (data not shown). We have found greater than 2.5 fold higher levels of IL-13 in IL-13Rα2 siRNA treated cells as compared to control cells. 15-LOX-1 protein was also significantly up-regulated by treatment with IL-13Rα2 siRNA (Figure 2C).
**PPARγ is expressed in GBM cells and PPARγ-ligands induce apoptosis in GBM cells.**

To determine whether PPARγ is present in GBM cells, PPARγ expression in A172 cells was examined by Western analysis. PPARγ is expressed in A172 cells (Figure 3A). To establish PPARγ function in the apoptotic process, the PPARγ ligands troglitizone or rosiglitizone were tested to determine whether they could induce apoptosis in GBM cells and whether the PPARγ antagonist, GW9662, could alter this effect. A172 cells were treated 24-72 hours and apoptosis was assessed by Annexin V/PI staining followed by FACScan flow cytometry. Both PPARγ ligands induced apoptosis in a time-dependent manner (greater than 60% total cell death by 72 hours) in A172 cells and treatment with GW9662 (5µM) significantly reduced this effect (Figure 3B). Control cells or cells treated with GW9662 alone or IL-13 did not have increased apoptosis.

**13-HpODE, the 15-LOX-1 metabolite, induces apoptosis in GBM cells.**

To determine whether the 15-LOX-1 metabolite 13-HpODE can induce apoptosis in GBM cells, we treated A172 cells with 13-HpODE and assessed apoptosis. Cells were treated with 13-HpODE for four hours and then microscopically examined to identify morphological changes associated with apoptosis (Figure 4A). Cells treated with 13-HpODE were clearly altered, with morphological changes that indicate apoptosis, including cytoplasmic and nuclear shrinkage. Cells were also treated with linoleic acid (LA), the substrate for 15-LOX-1, which is metabolized to 13-HpODE. Cells treated with LA were not affected. Control cells and vehicle treated cells were also not affected. After 24 hours of treatment, apoptosis of the A172 cells was measured by ELISA cell death detection (Figure 4B). Cells treated with 13-HpODE underwent apoptosis while control cells, vehicle treated cells, and LA treated cells were not affected. Apoptosis was also confirmed by Annexin V/PI staining, followed by FACScan flow cytometry (Figure 4C). A172 cells were treated with IL-13, linoleic acid, or 13-HpODE for 48 and 72 hours. Apoptosis
occurred with 13-HpODE treatment, but not with the other treatments. Pre-treatment with the PPARγ antagonist GW9662 for 30 minutes attenuated the 13-HpODE-induced apoptosis.

**Treatment with IL-13Rα2 siRNA promotes 15-LOX-1 expression in U87 GBM cells.**

We next examined the production of 15-LOX-1 in U87 cells. We were interested in the U87 cell line for two reasons. First, it is important to confirm our findings in different glioblastoma cell lines for validity. Secondly, the U87 cell line has previously been shown to grow well in both heterotopic and orthotopic *in vivo* GBM models, while the A172 cell line has been shown to grow well only in a heterotopic *in vivo* GBM model. (M.A. Vogelbaum, personal communication). Thus, it is important to confirm our results in a cell line like U87 in order to move forward with orthotopic *in vivo* studies. It is vital to be able to extend our studies to an orthotopic GBM model system due to the potential impact of a functional blood brain barrier and other local environment factors that may be present in the brain. To explore the effects in U87 cells, the IL-13Rα2 decoy receptor was inhibited using IL-13Rα2 siRNA. Inhibition of IL-13Rα2 by 5nM IL-13Rα2 siRNA increased the production of 15-LOX-1 protein in U87 cells (Figure 5A).

**IL-13Rα2 siRNA treatment induces apoptosis in U87 GBM cells.**

To determine whether IL-13Rα2 blocks IL-13-induced apoptosis in U87 cells, we treated U87 cells with IL-13Rα2 siRNA (5nM) for 72 hours. Apoptosis was assessed by Annexin V/PI staining followed by FACScan flow cytometry. Treatment with IL-13Rα2 siRNA (5nM) induced apoptosis with 30% cell death at 72 hours (Figure 5B). Treatment with GW9662 (5μM), the PPARγ antagonist, reduced this effect. Control cells, cells treated with GW9662 alone, lipofectamine, or non-target siRNA controls did not have increased apoptosis. To establish PPARγ function, we tested whether the PPARγ ligand rosiglitizone could induce apoptosis in U87 cells and whether GW9662, could alter this effect. U87 cells were treated 72 hours and
apoptosis assessed by Annexin V/PI staining followed by FACScan flow cytometry. The PPARγ ligand induced apoptosis (48% total cell death) in the U87 cells and treatment with GW9662 (5μM) reduced this effect (Figure 5B). Control cells or cells treated with GW9662 alone or IL-13 did not have increased apoptosis.

**IL-13Rα2 siRNA treatment in vivo reduces GBM tumor growth.**

To determine whether IL-13Rα2 siRNA can inhibit tumor growth in vivo, we injected U87 GBM cells into the flank of athymic mice. Because A172 cells grow very slowly to form a subcutaneous solid tumor, we have used U87 cells to study subcutaneous solid tumor development. We injected a U87 cell suspension in Matrigel under the dorsal skin of nude mice. The animals were left for 2 weeks without any treatment for development of visible and palpable tumors. Animals were then divided into four groups of ten mice each. Tumor volume was measured beginning from week 2. Intratumoral injections with either IL-13Rα2 siRNA (5 or 10nM), non-target (NTC) siRNA, or vehicle were done two times per week for four weeks. Tumor volume was measured prior to each injection. After the four weeks, animals were photographed (Figure 6A) and sacrificed, tumors were measured (Figure 6B) and then surgically removed, photographed (Figure 6C), and weighed (Figure 6D). Inhibition of IL-13Rα2 by 5 or 10nM IL-13Rα2 siRNA decreased tumor growth by 44 and 48 percent respectively compared to control tumors after four weeks of treatment (Figure 6B). There was a decrease in tumor growth observed with non target (NTC) siRNA (31%), however, the effect observed with IL-13Rα2 siRNA was significantly greater. The effect observed with NTC siRNA is most likely due to an antiviral response elicited by the presence of dsRNA in the cell (37-38). A decrease in tumor weight was also observed after four weeks of treatment with IL-13Rα2 siRNA. Inhibition of IL-13Rα2 by 5 or 10nM IL-13Rα2 siRNA decreased tumor weight by 43 and 59 percent respectively compared to controls (Figure 6D).
Discussion

Our data suggest that the unique expression of a decoy IL-13Rα2 receptor in GBM prevents IL-13 from initiating an intracellular cascade that can lead to apoptosis of GBM cells and thereby contributes to tumor growth. We show for the first time, that the down-regulation of the expression of this decoy receptor allows induction of 15-LOX-1-mediated cell death. From our studies, we establish a molecular mechanism and confirm a role for 15-LOX-1 in apoptosis in GBM cells. We demonstrate that when the expression of the IL-13 decoy receptor is inhibited, 15-LOX-1 is induced in GBM, which in turn can generate metabolites such as 13-HpODE, which can activate PPARγ and lead to apoptosis. This mechanism provides an explanation for how the expression of the decoy receptor IL-13Rα2 may prevent GBM apoptosis and contribute to tumor growth (Figure 7). Thus, we have identified both a novel approach and pathway for inducing apoptosis in GBM cells and perhaps for therapeutic treatment of GBM.

The utilization of RNA interference (RNAi) has been a major breakthrough in cellular biology. RNAi has become the technique of choice for the analysis of gene function and created the possibility for therapeutic gene silencing for cancer treatment, especially for drug-resistant cancers (39). RNAi is potent, as only a few siRNA molecules per cell are required to produce effective gene silencing (40-41). In the case of GBM, IL-13Rα2 may serve as an important therapeutic siRNA target since it is highly expressed in gliomas but not in normal brain. The advantage of using IL-13Rα2 siRNA to specifically silence the receptor compared to other IL-13-based treatment strategies is that we specifically knock down only the IL-13Rα2 receptor and are not targeting functional IL-13 receptors more generally as other current treatments have done and take advantage of an endogenous signaling pathway involving 15-LOX-1 which leads to glioma cell apoptosis and tumor inhibition. For example, the recombinant cytotoxin composed of IL-13 and a truncated form of Pseudomonas aeruginosa exotoxin A (IL13-PE38QQR) targets both IL-13 receptors (7). We believe the IL-13Rα1 receptor is needed
to allow normal endogenous signaling mechanisms to occur which can ultimately lead to cell
death of GBM cells.

Although IL-13Rα2 has been shown to be overexpressed on certain types of human cancer including glioblastoma, head and neck cancer, kidney cancer, ovarian cancer, medulloblastoma, and Kaposi’s sarcoma (42), its role in signal transduction in cancer is unknown. By specifically targeting and silencing the IL-13Rα2 receptor only, we now allow endogenous signaling through 15-LOX-1 to occur which can then lead to GBM tumor cell death. This is particularly promising given that fact that new treatments are urgently needed for this deadly disease. Use of IL-13Rα2 siRNA is a novel approach and could be an effective treatment for GBM with minimal side effects since we are allowing normal endogenous signaling cascades to now initiate.

Further investigations are needed to advance the implementation of this novel therapeutic approach. While initial studies using the in vivo flank model are promising, many heterotopic GBM models, such as the mouse flank tumor model, do not take into account the impact of a functional blood brain barrier and other local environment factors that may be present in the brain. Use of an intracranial GBM model will allow us to take into account the different environments found in the brain. In our flank model studies, we observed continued tumor growth despite administration of the IL-13Rα2 siRNA. This effect may be due to inadequate dosing and/or delivery of the IL-13Rα2 siRNA. Due to the nature of direct injections, there is potential difficulty in obtaining uniform delivery of siRNA to all tumor tissue. The distribution of the siRNA can be affected by several factors: needle placement into the tumor, needle-induced tissue damage, tumor tissue structure, infusion rate of the siRNA, and prevention of backflow. These challenges have limited the progress of drug/agent delivery in the clinical setting and have pointed to the need to refine and develop new delivery methods.
The next challenges are to validate our findings in orthotopic models and to optimize dosing and delivery. Both in vivo animal studies and subsequent clinical trials require an effective mode of delivery for siRNA therapy such as IL-13Ra2 siRNA. siRNA has a large enough molecular weight that it is unlikely to cross the blood-brain barrier following intravenous injection without a delivery system. Direct administration can be an effective method to deliver macromolecules to the brain parenchyma; however, as discussed, with direct administration, there is potential difficulty in obtaining uniform delivery to all tumor tissue. Currently, chemotherapeutic drug-impregnated biodegradable wafers and bolus injection rely upon simple diffusion to drive the drug into the brain parenchyma (43-44). Convection-enhanced delivery (CED) supplements diffusion and allows effective delivery of therapeutic agents at a relatively uniform concentration (44-45). This technique uses positive pressure to generate a continuous pressure gradient over a given time period. The trials of IL13-PE38QQR delivered by CED in the setting of newly diagnosed and recurrent GBM provide proof of principle that a targeted macromolecule can be delivered. CED provides homogenous, clinically significant, reproducible delivery of macromolecules into the brain parenchyma. This method of delivery may also be suitable for siRNA targeted therapy.

Other methods of RNA interference delivery which facilitate delivery of therapeutically significant quantities of RNA to the central nervous system via the systemic route have been explored. One approach has used liposomes constructed with antibodies to recognize two specific proteins (46). One antibody that is engineered into the liposomes recognizes only the transferrin receptor, a protein common to the blood brain barrier. By binding the transferring receptor, the liposomes gain entry to the compartment that houses and protects the brain. Once inside the compartment, a second liposome-embedded antibody binds to the human insulin receptor found in the membranes of brain cancer cells. Now within the tumor cell, the liposomes release the plasmid they carry which encodes a short hairpin RNA (shRNA) designed to interfere with the expression of epidermal growth factor receptor (EGFR), a proponent of
tumor cell proliferation. Without EGFR to encourage cell proliferation, the tumor growth is held in check. Pre-clinical studies on mice using this approach have shown encouraging results (46). Another transvascular method to deliver siRNA across the blood-brain barrier to the brain via intravenous injection has been explored. This method involves complexing siRNA to a short peptide derived from the rabies virus glycoprotein that binds specifically to acetylcholine receptors on neuronal cells (47-48) and encapsulating the siRNA with liposomes to protect the siRNA from degradation and improve delivery through the vasculature. Use of antibodies or peptides with liposomes to traverse both the blood brain barrier and the tumor cell membrane may prove to be an effective means of delivering IL-13Rα2 RNAi for GBM.

Another approach for siRNA delivery that has recently been examined is use of a multistage vector composed of mesoporous silicon particles (stage 1 microparticles, S1MP) loaded with neutral nanoliposomes (dioleoyl phosphatidylcholine, DOPC) containing small interfering RNA (siRNA) targeted against the EphA2 oncoprotein, which is overexpressed in most cancers, including ovarian (49). This delivery method resulted in sustained EphA2 gene silencing for at least three weeks following a single intravenous administration of S1MP loaded with EphA2-siRNA-DOPC. Furthermore, tumor burden, angiogenesis, and cell proliferation were substantially reduced compared with a noncoding control siRNA alone (49). This novel, multistage siRNA delivery system for sustained gene silencing may have broad applicability to pathologies beyond ovarian neoplasms and perhaps may be useful for GBM.

The effects of silencing the IL-13Rα2 receptor with IL-13Rα2 siRNA on 15-LOX-1 expression and apoptosis in our study support the potential promise of siRNA directed targeting for GBM therapy. While we are optimistic about the potential of siRNA directed targeting to IL-13Rα2, we must also keep in mind the possibility of combining this approach with other established therapies to further enhance therapeutic effects. Given the polygenic nature of GBM, the efficacy and specificity of GBM treatment will likely be further enhanced by using a
combination approach of siRNA with another traditional therapy such as chemotherapy, radiotherapy, or immunotherapy. Recently, combinatorial approaches have been explored and shown to be successful for improving efficacy of treatment of GBM. For example, the combination of temozolomide with surgery and radiotherapy has greatly enhanced the treatment of high grade gliomas (2, 50). In the future, IL-13Rα2 siRNA should be explored as a tantalizing new approach to be used in combination with other agents to improve the efficacy of GBM treatment.
Acknowledgement

We wish to thank the staff of the Flow Core Facility and the Center for Medical Art & Photography for providing technical assistance. This research was supported in part by ACS-ODSR2009-2 from the American Cancer Society (LCH) and PO1-HL087018 from the NIH (MKC).
References


33. Grommes C, Landreth GE, Schlegel U, Heneka MT. The nonthiazolidinedione
tyrosine-based peroxisome proliferator-activated receptor gamma ligand


Figure Legends

Figure 1. IL-13Rα2 siRNA inhibits the expression of IL-13Rα2 decoy receptor mRNA and protein in A172 cells.  (A) IL-13Rα2 mRNA was measured by RT-PCR at 48hr in A172 cells either treated with 5nM or 10nM IL-13Rα2 siRNA, oligofectamine alone, or untreated control cells. The data shown represent one of two separate experiments with similar results. (B) IL-13Rα2 protein was measured by Western analysis of A172 cell lysates at 48 or 72 hours. Lane 1: Control cells 48hr. Lane 2: IL-13Rα2 siRNA (5nM) 48hr. Lane 3: Non-target control siRNA (NTC si) (5nM) 48hr. Lane 4: Oligofectamine alone 48hr. Lane 5: Control cells 72hr. Lane 6: IL-13Rα2 siRNA (5nM) 72hr. Lane 7: Non-target control siRNA (NTC si) (5nM) 72hr. Lane 8: Oligofectamine alone 72hr. 30 μg of total protein loaded per lane.  β-tubulin was used as a loading control. The data shown represent one of three separate experiments giving similar results.

Figure 2. Inhibition of IL-13Rα2 decoy receptor expression by IL-13Rα2 siRNA induces apoptosis and promotes induction of 15-LOX-1 in A172 cells.  (A) Cells were transfected with IL-13Rα2 siRNA (5nM), non-target siRNA control (5nM), or oligofectamine alone and apoptosis assessed at 48 and 72 hours. Cells were pre-treated with GW9662 (5μM) for 30 minutes. Apoptosis was assessed by Annexin V/PI staining, followed by FACSscan flow cytometry. The data shown represent one of three separate experiments giving similar results. (B) 15-LOX-1 mRNA was measured by RT-PCR at 48hr in A172 cells either treated with 5nM or 10nM IL-13Rα2 siRNA,
oligofectamine alone, or untreated control cells. The data shown represent one of two separate experiments with similar results. (C) 15-LOX-1 protein was measured by Western analysis of A172 cell lysates at 48 or 72 hours. Lane 1: 15-LOX-1 standard. Lane 2: Control cells 48hr. Lane 3: IL-13Rα2 siRNA (5nM) 48hr. Lane 4: Non-target control siRNA (NTC si) (5nM) 48hr. Lane 5: Oligofectamine alone 48hr. Lane 6: Control cells 72hr. Lane 7: IL-13Rα2 siRNA (5nM) 72hr. Lane 8: Non-target control siRNA (NTCs) (5nM) 72hr. Lane 9: Oligofectamine alone 72hr. 30 μg of total protein loaded per lane. b-tubulin was used as a loading control. The data shown represent one of three separate experiments giving similar results.

Figure 3. PPARγ is expressed by A172 cells and PPARγ ligands induce apoptosis. (A) PPARγ was measured by Western analysis of cell lysates at 24 hours. Lane 1: Control cells. Lane 2: IL-13 (50μg/ml) 24hr. 30 μg of total protein loaded per lane. The data shown represent one of three separate experiments giving similar results. (B) A172 cells were treated with IL-13 (50μg/ml), troglitizone (5μM) or rosiglitizone (5μM) for 24, 48, and 72 hours. Pre-treatment with GW9662 (5μM), a PPARγ antagonist, was done for 30 minutes. Apoptosis was assessed by Annexin V/PI staining, followed by FACScan flow cytometry. The data shown represent one of three separate experiments giving similar results.

Figure 4. 13-HpODE, a 15-LOX-1 product and PPARγ ligand, induces apoptosis in A172 cells. (A) Morphology of A172 cells either untreated (panel 1), vehicle treated (panel 2), treated with 32μM 13-HpODE (panel 3), or 32μM linoleic acid (LA) (panel 4)
for 4 hours was examined. 13-HpODE induced morphological changes that indicate apoptosis, including cytoplasmic and nuclear shrinkage. (B) The induction of apoptosis in A172 cells determined by ELISA of cells either untreated, vehicle treated, treated with 32µM 13-HpODE, or 32µM linoleic acid (LA) for 24 hours. (C) A172 cells were treated with IL-13 (50µg/ml), linoleic acid (10µM) or 13-HpODE (10µM) for 48 and 72 hours. Pre-treatment with GW9662 (5µM), a PPARγ antagonist, was done for 30 minutes. Apoptosis was assessed by Annexin V/PI staining, followed by FACScan flow cytometry. The data shown represent one of three separate experiments giving similar results.

**Figure 5. Inhibition of IL-13Rα2 decoy receptor promotes induction of 15-LOX-1 expression and apoptosis in U87 cells.** (A) 15-LOX-1 protein was measured by Western analysis of U87 cell lysates at 48 hours. Lane 1: 15-LOX-1 standard. Lane 2: Control cells. Lane 3: Oligofectamine alone. Lane 4: Non-target control siRNA (NTC si) (5nM). Lane 5: IL-13Rα2 siRNA (5nM). 30 µg of total protein loaded per lane. b-tubulin was used as a loading control. The data shown represent one of three separate experiments giving similar results. (B) Cells were transfected with IL-13Rα2 siRNA (5nM) or rosiglitizone (5µM) and apoptosis assessed at 72 hours. Pre-treatment with GW9662 (5µM), a PPARγ antagonist, was done for 30 minutes. Apoptosis was assessed by Annexin V/PI staining followed by FACScan flow cytometry. The data shown represent one of three separate experiments giving similar results.
Figure 6. Inhibition of subcutaneous solid tumor development in nude mice after treatment with IL-13Rα2 siRNA. Mice were injected at the tumor site with IL13Ra2 siRNA (5 or 10μM), non-target siRNA (10μM), or vehicle alone twice per week for four weeks. (A) At the end of the fourth week, the tumors were measured and the animals were sacrificed and then photographed. (B) Measurement of tumor volume using a digital vernier caliper in nude mice. Tumor volume measurements were taken prior to each injection and prior to sacrifice. Data are means ± SEM of 10 animals in each group. *, P<0.05 compared with the control mean values; #, P<0.05 compared with the NTC si mean values. (C) The tumors were surgically removed, weighed, and photographed. The data are representative of 10 animals in each group. (D) Quantitation of tumor weight. Data are means ± SD of 10 animals in each group. *, P<0.05 compared with the control mean values; #, P<0.05 compared with the NTC si mean values.

Figure 7. Proposed mechanism for IL-13-induced apoptosis via 15-LOX-1 in GBM. We hypothesize that IL-13 initiates an intracellular cascade that works via 15-LOX-1 to activate PPARγ and regulate cell growth/apoptosis in GBM. The expression of the decoy receptor IL-13Rα2, which has a high affinity for IL-13, can inhibit the normal cellular responses initiated by IL-13 and may prevent GBM apoptosis and contribute to tumor growth.
Figure 1

A

% Inhibition of IL-13Rα2 mRNA

0 20 40 60 80 100
Oligofectamine 5nM 10nM
IL-13Rα2 siRNA

B

48hr 72hr

Control IL-13Rα2 siRNA NTC siRNA Oligofectamine Control IL-13Rα2 siRNA NTC siRNA Oligofectamine

IL-13Rα2

β-Actin
Figure 2

A

Percent Total Cell Death

Control  GW9662  Oligofect  Control siRNA  IL-13Rα2 siRNA  GW9662-IL-13Rα2 siRNA

B

Fold Induction of 15-LOX-1 mRNA

Control  Oligofect  5nM IL-13Rα2 siRNA  10nM IL-13Rα2 siRNA

C

48hr  72hr

Std.  Control  IL-13Rα2 siRNA  NTC siRNA  Oligofectamine  Control  IL-13Rα2 siRNA  NTC siRNA  Oligofectamine

15-LOX-1  β-tubulin
Figure 4

A

\[ \text{Untreated} \quad , \quad 0.2\% \text{ Ethanol 4 hr} \]

\[ 32 \mu M \text{ 13-HpODE 4 hr} \quad , \quad 32 \mu M \text{ LA 4 hr} \]

B

Absorbance (405 nm)

\[ \text{Control} \quad , \quad 32 \mu M \text{ 13-HpODE} \quad , \quad 32 \mu M \text{ LA} \quad , \quad 0.2\% \text{ Ethanol} \]

C

Percent Total Cell Death

\[ 48 \text{ Hours} \quad , \quad 72 \text{ Hours} \]

\[ \text{Control} \quad , \quad \text{GW9662} \quad , \quad \text{IL-13} \quad , \quad \text{LA} \quad , \quad 13-\text{HpODE} \quad , \quad \text{GW+13-HpODE} \]
Silencing IL-13Rα2 promotes glioblastoma cell death via endogenous signaling

Linda C. Hsi, Suman Kundu, Juan Palomo, et al.

Mol Cancer Ther Published OnlineFirst May 19, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-10-1064

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.