

Silencing IL-13R α 2 Promotes Glioblastoma Cell Death via Endogenous Signaling

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Abstract

Glioblastoma multiforme (GBM) is one of the most lethal forms of cancer, with a survival rate of only 13% to 27% within 2 years of diagnosis despite optimal medical treatment. We hypothesize that the presence of a unique IL-13R α 2 decoy receptor prevents GBM apoptosis. This receptor has a high affinity for interleukin-13 (IL-13), binds the cytokine, and competitively inhibits the intracellular signaling cascade initiated by IL-13. In cells lacking the IL-13R α 2 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 PPAR γ . 15-LOX-1 metabolites can modulate PPAR γ and activation of PPAR γ can suppress tumor growth. We hypothesize that in GBM, IL-13 can induce 15-LOX-1, which regulates cell apoptosis via signaling through PPAR γ and that expression of IL-13R α 2 prevents apoptosis and contributes to tumor growth. Our *in vitro* and *in vivo* data support this. Knocking down IL-13R α 2 with short interfering RNA 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 for promotion of apoptosis, and characterize a role for 15-LOX-1 in GBM apoptosis. Identifying a mechanistic pathway that can be targeted for pharmacologic intervention will have applied implications to developing novel and effective treatments of GBM. *Mol Cancer Ther*; 10(7); 1149–60. ©2011 AACR.

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% to 27% within 2 years of diagnosis, despite optimal medical treatment [i.e., surgery, radiotherapy, and temozolomide; refs. 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 phosphoinositide 3-kinase (PI3K), Akt, mTOR, or NF- κ 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 has greatly enhanced the treatment of high-grade gliomas when used in combination with surgery and radiotherapy (2). Nevertheless, less than 10% of patients remain alive 5 years postdiagnosis. This dismal prognosis compares unfavorably with other cancers such as breast, colon, lung, and prostate cancer. New treatments of 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. Proapoptotic gene therapy approaches, with targets such as TNF α , FasL, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) have been proposed; however, these approaches have shown increased neurotoxicity due to nonspecific killing of nontumoral 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

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doi: 10.1158/1535-7163.MCT-10-1064

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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 because of binding of the toxin to immune cells bearing the physiologic receptor (11, 12).

GBM expresses interleukin-13 (IL-13) receptors (13, 14). IL-13 is a proinflammatory, Th2-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-13R α 2 receptor subunit by GBM. IL-13R α 2 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 2 receptor subunits, IL-13R α 1 and IL-13R α 2 (18). IL-13R α 1 is a ubiquitously expressed low-affinity IL-13R, but after binding to IL-13, it recruits IL-4R α and forms a high-affinity IL-13R complex (type II IL-13R) and mediates signal transduction through the Janus-activated kinase (JAK)-STAT pathway (19, 20). IL-13R α 2, 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-13R α 2 may serve as a decoy receptor for the type II IL-13R complex (22, 23).

GBM has high levels of IL-13R α 2 receptor present compared with normal tissue. For example, approximately 80% glioblastoma tumors express high levels of IL-13R α 2 whereas corresponding normal brain tissues do not show detectable levels of this receptor (14). IL-13R α 1 is a normal receptor for IL-13 and, once bound, initiates a signaling cascade that 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 esterified 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 carcinogenesis (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-13R α 2 acts like a decoy receptor and does not allow normal IL-13 signaling to occur.

15-LOX-1 catalyzes the oxidation of arachidonic acid and linoleic acid (LA) into 15-(S)-hydroperoxyeicosatetraenoic acid and 13-(S)-hydroperoxide octadecadienoic acid (13-HpODE), respectively. Interestingly, these are the metabolic precursors to 15-(S)-hydroxyeicosatetraenoic acid and 13-(S)-hydroxy-octadecadienoic acid, both of which are known to activate the ligand-dependent

nuclear receptor PPAR γ . Recently, PPAR γ has emerged as a potential player in the IL-13-induced apoptosis pathway (30). The mechanism by which PPAR γ inhibits proliferation varies greatly among cell types and is not fully understood in GBM cells (31, 32); however, it does seem that some PPAR γ ligands 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-PE38QQR, clinical toxicity that limited the doses that could safely be delivered to patients, and little control over toxin administration. The clinical toxicity was likely because of binding of the toxin to the physiologic IL-13R α 1/IL-4 α type II receptor. This toxin would be predicted to be harmful for normal immune cells bearing the IL-13R α 1 receptor.

Rather than pursuing the targeting of the IL-13R α 2 receptor with a toxin or blocking IL-13 interaction with its receptors, we have taken an alternative approach to specifically inhibit expression of IL-13R α 2 in glioma cells by using short interfering RNA (siRNA). The siRNA does not inhibit the expression of the physiologic IL-13R α 1/IL-4 α receptor. With this novel approach, by specifically targeting and silencing the IL-13R α 2 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-13R α 2 will effectively and specifically eliminate IL-13R α 2-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 to be *Mycoplasma* free by PCR methods before this study. The culture medium for U87 cells was Eagle's Minimal Essential Medium (EMEM; 1 \times ; Life Technologies) with Earle's salts supplemented with 10% FBS, sodium pyruvate, nonessential amino acids (Invitrogen), penicillin, and streptomycin. The culture medium for A172 cells was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 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 on the basis of previous reports utilizing this compound (34).

RNA interference

Smartpool siGENOME siRNA reagents for IL-13R α 2 siRNA were obtained from Dharmacon 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 nontarget siRNA were used as controls. Nontarget siRNAs 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, PPAR γ , and IL-13R α 2 protein

Following appropriate treatment protocols, we analyzed cell lysates for 15-LOX-1, PPAR γ , and IL-13R α 2 levels. Cells were lysed in lysis buffer containing protease and phosphatase inhibitors. Protein concentration was measured by Bradford's method. Western blot analysis was conducted using standard Western blotting techniques, with specific antibodies directed against human cellular proteins. 15-LOX-1, PPAR γ , and IL-13R α 2 Western blot analysis was conducted using a 15-LOX-1 primary antibody (1:1,000; antibody supplied by J. Cornicelli), a PPAR γ primary antibody (Santa Cruz Biotechnology SC-7273) at a dilution of 1:2,000, and an IL-13R α 2 primary antibody (R&D Systems MAB614) at a dilution of 1:2,000, 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% to 12% gradient gel (Novex) according to the manufacturer's instructions. Proteins were transferred onto nitrocellulose membranes (Invitrogen). Blots were blocked with 10% nonfat dry milk in 20 mmol/L TBS (pH 7.4) 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.

Real-time PCR analysis

The real-time PCR (RT-PCR) conditions, primers, and sequences for 15-LOX-1 were done 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 2 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 lysates, according to the manufacturer's specifications. Each assay was carried out in at least 3 independent experiments. Annexin V/propidium iodide (PI) staining was done, followed by FACScan flow cytometry. Equal numbers of cells (1×10^5) were plated onto 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 μ L Annexin V-fluorescein isothiocyanate and 10 μ L PI. After incubation in the dark at room temperature for 15 minutes, the cells were immediately analyzed by FACScan flow cytometry. For statistical analysis, Student's *t* test was used to determine the statistical differences between various experimental and control groups. Data were reported as the mean \pm SD. A value of *P* < 0.05 was considered significant.

Treatment with IL-13, PPAR γ agonists, or PPAR γ antagonists

Treatment groups were compared with vehicle controls. IL-13 (50 μ g/mL; BioSource), rosiglitazone (5 μ mol/L; Cayman Chemical), or troglitazone (5 μ mol/L; Cayman Chemical) was used for treatment. Cells were treated from 0 (control) to 96 hours as noted. Cells were pretreated with the PPAR γ antagonist GW9662 (5 μ mol/L; Cayman) for 30 minutes before any additional treatment. Dose and time ranges were selected on the basis of previous reports utilizing these compounds (36). Each assay was carried out in at least 3 independent experiments.

Tumorigenesis in nude mice

U87 cells were harvested, counted, and suspended in an equal volume of Matrigel. A total of 2.5×10^6 cells in 500 μ L Matrigel were injected under the dorsal skin of

nude mice. For uniform development of visible tumors, the animals were left for 2 weeks without any treatment. 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 diameter² × 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 mm³; nontarget siRNA, 198–353 mm³; IL-13Rα2 5 μmol/L siRNA, 151–309 mm³; IL-13Rα2 10 μmol/L siRNA, 158–341 mm³. The mice were then injected at the tumor site with IL-13Rα2 siRNA (5 or 10 μmol/L), nontarget siRNA (10 μmol/L), or vehicle alone twice per week for 4 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 minute postinjection before removal to prevent backflow. Animals were assessed visually every day thereafter. Tumor volume measurements were taken before 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 was euthanized according to approved protocols. Animals with ulcerated tumors were also euthanized according to the 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 RT-PCR, 5 and 10 nmol/L IL-13Rα2 siRNA inhibited approximately 65% and 80% of the IL-13Rα2 mRNA, respectively (Fig. 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 downregulated by treatment with IL-13Rα2 siRNA at both 48 and 72 hours (Fig. 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 (5 nmol/L) for 48 and 72 hours. Apoptosis was assessed by Annexin V/PI staining, followed by FACScan flow cytometry. Treatment with IL-13Rα2 siRNA (5 nmol/L) induced

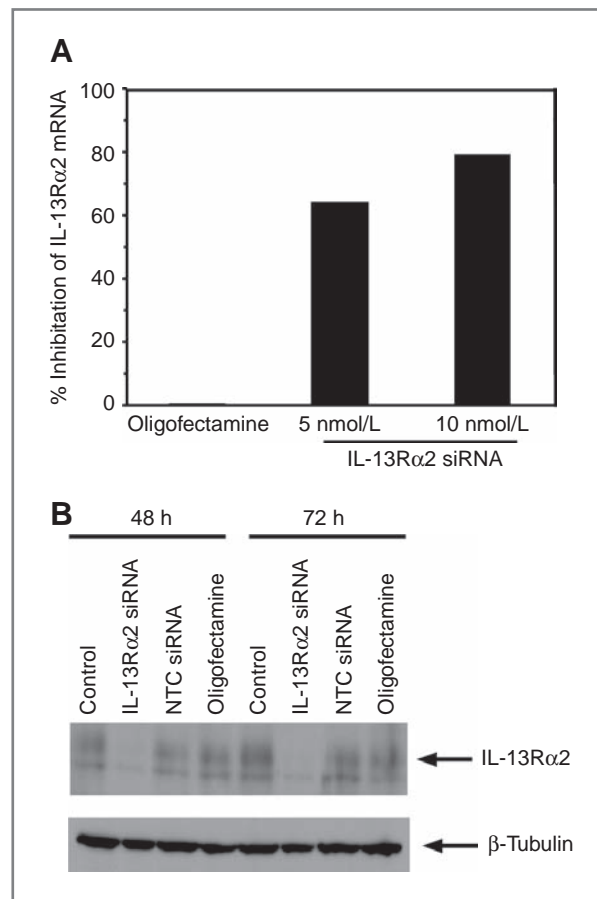


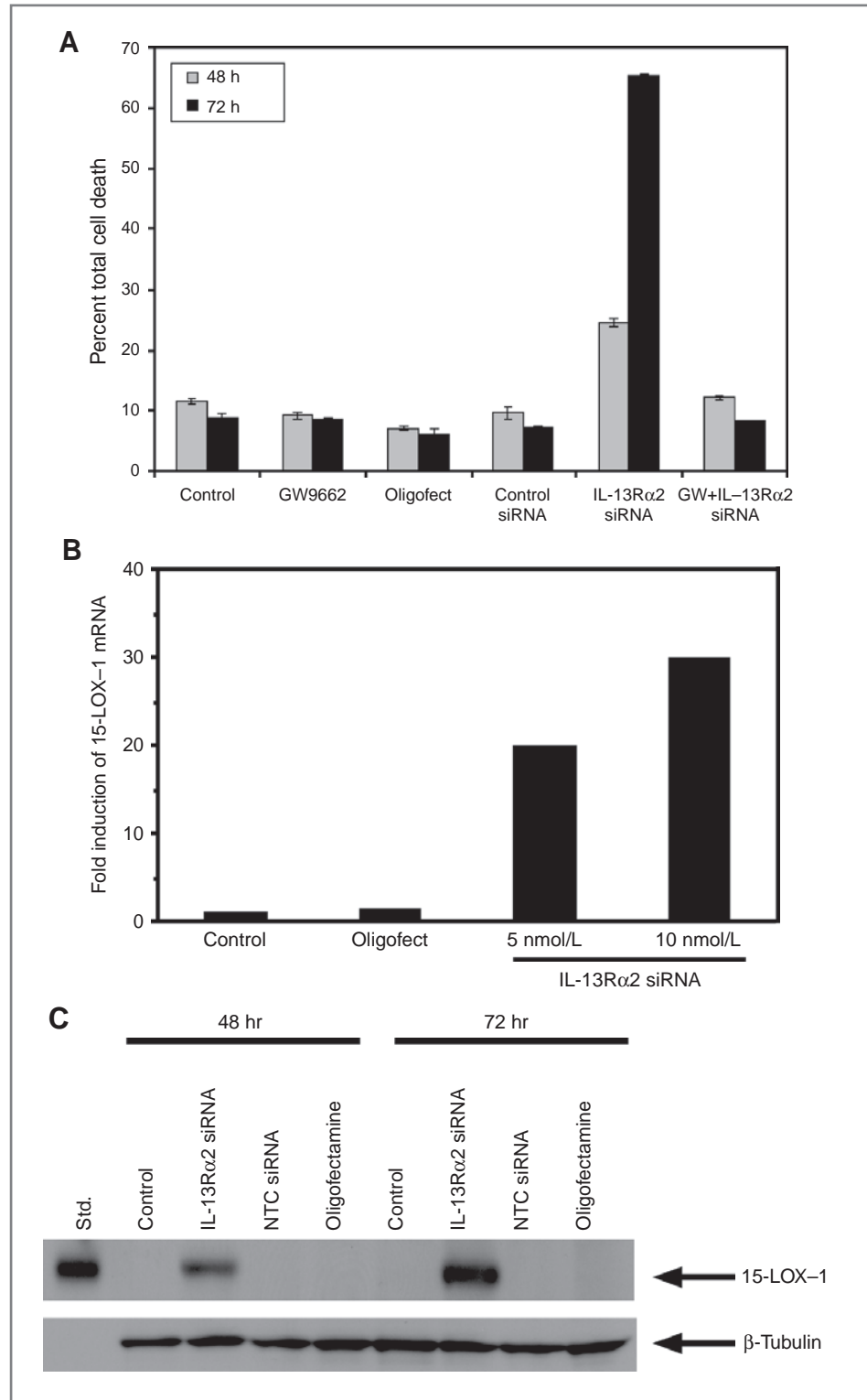
Figure 1. IL-13Rα2 siRNA inhibits the expression of IL-13Rα2 decoy receptor mRNA and protein in A172 cells. **A**, IL-13Rα2 mRNA was measured by RT-PCR at 48 hours in A172 cells treated with 5 or 10 nmol/L IL-13Rα2 siRNA, oligofectamine alone, or untreated control cells. The data shown represent 1 of 2 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 48 hours. Lane 2, IL-13Rα2 siRNA (5 nmol/L) 48 hours. Lane 3, NTC siRNA (5 nmol/L) 48 hours. Lane 4, oligofectamine alone 48 hours. Lane 5, control cells 72 hours. Lane 6, IL-13Rα2 siRNA (5 nmol/L) 72 hours. Lane 7, NTC siRNA (5 nmol/L) 72 hours. Lane 8, oligofectamine alone 72 hours. Total protein (30 μg) was loaded per lane. β-Tubulin was used as a loading control. The data shown represent 1 of 3 separate experiments giving similar results.

apoptosis in a time-dependent manner, with 25% and 65% cell death at 48 and 72 hours, respectively (Fig. 2A). Treatment with the PPARγ antagonist GW9662 (5 μmol/L) reduced this effect, suggesting the involvement of PPARγ in GBM apoptosis. Control cells, cells treated with GW9662 alone, oligofectamine, or nontarget 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

Figure 2. Inhibition of IL-13R α 2 decoy receptor expression by IL-13R α 2 siRNA induces apoptosis and promotes induction of 15-LOX-1 in A172 cells. **A**, cells were transfected with IL-13R α 2 siRNA (5 nmol/L), nontarget siRNA control 5 nmol/L, or oligofectamine (Oligofect) alone and apoptosis assessed at 48 and 72 hours. Cells were pretreated with GW9662 (GW; 5 μ mol/L) for 30 minutes. Apoptosis was assessed by Annexin V/PI staining, followed by FACScan flow cytometry. The data shown represent 1 of 3 separate experiments giving similar results. **B**, 15-LOX-1 mRNA was measured by RT-PCR at 48 hours in A172 cells either treated with 5 or 10 nmol/L IL-13R α 2 siRNA, oligofectamine alone, or untreated control cells. The data shown represent 1 of 2 separate experiments with similar results. **C**, 15-LOX-1 protein was measured by Western blot analysis of A172 cell lysates at 48 or 72 hours. Lane 1, 15-LOX-1 standard. Lane 2, control cells 48 hours. Lane 3, IL-13R α 2 siRNA (5 nmol/L) 48 hours. Lane 4, NTC siRNA (5 nmol/L) 48 hours. Lane 5, oligofectamine alone 48 hours. Lane 6, control cells 72 hours. Lane 7, IL-13R α 2 siRNA (5 nmol/L) 72 hours. Lane 8, NTC siRNA (5 nmol/L) 72 hours. Lane 9, oligofectamine alone 72 hours. Total protein (30 μ g) was loaded per lane. β -Tubulin was used as a loading control. The data shown represent 1 of 3 separate experiments giving similar results.



and apoptosis, was examined next. To explore this, the IL-13R α 2 decoy receptor was inhibited using IL-13R α 2 siRNA. RT-PCR was used to determine the effects of siRNA on the production of 15-LOX-1 mRNA levels.

Inhibition of IL-13R α 2 by 5 and 10 nmol/L IL-13R α 2 siRNA increased the production of 15-LOX-1 20- and 30-fold, respectively (Fig. 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 upregulation 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-13R α 2 siRNA treated cells than in control cells. 15-LOX-1 protein was also significantly upregulated by treatment with IL-13R α 2 siRNA (Fig. 2C).

PPAR γ is expressed in GBM cells and PPAR γ ligands induce apoptosis in GBM cells

To determine whether PPAR γ is present in GBM cells, PPAR γ expression in A172 cells was examined by Western analysis. PPAR γ is expressed in A172 cells (Fig. 3A). To establish PPAR γ function in the apoptotic process, the PPAR γ ligand troglitzone or rosiglitzone was tested to determine whether they could induce apoptosis in GBM cells and whether the PPAR γ antagonist GW9662 could alter this effect. A172 cells were treated for 24 to 72 hours, and apoptosis was assessed by Annexin V/PI staining, followed by FACScan flow cytometry. Both PPAR γ ligands induced apoptosis in a time-dependent manner (>60% total cell death by

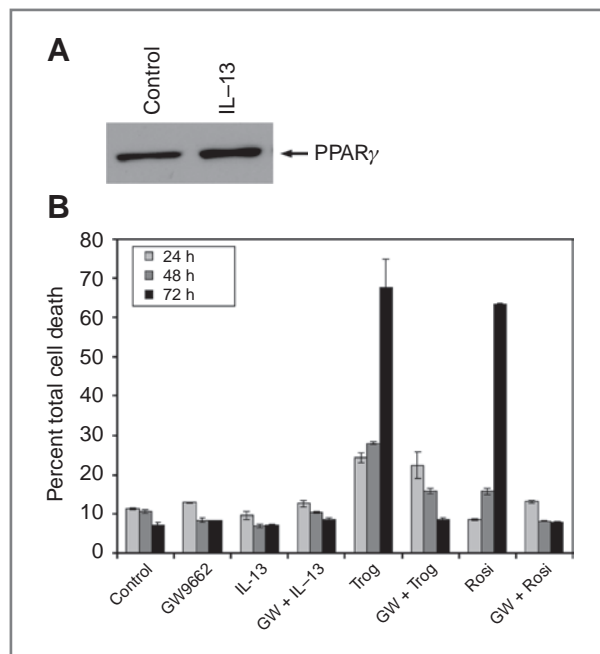


Figure 3. PPAR γ is expressed by A172 cells, and PPAR γ ligands induce apoptosis. A, PPAR γ was measured by Western blot analysis of cell lysates at 24 hours. Lane 1, control cells. Lane 2, IL-13 (50 μ g/mL) 24 hours. Total protein (30 μ g) was loaded per lane. The data shown represent 1 of 3 separate experiments giving similar results. B, A172 cells were treated with IL-13 (50 μ g/mL), troglitzone (Trog; 5 μ mol/L), or rosiglitzone (Rosi; 5 μ mol/L) for 24, 48, and 72 hours. Pretreatment with GW9662 (GW; 5 μ mol/L), a PPAR γ antagonist, was done for 30 minutes. Apoptosis was assessed by Annexin V/PI staining, followed by FACScan flow cytometry. The data shown represent 1 of 3 separate experiments giving similar results.

72 hours) in A172 cells and treatment with GW9662 (5 μ mol/L) significantly reduced this effect (Fig. 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 4 hours and then microscopically examined to identify morphologic changes associated with apoptosis (Fig. 4A). Cells treated with 13-HpODE were clearly altered, with morphologic changes that indicate apoptosis, including cytoplasmic and nuclear shrinkage. Cells were also treated with 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 (Fig. 4B). Cells treated with 13-HpODE underwent apoptosis, whereas 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 (Fig. 4C). A172 cells were treated with IL-13, LA, or 13-HpODE for 48 and 72 hours. Apoptosis occurred with 13-HpODE treatment but not with the other treatments. Pretreatment with the PPAR γ antagonist GW9662 for 30 minutes attenuated the 13-HpODE-induced apoptosis.

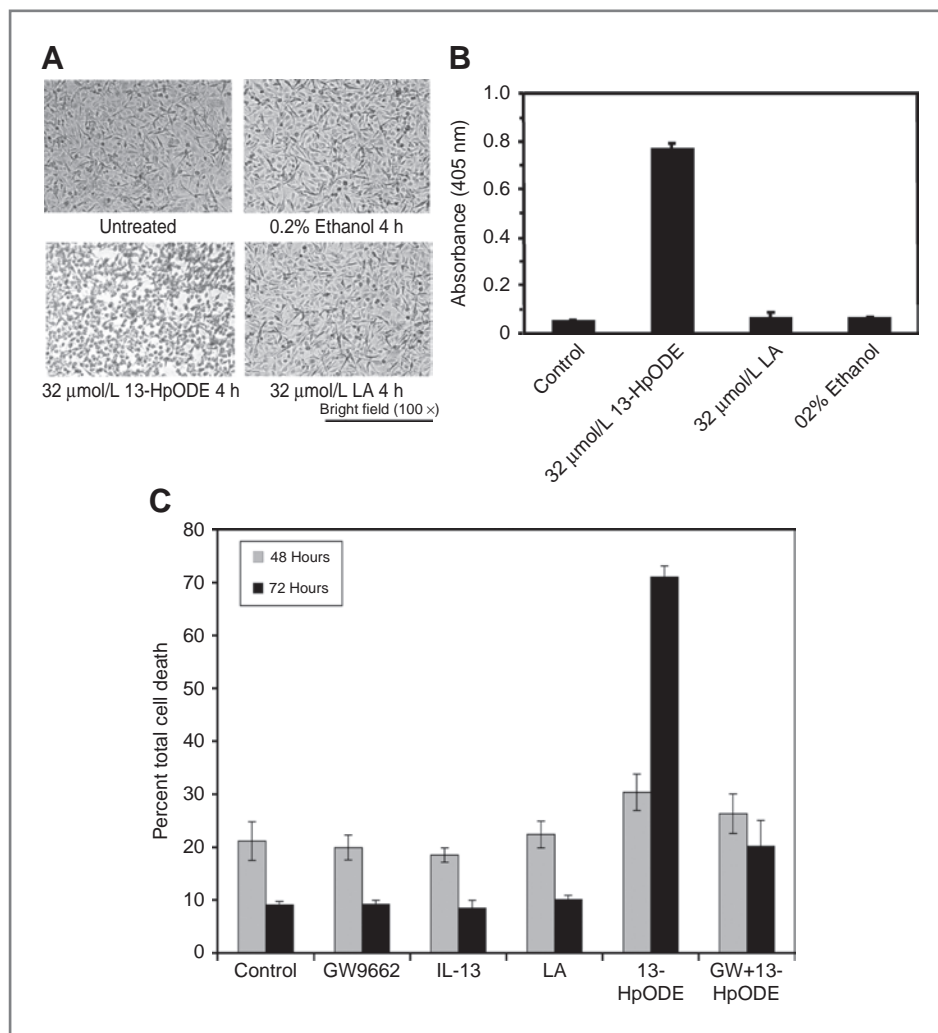
Treatment with IL-13R α 2 siRNA promotes 15-LOX-1 expression in U87 GBM cells

We next examined the production of 15-LOX-1 in U87 cells. We were interested in the U87 cell line for 2 reasons. First, it is important to confirm our findings in different glioblastoma cell lines for validity. Second, the U87 cell line has previously been shown to grow well in both heterotopic and orthotopic *in vivo* GBM models, whereas 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 such as U87 to move forward with orthotopic *in vivo* studies. It is vital to be able to extend our studies to an orthotopic GBM model system because of 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-13R α 2 decoy receptor was inhibited using IL-13R α 2 siRNA. Inhibition of IL-13R α 2 by 5 nmol/L IL-13R α 2 siRNA increased the production of 15-LOX-1 protein in U87 cells (Fig. 5A).

IL-13R α 2 siRNA treatment induces apoptosis in U87 GBM cells

To determine whether IL-13R α 2 blocks IL-13-induced apoptosis in U87 cells, we treated U87 cells

Figure 4. 13-HpODE, a 15-LOX-1 product and PPAR γ ligand, induces apoptosis in A172 cells. A, morphology of A172 cells untreated (top left), vehicle treated (top right), treated with 32 μ mol/L 13-HpODE (bottom left), or 32 μ mol/L LA (bottom right) for 4 hours was examined. 13-HpODE induced morphologic changes that indicate apoptosis, including cytoplasmic and nuclear shrinkage. B, the induction of apoptosis in A172 cells determined by ELISA of cells untreated, vehicle treated, treated with 32 μ M 13-HpODE, or 32 μ mol/L LA for 24 hours. C, A172 cells were treated with IL-13 (50 μ g/mL), LA (10 μ mol/L), or 13-HpODE (10 μ mol/L) for 48 and 72 hours. Pretreatment with GW9662 (GW; 5 μ mol/L), a PPAR γ antagonist, was done for 30 minutes. Apoptosis was assessed by Annexin V/PI staining, followed by FACScan flow cytometry. The data shown represent 1 of 3 separate experiments giving similar results.



with IL-13R α 2 siRNA (5 nmol/L) for 72 hours. Apoptosis was assessed by Annexin V/PI staining, followed by FACScan flow cytometry. Treatment with IL-13R α 2 siRNA (5 nmol/L) induced apoptosis, with 30% cell death at 72 hours (Fig. 5B). Treatment with GW9662 (5 μ mol/L), the PPAR γ antagonist, reduced this effect. Control cells, cells treated with GW9662 alone, Lipofectamine, or nontarget siRNA controls did not have increased apoptosis. To establish PPAR γ function, we tested whether the PPAR γ ligand rosiglitazone could induce apoptosis in U87 cells and whether GW9662 could alter this effect. U87 cells were treated for 72 hours, and apoptosis was assessed by Annexin V/PI staining, followed by FACScan flow cytometry. The PPAR γ ligand induced apoptosis (48% total cell death) in the U87 cells and treatment with GW9662 (5 μ mol/L) reduced this effect (Fig. 5B). Control cells or cells treated with GW9662 alone or IL-13 did not have increased apoptosis.

IL-13R α 2 siRNA treatment *in vivo* reduces GBM tumor growth

To determine whether IL-13R α 2 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 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 the development of visible and palpable tumors. Animals were then divided into 4 groups of 10 mice each. Tumor volume was measured beginning from week 2. Intratumoral injections with IL-13R α 2 siRNA (5 or 10 nmol/L), nontarget control (NTC) siRNA, or vehicle were done 2 times per week for 4 weeks. Tumor volume was measured before each injection. After the fourth week, animals were photographed (Fig. 6A) and sacrificed, tumors were measured (Fig. 6B), and then

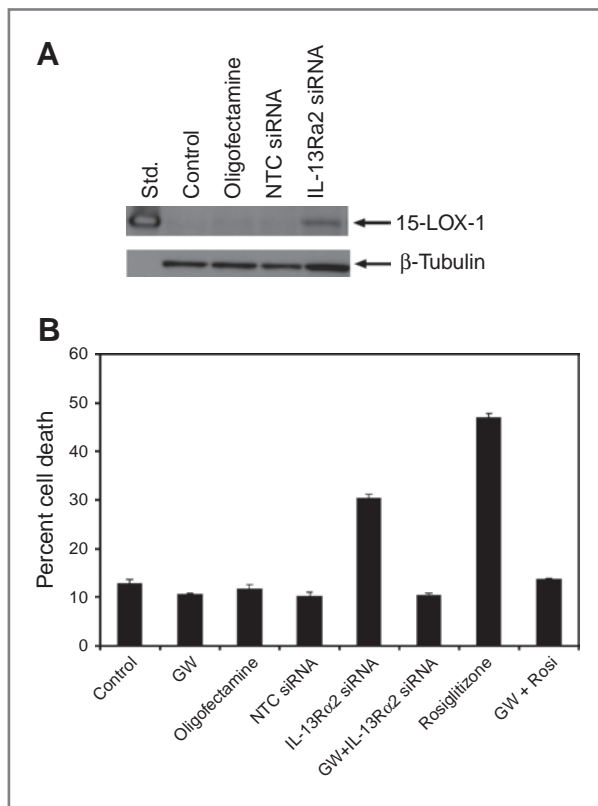


Figure 5. Inhibition of IL-13R α 2 decoy receptor promotes induction of 15-LOX-1 expression and apoptosis in U87 cells. **A**, 15-LOX-1 protein was measured by Western blot analysis of U87 cell lysates at 48 hours. Lane 1, 15-LOX-1 standard (Std.). Lane 2, control cells. Lane 3, oligofectamine alone. Lane 4, NTC siRNA (5 nmol/L). Lane 5, IL-13R α 2 siRNA (5 nmol/L). Total protein (30 μ g) was loaded per lane. β -Tubulin was used as a loading control. The data shown represent 1 of 3 separate experiments giving similar results. **B**, cells were transfected with IL-13R α 2 siRNA (5 nmol/L) or rosiglitazone (Rosi; 5 μ mol/L), and apoptosis was assessed at 72 hours. Pretreatment with GW9662 (GW; 5 μ mol/L), a PPAR γ antagonist, was done for 30 minutes. Apoptosis was assessed by Annexin V/PI staining, followed by FACSscan flow cytometry. The data shown represent 1 of 3 separate experiments giving similar results.

surgically removed, photographed (Fig. 6C), and weighed (Fig. 6D). Inhibition of IL-13R α 2 by 5 or 10 nmol/L IL-13R α 2 siRNA decreased tumor growth by 44% and 48%, respectively, compared with control tumors after 4 weeks of treatment (Fig. 6B). There was a decrease in tumor growth observed with NTC siRNA (31%); however, the effect observed with IL-13R α 2 siRNA was significantly greater. The effect observed with NTC siRNA is most likely because of an antiviral response elicited by the presence of double-stranded RNA in the cell (37, 38). A decrease in tumor weight was also observed after 4 weeks of treatment with IL-13R α 2 siRNA. Inhibition of IL-13R α 2 by 5 or 10 nmol/L IL-13R α 2 siRNA decreased tumor weight by 43% and 59%, respectively, compared with controls (Fig. 6D).

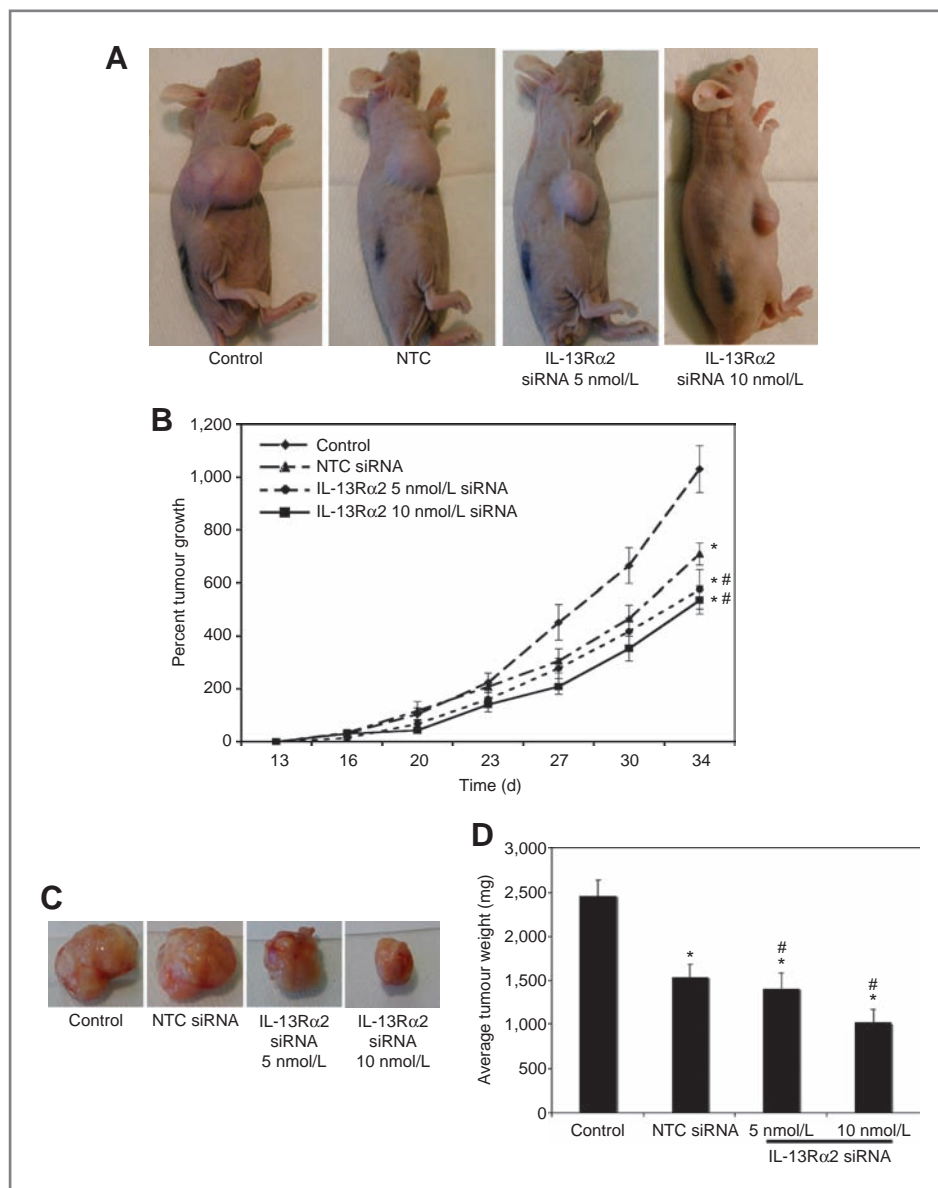
Discussion

Our data suggest that the unique expression of an IL-13R α 2 decoy 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 show 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 PPAR γ and lead to apoptosis. This mechanism provides an explanation for how the expression of the decoy receptor IL-13R α 2 may prevent GBM apoptosis and contribute to tumor growth (Fig. 7). Thus, we have identified both a novel approach and a 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-13R α 2 may serve as an important therapeutic siRNA target, as it is highly expressed in gliomas but not in normal brain. The advantage of using IL-13R α 2 siRNA to specifically silence the receptor compared with other IL-13-based treatment strategies is that we specifically knock down only the IL-13R α 2 receptor and do not target functional IL-13 receptors more generally as other current treatments have done and take advantage of an endogenous signaling pathway involving 15-LOX-1 that leads to glioma cell apoptosis and tumor inhibition. For example, the recombinant cytotoxin composed of IL-13 and a truncated form of *P. aeruginosa* exotoxin A (IL13-PE38QQR) targets both IL-13 receptors (7). We believe that the IL-13R α 1 receptor is needed to allow normal endogenous signaling mechanisms to occur, which can ultimately lead to cell death of GBM cells.

Although IL-13R α 2 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-13R α 2 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-13R α 2 siRNA is a novel approach and could be an effective treatment option for GBM with minimal side

Figure 6. Inhibition of subcutaneous solid tumor development in nude mice after treatment with IL-13R α 2 siRNA. Mice were injected at the tumor site with IL-13R α 2 siRNA (5 or 10 μ mol/L), nontarget siRNA (10 μ mol/L), or vehicle alone twice per week for 4 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 by using a digital vernier caliper in nude mice. Tumor volume measurements were taken both before each injection and before sacrifice. Data are means \pm SEM of 10 animals in each group. *, $P < 0.05$ compared with the control mean values; #, $P < 0.05$ compared with the NTC siRNA 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 \pm SD of 10 animals in each group. *, $P < 0.05$ compared with the control mean values; #, $P < 0.05$ compared with the NTC siRNA mean values.



effects, as we are now allowing normal endogenous signaling cascades to initiate.

Further investigations are needed to advance the implementation of this novel therapeutic approach. Although 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-13R α 2 siRNA. This effect may be due to inadequate dosing and/or delivery of the IL-13R α 2 siRNA.

Because of the nature of direct injections, there is potential difficulty in obtaining uniform delivery of siRNA to all tumor tissues. 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-13R α 2 siRNA. siRNA has a

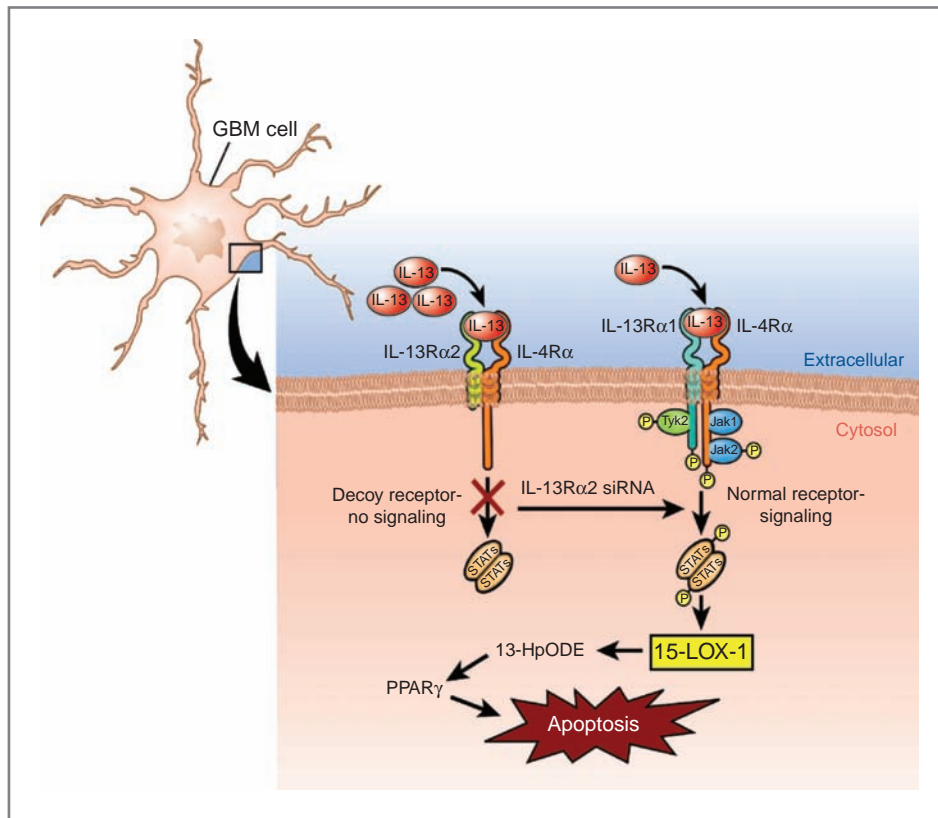


Figure 7. Proposed mechanism for IL-13-induced apoptosis via 15-LOX-1 in GBM. We hypothesize that IL-13 initiates an intracellular cascade that works via 15-LOX-1 to activate PPAR γ and regulate cell growth/apoptosis in GBM. The expression of the IL-13R α 2 decoy receptor, which has a high affinity for IL-13, can inhibit the normal cellular responses initiated by IL-13 and may prevent GBM apoptosis and contribute to tumor growth.

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 RNAi delivery that 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 con-

structed with antibodies to recognize 2 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 transferrin 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 that encodes a short hairpin RNA 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. Preclinical studies on mice by 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-13R α 2 RNAi for the treatment of GBM.

Another approach for siRNA delivery that has recently been examined is the use of a multistage vector composed of mesoporous silicon particles (stage 1 microparticles) loaded with neutral nanoliposomes [dioleoyl phosphatidylcholine (DOPC)] containing 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 3 weeks following a single intravenous administration of stage 1 microparticles 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 the treatment of GBM.

The effects of silencing the IL-13R α 2 receptor with IL-13R α 2 siRNA on 15-LOX-1 expression and apoptosis in our study support the potential promise of siRNA-directed targeting for GBM therapy. Although we are optimistic about the potential of siRNA-directed targeting to IL-13R α 2, 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 the 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-13R α 2 siRNA should be explored as a tantalizing new approach to be used in combination with other agents to improve the efficacy of GBM treatment.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank the staff of the Flow Core Facility and the Center for Medical Art & Photography for providing technical assistance.

Grant Support

This research was supported in part by grant ACSODSR2009-2 from the American Cancer Society (L.C. Hsi) and PO1-HL087018 from the NIH (M.K. Cathcart).

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Received November 23, 2010; revised March 11, 2011; accepted May 13, 2011; published OnlineFirst May 19, 2011.

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