The Excitatory Amino Acid Transporter-2 Induces Apoptosis and Decreases Glioma Growth In vitro and In vivo

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Abstract

Accumulating evidence suggests that glutamate plays a key role in the proliferation and invasion of glioblastoma tumors. Astrocytic tumors have been shown to release glutamate at high levels, which may stimulate tumor cell proliferation and motility via activation of glutamate receptors. Excess glutamate has also been found to facilitate tumor invasion by causing excitotoxic damage to normal brain thereby paving a pathway for tumor migration. Results from tissue microarray analyses showed decreased excitatory amino acid transporter-2 (EAAT-2) expression in high-grade glial tumors compared with low-grade astrocytomas and normal brain. EAAT-2 expression was inversely correlated with tumor grade, implicating its potential role in glial tumor progression, which was reflected by an undetectable level of EAAT-2 protein in glioma cell lines. In this study, we sought to investigate the effect of reconstituted EAAT-2 on glioma cell growth in vitro and in vivo by adeno viral-mediated gene transfer. Infection of glioma cells with Ad-EAAT-2 resulted in a physiologic level of functional EAAT-2, and a subsequent dose-dependent reduction in cell proliferation in all glioma cell lines tested compared with controls. Interestingly, results from analyses of Annexin V staining, detection of poly(ADP-ribose)polymerase cleavage and caspase-3 activation all indicated that Ad-EAAT-2 infection elicited apoptosis in glioma cells. Ex vivo experiments in nude mice showed a total suppression of tumor growth at sites that received Ad-EAAT-2-infected cells. Collectively, our results uncovered a new function of EAAT-2 in controlling glioma proliferation. Further studies will improve our knowledge of the role of glutamate in glioma growth and may provide useful prognostic information and alternative therapeutic targets for the treatment of glioma. (Cancer Res 2005; 65(5): 1934-40)

Introduction

There is accumulating evidence that glutamate plays a key role in the proliferation and invasion of glioblastoma tumors. Glutamate is a ubiquitous and important neuronal excitatory neurotransmitter in the central nervous system where it acts on the ligand-gated N-methyl-D-aspartate channel, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, and G protein-coupled glutamate receptors. In addition to its role in synaptic transmission, activation of ligand-gated N-methyl-D-aspartate and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid ion channels and G protein-coupled receptors is important for neuronal migration, survival, and differentiation (1–3). Glutamate also seems to be important for neuronal precursor cell proliferation (4, 5). Extracellular glutamate levels are regulated by glutamate transporters, which play a central role in the uptake of synthetically released glutamate, thereby preventing excitotoxicity and neuronal death. Inactivation of synthetically released glutamate is regulated through reuptake by glutamate transporters, of which the astrocytic excitatory amino acid transporter-2 (EAAT-2) is the most abundant. Loss of glutamate transporters can cause elevated levels of extracellular glutamate, which has been linked to the pathogenesis of several neurologic disorders, including motor neuron disease (6) and Alzheimer’s disease (7).

Surprisingly, elevated extracellular glutamate levels have been shown in glioma (8). The liberation of glutamate depends on de novo synthesis of glutamate from glutamine, which is released across the cell membrane in exchange for cystine via a glutamate-cysteine exchanger termed “system Xc” (8, 9). Over time, glioma cells in culture and in xenograft models release levels of glutamate that are toxic to normal neurons and glia (8, 10, 11). A second important reason that glioma cells have elevated extracellular glutamate levels is because glioma cells lack glutamate transporter expression (11, 12), which also contributes to glutamate excess.

It is not known why glioma cells release glutamate. Several studies have shown that N-methyl-D-aspartate, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, and G protein-coupled glutamate receptors are located on tumor cells with an astrocytic lineage (13–16). Astrocytic tumor cells that release glutamate might stimulate tumor cell proliferation and motility via autocrine or paracrine activation of glutamate receptors. Antagonism of N-methyl-D-aspartate and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid glutamate receptors has been shown to decrease tumor cell proliferation via decreased cell division (14). Likewise, pharmacologic antagonism of glutamate G protein-coupled receptors decreased glioma cell proliferation in vitro (15), suggesting that some tumors are sensitive to glutamate antagonists. Excess glutamate could also facilitate invasion by causing excitotoxic damage to normal brain. Tumors secreting high levels of glutamate grew faster and shortened animal survival in a rat C6 and RG2 glioma xenograft model, which may be due to excitotoxic damage to normal brain cells carving a pathway for tumor migration (10).

Our data show an inverse relationship between EAAT-2 expression and tumor grade; EAAT-2 is expressed at low levels in human high-grade glioma biopsy specimens, whereas its expression is greater in low-grade tumors. In the present study, we show that EAAT-2 expression decreases glioma cell proliferation in a time- and dose-dependent fashion in vitro. EAAT-2-induced growth suppression is due at least in part to induction of apoptosis. Consistent with stimulation of the apoptotic cascade, we observed induction of caspase-3 activation and...
Materials and Methods

Cell Culture and Culture Conditions. The human glioblastoma cell lines U87, U373, SNB19, and U251 were originally obtained from the American Type Tissue Culture Collection (Manassas, VA). All cell lines were grown in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin-streptomycin in a 37°C humidified incubator with 5% CO₂.

Construction of Adenoviral Vectors. Two replication-defective recombinant adenoviral vectors were used. The EAAT-2 adenoviral vector was generated using the AdEasy Adenoviral Vector System (Qbiogene, Carlsbad, CA). cDNA was obtained from Dr. Jeffrey Rothstein at the Johns Hopkins Hospital, Baltimore, MD. The EAAT-2 cDNA was subcloned into the pCMV-shuttle vector and cotransfected via electroporation with pAdEasy-1 adenoviral vector into BJ5183 electrocompliant cells and selected with kanamycin (50 μg/mL). Positive clones were isolated, expanded and digested with PstI prior to transfection into 293 cells using Fugene 6 (Roche Diagnostics Corp., Indianapolis, IN). Recombinant virus was amplified in the 293 cell line, and titers were determined by standard plaque assays (17) and purified using CsCl gradient centrifugation (18). Screening of recombinant EAAT-2 adenovirus was done with Western blotting. A control adenovirus with deletion of E1 (Ad-Δ-E1; ref. 19) was used as a control.

Adenoviral Infections. For infections, 1 × 10⁶ cells were plated in 10 cm tissue culture plates. The following day, the cells were washed and incubated with purified virus in 5 mL of DMEM/F12 media containing 10% fetal bovine serum for 1 hour at 37°C in a humidified atmosphere containing 5% CO₂/95% air, with brief agitation every 15 minutes. Cells were infected with 50 or 100 μl of dilution of infection (MOI) which was based on the original cell number plated in all experiments. After 1 hour, fresh DMEM supplemented with 10% fetal bovine serum was added to each dish.

Western Blot Analysis. Glioblastoma cell cultures were harvested and homogenized by sonication in ice-cold radioimmunoprecipitation assay buffer (50 mmol Tris-HCl, 20 μg antipain, 20 μg leupeptin, 1 mmol EDTA, and 5 mmol EGTA). Protein concentration was determined against control protein levels (bovine serum albumin) using the Bio-Rad Laboratories (Hercules, CA) protein assay kit, and aliquots of protein were separated using SDS-PAGE on 10% gels. Proteins were transferred by electroblotting to polyvinylidene fluoride membranes. Blots were blocked with 5% nonfat dry milk in 0.1% Tween 20, 50 μmol PBS at room temperature and incubated with primary antibody [rabbit anti-rat GLT-1/(EAAT-2) courtesy of Dr. Rothstein], rabbit anti-human PARP antibody (Promega, Madison, WI), mouse actin antibody (Chemicon, Temecula, CA) overnight at 4°C. Blots were washed, incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody, and immunoreactive proteins were observed via enhanced chemiluminescence (Bio-Rad).

Cell Proliferation/Survival Assay. Cells were infected as described above and 24 hours following infection, cells were washed gently, trypsinized, and counted in a Coulter counter using the trypan blue dye exclusion method (BD Biosciences, San Jose, CA). Viable cells were plated in 96-well plates at a density of 1 to 2 × 10⁵ in 0.2 mL DMEM/F-12 culture medium supplemented with fetal bovine serum. The cells were then allowed to grow. At the times specified, cells were washed with PBS and 100 μL of 2 mg/mL tetrazolium salt 3-[4,5]-dithiobis(2-nitrobenzoic acid) at 37°C overnight. Secondary antibody incubation was done at room temperature for 30 minutes. Mayer's hematoxylin nuclear staining was used as a counterstain. Next, an intensity score was assigned to each sample that represented the average intensity of the positive tumors on an arbitrary scale of 0 to 4+ in reference to average background staining intensity. Statistical analysis was done using a Kruskal-Wallis analysis of ranks.

Antitumor Activity in an Ex vivo Nude Mouse Tumor Xenograft Model

For the tumorigenicity experiments, U87 cells were grown, counted, and plated as described above. Cultured cells were infected with 1 × 10⁶ plaque forming units of Ad-Δ-E1 or Ad-EAAT-2 and at 24 hours post-infection, 2 × 10⁶ cells in 100 μL of serum containing media were injected s.c. into bilateral posterior flanks of 8-week-old female nude mice. Animals were followed daily and when flank tumor was visible, tumor size was recorded every 7 days. Animals were sacrificed at 35 days post-inoculation or when tumor burden reached 10 mm in diameter. Tumor sizes from animals that were inoculated with EAAT-2-infected U87 cells were compared with those from uninfected (mock) and Ad-Δ-E1-infected cells. Tumor volume was calculated for each flank tumor using the formula $V = \frac{d^2 \cdot h}{2}$, where $d$ is the diameter and $h$ is the half of the height. A nonparametric Friedman's two-way ANOVA was used to test the difference between the mean tumor volumes from each group.
Results

Human High-Grade Glioma Lack EAAT-2 Expression. A tissue microarray containing primary brain tumors of different grades was used to assess EAAT-2 expression by immunostaining. An array containing 44 primary brain tumors was prepared from formalin-fixed, paraffin-embedded archival tissue blocks as previously described (20–22). Tumor histologic diagnosis was confirmed by a neuropathologist (G.N. Fuller) and included 12 glioblastoma, 8 anaplastic astrocytomas, 11 anaplastic oligodendrogliomas, 8 oligodendrogliomas, and 3 low-grade astrocytomas. Examples of representative tumor specimens and positive control (normal cortex) from the microarray are shown in Fig. 1A. Tumors were grouped into their respective WHO 2000 histologic tumor grade (glioblastoma, grade 4; anaplastic astrocytoma and anaplastic oligodendroglioma, grade 3; oligodendroglioma and low-grade astrocytoma, grade 2) for analysis. Quantification and scoring of staining intensity revealed that high-grade glioblastoma had very faint or no EAAT-2 expression, whereas anaplastic astrocytoma and anaplastic oligodendroglioma had variable EAAT-2 expression, and the low-grade oligodendroglioma and low-grade astrocytoma tumors had the highest expression (Fig. 1B). Kruskal-Wallis analysis of ranks confirmed these results to be highly significant (P = 0.0003). These data suggested that EAAT-2 might be involved in glioma progression.

Ad-EAAT-2 Restores EAAT-2 Protein Expression in Cultured Glioma Cell Lines. Consistent with findings from tumor tissues and previous studies (1, 23), Western blot analyses of 14 glioma cell lines revealed the lack of EAAT-2 expression in all cell lines. Figure 2A showed the expression of EAAT-2 in four representative cell lines. Based on these observations, we sought to investigate the effect of econstituted EAAT-2 expression on glioma cell proliferation in vitro and in vivo. A recombinant EAAT-2 adenovirus was generated for this study. Following infection with 100 MOI of Ad-EAAT-2, glioma cell lines expressed elevated levels of EAAT-2, which were similar to normal rat cortex control levels, as determined by Western blotting (Fig. 2A). The transduced EAAT-2 protein localized mainly on the cell surface, as shown by immunohistochemical analysis (Fig. 2B).
EAAT-2 Expression Decreases Proliferation of Glioma Cell Lines In vitro. The effect of adenoviral delivery of EAAT-2 on glioma cell proliferation was determined for U251, U87, U373, and SNB19 glioma cell lines using the tetrazolium salt 3-[4,5-yl] 1 2,5 bromide assay and was defined as the percentage of viable Ad-EAAT-2-infected cells compared with adenoviral deleted E1 (Ad-Δ-E1)-infected cells and mock-infected controls. Twenty-four hours following infection with 50 and 100 MOI of adenoviruses, cells were plated in 96-well plates and viability was assayed at days 1, 2, 3, 5, and 7. As shown in Fig. 3A-D, EAAT-2 induced a dose- and time-dependent decrease in cell viability compared with Ad-Δ-E1-infected controls in U251 (A), U87 (B), U373 (C), and SNB19 (D) cells. There was no significant difference in cell viability between mock- and virus-infected (Ad-Δ-E1) controls (data not shown). U251 cells were less sensitive to the effects of EAAT-2 expression than U87, SNB19, and U373.

EAAT-2 Induces Apoptosis in U251, U87, U373, and SNB19 Glioma Cell Lines. The growth inhibition of glioma cell lines in vitro was further investigated to determine if cell death occurred via apoptosis or necrosis. We did costaining with Annexin V and PI, which detects apoptosis and necrosis, respectively (24, 25). Within the apoptotic cell population, cells in the early stages of apoptosis were Annexin V-positive and PI-negative. The population of cells in late stage apoptosis were Annexin V-positive and PI-positive. As shown in Fig. 4, at 72 hours post-Ad-EAAT-2 infection, analysis by flow cytometry of Annexin V- and PI-stained positive cells showed that U251, U87, U373, and SNB19 glioma cells were undergoing programmed cell death. Approximately 13% to 22% of cells were undergoing apoptosis at the time of the flow cytometric evaluation. The U87 cell line seemed to be less sensitive to the apoptosis-inducing effects of EAAT-2 because viability was only decreased to 60% at 7 days compared with 50%, 10%, and 20% in U251, U373, and SNB19 cell lines, respectively. U87 had the least amount of Annexin V staining as well as the least amount of growth suppression as seen in the tetrazolium salt 3-[4,5-yl] 1 2,5 bromide assay, suggesting that an alternate mechanism of cell death such as necrosis was involved. U87 contains wild-type p53 and U251, U373, and SNB19 have a mutant copy of the tumor suppressor p53. To determine if p53 status plays a role in the mechanism of cell death following EAAT-2 expression, D54 glioma cells, which also contain wild-type p53, were evaluated as described. More than 20% of cells were Annexin V-positive, showing activation of programmed cell death as seen in the other cell lines (data not shown), suggesting that p53 does not determine sensitivity to EAAT-2-induced apoptosis. Confirmation of cell death induction via apoptosis was determined by evaluating caspase-3 activation and PARP cleavage. Confirming the flow cytometry data, U251, U373, and SNB19 cell lines showed a time-dependent increase in caspase-3 activation over mock- and Δ-E1-infected controls in spectrophotometric assay results (Fig. 5A). Likewise, Western analysis using anti-PARP antibodies showed a time-dependent increase in PARP cleavage that was not seen in the control lanes. The U87 cell line had a large increase in caspase-3 activation but did not show observable PARP cleavage (Fig. 5B).

Ex vivo EAAT-2 Infection Prevents U87 Tumorigenicity in a Flank Tumor Model. Expression of EAAT-2 caused growth arrest and induced apoptosis in glioma cell lines in vitro. To evaluate the effect of EAAT-2 on in vivo tumorigenicity, we infected U87 cells with Ad-EAAT-2 ex vivo and subcutaneously implanted the cells in nude mice 24 hours following infection. Tumor size was measured serially over time. Tumors were observed in mock- and Ad-Δ-E1-infected controls starting at 1 week post-inoculation. By 2 weeks, tumor growth was evident in the control groups but unmeasurable in the EAAT-2-expressing tumors. Average tumor volume for the mock- and Ad-Δ-E1-infected tumor cells was significantly higher than for Ad-EAAT-2-infected U87 cells (P < 0.001). Figure 6 shows the average tumor volume over time Ad-EAAT-2-infected tumor cells did not show any evidence of tumor. Only 30% to 40% of Ad-EAAT-2-infected U87 cells underwent apoptosis in vitro, suggesting that Ad-EAAT-2 also decreases the ability of tumors to form in vivo. Taken together, these results suggest that EAAT-2 suppresses tumor formation of U87 cells in an ex vivo flank tumor model.

Figure 3. Ad-EAAT-2 decreases glioma proliferation in a dose- and time-dependent fashion. Glioma cell lines were treated with 50 or 100 MOI Ad-EAAT-2 and proliferation was determined at 1, 2, 3, 5, and 7 days post-infection using the tetrazolium salt 3-[4,5-yl] 1 2,5 bromide assay, as described in Materials and Methods.
Discussion

Recent reports show that glutamate and its receptors are important in glioma proliferation and invasion (12, 14, 15). Glutamate levels are elevated following its release in exchange for cystine uptake (11, 12), which is exacerbated by the lack of glutamate uptake due to a lack of EAAT-2 expression (11). Although the participation of EAAT-2 in regulating extracellular glutamate levels and preventing excitotoxic injury in normal brain is well characterized, its function in tumor proliferation and invasion is unknown. Here we show that EAAT-2 is differentially expressed in primary brain tumors, and that there is a highly significant association between higher tumor grades and lack of EAAT-2 expression. This association highlights its effect on the functional pathways that influence the proliferative and invasive phenotype of high-grade tumors. To examine EAAT-2 transporter function in glioma biology, we developed an adenovirus vector for its delivery to multiple glioma cell lines. Ad-EAAT-2 infection induced time- and dose-dependent growth inhibition in several glioma cell lines via induction of the apoptotic cascade, similar to the effects seen following transient transfection of U251 glioma cells (26), ultimately resulting in caspase-3 activation and PARP cleavage. Additionally, when tumor cells were infected with Ad-EAAT-2 before being injected into the flank of nude mice, the cells' ability to form tumors in vivo was significantly inhibited ($P = 0.0003$).

The loss of EAAT-2 glutamate transporter expression is one of the putatively responsible mechanisms leading to elevated levels of glutamate in glioma (11). In addition to its potential role in seizure induction (8, 27), glutamate release is thought to provide a growth advantage for glioma via activation of glutamate receptors (14, 15) and/or destruction of normal brain tissue (10), leading to enhanced tumor migration and invasion. Our demonstration of inhibited tumor cell growth in vitro and in vivo resulting from reintroducing the EAAT-2 transporter into glioma cell lines suggests that EAAT-2 has a novel role in the growth regulation of glial tumor cells. The activation of apoptotic pathways mediates the growth inhibition induced by EAAT-2, and can be potentially explained by more than one mechanism. One mechanism is that EAAT-2 could mimic tumor suppressor gene activity. Several tumor suppressor genes, including p53 and pRb, are well known inhibitors of cell proliferation and cell death via mediation of cell cycle progression and apoptosis. Tumor suppressor gene activity is dysregulated in cancer where inactivation of tumor suppressor genes results in altered cell proliferation and tumorigenesis (23, 28). As our results suggest, if EAAT-2's previously unidentified function involving growth suppression is actually at play, the lack of EAAT-2 expression could promulgate the uncontrolled growth of tumor cells. The tissue microarray data presented in this paper corroborate this hypothesis and show that the expression of EAAT-2 is progressively lost with higher grades of tumor. The lower levels of EAAT-2 expression found in high-grade glioma could contribute significantly in the transition of a lower-grade tumor to a more malignant phenotype. This loss of EAAT-2 expression in higher-grade tumors points to the possible effect of EAAT-2 on the growth-inhibitory pathways that are important in advanced tumor stages. In support of this concept, reintroducing EAAT-2 protein in our experiments apparently restored a growth-regulatory function in proliferating tumor cells, which ultimately led to cell death.

A second mechanism by which EAAT-2 could induce apoptosis in glioma is in providing cellular stress by directly or indirectly activating proapoptotic signaling pathways. EAAT-2 expression in tumor cells could result in the generation of reactive oxygen species (29, 30), increase Ca$^{2+}$ influx (31), or result in loss of the mitochondrial membrane potential leading to a depletion of essential intracellular nutrients (adenosine triphosphate; ref. 32), eventually altering cellular homeostasis and promoting apoptosis. Functional studies of the EAAT-2 glutamate transporter show that H$^{+}$ ions are cotransported into the cell in addition to glutamate uptake (33). Accumulation of intracellular H$^{+}$ ions may markedly decrease intracellular pH, which has been shown to induce apoptosis in some tumor cell lines (34). Tumor cells that release more glutamate might be more sensitive to EAAT-2 expression because glutamate and H$^{+}$ ions are cotransported. It also seems that tumor cells are more susceptible to decreases in intracellular pH than normal cells (35) so that EAAT-2 might directly induce apoptosis by decreasing intracellular pH. Additionally, although there are no known interactions between glutamate transporters and mitochondrial or death receptor pathways, EAAT-2 expression could alter the balance between proapoptotic and antiapoptotic signals to favor apoptosis in glioma cells. We are currently examining the intracellular events preceding the initiation of apoptosis in a glioma cell population.

In normal astrocytes, EAAT-2 plays a vital role in preventing accumulation of excitotoxic levels of glutamate (36–38). Loss of EAAT-2 expression in astrocytic tumors contributes to the elevated levels of glutamate that have been documented within and surrounding human gliomas (39, 40). Although the relevance of these findings is unknown, studies suggest that glutamate is
directly responsible for tumor growth by destroying normal brain, thereby enhancing tumor growth and invasion (10). Our results show that EAAT-2 expression is growth-inhibitory in vitro where it significantly lowered extracellular glutamate levels. Loss of the EAAT-2 transporter in glioma may not only enhance tumor cell survival in vivo through elevating glutamate levels, but may also be intrinsically advantageous for tumor cell survival. Although we do not know the relationship between EAAT-2-mediated lowering of extracellular glutamate levels in vitro and the induction of tumor cell apoptosis, this observed effect is independent of proposed cell survival mechanisms in vivo. Thus, not only might loss of EAAT-2 expression provide a growth advantage in vivo, it may also, in some way, be harmful to tumor cells.

Glioma cell lines in culture were not equally sensitive to the growth-inhibitory effects of Ad-EAAT-2 infection. The U373 and SNB19 cell lines were more sensitive than U251 and U87 cells, as shown by the more rapid and greater decrease in cell viability over time in these cell lines, which could be due to different levels of EAAT-2 expression. For example, as shown in Western blotting, U373 has the highest level of EAAT-2 expression and was the most sensitive to Ad-EAAT-2 infection. However, the U87 and SNB19 cell lines have similar levels of EAAT-2 expression, but the SNB19 cell line was more sensitive to the effects of EAAT-2 expression. The reason for this paradoxical relationship is unclear but it may be due to different genetic backgrounds in diverse cell lines. For example, in our studies, p53 status did not seem to influence the ability of EAAT-2 to induce apoptosis. Another explanation for the variable growth-inhibitory response to EAAT-2 may involve glutamate itself. Several groups have shown that glioma cells...
have varying levels of glutamate release (8, 10). It is possible that higher levels of glutamate release in different tumor types reflects a greater reliance on glutamate release per se. Such tumors are thus more sensitive to the effects of reintroducing the EAAT-2 gene, which removes glutamate from the extracellular space. If glioma cells have a mechanism by which glutamate receptor activation enhances proliferation, then decrements in extracellular glutamate would result in removing the growth-stimulatory actions of glutamate.

In summary, our results show that human high-grade glioblastoma tumors do not express EAAT-2. Adenoviral-mediated delivery of the EAAT-2 transporter at levels comparable to those seen in normal tissue resulted in growth inhibition in vitro via induction of apoptosis as well as inhibited tumorigenesis in vivo. Thus, our study suggests that loss of EAAT-2 may play a permissive role as tumors become more aggressive. Our study lays the foundation for further studies examining the role of this important transporter. A better understanding of the importance of glutamate release, and the uptake and regulation of glutamate transporters may provide greater insight into the molecular mechanisms involved in glioma progression.

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References

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