Evaluation of Recombinant Cytotoxins for the Therapy of CNS Tumors in Experimental Mouse Models

Shrila T. Shah

1. Yorktown High School, 3061 Chen Court, Yorktown Heights, New York, 10598, USA

shrila.shah@yorktown.org

Abstract

Malignant brain tumors, continue to be the cause of a disproportionate level of mortality, killing over 17,000 people each year. For metastasis, blood vessels are constructed (angiogenesis) to provide cancerous tissue with nutrients/oxygen. Blocking this would prevent the supply of factors essential for tumor growth. Angiogenesis is controlled by a balance of pro and anti-angiogenic factors in endothelial cells. Vascular endothelial growth factor (VEGF) is a key proangiogenic molecule that can be targeted with small molecule inhibitors such as Sunitinib, which have demonstrated, with some success, down regulation of tumor angiogenesis. However, benefits are often transitory, followed by restoration of tumor progression. The goal of this study was to determine if introducing an SLT-VEGF fusion protein would selectively target VEGFR-2 being overexpressed in vasculature, preventing “rebound” of vessel angiogenesis. SLT-VEGF is comprised of the subunit A Shiga-like toxin, which binds to VEGFR-2, inhibiting protein synthesis in cells. Cell lines glioma and glioblastoma were grown in flanks of nude mice as model systems to analyze the effects of treatment with Sunitinib (followed by growth without treatment), and SLT-VEGF (followed by growth without treatment). Cyrosections were prepared for immunohistochemical staining to assess the degree of angiogenesis. Results showed that SLT-VEGF treatment significantly blocked the rebound of new blood vessel formation around the tumor.

Keywords

Medulloblastoma; Angiogenesis; Sunitinib; Shiga-like Toxin; VEGFR-2; CD31; cyrosections; immunohistochemical
Introduction

About 80,000 new cases of primary brain tumors are diagnosed annually in the United States and approximately 32% of these tumors are malignant. This year, it is anticipated that nearly 17,000 people will die from a primary malignant central nervous system tumor of one form or another. Glioblastomas, which are a subtype of gliomas that arise from the supportive tissue of the brain, are one of the most common and aggressive brain tumors with about 12,390 new cases expected this year. The prognosis for glioblastoma patients is still poor with a median survival time of 15 months despite current treatment approaches. Conventional methods include: surgical resection, radiotherapy, and chemotherapy. Unfortunately, these therapies are often not effective in treating glioblastomas and they have long-lasting adverse effects, including impaired neural development (especially for children). Specifically, radiation therapy causes late effects such as neurocognitive deficiencies, hormone deficits, and growth impairment. The most common chemotherapy-related late effects are ototoxicity caused by platinum drugs, secondary leukemia, and infertility following exposure to alkylating agents. Therefore, since present therapy is relatively ineffective in treating this disease and puts the surviving patient at risk of severe side effects, a less toxic and more effective therapy is needed.

Angiogenesis in Tumor Growth

One current alternative treatment researched focuses on inhibiting the process of angiogenesis. Angiogenesis, the formation of a vascular network, is necessary for tumor formation in solid malignancies. As shown in figure 1, a tumor mass requires blood vessel networks to provide it with oxygen and metabolites in order to grow. This angiogenic process is far from simple, as it involves a multitude of interacting factors whose final outcome is determined by the balance of the pro- and anti-angiogenic factors produced by the tumor cells. It is seen that the ‘angiogenic switch’ is ‘turned off’ when the effects of proangiogenic molecules are balanced by that of anti-angiogenic molecules, and it is ‘turned on’ when the net balance is in favor of angiogenesis. Further studies have shown that vascular endothelial growth factor (VEGF) is a key driver of angiogenesis. One main VEGF receptor, VEGFR-2, is commonly overexpressed on the vasculature of most solid cancers including glioblastomas. As illustrated in Figure 2, an inhibition of VEGF-driven tumor angiogenesis has been shown to suppress tumor growth in animal models. Thus, given the characteristic high degree of endothelial proliferation, vascular permeability and ubiquity of proangiogenic growth factors, the targeting of blood vessels in brain tumors is a particularly attractive therapeutic strategy.

Mechanisms of Response and Resistance to VEGF-targeted Therapy

Today, there are two main strategies to target the tumor vasculature; anti-angiogenic drugs aimed at preventing the process of angiogenesis, thus stopping the formation of new blood vessels that are necessary for the growth and progression of the tumor and metastasis; and an antivascular therapy targeted to the already existing tumor vasculature. Although anti-angiogenic therapies such as Bevacizumab or Sunitinib have been shown to be successful in inhibiting the growth of tumor associated blood vessels, recent studies have demonstrated several important problems with these treatments. One of the most prominent issues is the development of resistance to anti-angiogenic therapy that most likely explains the varying results obtained in these studies. Resistance can essentially be broadly classified into inherent resistance, where the tumors fail to respond from the beginning of a treatment and acquired resistance, where tumors initially respond and then progress while still on treatment. Since anti-angiogenic therapy targets tumor cells indirectly by acting on tumor blood vessels, mechanisms that determine its response and resistance are likely to stem from a complex interaction between tumor cells. Acquired resistance
may perhaps occur because the tumor finds an alternative means to drive tumor vascularization and therefore is insensitive to the therapy, or because tumor cells become adapted so that they can grow despite the reduced vascular supply. Although inhibitors of VEGF/VEGFR signaling were expected to inhibit tumor growth via inhibition of endothelial cell proliferation, experimental evidence indicates that it induces vascular regression, presumably by inhibiting pro-survival VEGF functions. However, this vascular regression may lead to hypoxia in tumors and upregulation of VEGF production, which stimulates tumor revascularization after either prolonged exposure to the drugs or during interruptions in treatment. Essentially, a blockade of angiogenic signaling therapy suppresses the growth of newly formed tumor vessels, but is less effective against the more established tumor vasculature.

An alternative approach to anti-angiogenic signaling therapy is to target VEGFR for selective delivery of highly cytotoxic agents into endothelial cells; with the expectation that only tumor endothelial cells overexpressing such receptors will internalize therapeutically significant amounts of VEGFR-targeted cytotoxins. Since alternative signal transduction pathways cannot prevent or reverse the cytotoxic activity of plant or bacterial toxins, several groups have investigated recombinant proteins fused to VEGF for targeting such toxins to tumor vasculature. One such recombinant protein, known as SLT-VEGF, is comprised of a human VEGF121 protein fused to the A subunit of the Shiga-like toxin (SLT), a site-specific N-glycosidase that when internalized, cleaves off adenosine at position 4324 in 28S rRNA. This modification prevents the ribosome from properly interacting with elongation factors thus blocking protein synthesis and eventually leading to cell death. SLT-VEGF is highly cytotoxic to VEGFR-2 overexpressing cells in vitro, and selectively reduces such cells in tumor vasculature. The aim of this study is to test SLT-VEGF for its ability to sustain inhibition of tumor angiogenesis when used in combination with an angiogenesis signaling pathway inhibitor such as the small molecular drug Sutent (Sunitinib).

Problem:

As conventional therapies for treating CNS tumors have been shown to be ineffective, an approach to target tumors by depriving them of their blood supply should be evaluated as a method that avoids both intrinsic and acquired resistance.

Goal:

The goal was to...

1. Follow the dynamics of vascular remodeling after 5-day Sutent treatment and then determine if there is a correlation between the activity of VEGFR-2 promoter (as shown by luciferase activity) and vascular remodeling as shown by in vivo cell tagging and fluorescent microscopy;
2. Test SLT-VEGF for its ability to sustain inhibition of tumor angiogenesis when used in combination with an angiogenesis signaling pathway inhibitor, such as the small molecule drug Sutent.

Hypothesis:

It was hypothesized that...

1. There will be a correlation between VEGFR-2 promoter activity and vascular remodeling because VEGF is considered to be a key regulatory molecule in angiogenesis that induces vascular growth and permeability and acts as a survival factor for newly formed vessels;
2. SLT-VEGF will selectively target and inhibit protein synthesis of cells with high VEGFR-2 expression.

Results & Discussion

BLI and NIRF imaging was performed on mice anesthetized with a ketamine/ xylazine mixture (30 ul/mouse i.m). This amount was sufficient to anesthetize mice for one hour. After mice were anesthetized they receive i.p. injections of 100 ul of scVEGF/Cy (50 uM) followed 10-15 minutes later with 0.6 ml aqueous luciferin (3 mg/ml).

FaDu and U87 tumors grew despite Sutent or SLT-VEGF treatment. BLI showed half as much intensity in tumors of Sutent-treated mice as untreated control mice while BLI tumors of SLT-VEGF treated mice were as strong as in the control mice. NIRF was barely detectable in either control or Sutent-treated mice. After imaging mice were sacrificed and their tumors excised for immunohistochemical analysis of the tissue. The statistics we did on the image analysis was a one-way analysis of variance (ANOVA) on the ratios of pixel intensity of CD31 staining over DAPI (diamidino-2-
phenylindole; used for blue-florescent DNA) staining. Staining ratios of the three groups were compared for statistical significance by the Bonferroni multiple comparison procedure. After treatment was administered (SLT in combination with Sutent, and just Sutent), data was shown as seen in Figures 3, 4 and 5. As shown in the figures, the recovery group was statistically higher than the control group throughout both FaDu and U87 groups.

Anti-angiogenic therapy represents a promising alternative to the treatment of malignant brain tumors that avoid the problems of conventional therapies. It seems likely that a combination of anti-angiogenic agents, with other cytotoxic therapies, will be required to achieve maximal efficacy. In this study the combination of SLT-VEGF, a fusion toxin that
enters the cell via VEGF receptor mediated endocytosis, and depletes VEGFR-2+/CD31+ endothelial cells from the vasculature, and Sutent, a receptor protein-tyrosine kinase inhibitor, was evaluated for inhibiting tumor angiogenesis in two models of human and rat brain tumors. The treatment inhibits the actions of vascular endothelial growth factor (VEGF) and is an angiogenesis inhibitor. In this experiment, four main groups of treatments were created, and given to nude mice (medulloblastoma tumors). In the group with no treatment, the tumor had maximum amounts of blood vessel networks (figure 4). The Sutent treatment has slightly fewer networks however, as expected, after Sutent treated issues were allowed to “recover,” the vessel networks were re-established. One of the main reasons this may have occurred (other than the previously predicted signaling pathway resistance) could have been through the formation of pericytes. Pericytes, or contractile cells that wrap around the endothelial cells that line capillaries and venules throughout the body, were formed on the edge of these CNS Sutent treated tumors. Thus, an acquired resistance was formed, and a need for a drug with two preventative behaviors is necessary. Thus, when SLT was administered, and given a time period for recovery, the pixel count was significantly less. Administering SLT-VEGF with Sutent effectively prevented the rebound effect.

Conclusions

The key issues here are that glioblastoma, medulloblastoma, and other CNS tumors (the most malignant forms of infiltrating astrocytoma’s) can evolve from lower-grade precursor tumors to higher grade lesions. Underlying genetic alterations in these CNS tumors may tilt the balance in favor of an angiogenic phenotype by upregulation of proangiogenic factors and downregulation of angiogenesis inhibitors. Increased vascularity and endothelial cell proliferation are also driven by hypoxia-induced expression of proangiogenic cytokines, such as VEGF. Selective targeting of VEGFR (overexpressed receptor of VEGF) in tumor vasculature with VEGF conjugated with a radioactive isotope 177/Lu has been shown to be effective in orthotopic breast cancer models. As high levels of VEGFR expression in tumor vasculature are a common feature in a variety of cancers, included glioblastoma and other brain tumors, we found that non-radioactive VEGFR-targeting compounds was an effective anti-tumor therapy in anti-angiogenic therapy.

Methods

Animals and cell lines

Transgenic VEGFR2/Luc mice were obtained from Caliper and maintained in the Comparative Medicine Department of New York Medical College under an approved IUCAC protocol. The rat glioblastoma cell line FaDu (Catalog number HTB-437) and human Uppsala 87 Malignant Glioma (U87) were obtained from the American Type Culture Collection (ATCC) and maintained in culture with RPMI-1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine.

Sunitinib for Oral administration

90 ml of sterile DI was warmed in a glass beaker to ~80°C on a magnetic stirrer/ hot plate. 0.5 g of CMC was added and stirred vigorously for five minutes. The heat was turned off, and CMC stirred slowly for 16-18 hours until dissolved, at that point 1.9 g of NaCl, 4 ml 10% Tween 80, and 0.9 ml of benzyl alcohol were added and stirred until complete homogeneity was achieved. The volume was adjusted to 100ml with sterile DI and the resulting CMC stock solution was distributed in 10 ml aliquots in 15-ml conical tubes and stored at RT. 100 mg of Sutent was weighed and distributed into six 1.5-ml tubes containing approximately 1 ml of CMC. The Sutent mixture was vortexed at high speed for five min then all tubes were placed in a water bath sonicator, and sonicated on a high setting for 10 min. When all tubes became uniformly an orange-yellow color, the suspensions were collected in the original 15ml conical tube with the leftover CMC. The final concentration of the Sutent was 10 mg/ml. After sterilization by autoclaving the Sutent suspension was stable for 1-2 weeks in a refrigerator under constant agitation. The final concentration of ingredients included 0.5% CMC, 1.8% NaCl, 0.4% Tween 80 (Acros Organics, Cat #278632500, Belgium), and 0.9% Benzyl alcohol (Fluka, Cat #77013, Canada).

Reagents and Kits

Rat Anti-mouse Fik1 and PECAM/CD31 antibodies (BD Bioscience), rat IgG (Zymed), normal rabbit serum and biotinylated rabbit anti-rat IgG (Vector Labs), a biotin blocking kit and TSA-488 HRP-streptavidin kit (Molecular Probes) were all refrigerated at -20°C. 16% Formaldehyde (Polysciences) was stored at RT and Vector Shield Mounting medium (Vector Labs) was refrigerated. Tyramide stock was used to dissolve component A in 150 µL of DMSO (component B) and stored in small aliquots at 20°C. The HRP-streptavidin conjugate was reconstituted

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in 200 µL PBS which was stable for up to 9 months at 4°C. The 10x blocking reagent was made in PBS, and 1-ml aliquots were stored at 20°C. The 10x concentrate was diluted with 1xPBS immediately before staining. The 10xPBS concentrate was obtained from USB Corporation (75889), and the antibody diluent was obtained from BB Bioscience (55914).

**Sunitinib treatment of tumor bearing mice**

Mice bearing FaDu and U87 tumors were dosed with Sunitinib via gastric gavage. Sunitinib malate salt (Sutent, LC Laboratories, S-8803 Lot BST-102, City, State) was prepared as a suspension with carboxymethylcellulose (CMC, Fluka, Cat #21904, City, State) as described above.

**Bioluminescent Imaging (BLI) and Near Infrared Fluorescence (NIRF) of tumor bearing mice**

BLI and NIRF were used to detect endogenous luciferase in transgenic mice under control of a VEGFR-2 promoter. Tumors were established by inoculating 2x10^6 U87 cells subcutaneously in the right flank and 2 x10^6 FaDu cells subcutaneously in the left flank of mice. After tumors had reached a diameter of approximately 0.5 cm in diameter, mice were anesthetized with an intramuscular (i.m.) injection of 40 ul of a mixture of Ketamine (100 mg/ml, Fort Dodge Animal Health, Fort Dodge, KS) and Xylazine (100 mg/ml, Akron Incorporation, Akron, OH). Mouse eyes were covered with an ocular lubricant (Pharmaderm) during the procedure. BLI and NIRF images were acquired with a LAS 4000 Imager (Fujifilm, City, State). Prior to imaging mice were anesthetized as described above and injected i.p. with 1 ml of aqueous luciferin (5 mg/ml, Molecular Imaging Cat. # LUC 00, City, State). For NIRF a solution of ScV/Al 594 (Sibtech, Lot #3) reconstituted in PBS to 50 uM and scVEGF/Cy (Sibtech, Lot #27) were injected in a 0.1 ml volume per mouse via the retro-orbital plexus. After imaging experiments were completed, mice were sacrificed and tumors were removed and frozen in O.C.T Compound (Tissue-Tek) for immunohistological studies.

**Fluorescent Staining of Tissue Cryosections**

The frozen slides of tumor tissue cut with a cryostat were removed from the freezer and allowed to come to room temperature (10-30 min). A circle was drawn around the tissue section with a PAP pen and let dry for 1-2 min. Slides were labeled with permanent, solvent-resistant Marker/Superfrost. A 1% blocking solution in PBS (1 ml 10% protein concentrate stored at -20°C + 9 ml PBS) and a 1% formaldehyde solution (one 10ml vial of 16% formaldehyde + 150ml PBS) were freshly prepared. The working rat IgG (specificity control) was made by diluting rat IgG (2.5mg/ml) 1:25 with antibody diluent to give a final 0.1 mg/ml concentration. It was kept at +4°C. Slides were fixed in 1% formaldehyde 10 min at RT. Excess liquid was drained off and it was washed in PBS, 2x5 min. A stock 30% H_2O_2 solution was diluted 1:100 to a final concentration of 0.3% in a coplin jar (0.5 ml + 50 ml PBS). Slides were put in the jar for 10 min, after which, H_2O_2 was dumped off and slides were washed in PBS, 2x5 min. Slides were quenched once more with 30% H_2O_2 stock diluted to 1% (1.5ml H_2O_2 + 45 ml PBS).

**ImageJ Analysis**

ImageJ program was used (which is freely available from NIH) to determine the percentage of pixels that were positive for staining of CD31 and DAPI separately in a defined region of interest (ROI) for each image. DAPI staining represented the number cells within a ROI and the ratio of CD31 percent staining/DAPI percent staining was calculated to estimate the total percent of cells stained with CD31 in each image. These ratios were then compared by one-way ANOVA (to determine whether there are any statistically significant differences between the means of two or more independent (unrelated) groups) in CD31 expression between each treatment group.

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References


Author

Shrila is grateful to have conducted a project that closely regards her passion in entering the medical field and majoring in biology. Shrila has always been interested in research and hopes to continue studying brain tumors along with learning about other types of cancer.