Efficacy of direct intratumoral therapy with targeted protein toxins for solid human gliomas in nude mice

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Targeted protein toxins are a new class of reagents with the potential for great tumor selectivity and cytotoxic potency. Two such compounds were studied: 1) Tf-CRM107, a conjugate of human transferrin (Tf) and diphtheria toxin with a point mutation (CRM107); and 2) 454A12-rRA, a conjugate of a monoclonal antibody (454A12) to the human Tf receptor and recombinant ricin A chain (rRA). Both compounds are potent and specific in killing human glioblastoma cell lines in vitro. The authors investigated the activity of these reagents administered intratumorally against solid U251 MG human gliomas in vivo.

Nude mice with established U251 MG flank tumors (0.5 to 1.0 cm in diameter) were randomly assigned to be treated with 100-µl intratumoral injections of Tf-CRM107 (10 µg) or 454A12-rRA (10 µg), equimolar doses of CRM107 (4.3 µg), 454A12 antibody (7.5 µg), or rRA (1.5 µg), or phosphate-buffered saline (PBS) every 2 days for a total of four doses. Tumor volume and animal weight were assessed by a blinded observer before each treatment and biweekly for 30 days after initiating therapy. With Tf-CRM107 administration, tumor regression of greater than 95% occurred by Day 14 (p < 0.01) and tumors did not recur by Day 30. Treatment with 454A12-rRA caused a 30% decrease in tumor volume by Day 14 (p < 0.01). Treatment with equimolar doses of the unconjugated targeted protein toxin components CRM107, 454A12, or rRA caused significant U251 MG tumor growth inhibition, but the effects were less potent than the antitumor effects of the conjugates. This study also characterized the dose-response effect of Tf-CRM107 on tumor growth and tumor weight on Day 30. Nude mice with established U251 MG flank tumors (0.5 to 1.0 cm in diameter) were treated with 100-µl intratumoral injections of 10, 1.0, or 0.1 µg of Tf-CRM107 or PBS every 2 days for a total of four doses. All three doses of Tf-CRM107 significantly inhibited tumor growth by Day 14 (p < 0.01) and at Day 30 (p < 0.05), with a significant dose-response relationship. This study demonstrated in vivo efficacy of the targeted toxins Tf-CRM107 and 454A12-rRA against a human glioma. With intratumoral administration, the effect of Tf-CRM107 was tumor-specific and in some animals curative. Regional therapy with these potent tumor-specific agents using direct intratumoral infusion should limit systemic toxicity and may be efficacious against brain tumors.

KEY WORDS - glioma - protein toxin - transferrin receptor - mouse

The poor prognosis for patients with malignant brain tumors is in part related to a lack of potent agents with adequate tumor specificity. The development of monoclonal antibodies provides the possibility of creating novel therapeutic agents with greater tumor selectivity than conventional chemotherapy. Monoclonal antibodies against tumor-associated antigens and other binding moieties that provide tumor selectivity have been conjugated with radionuclides and with various toxins.1-7 We have studied two targeted protein toxins: 1) Tf-CRM107, a conjugate of human transferrin (Tf) and diphtheria toxin with a point mutation (CRM107); and 2) 454A12-rRA, a conjugate of a monoclonal antibody (454A12) to the human Tf receptor and recombinant ricin A chain (rRA), the enzymatically active subunit of the ricin protein toxin. Both conjugates are targeted to the human Tf receptor.

Transferrin receptors transport iron into cells and are typically overexpressed on tumor cells, most likely reflecting the increased iron requirements of rapidly dividing cells.8,9,10,11,12,13,14 Previous work in our laboratory demonstrated high expression of Tf receptors on glioblastoma and medulloblastoma cell lines.15 Immunohistochemical studies of tumors in the human central nervous system (CNS) also reveal high levels of Tf.
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In contrast to this, Tf receptors in normal brain are sparse and are largely restricted to the luminal surface of brain capillaries.28,32,37,41,44 Diphtheria toxin and ricin are protein toxins that consist of two subunits. The A subunit catalyzes the inactivation of protein synthesis, which ultimately results in cell death, and the B subunit is responsible for toxin binding to the cell surface and for translocation of the A chain into the cytosol, where it causes cell death. The CRM107 mutant diphtheria toxin contains one amino acid change in the B chain that reduces toxin binding 8000-fold, but leaves the translocation and enzymatic functions intact.20 In the Tf-CRM107 conjugate, CRM107 and human Tf are joined by a stable, nonreducible thioether bond.28 The 454A12-rRA conjugate joins a monoclonal antibody to the human Tf receptor by a disulfide bond to rRA, the enzymatically active A subunit of ricin.12 Both Tf-CRM107 and 454A12-rRA exhibit potent and specific killing of human glioblastoma, medulloblastoma, and breast carcinoma-derived cell lines in vitro.28 Free ricin A chain and CRM107 are 1000- to 10,000-fold less toxic than the targeted toxin conjugates. In addition, further study of CRM107 conjugates revealed that cells lacking the appropriate target receptor are 200,000 times less sensitive to CRM107 conjugates than are cells with the target receptor. Both Tf-CRM107 and 454A12-rRA efficiently kill human tumor cells at concentrations between $3.9 \times 10^{-12}$ M and $3.8 \times 10^{-10}$ M, well below the levels of conjugates required in brain or in cerebrospinal fluid (CSF) to produce CNS or systemic toxicity (O. Ilereil, et al., unpublished data).28,45

In vitro cytotoxicity of targeted protein toxins does not always correlate with in vivo efficacy or clinical applicability. Many factors may limit the antitumor efficacy of targeted toxins in vivo, including inadequate localization to tumor, poor distribution in tumor, heterogeneity of tumor-associated antigens, nonspecific binding to normal tissues, rapid clearance, instability of the agent, and systemic toxicity.19,28,32,37,38,41 Particularly for brain tumors, the blood-brain barrier (BBB) limits access of systemically administered agents to tumor infiltrating normal brain.34,38,42,43 One strategy to circumvent the BBB and eliminate systemic toxicity is regional therapy for localized primary brain tumors with direct intratumoral and peritumoral drug delivery. We investigated the activity of Tf-CRM107 and 454A12-rRA administered intratumorally against solid human gliomas (U251 MG) in nude mice.

Materials and Methods

Tumor Model

Solid U251 MG human gliomas were initially established in nude mice by injecting $10^7$ cultured U251 MG glioblastoma cells subcutaneously into the flanks of National Institutes of Health nu/nu female mice aged 4 to 6 weeks. The flank tumors were harvested and tumor fragments were transplanted into additional nude mice for the experimental groups. Palpable tumors were detected after 3 to 4 weeks and reached 0.5 to 1.0 cm in diameter by 4 to 6 weeks. Subcutaneous U251 MG tumors showed high cellularity and pleomorphism on routine histological testing (Fig. 1). Tumor size was evaluated by measuring two perpendicular diameters with Vernier calipers and using the formula $\frac{\pi}{4} LW^2$, where L is the longest diameter and W is the diameter perpendicular to L.8,13 These measurements were made by a blinded observer on each treatment day and at biweekly intervals thereafter for 30 days. The animals were sacrificed on Day 30 in order to determine the tumor weight. Statistical comparisons of tumor volumes and weights were performed by analysis of variance at different time points after initiating treatment. Differences in animal weights before and after treatment were analyzed by the Student t-test.

Targeted Protein Toxins

Preparation of the diphtheria toxin mutant CRM107 and its conjugation to human Tf were performed as pre-

FIG. 1. Photomicrographs of subcutaneous human U251 MG glial tumors in nude mice showing high cellularity and pleomorphism. Left: H & E, × 9. Right: H & E, × 74.
Previously described, the toxin and Tf are linked via a thioether bond. The 454A12-rRA was obtained commercially.*

Treatment With Tf-CRM107 and 454A12-rRA

Groups of five to 10 nude mice with established U251 MG flank tumors (0.5 to 1.0 cm in diameter) were randomly assigned to be treated with 100-μl intratumoral injections of Tf-CRM107 (10 μg, five mice), equimolar doses of CRM107 (4.3 μg, five mice), or phosphate-buffered saline (PBS, 10 mice) every 2 days for a total of four doses (on Days 0, 2, 4, and 6). Tumor volume was assessed for 30 days and are expressed as means ± standard error of the mean (SEM).

Results

In Vivo Treatment With Intratumoral Tf-CRM107

Treatment of U251 MG flank tumors with 10 μg of Tf-CRM107 administered intratumorally caused tumor regression in five of five mice (Fig. 2). Tumor regression was evident by Day 4, and the mean response was a greater than 95% decrease in tumor volume for the group by Day 14. In contrast, the tumors in animals that received injections of PBS alone continued exponential volume growth. Tumor volume on Day 14 was significantly different between the 10-μg Tf-CRM107 treatment group and the control animals (p < 0.01). Tumors did not recur by Day 30 (p < 0.01), and necropsy on Day 30 revealed no evidence of tumor in three of five mice. Treatment with CRM107 (the nontargeted toxin) alone had less potent antitumor effects than Tf-CRM107 (the targeted toxin) (Fig. 2).

Treatment with 10-, 1-, or 0.1-μg doses of Tf-CRM107 significantly inhibited U251 MG tumor growth in a dose-dependent fashion (Fig. 3 left). The reduction in tumor volume reached significance on Day 14 (p < 0.01) and was maintained on Day 30 (p < 0.05) for all three dose levels. Tumor weights on Day 30 were significantly reduced in Tf-CRM107-treated animals, with all three groups having reduced tumor weights compared to control animals (p < 0.01 for the 10-μg and 1.0-μg doses, p < 0.05 for the 0.1-μg dose) (Fig. 3 right). Comparing the dose of Tf-CRM107 that yields tumor growth inhibition similar to that of CRM107 alone (Fig. 2) shows that Tf-CRM107 is between 10 and 100 times more potent than the unconjugated toxin in tumor cell kill in vivo.

Treatment With Intratumoral 454A12-rRA

Treatment with intratumoral 454A12-rRA on alternate days for a total of four doses also caused tumor regression, with a 30% reduction in mean tumor volume by Day 14 (p < 0.01, compared to control animals) (Fig. 4). Despite tumor regression, tumor growth rebounded in this group by 10 days after the last treatment; however, tumor volumes remained smaller than those in control animals at 30 days (p < 0.01). Treatment with an individual conjugate component 454A12 or rRA also inhibited U251 MG tumor growth, but was less effective than the conjugate 454A12-rRA (Fig. 4).

Weight Loss

When compared to pretreatment weights, neither Tf-CRM107- nor 454A12-rRA-treated mice displayed a weight loss by 30 days (p = 0.28 and p = 0.58, respectively, at the 10-μg dose level).

Discussion

Potent cytotoxicity of Tf-CRM107 and 454A12-rRA against human glioblastoma, medulloblastoma, and breast carcinoma-derived cell lines in vitro was previously demonstrated by our laboratory. Subsequent work has focused on developing targeted protein toxins for regional therapy of human CNS tumors. Therapeutic efficacy of a monoclonal antibody-rin conjugate was demonstrated in an animal model of leptomeningeal neoplasia when the conjugate was delivered directly into the CSF. We now report that regional intratumoral administration of Tf-CRM107 and 454A12-rRA causes growth inhibition and tumor regression of solid U251 MG human glioblastomas grown in nude mice.

Intratumoral Tf-CRM107 treatment every other day, for a total of four doses, produced dose-dependent inhibition of U251 MG tumor growth in the flanks of nude mice. The highest dose (10 μg) affected tumor regression as early as Day 4 after the initial dose, and the tumor regression was greater than 95% for that group by Day 14. At 30 days, necropsy revealed only a fibrotic scar and no tumor in three of the five mice. Treatment with CRM107 (the nontargeted toxin) alone

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* 454A12-rRA conjugate supplied by Cetus Corp., Emeryville, California.
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![Graphs showing the dose-dependent inhibitory response of U251 MG gliomas to transferrin (Tf)-CRM107 treatment.](image)

Fig. 3. Graphs showing the dose-dependent inhibitory response of U251 MG gliomas to transferrin (Tf)-CRM107 treatment. Nude mice with established U251 MG flank tumors (0.5 to 1.0 cm in diameter) were randomly assigned to be treated with 100-μl intratumoral injections of Tf-CRM107 (10 μg, five mice; 1.0 μg, five mice; 0.1 μg, 10 mice) or phosphate-buffered saline (PBS, 10 mice) every 2 days for a total of four doses (on Days 0, 2, 4, and 6). Tumor volumes during the 30 days following the initial dose of Tf-CRM107 (left) and tumor weights after 30 days (right) are expressed as means ± standard error of the mean (SEM).

![Graph showing U251 MG glioma volume regression after treatment with 454A12-recombinant ricin A chain (rRA).](image)

Fig. 4. Graph showing U251 MG glioma volume regression after treatment with 454A12-recombinant ricin A chain (rRA). Nude mice with established U251 MG flank tumors (0.5 to 1.0 cm in diameter) were randomly assigned to be treated with 100-μl intratumoral injections of 454A12-rRA (10 μg, 10 mice), equimolar doses of 454A12 (7.5 μg, five mice) or rRA (1.5 μg, five mice), or phosphate-buffered saline (PBS, 10 mice) every 2 days for a total of four doses (on Days 0, 2, 4, and 6). Tumor volumes were assessed for 30 days and are expressed as means ± standard error of the mean (SEM).

CRM107 conjugates *in vivo* may reflect a potentiating effect provided by the B chain entry function which is preserved in CRM107 but not in rRA.5 Equimolar doses of rRA alone inhibited growth but were less potent than the targeted conjugate, as predicted from *in vitro* data. Equimolar doses of the antibody 454A12 alone inhibited growth but to a lesser degree than the conjugate 454A12-rRA. Previous studies have demonstrated that some monoclonal antibodies directed against the Tf receptor can inhibit tumor growth *in vivo*, possibly by decreasing the binding of Tf-iron complexes.56

Previous *in vivo* studies have indicated that targeted protein toxins are least effective when there are pharmacological barriers limiting access of the toxin to tumor cells. Several studies have demonstrated that blood-borne malignancies can be susceptible to antibody-directed toxins (immunotoxins) in animal models.2,23,57 Subsequent clinical trials have demonstrated partial responses to immunotoxins in patients with B-cell lymphoma refractory to conventional therapy.39 However, animal models and clinical trials of immunotoxin therapy for other solid tumors have been less successful.19,40,41 Systemically administered protein toxin conjugates often penetrate solid tumors poorly because of their high molecular weight, the relatively poor blood supply to areas of tumor, and rapid clearance from the blood.

Successful treatment of solid tumors has been demonstrated by using regional therapy with targeted toxins. Immunotoxins have efficacy when delivered directly into relatively confined body compartments, including after intraperitoneal and intrathecal injections for tumors involving these compartments in animal models.22,52,58 In addition, regional intratumoral injection of ricin-antibody conjugates has been used to eradicate human tumor xenografts in nude mice, including a Tf receptor-positive tumor (the human T-cell leukemia CEM) treated with a ricin-anti-Tf receptor antibody conjugate.29,49
Malignant brain tumors may be amenable to regional therapy with targeted protein toxins. These tumors, including primary brain tumors and secondary metastases from systemic cancers, have a grim prognosis, with patient survival measured in months.\(^5\)\(^6\)\(^8\)\(^9\) The most common primary malignant brain tumor, glioblastoma multiforme, is associated with a median survival of less that 1 year despite the best conventional therapy, radical surgery, and high-dose radiation therapy.\(^5\)\(^6\) When this tumor recurs, there is close to 100% mortality within a few months.\(^1\)\(^6\) At least 80% of glioblastomas recur within 2 cm of the primary tumor site.\(^2\)\(^4\)\(^5\)\(^6\) This suggests that improved local control may improve survival in these patients. However, standard chemotherapeutic agents have not made a significant impact on the poor prognosis for glioblastoma patients. One reason is the lack of potent agents with adequate tumor specificity for malignant brain tumors. Regional intratumoral therapy with targeted protein toxins with high potency and tumor specificity may improve local control of tumor growth.

The advantages of direct intratumoral therapy for brain tumors include circumvention of the BBB, achievement of high local drug concentrations in the tumor, and reduced systemic toxicity. A variety of methods have been used to deliver intratumoral chemotherapy for brain tumors including direct injection, intracavitary instillation, intracavitary topical application, chronic microinjection, and controlled release from polymer implants.\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\)\(^13\)\(^14\)\(^15\)\(^16\)\(^17\)\(^18\)\(^19\)\(^20\)\(^21\)\(^22\) These approaches have had only limited success, in part because of slow diffusion of drug through tumor and brain intersitial fluid relative to tissue clearance, thus only a small volume of tissue surrounding the drug source is treated.\(^15\)\(^16\)\(^17\)\(^18\) We have developed an acute high-flow microinfusion technique in which a pressure gradient is maintained during interstitial infusion to establish bulk flow in the extracellular space. This greatly enhances the distribution of small and large molecules, including high-molecular-weight proteins (RH Bobo, DW Laske, A Akbasak, et al., in preparation). The intratumoral delivery of targeted protein toxins performed in this study in nude mice directly reflects the drug delivery strategy that we are currently employing in patients with brain tumors in our department.

In order for regional therapy of brain tumors with protein toxins targeted to the human T\(\text{f}\) receptor to be selective, there needs to be higher T\(\text{f}\) receptor expression on brain tumor cells compared with surrounding normal brain cells. Transferrin receptor distribution and density in normal human brain tissue obtained at autopsy and in brain tumor biopsy specimens have been studied by immunohistochemistry using monoclonal antibodies against the human T\(\text{f}\) receptor.\(^21\)\(^22\) The majority of brain tumors contained T\(\text{f}\) receptor-positive cells and, among gliomas, the most intense staining occurred in glioblastomas, particularly in areas of pseudopalisading where virtually all cells were stained. By contrast, T\(\text{f}\) receptor staining in normal brain was limited to capillary endothelial cells, with only rare staining of glial cells and no staining of neurons.

The toxicity of Tf-CRM107, which cross-reacts with the rat T\(\text{f}\) receptor, injected intracerebrally was studied in our laboratory by stereotactically infusing Tf-CRM107 solutions of increasing concentration into the white matter of the right frontal lobe of adult Fischer 344 rats (O. Ilercil, et al., unpublished data). Infusate concentrations below 3.5 x 10^{-9} M resulted in no histological changes and the rats remained neurologically normal. Infusate concentrations of 3.5 x 10^{-9} M or higher caused varying degrees of right frontal lobe encephalomalacia, but no changes at distant brain sites. Histological evidence of systemic organ damage was not seen with any of the Tf-CRM107 doses tested. The infused concentration of Tf-CRM107 required to cause histological changes indicative of damage in normal rat brain is 100- to 10,000-fold higher than levels demonstrating antitumor activity in vitro.\(^25\) These data indicate that Tf-CRM107 concentrations with antitumor activity may be safely reached in normal brain.

Our results support the concept that targeted protein toxins are effective reagents for regional therapy of neoplastic disease. The antitumor efficacy demonstrated for Tf-CRM107 and 45A12-rRA against several tumor cell lines in vitro and the in vivo efficacy against the human U251 MG glioblastoma demonstrated in this report suggest that these agents may be useful for regional therapy of malignant brain tumors.

References
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