

ORIGINAL ARTICLE

Phase I clinical trial of a TGF- β antisense-modified tumor cell vaccine in patients with advanced glioma

H Fakhrai¹, JC Mantil², L Liu³, GL Nicholson², CS Murphy-Satter², J Ruppert² and DL Shawler¹

¹Advanced Biotherapies, Inc., San Diego, CA, USA; ²Wallace-Kettering Neuroscience Institute and Nuclear Medicine/PET, Kettering Medical Center, Kettering, OH, USA and ³NovaRx Corporation, San Diego, CA, USA

We performed a phase I clinical trial in grade IV astrocytoma to assess the safety of a whole-cell vaccine comprising autologous tumor cells genetically modified by a transforming growth factor- β 2 (TGF- β 2) antisense vector. Blocking secretion of the immunosuppressive molecule TGF- β in this manner should inhibit one of the major mechanisms by which tumor cells evade immune surveillance and should lead to clinically effective antitumor immunity. Six patients with progressive WHO grade IV astrocytoma were enrolled in the trial. Patients received 2–7 subcutaneous injections of 5×10^6 – 2×10^7 autologous tumor cells per injection. TGF- β 2 secretion by the tumor cells used to vaccinate patients was inhibited by 53–98%. Treatment was well tolerated with only low-grade, transient treatment-related toxicities reported. Two patients had partial regressions and two had stable disease following therapy. The overall median survival was 68 weeks. Median survival of the responding patients was 78 weeks, compared to a historic value of 47 weeks for glioma patients treated conventionally. There were indications of humoral and cellular immunity induced by the vaccine. These findings support further clinical evaluation of vaccines comprised of TGF- β antisense-modified tumor cells.

Cancer Gene Therapy advance online publication, 7 July 2006; doi:10.1038/sj.cgt.7700975

Keywords: TGF- β ; cancer immunotherapy; glioblastoma; gliosarcoma

Introduction

Brain tumors are responsible for significant morbidity and mortality in both pediatric and adult populations. Approximately 20 000 new cases of brain tumors are diagnosed in the US each year and recent evidence suggests an increase in the incidence of these tumors.^{1,2} Gliomas are diffuse within the brain and have boundaries that are impossible to recognize with current imaging modalities.³ Patients with glioblastoma multiforme (GBM) and gliosarcoma (GS) have a poor prognosis even when promptly treated with standard therapy, which consists of surgery followed by radiation and chemotherapy. Approximately 60% of newly diagnosed glioblastoma patients die in the first year, 90% die by the second year.^{4,5} Five-year survival rates are reported to be as low as 2%.⁶ Median survival of GBM patients with currently approved therapies is only 47 weeks.⁷ Thus, exploration

of new approaches for treatment of this devastating disease is essential.

Tumors utilize different mechanisms to evade immune surveillance. Many tumors, including gliomas, produce a variety of immunosuppressive molecules including transforming growth factor- β 1 (TGF- β 1) and TGF- β 2 (collectively referred to as TGF- β),^{8–10} prostaglandin E₂,¹¹ interleukin-6 and -10,¹² and cyclooxygenase-2.¹³ Cancer patients frequently demonstrate impaired immune function.^{10,14–16} As an example, elevated levels of TGF- β in patients with glioblastoma are associated with immunosuppression.¹⁵ In these patients, surgical removal of the tumor reduces serum levels of TGF- β and partially reverses immune suppression. Abnormal levels of TGF- β , closely followed by immune suppression, reappear in patients before clinical signs of recurrence are detected by clinical or radiographic means.¹⁷

We have previously demonstrated that TGF- β production by tumor cells may be blocked by antisense gene modification.¹⁸ Using the aggressive rat GS tumor model 9L, we showed that GS cells modified in this manner were rendered more immunogenic than parental cells. We further showed that subcutaneous immunization of rats with these TGF- β antisense-modified tumor cells resulted in the eradication of previously implanted, intracranial,

Correspondence: D Shawler, NovaRx Corporation, 6828 Nancy Ridge Dr., Suite 100, San Diego, CA 92121, USA.

E-mail: dshawler@novarx.com

Received 30 September 2005; revised 21 April 2006; accepted 14 May 2006

parental 9L tumors. The results of this study suggested that glioma may be an ideal candidate for a therapy approach designed to bypass general immunosuppression associated with cancer.

Based on these data, we performed a phase I clinical trial in patients with grade IV astrocytoma (GBM and GS). Patients were treated with multiple intradermal injections of autologous TGF- β 2 antisense-modified tumor cells. The results of the study were very encouraging and demonstrated that this vaccine regimen is well tolerated and resulted in improved survival and enhanced cellular and humoral antitumor immunity in some patients.

Patients and methods

Clinical protocol

The primary objective of the study was to evaluate the safety of intradermal injections of irradiated TGF- β 2 antisense gene-modified autologous tumor cells in patients with WHO grade IV astrocytomas who had completed a course of surgical intervention and radiation therapy. Secondary objectives were to monitor tumor progression, humoral and cellular immunity in these patients, to monitor the nature of immune infiltrates in needle biopsies obtained from the injection sites 24 h post inoculation, and to use immunohistology procedures to evaluate the nature of immune infiltrates in pre- and post-treatment tumor biopsies.

Eligible patients had pathologically confirmed WHO grade IV astrocytoma and who had undergone surgical intervention and radiation therapy (4500 cGy to the tumor with 3 cm margins and a 1500 cGy boost to the tumor bed). Patients were 18 years or older, had a Karnofsky performance status of 60%, and were able to give informed consent. Patients had to have hemoglobin ≥ 9.9 gm/dl, an absolute granulocyte count $\geq 1000/\text{mm}^3$, a platelet count $\geq 60\,000/\text{mm}^3$, BUN ≤ 30 mg/dl, creatinine ≤ 2 mg/dl, alkaline phosphatase and SGOT $\leq 2 \times$ upper limit of normal, and a prothrombin time and activated thrombin time $\leq 1.4 \times$ control unless therapeutically warranted. Patients were excluded if they had an active Epstein-Barr virus infection, a positive HIV antibody titer, severe systemic disease, such as lung or heart disease, were pregnant, or had a prior history of other malignancies.

This was a dose escalation study and doses of 5×10^6 , 1×10^7 , and 2×10^7 cells per injection were employed. Patients received four initial subcutaneous injections of irradiated TGF- β 2 antisense gene-modified autologous tumor cells in monthly intervals. Patients 1–4 received an initial dose of 5×10^6 cells per injection. Patients 5 and 6 received an initial dose of 1×10^7 cells per injection. After four injections, responding patients were eligible for further injections at the next higher dose. Thus, patient 1 received a fifth injection at a dose of 1×10^7 cells per injection, patient 3 received a fifth and sixth injection at a dose of 1×10^7 cells per injection, and patient 5 received injections 5–7 at a dose of 2×10^7 cells per injection.

The following clinical evaluations were performed before each treatment, 1 month after the completion of

therapy, and at 3-month intervals thereafter: history and physical examination, complete blood count with differential, platelet count, prothrombin times (PT), partial thromboplastin times (PTT), glucose, biliary urea nitrogen, creatinine, electrolytes, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, bilirubin, uric acid, calcium, total protein, albumin, amylase and lipase, and urinalysis.

Patients were evaluated for tumor response by magnetic resonance imaging (MRI) and positron emitting tomography (PET) scans. Response was reported using standard outcome measures for clinical trials: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD).

CR involved resolution of all measurable disease, and no new lesions. PR was defined as a decrease in the total volume of all lesions by at least 50% and no new lesions. SD was defined as less than a 50% decrease in the total volume of all lesions and no new lesions. PD was defined as any increase in the volume of all measurable lesions or the presence of new lesions.

Five patients had histologically confirmed GBM and one patient had GS and had failed standard therapy for their disease. All had PD at the time of enrollment. Four men and two women were enrolled in the trial. The age of the patient population was 50 ± 13 years with a range from 37 to 63 years.

Signed informed consent was obtained from each patient for the clinical protocol approved by the Institutional Review Boards of the University of California, Los Angeles Medical Center and the Kettering Medical Center, the Recombinant DNA Advisory Committee, and reviewed by the Food and Drug administration.

Vaccine preparation and characterization

Establishment of tumor cell cultures from tumor biopsies. Tumor cell cultures were established from biopsies obtained from glioma patients undergoing clinically indicated procedures.^{19,20} The presence of glioma cells in each of the established cell lines was confirmed by immunofluorescence analyses with a panel of monoclonal antibodies. Each cell line was positive for glial fibrillary acidic protein (GFAP) and vimentin, and negative for neurofilament, galactocerebroside, and fibronectin, a pattern that is typical of glial cells.

Measurement of TGF- β 2 levels by enzyme linked immunosorbent assay (ELISA). Serum-free supernatants from cell cultures were collected after 24 h and assayed in triplicate for active TGF- β 2 using an ELISA kit (R&D Systems, Minneapolis, MN). Quantitation was achieved using a standard curve containing known concentrations of TGF- β 2. For TGF- β 2 antisense gene-modified cell, a test limit of less than 50% TGF- β 2 secretion, compared to the unmodified parental cells, was employed.

Vector

To generate the TGF- β 2 antisense plasmid vector, a DNA fragment containing base pairs 6–935 of the human

TGF- β 2 cDNA was ligated in reverse orientation in the *HindIII*–*XhoI* sites of the pCEP-4 vector. Expression of the antisense molecule in pCEP-4 (Invitrogen, San Diego, CA) is driven by the cytomegalovirus promoter of the vector. The pCEP-4 vector also contains the hygromycin-resistance gene driven by the herpes simplex virus thymidine kinase promoter, the Epstein–Barr virus origin of replication, and the gene for the Epstein–Barr virus nuclear-associated protein 1. This vector is identical to the vector we previously reported,¹⁸ except that a human cDNA fragment replaced the simian TGF- β 2 cDNA.

Genetic modification of tumor cells with pCEP-4/TGF- β 2 antisense vector. Glioma cell lines that expressed more than 200 pg TGF- β 2 per 10⁶ cells per 24 h were genetically modified with the pCEP-4/TGF- β 2 antisense vector by electroporation (Genetronics, San Diego, CA) immediately after the cultures were established. Cells at a concentration of 1 \times 10⁷ cells/ml were mixed with vector DNA at a concentration of 50 μ g/ml and were subjected to a square wave electroporation regimen consisting of three waves at 2000–3000 V with each wave lasting 35 μ s. Following electroporation, cell viability was 87 \pm 5% (range from 78 to 95%). Selection with hygromycin was initiated 2 days later and was continued until colonies were established.

The genetically modified tumor cells were carried in continuous culture until there were sufficient cells for use in therapeutic applications or for cryopreservation. For inoculation, the gene-modified tumor cells were irradiated with 7000 cGy, resuspended in lactated Ringer’s solution, and injected intradermally in the upper arm.

All TGF- β 2 antisense gene-modified autologous tumor cells were tested for bacterial, mycoplasma, and fungal contamination before injection. The proliferative potential of the cells was evaluated using a clonogenicity assay. The TGF- β 2 secretion by the pCEP-4/TGF- β 2-transduced cells was confirmed to be less than 50% of the unmodified parental cells using an ELISA kit (R&D Systems, Minneapolis, MN).

Assays of humoral and cellular immunity

Humoral immunity. Antibodies reactive against the immunizing cell lines were evaluated by using an ELISA to compare pretreatment sera with sera obtained 4 months after the start of therapy. Briefly, 2 \times 10⁴ autologous tumor cells were plated on flat-bottom 96-well plates (Costar, Inc., Cambridge, MA) and incubated overnight at 37°C in a humidified 10% CO₂ atmosphere. The next day, the cells were incubated at 4°C for 1 h with the test sera. The plates were washed and incubated with an enzyme-conjugated anti-human immunoglobulin (Ig) M or IgG (Sigma Aldrich, St Louis, MO) for 30 min at 23°C, followed by a chromogenic substrate (30–60 min) with washings in between. Bound antibodies were quantified by spectrophotometric OD measurements, using an ELISA plate reader (SpectraMax 340, Molecular Devices). The level of colored product is proportional to the amount of analyte and detection reagent that is specifically bound in ELISA. A stimulation index was

calculated by dividing the absorbance of the post-treatment sample by the absorbance of the pretreatment sample.

Immunohistology. Standard hematoxylin and eosin staining and immunohistochemical methods were employed to characterize the immune infiltrates observed in skin biopsies at immunization sites and in tumor biopsies. Immune infiltrates were characterized by immune staining with monoclonal antibodies to T cells (CD3, CD4, CD8, and CD45RO), monocyte/macrophages (CD68), dendritic cells (CD86), natural killer cells (CD56), and B cells (CD20) using routine immune staining procedures. Incubation of sections with isotype-matched control antibodies were used as negative controls.

Results

Blocking of TGF- β 2 secretion

In total, 21 tumor biopsies from patients with advanced GBM or GS, obtained at the time of surgical resection, were processed to generate autologous glioma cell lines. While cell lines were established for all 21, gene modification with the pCEP-4/TGF- β 2 antisense vector was only performed in eight cell lines. All eight were successfully transfected. Three of the remaining 16 cell lines grew too slowly to be used in the trial and the remaining patients went on to other therapies making them ineligible for the trial. Table 1 shows the effect of TGF- β 2 antisense gene modification on the secretion of TGF- β 2 by six autologous glioma cell lines at the time of their use in the trial. The mean TGF- β 2 secretion by the unmodified tumor cells was 6.7 \pm 1.6 ng TGF- β 2/10⁶ cells/24 h (range, 2.1–12.5 ng TGF- β 2/10⁶ cells/24 h). The mean secretion by TGF- β 2 antisense-modified tumor cells was 1.15 \pm 0.25 ng TGF- β 2/10⁶ cells/24 h (range, 0.63–2.3 ng TGF- β 2/10⁶ cells/24 h). Gene modification with TGF- β 2 antisense blocked TGF- β 2 secretion by 79 \pm 9% (range; 68–95%). TGF- β 2 blocking efficiency decreased in correlation with the number of cell passages. In patient 1, TGF- β 2 secretion was initially blocked by 93%. By the time of the fifth injection for this individual, TGF- β 2 secretion was blocked by 70%. Because of this observation, TGF- β 2 secretion was measured before each injection and only cells in which TGF- β 2 secretion was blocked by at least 50% were used for therapy.

Table 1 Antisense gene modification of autologous glioma cells

Patient	TGF- β 2 (pg/10 ⁶ cells/24 h)		Inhibition (%)
	Unmodified	Antisense gene modified	
1	4200	1100	74
2	12500	630	95
3	7400	1256	83
4	2140	685	68
5	3970	937	76
6	10110	2313	77

TGF- β 2, transforming growth factor- β 2.

Clinical course

All six patients enrolled in the study had at least one relapse and had PD at the time of the first injection. A summary of patient demographics is shown in Table 2. Five patients presented with GBM and one patient presented with GS. The age of patients ranged from 37 to 63 years with a median of 51 years. There were no correlations between response to therapy and patient age.

Adverse events. Adverse events are shown in Table 2. The most common treatment-related adverse events were delayed type hypersensitivity- (DTH-) like reactions observed at the sites of the second and subsequent vaccinations in all patients. These reactions occurred 48–72 h following administration with areas of induration that ranged from approximately 1–5 cm². The DTH-like reactions generally resolved over a 1-week period. The timing of the occurrence after the second and subsequent vaccinations, but never after the first vaccination, suggests induction of immunological memory responses to the vaccine.

In addition to the DTH-like reactions, some patients experienced transient, flu-like symptoms during the course of treatment. These symptoms consisted of musculoskeletal aches and pains and fatigue. The symptoms were less than grade 2, did not result in

significant alterations in activities of daily living, and resolved within 2 weeks

There were no significant treatment-related changes observed in the blood counts and serum chemistries nor were there any treatment-related deaths in this study. All patients experienced adverse events related to tumor progression. These included edema, incontinence, pneumonia, weakness, dizziness, and mental confusion.

Clinical responses. Clinical results are shown in Table 3. Median survival in the trial was 66 weeks. All patients had PD at the time of enrollment. Four of the six patients demonstrated either a PR or SD during therapy. The median survival of these four patients was 78 weeks. The remaining two patients had tumor progression during therapy and did not finish the course of four injections.

Patient 1 received four injections (5×10^6 cells per injection) followed by one injection (1×10^7 cells per injection). The patient had undergone two tumor resections 7 and 18 weeks before entering therapy. At 3 weeks following the last injection, the patient presented with an episode of vertigo. An MRI taken at that time showed increased contrast enhancement compared to a pretreatment MRI (Figure 1a). Because this suggested tumor progression, surgery was advised. During surgery, severe tumor inflammation was observed in the absence of tumor progression and the patient was not resected.

Table 2 Patient demographics and adverse events

Patient	Sex	Diagnosis	Age	No. of vaccinations/dose	Drug related adverse events	Other adverse events
1	M	GBM	37	4/5 $\times 10^6$ 1/1 $\times 10^7$	Injection site induration, nausea	Dizziness
2	F	GBM	55	3/5 $\times 10^6$	Injection site induration	Progressive disease during treatment
3	M	GBM	47	4/5 $\times 10^6$ 2/1 $\times 10^7$	Injection site induration	Seizures, right-side weakness
4	M	GBM	63	2/5 $\times 10^6$	Injection site induration	Seizures, dizziness, confusion, lethargy, progressive disease during treatment
5	M	Gliosarcoma	59	4/1 $\times 10^7$ 3/2 $\times 10^7$	Injection site induration	Edema, lethargy, incontinence, confusion, pneumonia
6	F	GBM	41	4/1 $\times 10^7$	Injection site induration	Left-side weakness

F, female; GBM, glioblastoma multiforme; M, male.

Table 3 Clinical summary

Patient	Best clinical response	Survival (weeks)	Cause of death	Cellular infiltrates	Humoral immunity
1	Stable disease	59	Disease progression	Positive	Not done
2	Progression	50	Disease progression	Positive	Not done
3	Partial response	72	Brain herniation	Positive	Not done
4	Progression	57	Disease progression	Not done	Negative
5	Partial response	85	Pneumonia	Positive	Positive
6	Stable disease	83	Disease progression	Not done	Positive

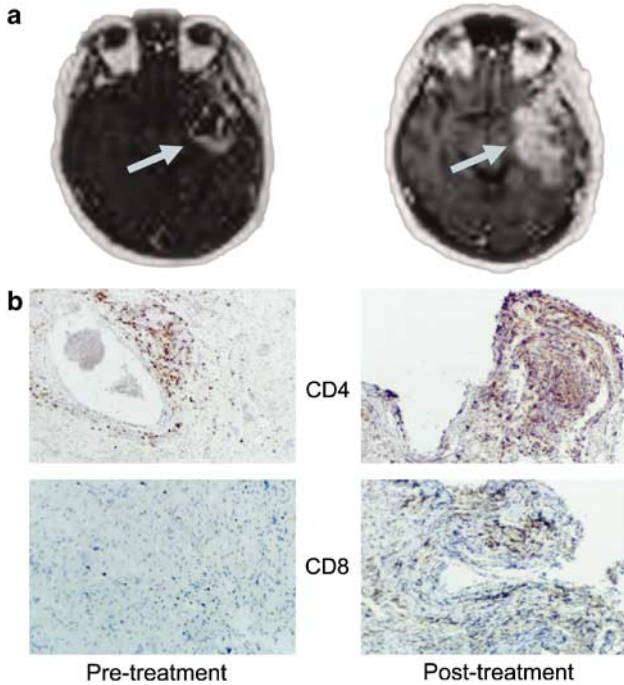


Figure 1 Pre- and post-treatment MRIs and immunohistochemistry in Patient 1. (a) Brain MRIs taken before (left) and after therapy (right) show areas of increased contrast enhancement (arrows). The increased contrast enhancement in the post-therapy MRI is larger than the same area in the pre-therapy MRI. (b) CD4 and CD8 immunohistochemistry shows that the increased contrast enhancement may have been caused by immune infiltration.

Histological evaluation of the biopsy obtained during surgery demonstrated a significant number of CD4+ and CD8+ immune infiltrates into the tumor compared to a biopsy obtained before vaccination (Figure 1b).

Patient 3 received four injections of 5×10^6 cells per injection followed by two injections of 1×10^7 cells per injection. As seen in Figure 2, the tumor volume increased following each set of injections and then decreased by the time of the next scan (4–8 weeks after the preceding injection). These data are consistent with the induction of an immune response against the tumor cells that gradually diminished with time.

The patient withdrew from the protocol after six injections and, after refusing all other therapies, was placed on decadron to control inflammation. Figure 3 shows scans obtained 3 weeks after his last injection and 3 months after his last injection. After 3 months of receiving only decadron, the patient's tumor showed a significant decrease in tumor volume. The patient died owing to complications of his disease 20 weeks after withdrawal from the protocol.

Patient 5 presented with an aggressive GS that was progressing after surgery, radiation therapy, chemotherapy, and γ -knife surgery. On entry into the trial, he had a poor quality of life. He received four injections (1×10^7 cells per injection) followed by three injections (2×10^7 cells per injection). Approximately 3 weeks following the

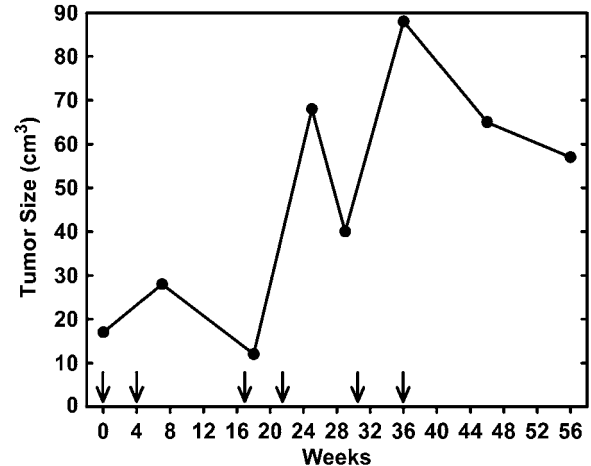


Figure 2 Changes in the tumor volume of patient 3 during vaccination with TGF- β 2 antisense-modified autologous tumor cells. Patient 3 received six injections of vaccine therapy with TGF- β 2 antisense-modified autologous tumor cells, shown by the arrows above the x-axis. The tumor volume measured by increased contrast enhancement in MRI scans increased following each injection. When there was more than an 8-week interval between injections, the tumor volume always decreased.

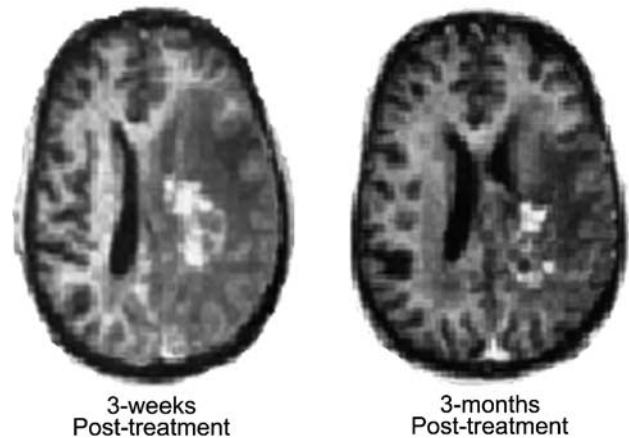


Figure 3 Post-treatment MRI scans of patient 3 following vaccination with TGF- β 2 antisense-modified autologous tumor cells. MRI scans obtained 3 weeks following treatment showed increased contrast enhancement at the tumor site (left panel). The patient declined further therapy and received only decadron to control inflammation. At 3 months following treatment, the increased contrast enhancement had resolved (right panel), suggesting either a delayed response to therapy or the resolution of inflammation following therapy.

last injection, he had an episode of vertigo and an MRI taken at the time showed increased contrast enhancement in the tumor. The tumor was partially debulked and analyzed histologically for CD3, CD4, CD8, CD45RO, and CD68 expression. An MRI taken approximately 1 month after the surgery showed improvement, suggesting that the increased contrast enhancement was not due to tumor progression. The patient's quality of life improved

during therapy and at one point his cognition score was 30 out of 30 points. The patient survived 85 weeks following disease presentation. During the course of his illness, the patient had numerous bouts of pneumonia and eventually died owing to complications of pneumonia.

Patients 2 and 4 had tumor progression after two injections each, they were treated by surgery and/or chemotherapy and were placed off study. The patient had tumor progression 1 month after the fourth injection, received other therapies and was placed on off-study status.

Immunologic responses

Cellular immune infiltrates. Tumor and injection-site biopsies from four patients were examined by immunohistochemistry for the presence of immune effector cells. Each of these patients showed an increase in the infiltration of immune effector cells following vaccination with TGF- β 2 antisense gene-modified tumor cells (Table 1). There was no correlation between the observed effector infiltrates and the clinical response of the patients. Both responding and non-responding patients had evidence of increased immune infiltration.

As described above, patient 1 showed significant increases in CD4+ and CD8+ immune infiltrates into the tumor following vaccination (Figure 1b). A tumor biopsy obtained 3 weeks following the last injection in patient 5 showed significant CD68+ (Figure 4b) and CD45RO+ (Figure 4c) effector cell infiltration. In contrast, these cells were rarely observed in the pretreatment tumor. The CD45RO+ cells were confined mainly to the region of viable tumor and few CD45RO+ cells were observed in the necrotic areas of the tumor bed.

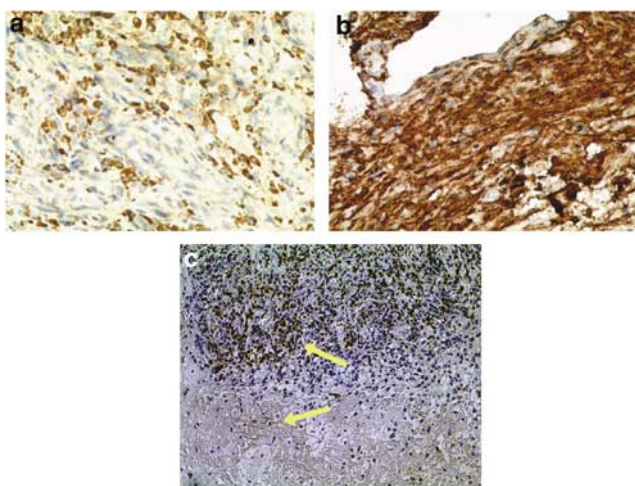


Figure 4 CD68 and CD45RO infiltration in the tumor bed of patient 5. CD68+ infiltration was increased in the post-therapy tumor (b) compared to the pretreatment tumor (a). CD45RO+ infiltrates were also observed in the post-therapy tumor biopsy (c). CD45RO+ cells were confined mainly to the region of viable tumor (upper arrow) and few CD45RO+ cells were observed in the necrotic areas of the tumor bed (lower arrow).

Humoral immunity. Pre- and post-treatment sera from three patients were examined by ELISA for immunoglobulin reactivity against autologous tumor cells (Figure 5). The post-treatment time points were 2 months post-treatment for patients 4 and 6 and 5 months post-treatment for patient 5. Patients 5 and 6 demonstrated increased IgG reactivity with autologous tumor cells following treatment. Patient 4 failed to demonstrate such an increase. None of the three patients showed an increase in IgM reactivity with autologous tumor cells following treatment. One patient with an IgG response had a PR to treatment, whereas the other patient had SD following treatment. The patient who failed to demonstrate an IgG response continued to progress while on protocol. Although the correlation between an IgG response and clinical response is interesting, the numbers of patients are too small to infer significance.

Discussion

Malignant gliomas are associated with systemic suppression of host immune competence that is caused, in part, by tumor cell secretion of the potent T-cell suppression factor TGF- β .^{10,15} We have previously demonstrated that injection of animals with syngeneic tumor cells in which TGF- β secretion is blocked by gene modification has shown exceptional efficacy in eliminating previously implanted tumors.¹⁸ In this phase I clinical trial for stage IV astrocytoma, we demonstrated that TGF- β 2 secretion may be blocked by antisense gene modification of tumor cells obtained from surgical biopsies, and that sufficient numbers of the gene-modified cells may be grown *in vitro*

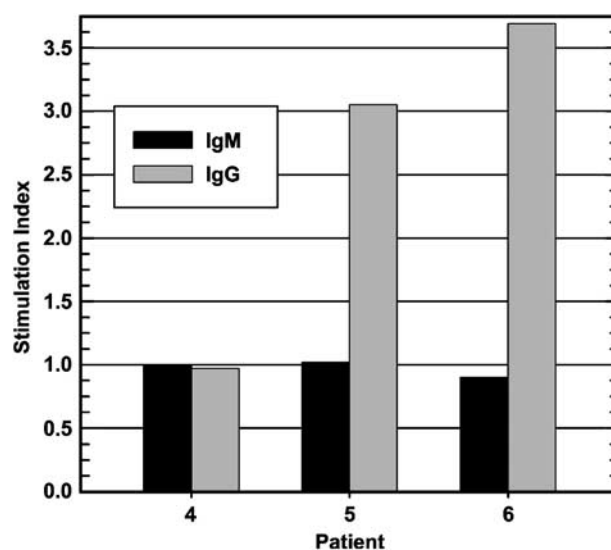


Figure 5 Humoral immunity in glioma patients vaccinated with TGF- β 2 antisense-modified autologous tumor cells. A 3–4-fold increase in the amount of serum IgG antibodies reactive with autologous tumor cells was observed in patients 5 and 6 but not in patient 4. Similar increases were not seen with serum IgM antibodies.

to administer to patients as part of a tumor cell vaccine regimen. We also demonstrated that this vaccine may be safely administered to glioma patients, and that treatment-related toxicities were less than grade 2 and resolved within 2 weeks. Survival in all patients exceeded the median survival of approved therapies. Evidence of antitumor immunity was also observed in these patients. Finally, we demonstrated that tumor inflammation caused by immune infiltration following administration of the vaccine may mimic tumor progression when observed on MRI scans.

Six patients with recurrent, progressive glioma were enrolled in the study. Of these, two patients demonstrated PRs, two demonstrated SD, and two continued to progress during therapy. The median survival of all patients was 65 weeks and the median survival of four responding patients was 78 weeks, which compares favorably to the median historic survival of 47 weeks for this disease. The age of the patient population was 50 ± 13 years with a range from 37 to 63 years. Age is associated with survival in glioma^{5,21}; patients 41–59 years have a 1-year survival of 45%, whereas patients greater than 60 years have a 1-year survival of 22%. Our study had four patients in the 41–59 year range, with a median survival of 78 weeks and one patient over the age of 60 years who survived 58 weeks.

Previously, we had demonstrated the safety of administering interleukin (IL)-2-transduced autologous tumor cells as a vaccine in a single patient with GBM.¹⁹ The vaccine induced a CD8⁺ cellular immune response in the patient. An MRI revealed marked tumor necrosis 4 weeks following the highest treatment dose. These findings were similar to the data reported in this study.

An important finding in this study was the observation that some patients may respond to immunogene therapy in a manner that mimics tumor progression. In patient 1, what appeared to be tumor progression in the MRI scan was demonstrated at surgery to be increased contrast enhancement caused by inflammation. Immune histology performed on this tumor showed that the inflammation had significant CD4⁺ and CD8⁺ components. Similar findings were observed in patients 3 and 5. These data suggest that caution must be exercised in interpreting the results of scans obtained during immunotherapy. It also suggests that patients should not necessarily be placed on other therapies as a result of increased contrast enhancement. In particular, immunosuppressive therapies such as chemotherapy should be avoided inasmuch as they may inhibit the antitumor activity of infiltrating immune effector cells.

The immune infiltrates observed in injection site and tumor biopsies, as well as the increase in IgG reactivity with autologous tumor cells, show that the TGF- β 2 antisense gene-modified tumor cell vaccine induces immunologic activity. Effector cell infiltration was observed in post-treatment tumor biopsies, while being virtually absent in pretreatment tumor biopsies. This finding is important for two reasons. First, it demonstrates that immune responses can be induced in glioma, a disease associated with severe systemic immunosuppres-

sion.^{15,17} Second, the finding of immune infiltrates in patients' tumors following therapy demonstrates that activated immune effector cells can pass through the blood–tumor barrier to target cells in the tumor. Although this has been observed by others,²² this is the first observation of the ability of a tumor cell vaccine to induce effector cells to cross the blood–brain barrier in human trials.

Initial studies of vaccination with tumor cells, which were designed to augment tumor antigen presentation and induce specific antitumor immunity, yielded promising but limited results.²³ As a result, a number of investigators have tried to increase the immunogenicity of whole tumor cell vaccines by genetically modifying the tumor cells with various immune modulators.^{24–27} Unfortunately, these studies fail to consider the immune impairment associated with malignancies such as glioma. It is a long established principle that most tumors escape immune surveillance by secreting immunosuppressive molecules.²⁸ Primary among these is TGF- β , which is produced by cancers of different histologic origins.

TGF- β suppresses immunity, in part, by inhibiting T- and B-cell activation in response to antigen stimulation. Immune suppression mediated by TGF- β appears to be due to impairment of IL-2R function and suppression of high affinity IL-2R expression,²⁹ inhibition of natural killer cells (NK) and lymphokine-activated killer cells (LAK) induction and proliferation,^{30,31} and the inhibition of maturation and antigen presentation of dendritic cells.^{32,33} However, effector cells, once they become activated, are refractory to the immune inhibitory properties of TGF- β and are capable of targeting and destroying tumor cells that continue to secrete TGF- β .^{18,34} In this manner, by blocking the production of TGF- β in the microenvironment of the vaccine site, we can activate T cells and dendritic cells, which are capable of evoking an antitumor immunity against the *in situ* tumor.

Before and during this trial, 21 tumor biopsies were obtained, but of the 21 cell lines, only eight were gene-modified. The growth of some of these tumors was too slow and could not be utilized for therapy and many patients opted for other therapies during the long waiting period while the cells were being prepared. Eventually, only six of these patients were treated on the study. This is a significant obstacle to the practical application of autologous tumor cell vaccines. In effect, significant time and resources can be expended to generate cell lines that eventually only treat a small fraction of patients.

The use of allogeneic tumor cell lines as components of whole tumor cells vaccines provide a practical alternative to the use of autologous tumor cells. In a phase I study, patients who were injected with a colon cancer vaccine comprised of CD-80 gene-modified allogeneic tumor cells admixed with allogeneic fibroblasts modified to secrete IL-2 mounted cytotoxic T-cell responses capable of targeting autologous tumor cells.³⁵ Similar results have been seen with other investigators utilizing allogeneic tumor cell vaccines.^{36–39}

In summary, our findings suggest that immunogene therapy involving vaccination of glioma patients with

autologous tumor cells genetically modified with a TGF- β 2 antisense vector was well tolerated and may be associated with increased survival and the generation of cellular and humoral antitumor immune responses. The trial also demonstrated that some patients responding to immunotherapy may show an increase in tumor volume caused by inflammation that may be mistaken for tumor progression by current imaging methods. Equally important, the trial also showed the impracticality of using autologous tumor cells as vaccine components, a fact previously demonstrated by us²⁰ and others.^{40–42} These findings support further clinical evaluation of vaccines comprised of TGF- β antisense-modified tumor cells in glioma and other tumors associated with systemic immunosuppression related to TGF- β 2 secretion, but they also suggest that the use of allogeneic tumor cells may provide a practical alternative to autologous tumor cells.

Acknowledgements

We thank Helen Lin, MD and Ali Haghghi for their helpful contributions to this project. This work was partially supported by grants no. CA96025 and CA105964 from the National Institutes of Health, Bethesda, MD to NovaRx Corporation, San Diego, CA.

References

- Legler JM, Ries LA, Smith MA, Warren JL, Heineman EF, Kaplan RS *et al*. Cancer surveillance series (corrected): brain and other central nervous system cancers: recent trends in incidence and mortality. *J Natl Cancer Inst* 1999; **91**: 1382–1390.
- Jukich PJ, McCarthy BJ, Surawicz TS, Freels S, Davis FG. Trends in incidence of primary brain tumors in the United States, 1985–1994. *Neuro-oncology* 2001; **3**: 141–151.
- Black KL, Emerick T, Hoh C, Hawkins RA, Mazziotta J, Becker DP. Thallium-201 SPECT and positron emission tomography equal predictors of glioma grade and recurrence. *Neurol Res* 1994; **16**: 93–96.
- Scott CB, Scarantino C, Urtasun R, Movsas B, Jones CU, Simpson JR *et al*. Validation and predictive power of Radiation Therapy Oncology Group (RTOG) recursive partitioning analysis classes for malignant glioma patients: a report using RTOG 90-06. *Int J Radiat Oncol Biol Phys* 1998; **40**: 51–55.
- Glioma Meta-analysis Trialists (GMT) Group. Chemotherapy for high-grade glioma. *Cochrane Database Syst Rev* 2002; **3**: CD003913-.
- McLendon RE, Halperin EC. Is the long-term survival of patients with intracranial glioblastoma multiforme overstated? *Cancer* 2003; **98**: 1745–1748.
- Padma MV, Said S, Jacobs M, Hwang DR, Dunigan K, Satter M *et al*. Prediction of pathology and survival by FDG PET in gliomas. *J Neurooncol* 2003; **64**: 227–237.
- Constam DB, Philipp J, Malipiero UV, ten Dijke P, Schachner M, Fontana A. Differential expression of transforming growth factor-beta 1, -beta 2, and -beta 3 by glioblastoma cells, astrocytes, and microglia. *J Immunol* 1992; **148**: 1404–1410.
- Fakhrai H, Gramatikova S, Safaei R. Downregulation of transforming growth factor-beta as therapeutic approach for brain tumors. In: Liao LM, Cloughsey T, Becker DP, Bigner DD (eds). *Brain Tumor Immunology*. Humana Press: Totowa, 2000, pp 289–305.
- Platten M, Wick W, Weller M. Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. *Microsc Res Tech* 2001; **52**: 401–410.
- Luo JS, Kammerer R, von Kleist S. Comparison of the effects of immunosuppressive factors from newly established colon carcinoma cell cultures on human lymphocyte proliferation and cytokine secretion. *Tumour Biol* 2000; **21**: 11–20.
- Ordemann J, Jacobi CA, Braumann C, Schwenk W, Volk HD, Muller JM. Immunomodulatory changes in patients with colorectal cancer. *Int J Colorectal Dis* 2002; **17**: 37–41.
- Konno H, Baba M, Shoji T, Ohta M, Suzuki S, Nakamura S. Cyclooxygenase-2 expression correlates with uPAR levels and is responsible for poor prognosis of colorectal cancer. *Clin Exp Metast* 2002; **19**: 527–534.
- Fischer JR, Schindel M, Stein N, Lahm H, Gallati H, Krammer PH *et al*. Selective suppression of cytokine secretion in patients with small-cell lung cancer. *Ann Oncol* 1995; **6**: 921–926.
- Dix AR, Brooks WH, Roszman TL, Morford LA. Immune defects observed in patients with primary malignant brain tumors. *J Neuroimmunol* 1999; **100**: 216–232.
- Shim KS, Kim KH, Han WS, Park EB. Elevated serum levels of transforming growth factor-beta1 in patients with colorectal carcinoma: its association with tumor progression and its significant decrease after curative surgical resection. *Cancer* 1999; **85**: 554–561.
- Roszman T, Elliott L, Brooks W. Modulation of T-cell function by gliomas. *Immunol Today* 1991; **12**: 370–374.
- Fakhrai H, Dorigo O, Shawler DL, Lin H, Mercola D, Black KL *et al*. Eradication of established intracranial rat gliomas by transforming growth factor beta antisense gene therapy. *Proc Natl Acad Sci USA* 1996; **93**: 2909–2914.
- Sobol RE, Fakhrai H, Shawler D, Gjerset R, Dorigo O, Carson C *et al*. Interleukin-2 gene therapy in a patient with glioblastoma. *Gene Therapy* 1995; **2**: 164–167.
- Sobol RE, Shawler DL, Carson C, Van Beveren C, Mercola D, Fakhrai H *et al*. Interleukin 2 gene therapy of colorectal carcinoma with autologous irradiated tumor cells and genetically engineered fibroblasts: a phase I study. *Clin Cancer Res* 1999; **5**: 2359–2365.
- Davis FG, Kupelian V, Freels S, McCarthy B, Surawicz T. Prevalence estimates for primary brain tumors in the United States by behavior and major histology groups. *Neuro-oncology* 2001; **3**: 152–158.
- Sampson JH, Archer GE, Ashley DM, Fuchs HE, Hale LP, Dranoff G *et al*. Subcutaneous vaccination with irradiated, cytokine-producing tumor cells stimulates CD8+ cell-mediated immunity against tumors located in the ‘immunologically privileged’ central nervous system. *Proc Natl Acad Sci USA* 1996; **93**: 10399–10404.
- Hoover Jr HC, Surdyke M, Dangel RB, Peters LC, Hanna Jr MG. Delayed cutaneous hypersensitivity to autologous tumor cells in colorectal cancer patients immunized with an autologous tumor cell: bacillus Calmette–Guerin vaccine. *Cancer Res* 1984; **44**: 1671–1676.
- Fakhrai H, Shawler DL, Gjerset R, Naviaux RK, Koziol J, Royston I *et al*. Cytokine gene therapy with interleukin-2-transduced fibroblasts: effects of IL-2 dose on anti-tumor immunity. *Hum Gene Ther* 1995; **6**: 591–601.

- 25 Watanabe Y, Kuribayashi K, Miyatake S, Nishihara K, Nakayama EL, Taniyama T *et al*. Exogenous expression of mouse interferon gamma cDNA in mouse neuroblastoma C1300 cells results in reduced tumorigenicity by augmented anti-tumor immunity. *Proc Natl Acad Sci USA* 1989; **86**: 9456–9460.
- 26 Tepper RI, Pattengale PK, Leder P. Murine interleukin-4 displays potent anti-tumor activity *in vivo*. *Cell* 1989; **57**: 503–512.
- 27 Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K *et al*. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci USA* 1993; **90**: 3539–3543.
- 28 Sulitzeanu D. Immunosuppressive factors in human cancer. *Adv Cancer Res* 1993; **60**: 247–267.
- 29 Miescher S, Whiteside TL, de Tribolet N, von FV. *In situ* characterization, clonogenic potential, and antitumor cytolytic activity of T lymphocytes infiltrating human brain cancers. *J Neurosurg* 1988; **68**: 438–448.
- 30 Hirte H, Clark DA. Generation of lymphokine-activated killer cells in human ovarian carcinoma ascitic fluid: identification of transforming growth factor-beta as a suppressive factor. *Cancer Immunol Immunother* 1991; **32**: 296–302.
- 31 Naganuma H, Sasaki A, Satoh E, Nagasaka M, Nakano S, Isoe S *et al*. Transforming growth factor-beta inhibits interferon-gamma secretion by lymphokine-activated killer cells stimulated with tumor cells. *Neurol Med Chir (Tokyo)* 1996; **36**: 789–795.
- 32 Kobie JJ, Wu RS, Kurt RA, Lou S, Adelman MK, Whitesell LJ *et al*. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. *Cancer Res* 2003; **63**: 1860–1864.
- 33 Kao JY, Gong Y, Chen CM, Zheng QD, Chen JJ. Tumor-derived TGF-Beta reduces the efficacy of dendritic cell/tumor fusion vaccine. *J Immunol* 2003; **170**: 3806–3811.
- 34 Hirte HW, Clark DA, O'Connell G, Rusthoven J, Mazurka J. Reversal of suppression of lymphokine-activated killer cells by transforming growth factor-beta in ovarian carcinoma ascitic fluid requires interleukin-2 combined with anti-CD3 antibody. *Cell Immunol* 1992; **142**: 207–216.
- 35 Sobol RE, Shawler DL, Garrett MA, Van Beveren C, Trojan J, Trauger RJ *et al*. Induction of T cell responses against autologous tumor following treatment of colorectal carcinoma patients with an IL-2/CD80 genetically modified allogeneic tumor cell vaccine. *Proc Am Assoc Cancer Res* 2001; **42**: 684.
- 36 Eaton JD, Perry MJ, Nicholson S, Guckian M, Russell N, Whelan M *et al*. Allogeneic whole-cell vaccine: a phase I/II study in men with hormone-refractory prostate cancer. *BJU Int* 2002; **89**: 19–26.
- 37 Jaffee EM, Hruban RH, Biedrzycki B, Laheru D, Schepers K, Sauter PR *et al*. Novel allogeneic granulocyte-macrophage colony-stimulating factor-secreting tumor vaccine for pancreatic cancer: a phase I trial of safety and immune activation. *J Clin Oncol* 2001; **19**: 145–156.
- 38 Maio M, Fonsatti E, Lamaj E, Altomonte M, Cattarossi I, Santantonio C *et al*. Vaccination of stage IV patients with allogeneic IL-4- or IL-2-gene-transduced melanoma cells generates functional antibodies against vaccinating and autologous melanoma cells. *Cancer Immunol Immunother* 2002; **51**: 9–14.
- 39 Osanto S, Schiphorst PP, Weijl NI, Dijkstra N, Van Wees A, Brouwenstein N *et al*. Vaccination of melanoma patients with an allogeneic, genetically modified interleukin 2-producing melanoma cell line. *Hum Gene Ther* 2000; **11**: 739–750.
- 40 Chang AE, Li Q, Bishop DK, Normolle DP, Redman BD, Nickoloff BJ. Immunogenetic therapy of human melanoma utilizing autologous tumor cells transduced to secrete granulocyte-macrophage colony-stimulating factor. *Hum Gene Ther* 2000; **11**: 839–850.
- 41 Dillman RO, Beutel LD, Barth NM, de Leon C, O'Connor AA, DePriest C *et al*. Irradiated cells from autologous tumor cell lines as patient-specific vaccine therapy in 125 patients with metastatic cancer: induction of delayed-type hypersensitivity to autologous tumor is associated with improved survival. *Cancer Biother Radiopharm* 2002; **17**: 51–66.
- 42 Simons JW, Mikhak B, Chang JF, DeMarzo AM, Carducci MA, Lim M *et al*. Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using *ex vivo* gene transfer. *Cancer Res* 1999; **59**: 5160–5168.