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LABORATORY INVESTIGATION

TGF-β2 inhibition augments the effect of tumor vaccine and improves the survival of animals with pre-established brain tumors

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Abstract TGF-β2 secretion by high grade gliomas has been implicated as one of the major factors contributing to tumor growth, alterations in the host immune response to tumor, and failure of gliomas to respond to current immunotherapy strategies. We hypothesized that targeted delivery and inhibition of TGF-β2 by TGF-β2 antisense oligonucleotides (AS-ODNs) would overcome tumor-induced immunosuppression and enhance the capacity of tumor vaccines to eradicate established brain tumors. Utilizing the mRNA sequences of TGF-β2, specific AS-ODNs were constructed and tested for their ability to inhibit TGF-β2 production in 9L glioma cells. The effect of combining local intracranial administration of antisense ODNs with systemic tumor vaccine was examined. Fisher 344 rats were vaccinated subcutaneously with irradiated 9L tumor cells 3 days after intracranial tumor implantation. Four days after vaccination, ODNs were administered into the tumor mass and survival was followed. ODNs delivered locally distributed widely within the brain tumor mass and inhibited TGF-β2 expression. Survival of tumor-bearing rats treated with the combination of local antisense and systemic tumor vaccine was significantly enhanced (mean survival time (MST): 48.0 days). In contrast, MST for animals treated with nonsense plus vaccine, vaccine alone, antisense alone or PBS showed no survival advantage and no statistical differences between groups (33.5 days, 29.0 days, 37.5 days, and 31.5 days, respectively). Our data supports the hypothesis that local administration of antisense TGF-β2 ODNs combined with systemic vaccination can increase efficacy of immunotherapy and is a novel, potentially clinically applicable, strategy for high-grade glioma treatment.

Keywords Glioma · Cancer vaccine · Antisense oligonucleotides · TGF-β2 · Immunotherapy · Immunosuppression

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Introduction

Malignant gliomas account for 65% of all primary brain tumors and affect approximately 17,000 Americans

every year. Despite current multimodal therapies of surgery, radiation therapy, and chemotherapy, the prognosis for patients with this tumor remains dismal. Poor prognosis is primarily attributable to the highly infiltrating nature of high-grade gliomas, which makes complete resection of the tumor impossible and local failure highly likely. The ability to substantially impact survival in this disease relies on the development of novel treatment strategies capable of eradicating residual individual infiltrating tumor cells. Tumor vaccine therapy is one such promising approach.

For several decades, vaccine strategies have focused on combining various forms of modified and unmodified tumor antigen with an immune adjuvant [1–7]. Although these attempts have succeeded in generating an effective antitumor immune response in the animal model, this response could only reliably be generated prior to tumor cell challenge (prevention regimen). Few experimental studies have demonstrated success in generating an effective antitumor immune response in the presence of well-established, viable tumor (treatment regimen) [3, 8]. This explains, in part, the universally poor results observed when these treatment strategies have been moved to the clinical arena [9–11]. A possible explanation for the effectiveness of immunotherapy in the ‘‘prevention'' as opposed to the ‘‘treatment'' regimen may lie in, among other factors, immunosuppressive activity mediated by the glioma itself [12]. Indeed, impaired cell-mediated immunity is well described in patients with gliomas [13–15]. These patients often exhibit cutaneous anergy with abnormal delayed hypersensitivity for common recall and tumor antigens, reduced circulating T lymphocytes, a depressed lymphocyte proliferative response to mitogens, decreased antibody response to various antigens, and deficient antibody-mediated and T cell mediated cytotoxicity in vitro. Since surgical removal of the tumor has been shown to result in a partial reversion of the immunosuppressive state, the glioma itself is thought to play a major role in inducing and maintaining this impaired cell-mediated immunity [16].

Malignant glial tumors are known to release several immunosuppressive factors, including prostaglandin E2 [17], IL-10 [18] and TGF-β2 [15]. Among these factors, TGF-β2 is one of the best characterized [19, 20]. TGF-β2 can inhibit T and B cell proliferation [21, 22], IL-2 receptor induction [23], cytokine production [24], natural killer cell activity [25], cytotoxic T lymphocyte development [24], and lymphokine-activated killer cell generation [26]. TGF-β2 has also been shown to directly hamper the cytotoxicity and proliferation of tumor-infiltrating lymphocytes [19, 27, 28] and to down-regulate the expression of the class II antigen HLA-DR [29], possibly contributing to the tumor cells ability to escape immune surveillance. Evidence also exists to its importance as a growth promoter of malignant glioma cells [30, 31]. Residual tumor cells may maintain the ability to secrete local immunosuppressive factors, including TGF-β2, even after radiation therapy [32]. Given these actions, TGF-β2 has been implicated as one of the major contributing factors to the failure of current immunotherapy strategies.

Despite these limitations, any therapeutic reduction in the TGF-β2 production in gliomas might be expected to reverse immunosuppression and enhance the effect of immunotherapy. Several investigators have already tested this hypothesis, in part. Animal studies by Fakhrai et al. have utilized glioma cells transduced with anti-TGF-β2 as a systemic vaccine; these studies showed promising efficacy in prolonging survival in rats bearing 9L gliomas and clearly demonstrated reduction in tumor size in survivors [33] In addition, preliminary results from a Phase I/II clinical trial by Bogdahn et al. utilizing intracranially administered anti-TGF-β2 antisense ODNs have shown a median survival following recurrence of 44.0 weeks in patient with GBMs and 97.4 weeks in patients with anaplastic astrocytoma [34]. Minimal toxicity was found in the dose escalation phase of the study, suggesting promising clinical utility for this regimen [34]. However, to our knowledge, no group has combined the use of intracranial antisense ODN administration with systemic vaccination as a therapeutic paradigm for gliomas.

Use of AS-ODNs to block the translation of particular gene products within cells is a powerful approach for specifically inhibiting the expression of target proteins [35, 36]. Indeed, direct intracranial AS-ODN infusion has been widely used to block the transcription of many different genes within the brain [37–39]. Furthermore, AS-ODNs injected intracranially have been shown to be preferentially taken up by tumors [40]. The advantage of using antisense oligonucleotides, as opposed to small molecule inhibitors of TGF-β, is that the latter exhibit a lower target specificity and show complete inhibition of all three human TGF-β isoforms. In addition, nonspecific cross-reaction with other ATP-kinases is possible. Hence, small molecule TGF-β inhibitors might be more likely to cause unexpected toxicity due to ‘‘off-target'' inhibition (personal communication, Dr. Karl-Hermann Schlingensiepen, chief operating officer, Anti-sense Pharma, Regensburg, Germany).

Utilizing a 9L intracranial glioma brain tumor model, which has been shown to secrete TGF-β2 and produce immunosuppression in the immunized rat [41], we conducted this study to investigate whether regional intracranial delivery of TGF-β2 ASODN could

overcome this tumor-induced immunosuppression and potentially enhance the antitumor effect of systemically administered tumor vaccine. Our data, for the first time has demonstrated the efficacy of treating pre-existing intracranial tumor with the combination of systemic tumor vaccine and the intracranial administration of anti-TGF-β2 antisense. These observations support the contention that enhancing the anti-tumor effect of active specific immunotherapy through reversal of tumor-induced immunosuppression is feasible.

Materials and methods

Cell culture and cell line

9L gliosarcoma cells were originally derived from a chemically-induced tumor in Fischer 344 rat. The malignant glioma cell line is known to secrete TGF-β2 [33], thus making it similar to human gliomas [42]. Although 9L is highly immunogenic, and ball-like with limited infiltrative growth, the model was chosen based on our extensive previous experience and the fact that the Fischer 344/9L rat glioma model is one of the only models where the tumor is syngeneic. It was felt that in this immunological experiment this fact outweighed the disadvantages of the model. The cells were cultured in RPMI-1640 medium (Life Technologies, Inc., Bethesda, MD), supplemented with 10% fetal bovine serum, 50 µM 2-mercaptoethanol and 2 mM glutamine in a humidified incubator at 37°C with a 5% CO_2/air atmosphere. For these experiments, 9L tumor cells were collected during the logarithmic growth phase and used as described.

Animals

All animal experiments were performed on Fischer 344 male rats (250 g) obtained from the Charles River Laboratories (Wilmington, MA). The care and use of all animals were in accordance with the guidelines of the animal welfare committee of the University of Colorado Health Sciences Center.

ODNs

The selection of the antisense oligonucleotide against TGF-β2 was based on the published nucleotide sequence coding for the rat's TGF-β2 gene [43]. Briefly, we designed multiple short phosphorothioate-modified antisense and nonsense ODNs directed against the 5′ and 3′ untranslated regions (UTR) and translated regions of rat TGF-β2 mRNA. The location of each of 19 ODNs generated relative to the rat TGF-β2 mRNA are shown in Fig. 1. Each of the ODNs designed was 20 mers in length and synthesized commercially by Integrated DNA Technologies (Coralville, IA). These AS-ODNs were screened for their efficacy to inhibit TGF-β2 production in vitro in 9L glioma cell culture. Because unmodified phosphodiester ODNs have a half-life of less than 15 min in the presence of serum [44], modified phosphorothioate ODNs were used in both the in vitro and in vivo experiments.

ODN sequences

Metabolic labeling and immunoprecipitations

9L glioma cells were plated at 2.5×10^5/well in 6-well plates and incubated overnight. For labeling of TGF-β2, the cells were washed and starved for 30 min in methionine-free DMEM medium containing 5% dialyzed FBS. The cells were then pulsed for 60-min with [^{35}S]Translabel (100 µCi) and chased at indicated time intervals. Time point "0" indicates the time point immediately following the labeling. To immunoprecipitate TGF-β2, cells were washed and lysed with 1 ml

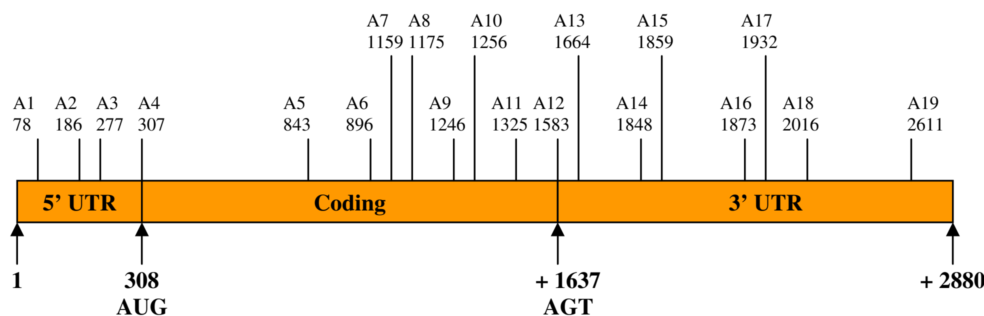

Fig. 1 Rat TGF-β2 mRNA sequence. Nineteen AS-ODNs were designed and synthesized. Each of the AS-ODNs were 20 bases in length and predicted to hybridize with rat TGF-β2 mRNA in the 5′-untranslated region, protein coding region and 3′-untranslated region, respectively. The numbers correspond to the approximate location of the antisense sequences generated

immunoprecipitation buffer (1% TX-100, 0.4% deoxycholic acid, 100 mM PMSF, and a complete protease inhibitor (Boehringer-Mannheim)) for 10 min, after which the lysate was clarified by centrifugation at 14,000 rpm for 10 min at 4°C. The lysate was then incubated at 4°C overnight with 25 μl of polyclonal anti-TGF-β2 antibody (SC-90, Santa Cruz Biotechnology, Santa Cruz, CA) followed by the addition of protein-A sepharose (Sigma) for 2 h This antibody recognizes both the latent and the activated forms of TGF-β2 according to the manufacturer's specifications. The complexes were then washed 4 times with 0.1% Tween-20 PBS buffer and eluted from the sepharose beads by SDS-loading buffer. Samples were resolved by electrophoresis through a 12% SDS-gel and visualized by autoradiography. Bands were quantitated with a Scion Image.

Treatment of cells in culture with ODNs

Antisense and nonsense ODNs were screened for their efficacy to inhibit TGF-β2 production in 9L glioma cells in culture. Briefly, 9L glioma cells were plated at 2.5×10^5/well in 6-well plates and incubated overnight. After reaching 60–80% confluency, the cells were transfected with a single administration of various concentrations of either antisense ODNs or nonsense ODNs using the LipofectAMINE-mediated liposome transfection technique. The efficiency of transfection was confirmed by monitoring cellular uptake of fluorescently-labeled (FITC) ODNs. Twelve hours following ODN treatment, the cell lysate was analysed for TGF-β2 protein production by the immunoprecipitation technique described earlier. Newly formed TGF-β2 was expressed as a percentage of the level of TGF-β2 produced in cultures of untreated control cells. From this screening, a single AS-ODN was identified as the most efficient at inhibiting TGF-β2 protein production.

Time course and dose response of TGF-β2 inhibition, with in vitro testing of concentrations of ODNs

9L glioma cells were used to investigate the time course and dose response of TGF-β2 inhibition by TGF-β2 AS-ODNs. Briefly, 9L glioma cells were plated at 2.5×10^5/well in 6-well plates and incubated overnight. After reaching 60–80% confluency, the cells were transfected with 200 nM of either antisense ODN or nonsense ODN using LipofectAMINE-mediated liposome transfection techniques. For the time course experiments; 3, 6, 12, 24 and 48 h following ODN treatment, the cell lysate was analyzed for TGF-β2 protein production. For the dose response experiments, the cells were transfected with various concentrations (50, 100, 200, and 400 nM) of either antisense ODN or nonsense ODN for 12 h using LipofectAMINE-mediated liposome transfection. The nonsense ODN transfected cells served as a control for the effects of the ODN itself. In both experiments, newly formed TGF-β2 protein was expressed as a percentage of the levels of TGF-β2 protein in untreated control cells.

Intracranial tumor implantation

Using a stereotactic frame (Kopf, USA), cannulas were permanently installed into the right frontal lobes of Fisher 344 rats on day 0. The coordinates used for cannula implantation were 2 mm to the right and 2 mm anterior to the bregma, and 3 mm deep to the outer table of the cranium. Following stereotactic implantation, the cannula (O.D. = 0.025 in. and I.D. = 0.013 in.) was secured to the skull using orthodontic resin. A sterile stylet (O.D. = 0.013 in.) was placed within the cannula. Seven days following cannula implantation, 9L glioma cells (5×10^3/10 μl phosphate buffered saline (PBS)) in logarithmic growth phase were sterilely introduced under general anesthesia into the right frontal lobes of the rats through the implanted cannulas. The number of glioma cells implanted was sufficient to cause death in 100% of the animals within 30 days [45]. From previous studies conducted in our laboratory, we have shown that the size of pre-established tumor 7 days following implantation is 3–4 mm in diameter, with an estimated cell number of 1.8×10^6 [46]. After receiving intracranial tumor cell injections, the animals were monitored twice a day for neurological signs and weighed every other day until death or sacrifice. All animals were sacrificed when moribund. At the time of death, the brains of all animals were harvested for histological evaluation.

Intracranial injection of ODNs

For analysis of ODN distribution within the brain, FITC-labeled AS-ODNs (1.0 nmol/10 μl PBS) were introduced into the frontal lobes of anesthetized rats via the permanently implanted cannulas 10 days after intracranial implantation of tumor cells. At 6, 12, 24, and 48 h after AS-ODN injection, the rats were anesthetized and perfused transcardially with PBS (100 mM) containing 0.1% heparin and 4% paraformaldehyde. The brain was then harvested and transferred to a 10% sucrose PBS solution for overnight incubation at 4°C. The brain was then embedded in Tissue Freezing Medium (Triangle Biomedical Sci-

ences, Durham, NC) by immersion in –70°C dry ice and prepared for frozen sectioning. About 40 μm thickness coronal brains sections were cut encompassing the level of the cannula. These sections were then thaw-mounted onto gelatin-coated slides. Individual sections were studied microscopically using the immuno-fluorescence microscope to examine the presence of FITC-labeled AS-ODNs, and then counter-staining with H&E to examine the relationship to tumor.

To evaluate the effect of the ODNs on TGF-β2 protein production, in vivo, antisense or nonsense anti-TGF-β2 ODNs or PBS was administered intracranially via the permanently implanted cannula over a 48 h period. These injections were administered manually over a 2-min interval rather than via positive pressure pump infusion. This was performed 10 days after tumor cell implantation. At 24 h and 2, 3, 4, and 5 days following the end of the infusion, the animals were sacrificed and the intracranial tumor harvested. The tumor tissue was homogenized and resolved on an SDS gel, followed by Western blotting to confirm the effect on TGF-β2 protein production.

To evaluate the combined effect of TGF-β2 antisense ODN and tumor vaccination, Fisher 344 rats were vaccinated 72 h after tumor implantation. Four days following vaccination, the antisense and nonsense ODNs were infused, again through the cannula, once a day for 5 days. Survival was then followed.

Western blot for in-vivo TGF-β2 protein production

Whole brains of control animals and animals treated with either antisense or nonsense ODN were sampled. The tumor from each of these animals was isolated from the surrounding normal brain using microsurgical dissection. The tumor was then washed three times with PBS. The tumor was subsequently homogenized in 200 μl of lysis buffer using a Fisher Scientific sonic dismembrator (Pittsburgh, PA). The resultant cell pellet was incubated at 4°C for 10 min and the cell lysate clarified by centrifugation for 10 min at 12,000 *g*. The protein concentration in the supernatant was determined using a BioRad protein assay kit (Biorad Laboratories, Hercules). SDS-loading buffer was then added to 50 μg of the clarified supernatant with the contained proteins subjected to SDS-PAGE (12%). The protein was then transferred onto Immobilon membranes (Millipore). Immunoblots were blocked with 2% BSA in 0.1% Tween-20 PBS for 1 h and then incubated with the anti-TGF-β2 primary antibody SC-90 for 1 h at room temperature. The immunoblots were washed three times (0.1% Tween-20 PBS), incubated with horseradish peroxidase-conjugated secondary antibody (Protein G) for 1 h, and revealed by using ECL, the enhanced chemiluminescence system (New England Nuclear).

Subcutaneous vaccination

About 5×10^6 irradiated 9L-glioma cells (i9L) (10,000 cGy) and 10,000 U interferon-gamma (IFN-γ) (R&D Systems, Minneapolis MN) in PBS (0.8 ml) was injected subcutaneously into the right hind leg of Fischer 344 rats. The rats received daily subcutaneous injections of IFN-γ (10,000 U in 0.3 ml) for 5 additional days at this same site. This strategy was previously shown to be effective in preventing the growth of intracranial tumor in 80% of animals immunized prior to tumor implantation [41]. In that study, IFN-gamma was utilized on the premise that it upregulates MHC class I surface antigens, making the tumor more immunogenic.

Histological detection of mononuclear cell infiltration

To detect mononuclear cell infiltration at the site of intracranial tumor cell challenge, the brain from animals treated with ODN and vaccination was removed, fixed in 10% formalin, and embedded in paraffin for histological examination. Four micron thick tissue sections were obtained and stained with hematoxylin and eosin. These sections were then evaluated for the presence of mononuclear cell infiltration by the study neuropathologist, who was blinded to the treatment groups.

Statistical analysis

Survival estimates and median survivals were determined using the method of Kaplan and Meier [47]. Survival data were compared using non-parametric log-rank analysis [48]. Statistical significance was determined at the 0.01 level.

Results

Screening of TGF-β2 antisense ODNs

In order to optimize the timing of TGF-β2 AS-ODN administration for maximal inhibition of TGF-β2 protein production, the turnover rate of TGF-β2 was determined. Using the standard metabolic labeling and immunoprecipitation procedures described earlier, we determined the stability of TGF-β2 in 9L glioma cells. TGF-β2 first appeared as a precursor that was then slowly converted to its mature form (Fig. 2). The

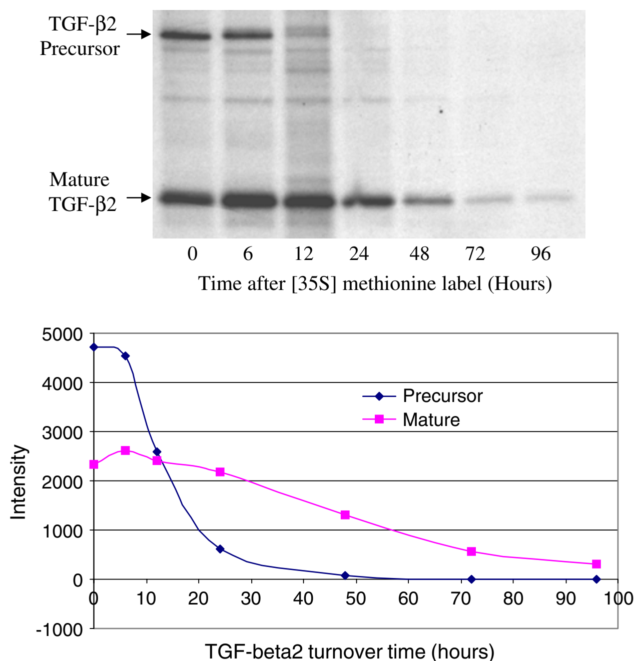

Fig. 2 Turnover rate of TGF-β2 protein. 9L glioma cells were pulse radiolabeled with [^{35}S] methionine for 1 h and chased at different intervals (from 1 to 48 h). The TGF-β2 protein was immunoprecipitated from 9L glioma cell lysate in each of the groups and resolved on SDS gels for autoradiography

half-life of the precursor form of TGF-β2 converting to the mature form of TGF-β2 was ~12 h, while the half-life of the mature form was ~48 h.

In order to maximize the transfection efficiency, a range of AS-ODN concentrations were investigated. Transfection of 200 nM ODN (1:3 ODN/LipofectAMINE ratio) yielded an almost 100% efficiency of delivery into cultured 9L glioma cells. The fluorescence was detectable in the nucleus and cytoplasm as early as 3 h after incubation and persisted for at least 48 h, with more ODN focusing in the nucleus (Fig. 3). The AS-ODNs were found to be successfully incorporated into the cells at concentrations as low as 50 nM. Ten-fold higher concentration of ODNs was needed to achieve equivalent 9L cell uptake in the absence of LipofectAMINE. However, most of the ODN in this latter experiment was located in the cell cytoplasm. Based on these results, the 200 nM ODN concentration was chosen to screen the activity of the AS-ODN library to inhibit TGF-β2 production in 9L glioma cells

Initial screening of the AS-ODNs generated was performed following a single administration of AS-ODN to cultured 9L glioma cells, as described in the Material and methods section. From this data the profile of TGF-β2 inhibition was determined. From a total of 19 candidate AS-ODNs tested, the TGF-β2 AS-ODN "A6" showed the greatest TGF-β2 protein inhibition (Fig. 4).

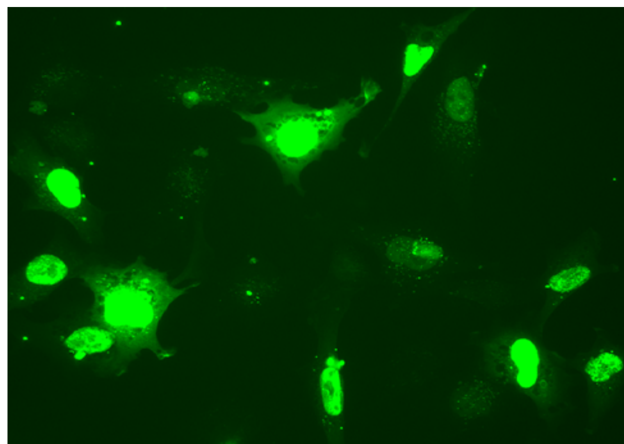

Fig. 3 Transfection of FITC-labeled AS-ODN into 9L glioma cells using LipofectAMINE. The day before transfection, 9L cells were seeded at a density of 5×10^5 (optimized density) in RPMI supplemented with 10% fetal bovine serum in 6-well plates. The cells were incubated with transfection complexes for 3 h at 37°C, after which the media was removed and the cells were washed once with 2 ml RPMI and replaced with RPMI containing 10% fetal bovine serum. Photo was taken after 6 h of transfection

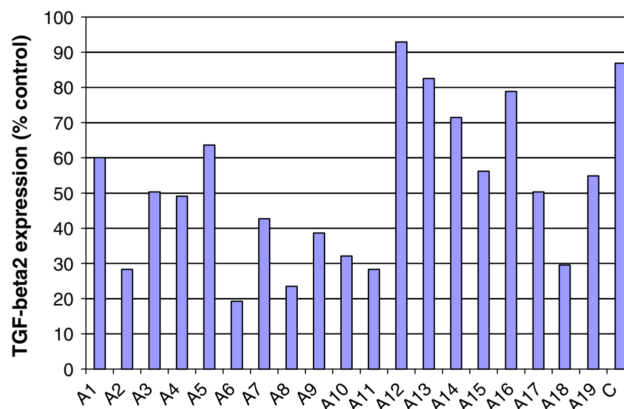

Fig. 4 Reduction of newly synthesized TGF-β2 protein in 9L glioma cells following treatment with appropriate phosphorothioate AS-ODNs. Cells were treated with the various AS-ODNs at a concentration of 200 nM and cells were lysed for immunoprecipitation 24 h later. TGF-β2 protein levels were analyzed, quantified and normalized to background level and are expressed as a percentage of the levels of TGF-β2 protein in untreated control cells

Time course and dose response of TGF-β2 inhibition in vitro

The duration and extent of TGF-β2 protein inhibition by treatment in vitro with AS-ODN A6 was evaluated. The results of AS-ODN TGF-β2 protein inhibition in vitro are shown in Fig. 5. We found that the inhibitory effect of AS-ODN A6 on TGF-β2 levels was seen as early as 6 h, reached a maximum effect at 12 h, with inhibition decreasing by 24 h. Testing for specificity, 9L

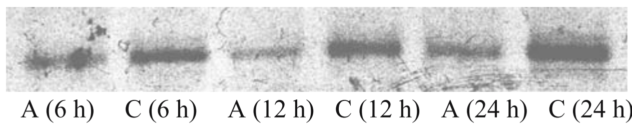

Fig. 5 Time course of TGF-β2 inhibition. Cells were treated with the AS-ODN ''A6'' at a concentration of 200 nM for 12 h at 37°C, washed once with RPMI and replaced with RPMI containing 10% fetal bovine serum. A6 treated cells (A) or untreated control cells (C) were lysed for immunoprecipitation after culturing for 6, 12, and 24 h

glioma cells were treated with an increasing concentration (50–400 nM) of AS-ODN A6 and a nonsense control ODN. Twelve hours after the addition of the AS-ODNs, the level of newly synthesized TGF-β2 protein was analyzed by immunoprecipitation and compared with that in an untransfected control (Fig. 6). At the highest concentration of AS-ODN tested (400 nM), the TGF-β2 protein level was reduced by almost 90%.

ODN distribution in vivo

A representative H&E and fluorescent photomicrograph showing the tissue distribution of FITC-labeled ODN, 24 h after infusion into intracranial 9L gliomas in vivo, is shown in Fig. 7 (4× magnification). A 10× magnification of a region outlined in the photomicrographs was also included to better assess cellular detail. FITC-labeled AS-ODNs delivered intratumorally were found to diffuse out from the viable tumor into normal surrounding brain tissues in a time-dependent manner. The diffusion appears to reach a maximum by 24 h, being virtually gone by 48 h. At 24 h, in our experimental model, the tumor appeared to exhibit fairly homogeneous fluorescence. Whether this fluorescence is associated with the intracellular or extracellular space could not be determined with certainty from this technique. However, based on the data from the in vitro FITC AS-ODN study (Fig. 2) and that of the in vivo suppression study of TGF-β2 protein production (Fig. 8), we believe that the fluorescence observed is most likely of intracellular origin. We cannot further speculate as to the precise subcellular localization of the AS-ODN.

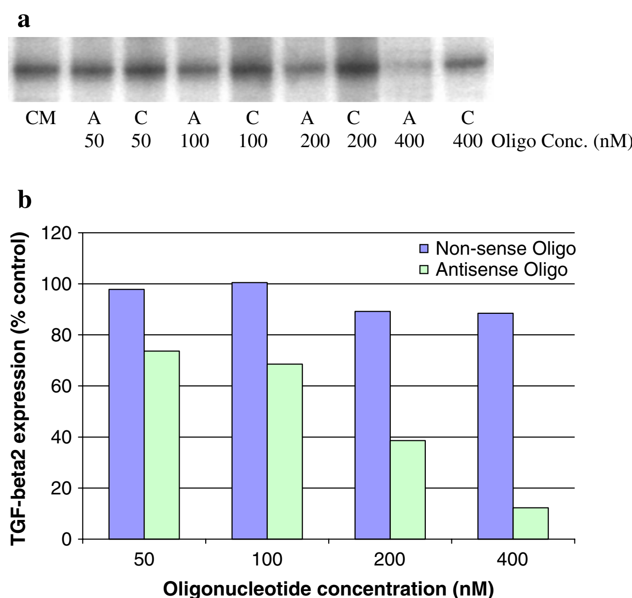

Fig. 6 Inhibition of TGF-β2 protein expression by AS-ODN A6 in 9L glioma cells. (**a**) 9L glioma cells were treated with increasing concentrations (50–400 nM) of AS-ODN A6 (A) or nonsense ODN (C) for 12 h and analyzed for TGF-β2 protein expression by immunoprecipitation. (**b**) Quantification of TGF-β2 protein levels after normalization of the background and compared to non-treatment control (CM)

In vivo ODN suppression of TGF-β2 protein production

The efficacy of AS-ODN A6 was tested in vivo. AS-ODN A6 was infused into the brain of tumor bearing rats and the tumors were harvested at various time intervals following injection. Infusion of AS-ODN A6 produced a dose dependent specific inhibition of TGF-β2 protein expression in the brain tumor as demonstrated by western blot (data not shown) The TGF-β2 protein level was consistently decreased by 70% (to 30% of control) at 72 h after initial antisense infusion and returned to normal at 5 days (Fig. 8). In contrast, the beta-actin protein level, an internal control, was unaltered.

Combined effect of AS-ODN administration and tumor vaccine on survival

Following the demonstration of an effective in vivo inhibition of TGF-β2 production by AS-ODN A6, the effect of combining local intracranial AS-ODN administration with a systemic tumor vaccine was examined. Previous work from our laboratory has indicated that following systemic vaccination, at least 5 days are required for an effective antitumor immune response to develop [41]. Our previous published study showed that animals treated with interferon gamma alone did not have increased survival; thus our historical ''interferon-gamma only'' treated group served as a control [41]. This previous study also showed that interferon gamma alone, combined with irradiated tumor cells was preferable to other cytokines (either alone or in combination) in increasing tumor vaccine immunogenicity.

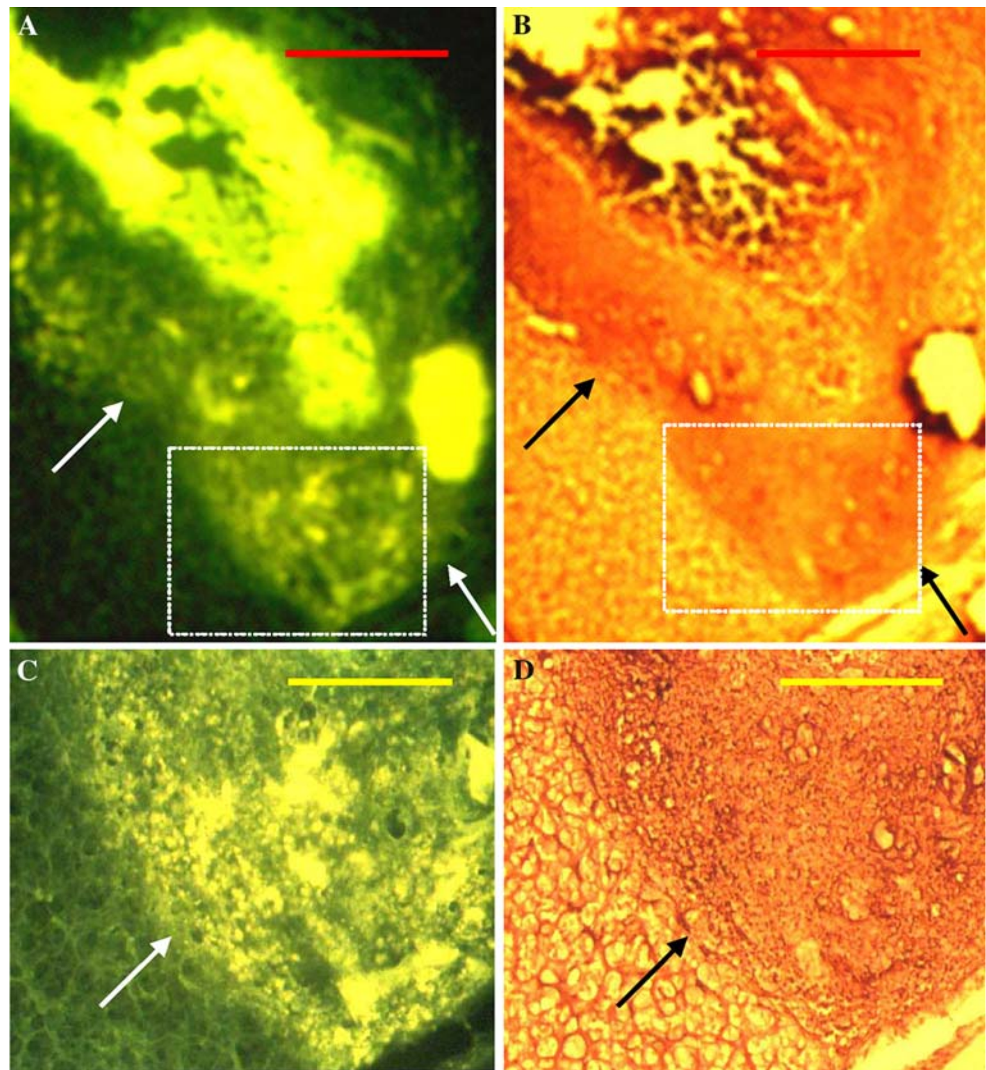

Fig. 7 Antisense uptake in vivo by intracranial 9L glioma. Photomicrographs showing the distribution of AS-ODN 24 h (**A** and **B**) after infusion of 1.0 nmol FITC-labeled AS-ODNs in a volume of 10 μl (4× power). The arrows indicate the tumor/brain interface. In section **A** and **B** the catheter tract is oriented superiorly and slightly to the left. Sections **C** and **D** are high power magnifications (10×) of the area outlined in **A** and **B**. These latter photomicrographs confirm the presence of AS-ODN fluorescence within viable tumor tissue. Red bar = 1 mm. Yellow bar = 400 μm

The optimal anti-tumor response was obtained when vaccination was applied 7 days prior to tumor cell challenge [41]. Given this information, and the finding that the half-life of the mature form of TGF-β2 is 48 h (Fig. 1), we initiated systemic vaccination at day 3 following intracranial tumor challenge. At day 7 following intracranial tumor challenge (4 days after systemic vaccination), AS-ODN A6 was then administered intratumorally every day for 5 days. The survival of tumor-bearing rats treated with the combination of intracranial AS-ODN and systemic tumor vaccine was significantly enhanced compared against all other groups (median survival time: 48.0 days, $P = 0.0022$). Survival for each group compared to PBS control (31.5 days) was: 48.0 days for anti-sense plus vaccine ($P = 0.0009$), 37.5 days for anti-sense alone ($P = 0.0667$), 35.0 days for non-sense alone ($P = 0.2544$), 33.5 days for non-sense plus vaccine ($P = 0.5842$), and 29.0 days for vaccine alone ($P = 0.8002$) (Fig. 9). Approximately 30% of animals survived until termination of the experiment at 60 days, when they were sacrificed (Fig. 9). Therefore, while inhibition of TGF-β2 expression alone showed mild benefit, combining TGF-β2 inhibition with

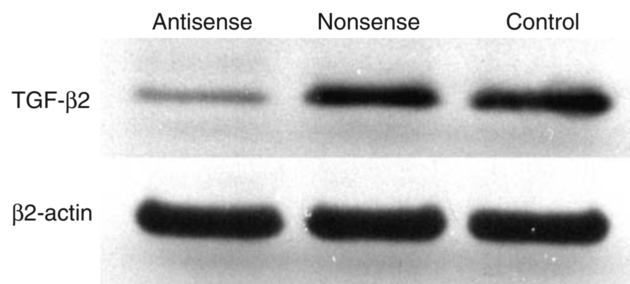

Fig. 8 In vivo inhibition of TGF-β2 expression by TGF-β2 antisense in the 9L glioma model. Fischer 344 rats with permanently installed right frontal intracranial cannulae, were administered 1×10^4 9L glioma cells, followed 10 days later by the administration of sterile 1.0 nmol TGF-β2 AS-ODN in a volume of 10 μl for 2 days. Animals were euthanized 72 h after initial antisense infusion. Tumors were isolated and lysed, and TGF-β2 protein depletion was demonstrated by western blot

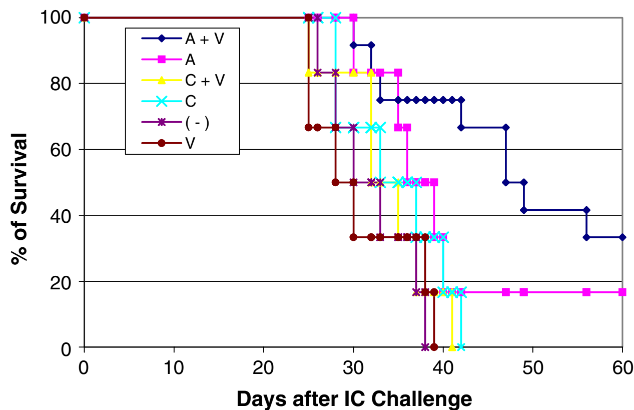

Fig. 9 Demonstration of the combined effect of local anti-TGF-β2 antisense ODN and systemic tumor vaccine on the survival of animals with pre-established intracranial tumor. Fisher 344 rats were vaccinated subcutaneously with irradiated 9L tumor cells plus INF-r 3 days after tumor implantation. Four days after vaccination, ODNs were administered intracranially into the tumor mass daily for 5 days. Survival was then followed. [A = antisense, V = vaccine, C = nonsense, (–) = PBS control; n = 12 in TGF-β2 antisense + vaccine group and 6 in others]

systemic anti-tumor vaccination demonstrated a significant benefit against intracranial glioma progression.

Pathology

Coronal brain sections of all animals in the treatment and control groups described above demonstrated well-demarcated, poorly differentiated tumor. Although there was a tendency for animals in the antisense plus vaccine group to have a slightly more prominent mononuclear cell infiltrate within the peripheral portions of the tumor mass, inflammation could be seen in at least some animals from each of the treatment, and control, groups. Hence, the extent of the inflammatory cell component was not felt to discriminate between treatment groups. The most distinctive and easily recognized difference between the various treatment groups was the number of days necessary to achieve growth of the otherwise histologically identical 9L tumor. As depicted in Fig. 10, an equivalent intracranial tumor size was achieved in a representative animal from the antisense plus vaccine treatment group in 85 days (right), as compared to a representative PBS control animal in 30 days (left). No animal in any of the groups showed neuronal necrosis, inflammation, hemorrhages, or infarctions in areas remote from the tumor. Hence, there was no evidence of autoimmune encephalitis or other immune damage to brain, at least in the time frame encompassed by this study. In the majority of the animals in the antisense and nonsense treatment groups, brain tissue uninvolved by tumor, but lying immediately adjacent to the tumor mass, did show small areas of basophilic stippling unassociated with granulomas, neutrophilic influx, macrophages, or tissue disruption. This focal basophilic material was positive on von Kossa staining, consistent with calcium deposition. Staining was negative for any bacterial or fungal organisms. No animals in the PBS or vaccine only groups showed this change and no reaction was seen on the part of the host to this material. This change was interpreted as focal dystrophic calcification occurring only in the vicinity of the injections; hence this was considered secondary to the sense/antisense vehicle since both antisense and nonsense treatment groups showed this change.

Discussion

Recent data suggests that the overexpression of TGF-β2 in gliomas contributes to the immunosuppressed state and may be a major factor responsible for the failure of current immunotherapy strategies [20, 48]. Therefore, a therapeutic reduction of TGF-β2 would be expected to reverse this tumor-induced immunosuppression and enhance immunotherapeutic strategies. Indeed, specific blockade of TGF-β2 secretion [49], T-cell specific TGF-β2 signaling [50], and TGF-β2 production [51, 52] have all been shown to exhibit an beneficial effect on lymphocyte function and have resulted in an enhanced immune response. Despite the success of these previously published interventions, no study has investigated the combined effect of TGF-β2 inhibition and vaccine therapy to enhance antitumor immunity.

The current study clearly demonstrates that inhibition of local intracranial TGF-β2 expression, when combined with systemic vaccination, was associated with an increase in survival of animals with pre-established brain tumor. Survival was significantly increased and approximately 30% of animals survived until day 60, the termination of the experiment (see Fig. 9). This provides proof-of-principle that the approach has merit and deserves further exploration.

We could not detect a dramatic difference in the number of lymphocytes in the antisense versus nonsense treatment groups, despite the clear-cut efficacy and overall tumor size reduction in the antisense treated animals. While TGF-β2 is known to reduce lymphocyte function and inhibit the immune response, this is due to down-regulation of lymphocyte efficacy and functional tumor kill, and not simply a reduction in

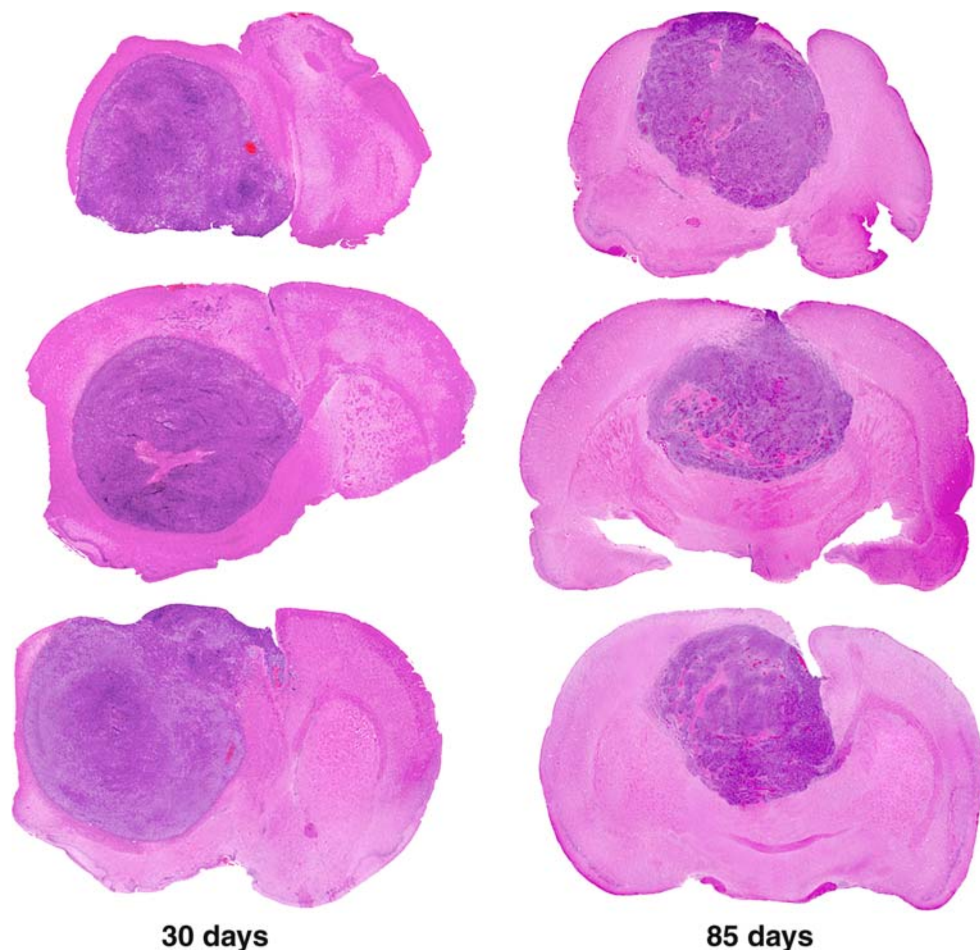

Fig. 10 Tumor size in representative animals treated with anti-TGF-β2 antisense ODN plus tumor vaccine (right) and control (left)

lymphocytic numbers within the tumor, as shown in our previous study [41].

TGF-β2 is secreted by a number of cells other than glioma cells, including reactive astrocytes, meningeal cells, and choroid plexus cells [53]. Since TGF-β2 also has the potential to promote tumor cell proliferation directly [54] and increase angiogenesis [55], it is possible that the beneficial effects seen in our experiments might be the result of direct TGF-β2 inhibition of blood vessels. This possibility deserves further investigation. However, the fact that survival was significantly improved in the vaccine plus antisense treated group, as opposed to the antisense treated group alone, argues for an enhanced synergistic immune effect. Therefore, reversal of local TGF-β2 induced immunosuppression appears to shift the balance of the immune response, favoring anti-tumor immunity.

Because AS-ODNs can specifically target mRNA sequences of interest and block the translation of specific gene products [35], AS-ODNs were used in this study to interfere with TGF-β2 mRNA translation and inhibit TGF-β2 protein production. Our in vitro data indicates that AS-ODNs designed against the 5′ and 3′untranslated and translated regions of rat TGF-β2 mRNA (Fig. 1) were effective in inhibiting TGF-β2 production by 9L glioma cells (Fig. 4). These results are consistent with the work of others, which has indicated that sequences in both the translating and non-translating regions are suitable targets for antisense therapy [51, 52]. We also demonstrated that inhibition of TGF-β2 production was both time-dependent (Fig. 5) and concentration-dependent (Fig. 6), with inhibition starting to appear at ODN concentrations as low as 50 nM and as early as 12 h after treatment. Our study has also indicated that, whereas transfection reagents were required to promote tumor uptake and nuclear distribution of AS-ODNs in vitro, ODNs introduced in vivo, without the use of a transfection reagent, readily entered the tumor cells.

Direct intracranial AS-ODN infusion has been widely used to block the transcription of many different gene products within the brain [35–37]. Grzanna et al. demonstrated rapid and extensive dose-depen-

dent brain tissue penetration and cellular uptake of labeled phosphorothioate AS-ODNs following either intrastriatal or intraventricular injections [56]. Other investigators have also confirmed the cellular uptake and bio-distribution of intracranially administered phosphorothioate-modified AS-ODNs, and have shown a lack of adverse side effects. ODNs administered intracranially have been shown to be stable even after 48 h in situ [57–59]. Our results are therefore consistent with previous reports demonstrating that cationic lipid-mediated ODN transfection is not required for ODN delivery in vivo, and that direct intracranial infusion of ODNs can lead to their preferential uptake by tumor cells and surrounding normal brain tissues (Fig. 7). Previous work in our laboratory has shown that opposed to a single infusion, multiple infusions over time appear to give the most uniform and complete ODN distribution within tumor (data not shown).

No adverse effects of this treatment paradigm were detected. Neither autoimmune encephalitis nor tissue infarction was present. Pathologic evaluation did reveal the presence of a focal basophilic material found in the brain adjacent to the tumor mass, which was present in the majority of the antisense and nonsense treated animals, but confined to the area immediately adjacent to the injection site. This change was interpreted as focal dystrophic calcification and may be related to the multiplicity of sense/antisense injections.

In order to effectively inhibit TGF-β2 protein production in 9L glioma cells by AS-ODNs, it is crucial to determine the half-life of TGF-β2. Our study shows that although the half-life of TGF-β2 mRNA is relatively short (65 min) [60], the half-life of its translation products is rather long, lasting up to 60 h (12 h for the precursor form and 48 h for the mature form). These results suggest that a continuous presence of AS-ODN is required to effectively maintain a low level of TGF-β2 secretion. Indeed, the intracranial infusion of AS-ODN daily for 2 days resulted in a substantial reduction of TGF-β2 expression in our 9L glioma animal model (Fig. 8). It should be noted that although TGF-β2 expression in our model was substantially reduced, it was not completely abrogated. These results can be attributed to the short biological half-life of ODNs [37, 39, 61] and implies that the efficient and adequate reduction of TGF-β2 protein expression depends on the development of an efficient ODN delivery system.

After establishing the effectiveness of our AS-ODN to decrease the level of TGF-β2 protein production, experiments were carried out to test the hypothesis whether direct inhibition of TGF-β2 by AS-ODNs could reverse TGF-β2-induced immunosuppression and enhance the effect of systemic tumor vaccines. The results reported in this study clearly demonstrate a synergistic effect (Fig. 9). Previous experiments from our laboratory have demonstrated that vaccination alone was inadequate in extending animal survival in the presence of pre-established tumor [41]. In this previous work, when animals with pre-existing tumor were compared against those with tumor introduced following systemic vaccination, both groups showed an abundance of intracranial tumor infiltrating lymphocytes. The primary difference between the groups was a significant down-regulation in peritumoral lymphocyte anti-tumor cytotoxicity in the group with pre-existing tumor. Therefore, it appears that the benefit of systemic tumor vaccine when combined with local intracranial TGF-β2 inhibition is based on the reversal of this local tumor-induced immunosuppression. We did not, however, further measure immune function in these animals, either in our previous work [41] or in this current experiment. It should also be acknowledged that spontaneous lymphocytic baseline inflammation to various implanted gliomas cell lines has been reported [62, 63]. However, whatever the mechanism, to our knowledge, this is the first study to demonstrate that reversal of tumor-induced immunosuppression by inhibition of TGF-β2 production is associated with an improved survival time following systemic vaccine therapy in immuno-competent animals with pre-established intracranial glioma.

An alternative interpretation of the results might be that the anti-sense therapy alone afforded some tumor growth inhibition, making the tumor more susceptible to vaccine therapy.

It should be noted that although animals treated in our study demonstrated prolonged survival, the immune system appeared incapable of completely eradicating tumor in the majority of the animals treated. The efficacy of this combined therapy undoubtedly is dependent on both the duration, and the degree, of TGF-β2 reduction, as well as the efficacy of the systemic vaccination. Work is currently in progress to better characterize the precise mechanisms of tumor suppression by TGF-β2 in this paradigm. We are also attempting to maximize the effect of both of these interventions, exploring the use of convection-enhanced delivery of AS-ODNs and the use of more potent dendritic cell vaccines.

In summary, our data supports the hypothesis that inhibition of local TGF-β2 production enhances the anti-tumor effect of systemic active specific immunotherapy. This work provides the basis for future studies

investigating novel strategies designed to inhibit tumor-induced immunosuppression as an adjunct to systemic vaccine therapy. In this treatment paradigm in the clinical setting, we foresee surgical debulking remaining an important treatment modality. Surgery along with treatment with antisense oligonucleotides and vaccine would result in a maximal inhibition of tumor related TGF-β secretion.

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