Targeting Urokinase-Type Plasminogen Activator Receptor on Human Glioblastoma Tumors With Diphtheria Toxin Fusion Protein DTAT

Daniel A. Vallera, Chunbin Li, Ni Jin, Angela Panoskaltsis-Mortari, Walter A. Hall

Background: The prognosis for patients with brain cancer is poor, and new therapies are urgently needed. Recombinant toxic proteins that specifically target tumor cells appear to be promising. Urokinase-type plasminogen activator (uPA) receptor (uPAR) is expressed on the surface of glioblastoma and some other tumor cells and endothelial cells. We synthesized a recombinant fusion protein, DTAT, which contains the catalytic portion of diphtheria toxin (DT) for cell killing fused to the noninternalizing amino-terminal (AT) fragment of uPA, and investigated its effectiveness in targeting uPAR-positive tumor cells. Methods: In vitro cytotoxicity of DTAT was measured by cell proliferation assays. For in vivo studies, athymic nude mice (four to five animals/group) bearing uPAR-expressing human glioblastoma (U118MG) cell-induced tumors were injected with DTAT or control protein. Tumor volume was assessed over time, and differences between treatments were analyzed by Student’s t test. Effects of DTAT on body organ systems were evaluated in normal, tumor-free C57BL/6 mice histologically and functionally by serum enzyme tests. All statistical tests were two-sided. Results: In vitro, DTAT was highly potent and selective in killing uPAR-expressing glioblastoma cells (U118MG, U373MG, and U87MG) and human umbilical vein endothelial cells. In vivo, compared with mice treated with control proteins, DTAT caused a statistically significant (P = .05) regression of small U118MG cell-induced tumors in all mice. Control fusion proteins that did not react with glioblastoma cells had no effect on tumor growth. DTAT given to tumor-free C57BL/6 mice had little effect on kidney, liver, heart, lung, and spleen histologies. Serum analysis in the same mice showed no elevation in blood urea nitrogen, indicating lack of effect on kidney function but a statistically significant (P = .046), albeit non-life-threatening, elevation in liver alanine aminotransferase levels. Conclusion: DTAT may have potential for intracranial glioblastoma therapy because of its ability to target tumor cells and tumor vasculature simultaneously and its apparent lack of systemic effects. [J Natl Cancer Inst 2002;94:597–606]
specifically cytotoxic (9). Recently, we showed that diphtheria toxin (DT)-IL-13 fusion toxin was effective against gliomas that express high levels of IL-13 receptor (IL-13R) (10). Despite its well-documented potency and selectivity, use of IL-13-FP is limited because all glioblastomas do not express IL-13R. Data in this paper, including nude mice studies, indicate that certain GBM tumors are unaffected by therapy with IL-13-FP. Therefore, based on an extensive body of literature supporting the expression of urokinase-type plasminogen activator (uPA) receptor (uPAR) on glioblastoma [reviewed in (11)], we devised an alternative strategy for DT-IL-13-unresponsive tumors. The uPAR was targeted because, in addition to its expression on tumor cells (12–16), it is also expressed on endothelial cells [reviewed in (17)]. Endothelial cell expression of uPAR is an advantage because other investigators have successfully targeted tumor neovascularure with FP, thus demonstrating that destruction of the tumor microvasculature inhibits tumor growth (18).

To simplify our approach, we cloned a hybrid molecule by using the amino-terminal (AT) fragment (ATF) of uPA. Urokinase is sequestered at the cancer cell surface by its receptor uPAR, thereby activating circulating plasminogen protease [reviewed in (19)]. This enhances proteolysis of extracellular adhesion molecules such as collagen and fibronectin, thus promoting tumor invasion. We chose the ATF domain because it includes the molecular binding region and a fusion protein made with ATF and saporin-inhibited human uPAR-expressing cells (20). The ATF domain completely lacks the catalytic domain of uPA but possesses an endothelial growth factor (EGF)-like or growth factor domain that comprises the receptor binding sequence of human uPA (21). Upstream of the ATF, DT’s catalytic region was attached to render the molecule cytotoxic. A single DT molecule in the cytosol can kill a cell (22). The translocation-enhancing region of DT was included in the design to enhance the toxicity of the FP. To test the idea that an agent with the ability to bind to both tumor and tumor vasculature would have potent anti-glioblastoma effects, we synthesized and tested in vivo and in vitro the effectiveness of DTAT, which can target uPAR on the cell surface. We hypothesize that the DTAT molecule will be highly active against glioblastoma cells.

MATERIALS AND METHODS

Recombinant DTAT

Recombinant DTAT was synthesized by a technique previously described (23). The hybrid gene was constructed by the method of gene splicing (Fig. 1, A and B). An Nco1/XhoI gene fragment was cloned by polymerase chain reaction (with a splice overlap extension encoding the 390-amino-acid portion of DT [DT390], an EASGGPE linker [black box in Fig. 1, panel A], and the downstream 135-amino-acid AT fragment from uPA) and ligated into the pET21d expression vector forming plasmid pDTAT.pET21d. An Nco1/XhoI gene fragment was cloned by polymerase chain reaction and splice overlap extension encoding DT390, an EASGGPE linker (black box), and the downstream 135-amino-acid AT fragment (ATF) from uPA. The gene was cloned into the pET21d expression vector forming the plasmid pDTAT.pET21d. B) Cartoon depicts DT390, the 193-amino-acid N-terminal A chain, the 342-amino-acid B chain, a disulfide bond between the two chains, and the native binding region that has been genetically removed (145 amino acids). The region between the two black arrows depicts the native binding region. The four bands on the B chain depict the hydrophobic translocation enhancing region (TER). The RVRR is a protease-sensitive site that must be nicked in low pH endosomes to activate the toxic protein.

Fig. 1. A) Construct encoding the DTAT (diphtheria toxin [DT]-amino terminal [AT] fragment of urokinase-type plasminogen activator [uPA]) gene fragment used in these studies. An Nco1/XhoI gene fragment was cloned by polymerase chain reaction and splice overlap extension encoding DT390, an EASGGPE linker (black box), and the downstream 135-amino-acid AT fragment (ATF) from uPA. The gene was cloned into the pET21d expression vector forming the plasmid pDTAT.pET21d. B) Cartoon depicts DT390, the 193-amino-acid N-terminal A chain, the 342-amino-acid B chain, a disulfide bond between the two chains, and the native binding region that has been genetically removed (145 amino acids). The region between the two black arrows depicts the native binding region. The four bands on the B chain depict the hydrophobic translocation enhancing region (TER). The RVRR is a protease-sensitive site that must be nicked in low pH endosomes to activate the toxic protein.

Recent advances in cell biology have provided new insights into the mechanisms by which tumors evade immune surveillance. Glioblastoma, the most common primary brain tumor, is characterized by a complex network of cells, including tumor cells, immune cells, and stromal cells. The tumor microenvironment plays a critical role in the growth and survival of glioblastoma cells. The presence of tumor-associated macrophages (TAMs) and regulatory T cells (Tregs) has been linked to the resistance of glioblastomas to immunotherapy.

The development of DTAT as a targeted therapeutic agent for glioblastoma is based on the premise that the DTAT molecule will selectively target glioblastoma cells. DTAT recognizes and internalizes uPAR, a cell surface receptor that is expressed in glioblastomas but not in other tissues. The internalized DTAT is then released from the cell, allowing it to kill neighboring glioblastoma cells. This selectivity for glioblastoma cells makes DTAT a promising therapeutic agent for the treatment of this aggressive cancer.

The effectiveness of DTAT was evaluated in a xenograft mouse model of glioblastoma. Tumor bearing nude mice were injected with DTAT, and the tumor growth was monitored over time. The results demonstrated that DTAT was effective in slowing tumor growth and extending the survival of the mice.

In conclusion, DTAT is a promising therapeutic agent for the treatment of glioblastoma. Further studies are needed to determine the optimal dosing and scheduling of DTAT, as well as to evaluate its effectiveness in clinical trials.

chemical Facility) were used to verify that the hybrid gene had been cloned in frame. Plasmid was transformed into the Escherichia coli strain BL21(DE3) (Novagen). Expression was induced and protein was refolded and purified from inclusion bodies. The pellets were washed three times with Triton X-100 buffer and four times with wash buffer (50 mM Tris, 50 mM NaCl, and 5 mM EDTA) buffer by briefly homogenizing with a tissuemizer and incubating for 5–10 minutes. Incubation bodies were collected by centrifugation at 24000g for 50 minutes. Solubilization of the inclusion body pellet was achieved by sonication in denaturant buffer consisting of 7 M guanidine, 0.1 M Tris (pH 8.0), and 2 mM EDTA. To remove insoluble material, the solution was centrifuged at 40000g for 10 minutes, and the supernatant was collected. Renaturation was initiated by a rapid 100-fold dilution of the denatured protein into chilled refolding buffer consisting of 0.1 M Tris (pH 8.0), 0.5 M L-arginine, and 2 mM EDTA. The samples were incubated at 10°C for 48 hours. Ultrafiltration was performed against 20 mM Tris (pH 7.8) with a spiral membrane ultrafiltration cartridge on Amicon’s CH2 system (Amicon, Beverly, MA). Samples were loaded on a Q-Sepharose (Sigma-Aldrich, St. Louis, MO) ion exchange column and eluted with 1 M NaCl in 20 mM Tris (pH 7.8). The protein was diluted fivefold and subsequently applied to a Resource Q column (Pharmacia Biotech, Uppsal, Sweden) and eluted with a linear salt gradient from 0 to 1 M NaCl in 20 mM Tris (pH 7.8) and dialyzed against 1× phosphate-buffered saline (PBS). Ion exchange chromatography conditions were based on the estimated isoelectric point of 6.1 derived with the use of ISEOLECTRIC (Wisconsin Package version 10.0-UNIX; Genetics Computer Group, Madison, WI). The main peak from the Resource Q column was further purified by size-exclusion chromatography on a TSK 250 column ( Tosohaas, Montgom-
eryville, PA). Standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was performed to assess purity. Preparations of control DT390 containing FPs with either mouse or human interleukins (e.g., DT-m-IL-4, DT-h-IL-2, DT-h-IL-13, and DT-m-IL-3) have been previously reported (23–25).

Cell Lines and Antibodies

The cell lines U118MG, U87MG, U373MG, and T98G (with suffix G) were derived from human patients diagnosed with GBM and were obtained from American Type Culture Collection (Manassas, VA). Neuro-2a (a murine neuroblastoma cell line), Daudi (derived from human Burkitt’s lymphoma), and SKBR3 (a human mammary gland adenocarcinoma line) were also obtained from ATCC. They were all maintained in RPMI-1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS, BioWhittaker), 2 mM L-glutamine (Life Technologies, Rockville, MD), 0.1 mM nonessential amino acids (Life Technologies), 1.0 mM sodium pyruvate, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies). Human umbilical vein endothelial cells (HUVECs), used within seven passages, were obtained from Dr. S. Ramakrishnan (University of Minnesota) and were maintained in Medium 199 (Life Technologies) containing 15% nonessential amino acids (Life Technologies), 0.5 mM sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1 mL epidermal growth factor (BioWhittaker). All the cells were maintained at 37 °C in a humidified incubator with 5% CO₂/95% air and were passaged two to three times per week.

Neutralization experiments were performed by using polyclonal rabbit anti-human urokinase immunoglobulin G (IgG) as well as a murine IgG2a monoclonal antibody against human uPAR obtained from American Diagnostica (Greenwich, CT). Anti-IL-4 antibody (rat anti-mouse IgG1 from clone 11B11) (26) was used as a control for the blocking experiments (described in Fig. 4, B). To facilitate human tumor growth in nude mice, rabbit anti-asialoGM1 (Wako Chemicals USA, Richmond, VA) was administered.

Proliferation Assay

The in vitro cytotoxicity of DTAT and other FPs was measured by inhibition of DNA synthesis (27). Cells at 10²/well were plated in 100 μL of culture medium in a 96-well flat-bottomed tissue culture plate and incubated overnight at 37 °C in a humidified atmosphere of 5% CO₂/95% air. Various concentrations of DTAT diluted in culture medium were added in 100-μL volumes and incubated for 48 or 72 hours. One μCi of [methyl-³H]thymidine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) was added at the beginning of the last 8 hours of incubation. The cells were washed and harvested on glass fiber filters, and the incorporation of radioactivity was quantified. All cytotoxicity assays were performed in triplicate and expressed as a percentage of control response, i.e., incorporation of [³H]thymidine in cells incubated without toxin. The assays were repeated at least two to four times, showing that the results were highly reproducible. The concentration of DTAT at which 50% inhibition of DNA synthesis (IC₅₀) occurred was calculated. For blocking experiments, antibodies were pre-incubated with the toxins for 30 minutes at 37 °C, and the experiment was performed as described above.

In Vivo Mouse Tumor Studies

Six- to eight-week-old female athymic nu/nu nude mice (for tumor studies) and C57BL/6 mice (for toxicity studies) were purchased from the National Institutes of Health (Bethesda, MD) and maintained in microisolator cages under specific pathogen-free conditions as set forth by the Department of Research Animal Resources (University of Minnesota, Minneapolis). On day 2 and day 4, 25 μL of anti-asialoGM1 diluted in 175 μL PBS was injected intraperitoneally into each nude mouse. Anti-asialoGM1 is an immunosuppressive agent that reacts with mouse natural killer (NK) cells, mouse monocytes, and fetal thymocytes, thereby enhancing tumor growth. On day 0, U118MG cells (6 × 10⁶/0.1 mL culture medium) were injected subcutaneously into the right flank of each mouse. Each treatment group consisted of four or five animals. Mice were examined every 2–3 days. Pulpal tumors with tumor volume larger than 0.15 cm³ were treated by intratumoral injection. Twenty micrograms of DTAT diluted in 50 μL of PBS was injected every other day. Five such treatments were given to each mouse. DTAT was injected from three different directions by using a 0.5-cc insulin syringe with a 28-gauge needle over a period of approximately 1 minute. Control mice received an irrelevant FP DT-h-IL-2 (FP containing DT and human IL-2) or PBS injections. Tumor size was measured by caliper every 2–3 days. The approximate tumor volume was calculated as a product of length, width, and height.

Histology

A group of normal C57BL/6 mice without tumors were treated in the same manner as in the nude mouse experiment. At the end of the treatment, the mice were sacrificed and autopsied and their tissues were taken for histopathologic analysis as described (28). All samples were imbedded in OCT compound (Miles Laboratories, Elkhart, IN), snap frozen in liquid nitrogen, and stored at −80 °C until sectioned. Four serial sections (4 μm thick) were cut, thaw-mounted onto glass slides, and fixed for 5 minutes in acetone. Slides were stained with hematoxylin and eosin (H & E) for histopathologic assessment.

Blood Urea Nitrogen (BUN) and Alanine Aminotransferase (ALT) Assays

Both assays were performed as previously described (29) on Kodak EKTACHEM clinical chemistry slides (Eastman Kodak, Rochester, NY) on a Kodak EKTACHEM 950 by the Fairview University Medical Center–University Campus (Minneapolis, MN). C57BL/6 mice were randomly grouped (n = 5) and injected with DTAT, PBS (no FP), or a control DTanti-CD3sFv fusion toxin. DTanti-CD3sFv has been previously shown to cause organ toxicity. On the day immediately following the last injection, mice were sacrificed and bled. Individual serum samples collected by bleeding the heart were studied for BUN and ALT. Minimum specimen volume was 11 μL for each assay. The BUN assay was read spectrophotometrically at 670 nm. In the ALT assay, the oxidation of NADH was used to measure ALT activity at 340 nm.

Statistical Analysis and Reproducibility

Groupwise comparisons of continuous data were made by Student’s t test. In vitro experiments were repeated at least twice,
and in vivo experiments were repeated at least once. All statistical tests were two-sided.

RESULTS

Purity of DTAT

To assess the purity of fractions containing DTAT collected from our chromatography procedure, SDS–PAGE analysis was performed. SDS–PAGE analysis indicated that the purification schema resulted in a purity exceeding 95% for DTAT and the isoelectric point of DTAT was estimated to be 6.1. Control FPs, including DT-IL-13, were prepared and tested for purity in a similar manner.

DT-IL-13 and Killing of Glioblastoma Cell Lines

To determine whether DT-IL-13 would kill human glioblastoma cells, DT-IL-13 was tested in a tritiated thymidine incorporation assay. A different report from our group (10) has previously addressed the high selectivity and potency of DT-IL-13. Although DT-IL-13 in picomolar concentrations killed some glioblastoma cell lines (Fig. 2, A and B), others were not affected. Fig. 2, B, shows that DT-IL-13 inhibited the U373MG glioblastoma line (IC_{50}<0.01 nanomoles). In contrast, another glioblastoma cell line, U87MG, was inhibited only 45% when treated with a 1000-fold higher (10 nM) concentration of the agent. A third glioblastoma cell line, T98G, was not inhibited at all. These findings appear to be related to the IL-13R expression (30); expression of IL-13 receptors in the U87MG cell line is not known. In other words, higher receptor numbers are associated with greater killing levels.

Comparison of Cytotoxicities of DTAT and DT-IL-13

Because uPAR is overexpressed on certain cancer cells, it was important to determine the ability of DTAT to kill glioblastoma and to discover whether it could kill certain glioblastomas that were not eliminated by DT-IL-13. In our hands, U118MG cells were not inhibited well by DT-IL-13 treatment. Fig. 2, A, shows that despite treatment with 10 nM DT-IL-13, roughly 27% of the proliferative activity was still evident at 48 hours, compared with that of the control cultures. This is in contrast to the U373MG cell line in Fig. 2, B, in which even 0.1 nM kills more than 95% (<5% proliferative activity compared with control cultures was evident) in 72 hours. In comparison, Fig. 2, A, shows that at 72 hours, 10 nM DTAT inhibited statistically significantly (P = .005) more (94%) of the U118MG cells than did 10 nM DT-IL-13. These findings indicated that in vitro, DTAT inhibited the U118MG glioblastoma better than did DT-IL-13. These differences were obvious even at 48 hours (P < .001).

DTAT Selectivity In Vitro

To study the selectivity of DTAT, it was first tested against various cell lines such as the B cell line Daudi and breast cancer line SKBR3 that do not express uPAR. Fig. 3, A, shows that growth of U87MG was inhibited by DTAT (IC_{50}<1 nM). In contrast, the growth of Daudi and SKBR3 cells was not inhibited. The growth of the murine neuroblastoma cell line Neuro-2a was slightly inhibited at the highest concentration (10 nM). To further study selectivity in a different experiment, U118MG cells were treated with an irrelevant FP control (Fig. 3, B). DT-m-IL-4 was used as a negative control, because mouse IL-4 is species-restricted and does not bind to human cells. Whereas DTAT inhibited U118MG cells (Fig. 2, A), DT-m-IL-4 had no effect. Fig. 3, A and B, indicates that DTAT is highly selective in its ability to kill uPAR-expressing cells.

To determine whether exposure of DTAT to its target for longer periods would heighten its ability to kill, DTAT was incubated with its U118MG target for periods of 24, 48, and 72 hours. Dose–response studies revealed that the maximum level of cytotoxicity was obtained after 48 hours (data not shown). A longer exposure of 72 hours did not enhance the cytotoxicity.
We concluded that a near maximum level of killing was reached after 48 hours of exposure to DTAT (data not shown).

**Ability of DTAT to Kill Endothelial Cells**

As mentioned earlier, the ability of DTAT to bind to the tumor and its microvasculature could provide a therapeutic advantage because tumor growth is dependent on a thriving vasculature. To measure the binding ability of DTAT, DTAT was incubated with human HUVEC in vitro. Fig. 4, A, shows that DTAT was able to inhibit the proliferation of HUVEC in a dose-dependent manner with an IC$_{50}$ of approximately 2 nM. In contrast, a number of control FPs (binding those receptors not found on HUVECs, including mouse IL-4 receptor [m-IL-4R], human IL-2 receptor [h-IL-2R], human IL-13 receptor [h-IL-13R], or mouse IL-3 receptor [m-IL-3R]) were not inhibitory.

To determine whether the activity of DTAT on HUVECs was mediated by the ATF, polyclonal anti-urokinase antibody was pre-incubated with DTAT before adding to cultured HUVECs (Fig. 4, B). Anti-urokinase blocked the inhibition by 1 and 10
nM DTAT. A control anti-mouse IL-4 antibody (11B11) had no effect on DTAT activity. Together, these data show that DTAT selectively kills endothelial cells in vitro through its ability to bind uPAR.

**In Vivo Anti-Tumor Activity of DTAT Measured Against U118MG Cell-Induced Tumors in Nude Mice**

To determine the effectiveness of DTAT, we established a nude mouse model of human glioblastoma. U118MG tumor cells were inoculated into nude mice. When the tumor had established on day 30, mice were given a course of DTAT. Fig. 5, A, shows that in a group of five mice, tumors that had reached the size of 0.2 cm$^3$ all regressed following a 5-dose course of DTAT with 20 μg/day given every other day. In contrast, tumors in groups of mice treated with control DT-h-IL-2 and PBS did not regress over the study period. Tumors in these groups continued to increase in size. There was a statistically significant difference ($P = .05$) in tumor growth curves on day 48 when the DTAT group was compared with the control groups. Also, DT-IL-13 did not inhibit the growth of U118MG-induced tumors in vivo. Thus, in vitro findings were similar to the in vivo results. These results also support the idea that DTAT might be a useful treatment of tumors that are not effectively treated with DT-IL-13.

Fig. 5, B, shows the tumor growth curves of each of the individual mice in the DTAT group. The tumors steadily diminish in size following DTAT treatment. Only one of the tumors did not entirely regress by day 65. These in vivo findings were reproduced in another experiment, again with five mice in each experimental group.

**Effects of DTAT on Body Organs**

To study the toxic effects of the tumor treatment dose of DTAT, C57BL/6 mice without tumors were given the same 5-dose course of DTAT with 20 mg/day given every other day by subcutaneous treatment as the tumor-bearing nude mice and then studied the day following the last injection. Frozen tissue sections were stained with H & E and examined microscopically. Functional assays were performed on serum. Kidney tissue appeared unaffected by DTAT treatment with the exception of some minor neutrophil infiltration. Fig. 6, A, shows that glomeruli appeared healthy. These studies were particularly important because similar doses of FP in other studies have induced glomerular destruction, rupture of renal tubules, and proximal tubular vacuolization. Liver effects were studied in the same group of mice (Fig. 6, B). Examination of tissues revealed a few areas of infiltration of peripheral mononuclear cells and neutrophils. Otherwise, tissues appeared relatively unaffected by treatment. Fig. 6, B (inset), shows that DTAT had no effect on heart; it also had no effect on the spleen (not shown). Minor neutrophil infiltration was observed in the lung compared with normal controls (not shown).

Functional analysis of tissue revealed a statistically significant ($P = .046$), albeit non-life-threatening, elevation in ALT level in serum (Fig. 7, A). Although these elevations were not indicative of liver failure and histology indicated that the liver was intact, it appeared that DTAT did affect the liver at this dose. To further study the effects of DTAT on kidney function, serum was analyzed from these same mice. Fig. 7, B, shows no significant fluctuations in BUN levels following DTAT treatment, indicating that treatment did not interfere with renal activity. As a positive control, a group of mice were treated with DTanti-CD3sFv, a fusion toxin that has been shown to mediate renal damage in past studies (28). This positive control caused a statistically significant ($P = .033$) elevation in BUN activity. Histology studies confirmed glomerular damage and tubular necrosis induced by DTanti-CD3sFv.
DISCUSSION

An original contribution of these studies is the description of DTAT, a diphtheria-toxin-based FP directed against the uPAR overexpressed on glioblastoma cells. Previous studies by other investigators (11, 31) have encouraged the development of new drugs targeting the uPAR, but we provide evidence for the first time that uPAR may serve as a valid target for FP-directed therapy against chemotherapy and radiation refractory glioblastoma. An important element of these studies was the use of the nude mouse model to assess the effect of DTAT on glioblastoma independently of effects on the tumor neovasculature. DTAT, which is not species cross-reactive (32), inhibited the human glioblastoma U118MG but could not be used to assess the antiendothelial cell effects because tumor vasculature was derived from the mouse, not from humans. Still, it appears that DTAT, even without the help of an antivascular effect, is delivering potent therapy and killing glioblastoma tumors in this model. This is also the first time that attachment of the catalytic domain of DT and its translocation-enhancing region has been used to enhance the ability of ATF to internalize and serve as a potent and selective IT. Clearly, these findings raise some important questions regarding the deployment of this agent for clinical use.

Interestingly, intratumoral administration of DTAT caused the regression of tumors, even after dosing was discontinued. Ramakrishnan et al. (18) reported that an immunotoxin made by chemically conjugating vascular endothelial growth factor (VEGF) to truncated diphtheria toxin successfully inhibited the growth of subcutaneous tumors in nude mice. In these studies, tumor growth in the treated group was delayed as a consequence of the VEGF FP treatment. When treatment was stopped, the tumor immediately began to grow. This difference may be due to the intratumoral route of administration of DTAT as compared with systemic delivery in their study. Less than 0.001% of a systemically administered biologic agent (such as an FP) distributes to the tumor (5). Thus, direct intratumoral therapy of glioblastoma, with an agent that binds both tumor and vasculature, may be an advantage in treating glioblastoma that seldom leaves the cranium. However, there could be other explanations for the differences, including the fact that two different model systems were used or that the two different FPs may be internalized differently.

Enzyme studies revealed that the dose of DTAT (5 doses × 20 μg/dose) that caused tumor regression had some effect on the liver but not on other organs of normal mice. It is possible that further elevations in dosage might be prohibitive. However, our time that uPAR may serve as a valid target for FP-directed therapy against chemotherapy and radiation refractory glioblastoma. An important element of these studies was the use of the nude mouse model to assess the effect of DTAT on glioblastoma independently of effects on the tumor neovasculature. DTAT, which is not species cross-reactive (32), inhibited the human glioblastoma U118MG but could not be used to assess the antiendothelial cell effects because tumor vasculature was derived from the mouse, not from humans. Still, it appears that DTAT, even without the help of an antivascular effect, is delivering potent therapy and killing glioblastoma tumors in this model. This is also the first time that attachment of the catalytic domain of DT and its translocation-enhancing region has been used to enhance the ability of ATF to internalize and serve as a potent and selective IT. Clearly, these findings raise some important questions regarding the deployment of this agent for clinical use.

Interestingly, intratumoral administration of DTAT caused the regression of tumors, even after dosing was discontinued. Ramakrishnan et al. (18) reported that an immunotoxin made by chemically conjugating vascular endothelial growth factor (VEGF) to truncated diphtheria toxin successfully inhibited the growth of subcutaneous tumors in nude mice. In these studies, tumor growth in the treated group was delayed as a consequence of the VEGF FP treatment. When treatment was stopped, the tumor immediately began to grow. This difference may be due to the intratumoral route of administration of DTAT as compared with systemic delivery in their study. Less than 0.001% of a systemically administered biologic agent (such as an FP) distributes to the tumor (5). Thus, direct intratumoral therapy of glioblastoma, with an agent that binds both tumor and vasculature, may be an advantage in treating glioblastoma that seldom leaves the cranium. However, there could be other explanations for the differences, including the fact that two different model systems were used or that the two different FPs may be internalized differently.

Enzyme studies revealed that the dose of DTAT (5 doses × 20 μg/dose) that caused tumor regression had some effect on the liver but not on other organs of normal mice. It is possible that further elevations in dosage might be prohibitive. However, our
toxicity studies were performed in a different model by giving normal mice without tumors subcutaneous injections. Thus, higher doses of DTAT may be tolerated in tumor-bearing mice because the tumor combined with its extensive vascular network could provide an expansive “antigenic sink” that absorbs injected DTAT and limits the amount that would leave the tumor and traffic to nontarget sites such as liver and kidney. In fact, we have recently found (Vallera DA, Buchsbaum DJ: unpublished data) that radiolabeled FP was remarkably localized in the tumor over time when FP was administered intratumorally. Twenty-four hours after administration, 12% of the injected dose/gram (ID/g) was found in glioblastoma, whereas less than 1% was found in kidney and none was found in the blood. In mice without tumor, the absence of tumor and this antigenic sink may put more toxic stress on the liver, which would explain our ALT elevations. Interestingly, we already have preliminary evidence that this may be the case, because mice with large (>6 cm³) subcutaneous tumors tolerated a 100 μg/dose course of DTAT, and the tumor underwent a 50% reduction in size. All together, this also indicates a reasonable therapeutic window that makes DTAT attractive for therapeutic use.

The difference in renal toxicity and liver toxicity of DTanti-CD3sFv and DTAT is difficult to explain. We do know that dimerizing DTanti-CD3sFv prevents the renal toxicity by preventing its filtration into the kidney (27). Size cannot be the only issue because DTAT is smaller than monomeric DTanti-CD3sFv but is not as toxic to the kidney. Since DTAT is not reactive with murine uPAR, DTAT toxicity is not likely to be attributed to specific activity. Since DTanti-CD3sFv is reactive with murine T cells, it is possible that when it is filtered into the kidney, DTanti-CD3sFv targets itinerant T cells. Perhaps, their destruction in the kidney promotes damage. It is also possible that because the DT moiety is identical in the two agents, toxicity differences are somehow attributable to physical differences in the ligand moiety of the molecules. Other DT-containing FPs directed to different cell surface determinants, such as CD25 and the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor, have exhibited renal and liver toxicity in rodents and in humans (33,34).

Future studies will emphasize toxicity in normal brain. Because DTAT cross-reacts with certain primate species (35), it could be tested in normal monkey brain. However, tumor cannot be grown in monkeys, and the presence of tumor may affect the outcome of DTAT administration. A better model might be a syngeneic mouse model in which we could intracranially implant mouse glioblastoma. Because mouse and human DTAT are not cross-reactive, we would assemble and purify murine DTAT. Unlike our current nude mouse model, murine DTAT could be injected into the brain of mice with syngeneic tumor and would bind to both tumor and its neovasculature.

Why would we expect that DTAT would kill endothelial cells in tumor vasculature and spare normal endothelial cells? First, DTAT may be selective for the rapidly expanding tumor endothelium as compared with normal endothelium. Thus, uPAR expression may be differentially high in the proliferating endothelium of the tumor neovasculature, although uPAR has not been convincingly demonstrated to be a proliferation-associated marker. Second, alteration in the endocytic/processing pathway of internalized cytotoxic conjugate has been observed and suggested as the mechanism to account for the differential sensitivity of endothelial cells to VEGF-toxin conjugates (36). The same could be true for the intracellular processing of DTAT, which has not yet been studied. Third, immunohistochemical studies revealed that uPAR is mostly expressed in tissues undergoing extensive remodeling, e.g., in trophoblast cells in the placenta, in keratinocytes at the edge of incisional wounds, and in cells of primary tumor and metastasis (37). Perhaps the more quiescent normal endothelium is not as readily affected by treatment.

The literature supports IL-13R and IL-4R, which share receptor components (8), as useful targets for immunotoxin therapy in brain tumors (9,30,38,39). However, DTAT was developed as an alternative for tumors that fail to respond to DT-IL-13 treatment. On the basis of the literature (30), IL-13R is also highly expressed on some brain tumors but minimally expressed on others; thus, IL-13-FP may show promise in certain instances. However, GBM is heterogeneous and some glioblastomas may not respond to DT-IL-13 either because they don’t express IL-13R or because it is not expressed in high enough density. DTAT represents a useful alternative therapy for these tumors. With two different FPs, it may also be possible to use combination therapy because some tumors may simultaneously express both receptors. In this instance, combinations of FPs may bind higher numbers of receptors on the cell surface. Presumably more toxin will be internalized. We have previously reported that kinetics of cell kill was far greater with FP combinations than kinetics observed with individual FP (40). More recently, the Vitetta group (41) observed that anti-CD19 and anti-CD22 IT are more effective against patient-derived B-acute lymphoblastic leukemia cells and found the combination to be more effective than the individual FP. Combination studies are under way.

Why did we target uPAR? Several tumor cell lines overexpress uPAR, including breast cancer (12,13), melanoma (14), colon cancer (15), and prostate carcinoma cells (16), and the literature describing its expression on glioblastoma is extensive (11). Others have targeted uPAR with FP (20,42). To simplify our approach, we cloned the ATF of uPA. This molecule binds to uPAR-expressing cells (19). However, the ATF domain completely lacks the catalytic domain of uPA but possesses an EGF-like domain that comprises the receptor binding sequence of human uPA. Therefore, ATF can target cell surface molecules of uPAR.

Glioblastoma therapy affords the advantage of local delivery, and clinical responses for FP therapy for this disease have exceeded responses for FP in any other disease. Response rates in excess of 50% have been reported (7). In the first GBM FP phase I clinical study, Youle’s group at the National Institutes of Health (NIH) (43) evaluated the toxicity of an FP consisting of transferrin as a ligand linked to a mutated form of diphtheria toxin called CRM107. A trial was conducted with regional therapy of Tf-CRM107 in 18 patients. At least a 50% decrease in tumor volume occurred in nine of 15 assessable patients. Reduction occurred no earlier than a month after completion of the first Tf-CRM107 infusion, and the response did not peak in four patients until 6–14 months after the first treatment. The median survival after treatment in the group of nine responders who had malignant glioblastomas was 74 weeks (three were still alive at 102–142 weeks after the first treatment compared with 36 weeks for the nonresponders). These findings are impressive and dramatic for a disease that is extremely difficult to treat. Similar results have been reported for leptomeningeal carcino-
matosis (44). Rand et al. (38) have investigated the safety and activity of directly infusing an IL-4-FP made by linking circularly permuted IL-4 and truncated Pseudomonas toxin, also with promising results.

In summary, DTAT is highly selective and causes the regression of human glioblastoma in a mouse model without undue toxicity. Because it is different from other brain tumor targeting agents in its ability to directly target glioblastoma while simultaneously targeting the vasculature, it should be pursued as a useful alternative for treatment of chemotherapy-resistant and radiation-resistant glioblastomas. Studies in the nude mouse have been informative but are somewhat limited, because human DTAT is not species cross-reactive. Thus, the effect of DTAT on tumor can be assessed but not its reactivity on tumor microvasculature derived from the mouse. The construction of a DTAT homologue that is reactive with mouse endothelial cells is currently under way.

REFERENCES

(8) Ohara NI, Leland P, Murata T, Debinski W, Puri RK. The IL-13 receptor structure differs on various cell types and may share more than one component with IL-4 receptor. J Immunol 1997;158:756–64.

Engelholm LH, Behrendt N. Differential binding of urokinase and peptide antagonists to the urokinase receptor: evidence from characterization of the receptor in four primate species. Biol Chem 2001;382:435–42.


Rajagopal V, Kreitman RJ. Recombinant toxins that bind to the urokinase receptor are cytotoxic without requiring binding to the alpha(2)-macroglobulin receptor. J Biol Chem 2000;275:7566–73.


NOTES

Supported in part by a grant from the Minnesota Medical Foundation to W. A. Hall.

We thank Dr. Robert Kreitman, National Cancer Institute, and Dr. S. Ramakrishnan, University of Minnesota, for their helpful discussions. We thank Sekou Doumbia, University of Minnesota, for his valuable technical assistance.

Manuscript received April 30, 2001; revised February 14, 2002; accepted February 20, 2002.