Oncogene AEG-1 Promotes Glioma-Induced Neurodegeneration by Increasing Glutamate Excitotoxicity

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Abstract

Aggressive tumor growth, diffuse tissue invasion, and neurodegeneration are hallmarks of malignant glioma. Although glutamate excitotoxicity is considered to play a key role in glioma-induced neurodegeneration, the mechanism(s) controlling this process is poorly understood. Astrocyte elevated gene-1 (AEG-1) is an oncogene that is overexpressed in several types of human cancers, including more than 90% of brain tumors. In addition, AEG-1 promotes gliomagenesis, particularly in the context of tumor growth and invasion, 2 primary characteristics of glioma. In the present study, we investigated the contribution of AEG-1 to glioma-induced neurodegeneration. Pearson correlation coefficient analysis in normal brain tissues and samples from glioma patients indicated a strong negative correlation between expression of AEG-1 and a primary glutamate transporter of astrocytes EAAT2. Gain- and loss-of-function studies in normal primary human fetal astrocytes and T98G glioblastoma multiforme cells revealed that AEG-1 repressed EAAT2 expression at a transcriptional level by inducing YY1 activity to inhibit CBP function as a coactivator on the EAAT2 promoter. In addition, AEG-1–mediated EAAT2 repression caused a reduction of glutamate uptake by glial cells, resulting in induction of neuronal cell death. These findings were also confirmed in samples from glioma patients showing that AEG-1 expression negatively correlated with NeuN expression. Taken together, our findings suggest that AEG-1 contributes to glioma-induced neurodegeneration, a hallmark of this fatal tumor, through regulation of EAAT2 expression. Cancer Res; 71(20); 6514–23. ©2011 AACR.

Introduction

Tumors of the central nervous system (CNS) are the most prevalent solid neoplasms of childhood and the second leading cancer-related cause of death in adults between the ages of 20 and 39 years (1, 2). Gliomas, the most common brain tumors of the adult CNS, originate from neuroepithelial tissue and are classified morphologically as astrocytic, oligodendroglial, ependymal, and choroid plexus tumors (2–4). Astrocytomas, composed predominantly of neoplastic astrocytes, account for 80% to 85% of all gliomas and are staged as low grade (grade I) to high grade (grade IV) on the basis of nuclear atypia, mitotic activity, endothelial hyperplasia, and necrosis (4). Glioblastoma multiforme (grade IV astrocytoma; GBM) is an extremely aggressive, invasive, and destructive malignancy with a proliferation rate that is 2 to 5 times faster than grade III tumors (2, 5). Extensive surgical resection is not curative due to the highly invasive capacity of GBM cells into normal brain parenchyma (3). Moreover, glioblastoma multiforme is largely resistant to currently available treatments that are based on cytotoxic approaches targeting replicating DNA, such as chemotherapy or radiotherapy (6–8). In addition to uncontrolled proliferation and diffuse tissue invasion, neurodegeneration is another attribute of malignant gliomas (2, 9–11).

The mechanisms of glioma-induced neurodegeneration are poorly understood although excitotoxic levels of glutamate play a key role in this phenomenon (10, 11). Although glutamate is a major neurotransmitter implicated in most aspects of normal brain functions, it is a potent neurotoxin at high concentration, indicating that glutamate must be constantly removed for maintenance at a low level (12, 13). The excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2), predominantly expressed on astrocytes, are responsible for the
clearance of excitotoxic levels of glutamate from synapses, and an impaired glutamate uptake by glial cells causes widespread neurodegeneration and lethal epilepsy (14–16). Furthermore, a number of studies have reported that glioma cells release high levels of glutamate, which causes neuronal cell death and promotes malignant glioma progression (17–20).

Astrocite elevated gene-1 (AEG-1) is a multifunctional oncogene that is overexpressed in a variety of human cancers, although it was originally isolated as a novel HIV-1- and TNF-α–induced transcript from primary human fetal astrocytes (PHFA; refs. 21, 22). As a target of Ras, AEG-1 activates multiple oncogenic signaling pathways including phosphoinositide-3 kinase (PI3K)–Akt, mitogen-activated protein kinase (MAPK), Wnt, and NF-κB involved in regulation of proliferation, invasion, chemoresistance, angiogenesis, and metastasis (21, 23–30). In particular, AEG-1 showed higher expression, compared with that in normal brain tissues, in tumors of the CNS, such as neuroblastoma, glioblastoma multiforme, and oligodendroglioma (28, 30–32). Gain- and loss-of-function studies in glioma cells revealed crucial roles in proliferation and invasive ability of glioma cells (28, 31). In addition, in vivo experiments using orthotopic glioma models confirmed the role of AEG-1 in glioma progression (28, 31). Furthermore, we observed an interesting inverse correlation between the expression levels of AEG-1 and EAAT2. AEG-1 expression is elevated following treatment of astrocytes, whereas EAAT2 expression is downregulated (22, 33–36). In the setting of glialoma progression, AEG-1 gradually increases as astrocytes evolve into malignant glioma while, in parallel, EAAT2 expression decreases (11, 17, 18, 28, 31, 32, 37). Both HIV-1 infection and glioma progression are associated with neurodegenerative changes, and glutamate excitotoxicity is one of the predominant mechanisms mediating neurodegeneration. On the basis of these considerations, we presently investigated the role of AEG-1 in glioma-induced neurodegeneration with a focus on regulation of the glutamate transporter and its concomitant control of glutamate levels.

Materials and Methods

Tissue array and immunostaining

Immunofluorescence analyses in human glioma tissue arrays (GL806) obtained from Tissue Array Networks were conducted as previously described (28). Anti-AEG-1, anti-EAAT1, and anti-EAAT2 antibodies were described (34, 38) and the anti-NeuN antibody was purchased from Millipore. Images were captured with a confocal laser scanning microscope LSM multiphton 510 META (Zeiss), and analyzed using ImageJ (NIH). For analyzing localization of AEG-1, PHFA cells seeded onto 4-well chamber slides were transfected with pcDNA, AEG-1, or each AEG-1 deletion construct. Two days later, the cells were fixed and immunostaining was carried out with anti-HA antibody (Covance) as described (27).

Cell lines

Normal PHFAs, human glioma cell lines H4 (neuroglioma), T98G (glioblastoma multiforme), and U251-MG (neuronal glioblastoma) cells were previously described (26, 28). PC-12 (rat pheochromocytoma) cells were purchased from the American Type Tissue Culture and Collection and cultured in Dulbecco’s modified Eagle medium (DMEM) with 5% FBS and 10% heat-inactivated horse serum at 37°C. The normal control sh (NCsh), AEG-1sh-2, and AEG-1sh-4 cell lines were established by transfection with control short-hairpin RNA (shRNA), AEG-1 shRNA #2, and AEG-1 shRNA #4 expression plasmids (SA Biosciences; catalogue no. KH18459H1) in T98G cells, respectively, and selected with hygromycin.

Recombinant adenovirus, siRNA, and plasmids

Both Ad.vec and Ad.AEG-1 have been previously described (26). Control and YY1 siRNAs were purchased from Santa Cruz Biotechnology. The expression plasmids of AEG-1 and AEG-1 deletion mutants tagged with hemagglutinin (HA) were described previously (25). The N′-deletion mutants N1–N5 include amino acids 71–582, 101–582, 205–582, 232–582, and 262–582, respectively. The C′-deletion mutants C1–C4 include amino acids 1–513, 1–404, 1–356, and 10–289, respectively. The 5′-deletion mutants of the human EAAT2 promoter–reporter (EAAT2Pro) constructs and NF-κB–Luc have been described previously (25, 35, 38). The EAAT2Pro-954mYY1 and EAAT2Prom-954mNFκB1 constructs were made using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) in the context of the EAAT2Pro-954 construct. The sequences used for PCR primers include: EAAT2 Pro-954mYY1, 5′-TGGAGCCCCCGGAGCTCCCCGCCAAGCATTATCCCGCG-3′, and EAAT2Prom-954mNFκB1, 5′-TCCGAGCCCCCGGAGCTCAAAAGCCAGCCGATCCCGCG-3′. The mutated sequences are underlined.

Western blotting and immunoprecipitation assays

Whole-cell lysates were prepared, and communoprecipitation and Western blot analyses were carried out as described previously (25, 38). The antibodies for YY1 (Santa Cruz Biotechnology), CBP (Abcam), and EF1α (Upstate) were purchased. Whole-cell lysates from human tissue samples were previously described (28).

Northern blotting, real-time PCR, and nuclear run-on assays

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). Real-time PCR (RT-PCR) was conducted using the ABI 7900 Fast Real-Time PCR System and TaqMan Gene Expression Assays for individual mRNAs (Applied Biosystems). The nuclei were extracted using NP40 lysis buffer, and nuclear run-on assays were conducted (38).

Transient transfection and luciferase assays

Cells were plated in 24-well plates, infected with Ad.vec or Ad.AEG-1, and transfected with the indicated plasmids and Renilla luciferase plasmid (Promega) together with 20 nmol/L of control or YY1 siRNA using LipofecAMINE 2000 (Invitrogen); luciferase activities were measured using a Dual-Luciferase Reporter Assay Kit (Promega). Firefly luciferase activity was normalized by Renilla luciferase activity.
Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were conducted as described (38). The sequences of oligonucleotide used as probe include 5'-CGCCAAGCCCATCCCGCG-3' (the YY1 binding site is underlined) and the mutant oligonucleotide 5'-CGCCAAGCATTCGCCGCG-3' (the mutated sequences are in bold type).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were conducted using the ChIP-IT Kit (Active Motif). The primers for the human EAAT2 promoter used are as following: sense, 5'-ATCGCTCTCTCGGGGAAGCCA-3'; antisense, 5'-TAAGCCCTTTAGCGCCTCAA-3'.

Glutamate uptake assays

Assays to determine glutamate uptake were conducted as described (38).

Cell viability assays

Cells were treated with 1% heat-inactivated horse serum containing 100 μmol/L of glutamate for 10 or less minutes, and the conditioned media were collected. PC-12 cells (1 x 10⁴ cells/well) were seeded in 24-well plates, and treated with neuronal differentiation media [DMEM with 1% heat-inactivated horse serum supplemented with 1 mmol/L di-butyryl cAMP, Sigma; and 50 ng/mL of nerve growth factor (NGF), Promega] for 3 to 6 days. Then, the PC-12–derived neuron cells were treated with conditioned media for 1 day. Cell viability was measured by an MTT assay (24).

Statistical analysis

Data were presented as mean ± SEM and analyzed for statistical significance using the unpaired Student’s t test. Pearson correlation coefficient (r) analysis was used to compare gene expressions between 2 genes.

Results

AEG-1 expression negatively correlates with EAAT2 expression and the number of neuronal cells in glioma patients

To examine a possible correlation between the expression of AEG-1 and EAAT2 in glioma, we first carried out immunofluorescence staining of AEG-1 and EAAT2 in samples from glioma patients using a tissue array containing 35 cases of glioma and 5 normal brain tissues in duplicate (Fig. 1A–C). The specificity of anti-AEG-1 and anti-EAAT2 antibodies for immunostaining was confirmed by competition with each immunogen (Supplementary Fig. S1). Although the expression of AEG-1 greatly increased in samples from glioma patients compared with that in normal human brains, EAAT2 expression significantly decreased (Fig. 1A and C). A scatter-plot and Pearson correlation coefficient analysis revealed a strong negative correlation (r = -0.725) between expression of AEG-1 and EAAT2 (Fig. 1B). However, analysis of expression patterns of EAAT1, another glial...
glutamate transporter, indicated almost no difference between glioma and normal brain tissues and little correlation between expressions of AEG-1 and EAAT1 (Supplementary Fig. S2A–C). These results were also confirmed in samples from glioblastoma multiforme patients compared with normal brain tissues by Western blot analysis (Fig. 1D). These results indicate an inverse correlation between expressions of AEG-1 and EAAT2, but not EAAT1 in glioma. Dysregulation of EAAT2 causes glutamate excitotoxicity, which is implicated in various types of neurodegenerative diseases and glioma-induced neurodegeneration (10–14, 17–20, 37). Accordingly, we further quantified and compared the expression of AEG-1 and EAAT2 with NeuN, a neuron-specific marker in glioma patient samples. NeuN expression was decreased in glioma samples compared with normal cerebrum tissues, and a scatter-plot of the data and Pearson correlation coefficient analysis showed a strong positive correlation ($r = 0.798$) between expression of EAAT2 and NeuN (Fig. 1E and F). Furthermore, the comparison analysis revealed a strong negative correlation ($r = -0.649$) between expression of AEG-1 and NeuN (Fig. 1G), indicating that samples from glioma patients with their higher levels of AEG-1 have fewer neurons. These observations and the negative correlation between AEG-1 and EAAT2 expression (Fig. 1B) together with previous studies showing that AEG-1 has the ability to regulate promoter activity of its target gene (21, 25, 31, 34) suggested that AEG-1 in glioma might negatively regulate EAAT2 expression and glutamate uptake, thereby causing neuronal cell death in patients with glioma.

**AEG-1 represses expression of EAAT2**

To determine whether AEG-1 could repress EAAT2 expression, we first confirmed the negative relationship between expression of AEG-1 and EAAT2 in PHFA and human glioma cell lines (Fig. 2A). Then, we analyzed PHFA cells infected with Ad.vec or Ad.AEG-1. As shown in Figure 2B (left), overexpression of AEG-1 significantly reduced EAAT2 expression in PHFA. In addition, EAAT2 mRNA was strongly reduced by AEG-1 (Fig. 2B, middle). This reduction in EAAT2 mRNA expression resulted from decreased transcription, as confirmed using nuclear run-on assays (Fig. 2B, right). These observations document that AEG-1 negatively regulates EAAT2 expression at a transcriptional level. To further confirm the negative regulation of EAAT2 by AEG-1 in glioma, we cloned AEG-1 knockdown glioma cell lines (NCsh, AEG-1sh-2, and AEG-1sh-4) by stable transfection with a control, AEG-1 shRNA-2 or AEG-1 shRNA-4 plasmid in T98G cells, a highly aggressive glioma cell line with high levels of AEG-1 and low levels of EAAT2 (as shown in Fig. 2A). RT-PCR and Western blot analyses verified AEG-1 knockdown in the clones. AEG-1 knockdown increased EAAT2 expression (Fig. 2C) and recovery of AEG-1 expression in the knockdown cells reduced EAAT2 expression (Supplementary Fig. S3), confirming that AEG-1 is a negative regulator of EAAT2. In addition, to

![Figure 2. AEG-1 represses EAAT2 expression. A, AEG-1 and EAAT2 expression in various cell lines. B, PHFA cells were infected with multiplicity of infection (MOI) of 20 for Ad.vec or Ad.AEG-1 and Western blot analysis was carried out with the indicated antibodies, and EF1α served as an internal control (left). Using total RNAs from the infected cells, Northern blotting assays were conducted, and the levels of GAPDH mRNA served as a control (middle). Nuclear run-on assays with nuclei from infected cells were conducted, and the transcription rate of GAPDH was used as a control (right). C, real-time PCR (RT-PCR) analysis of AEG-1 and EAAT2 mRNA expression in NCsh (normal control sh), AEG-1sh-2, or AEG-1sh-4 cells (left). *, $P < 0.01$ versus NCsh. Cell lysates from NCsh, AEG-1sh-2, and AEG-1sh-4 were immunoblotted with the indicated antibodies (right). D, Ad.vec- or Ad.AEG-1–infected PHFA cells were transfected with EAAT2Pro–Luc. Transient transfection and luciferase assays were conducted at least 3 times in triplicate. Data: fold-normalized activity relative to that of Ad.vec–infected PHFA was taken as 1. *, $P < 0.01$ versus Ad.vec.](cancerres.aacrjournals.org/article/doi/10.1158/0008-5472.CAN-11-0782/fig-2){/caption}
investigate the role of AEG-1 in regulating the EAAT2 promoter, an EAAT2 promoter–reporter plasmid (EAAT2Pro-Luc) was transiently transfected into Ad.vec- or Ad.AEG-1–infected PHFA cells. AEG-1 potently induced NF-kB activity as previously shown (25; Supplementary Fig. S4), whereas it repressed EAAT2 promoter activity (Fig. 2D). Taken together, these results show that AEG-1 negatively regulates EAAT2 expression at a transcriptional level.

YY-1 is responsible for AEG-1–mediated EAAT2 repression

To determine the mechanism(s) by which AEG-1 regulates transcription of EAAT2, a series of 5′-deletion mutants of the EAAT2 promoter–reporter construct were transfected into Ad. vec- or Ad.AEG-1–infected PHFA cells. Serial deletions from −964 to −37 showed a similar pattern of AEG-1–mediated repression in promoter activity (Fig. 3A). These results suggest that transcription factors binding to the −37/+43 region are capable of regulating a reduction of EAAT2 promoter activity in response to AEG-1. Accordingly, we analyzed the −37/+44 region of the EAAT2 promoter using Transcription Element Search System (www.cbil.upenn.edu/cgi-bin/tess) to identify putative transcription factor binding sites potentially responsible for AEG-1–mediated repression. This analysis located 2 putative transcription factor binding sites, YY1 and NF-kB1, which are well-known transcription factors that function as activators or repressors, depending on cellular binding context of a target promoter and the presence of other transcription factor binding sites in the promoter (39, 40). To determine which transcription factor was responsible for EAAT2 promoter repression, we engineered 2 mutant EAAT2 promoter–reporter plasmids containing a site-directed mutation of the putative YY1 or NF-kB binding site in the −954 EAAT2 promoter construct (mYY1 and mNF-kB). These constructs were transfected into PHFA cells, and each promoter activity was compared with that of the wild-type plasmid −954EAAT2Pro–Luc. As shown in Figure 3B, mutation in the YY1 binding site abolished the EAAT2 promoter response to AEG-1, whereas mutation in the NF-kB site had no effect. These experiments indicate that YY1 is responsible for AEG-1–mediated EAAT2 promoter repression. To further clarify the role of YY1, we conducted EMSA using a 20-bp double-stranded probe containing the YY1 binding site of the EAAT2 promoter. As shown in Figure 3C (lanes 2 and 3), the intensity of a DNA–protein complex was significantly higher in Ad.AEG-1–infected PHFA nuclear extracts in comparison with Ad.vec–infected PHFA nuclear extracts. To further characterize this nucleoprotein complex, competition assays were conducted using an unlabeled probe or a mutant probe containing mutations in the YY1 binding site. As shown in Figure 3C, the cold probe (lane 4) completely competed with the DNA–protein complex, whereas the mutant probe (lane 5) had

Figure 3. YY1 is responsible for AEG-1–mediated EAAT2 repression. A, Ad.vec- or Ad.AEG-1–infected PHFA cells were transfected with each deletion EAAT2 promoter–reporter construct. Data: fold-normalized activity relative to that of the −954 EAAT2Pro–Luc in Ad.vec–infected PHFA taken as 1. B, the infected PHFA cells were transfected with EAAT2Pro−954, mNFkB1, or mYY1. Data: fold-normalized activity relative to that of the −954 EAAT2Pro–Luc in Ad.vec–infected PHFA was taken as 1. C, nuclear extracts from the infected PHFA cells were mixed with the probe containing the YY1 site of EAAT2 promoter in the following order: 1, no extracts; 2, Ad.vec; 3, Ad.AEG-1; 4, Ad.AEG-1 + cold wild probe (100×); 5, Ad.AEG-1 + unlabeled mutant probe containing mutated YY1 site (100×); 6, Ad.AEG-1 + anti-YY1 antibody; and 7, Ad.AEG-1 + control immunoglobulin G (IgG). Data: fold-normalized activity relative to that of Ad.vec–infected and control siRNA–transfected PHFA was taken as 1. E, the infected and transfected PHFA cell lysates were immunoblotted with the indicated antibodies. All transient transfection and luciferase assays were conducted at least 3 times in triplicate. Data in graphs present mean ± SEM. *, P < 0.01 versus Ad.vec.
little effect on complex formation. In addition, supershift experiments with an anti-YY1 antibody resulted in a retarded mobility of the complex, whereas a control immunoglobulin G (IgG) did not (Fig. 3C, lanes 6 and 7). In addition, YY1 siRNA inhibited the AEG-1-mediated EAAT2 promoter repression (Fig. 3D). These results were also confirmed by Western blot analysis using PHFA cell lysates treated with Ad.AEG-1 and YY1 siRNA (Fig. 3E). In total, these results indicate that AEG-1 increases YY1 binding to the EAAT2 promoter, which is responsible for AEG-1-mediated repression of EAAT2 expression.

AEG-1 directly interacts with YY-1 and CBP

AEG-1 physically interacts with a coactivator, cyclic AMP-responsive element binding protein (CREB)-binding protein (CBP), and YY1 interaction with CBP is 1 mechanism by which YY1 negatively or positively regulates gene expression (25, 39). On the basis of this consideration, we hypothesized that AEG-1 might play a role as a bridge between YY1 and CBP on the EAAT2 promoter, causing YY1 to function as a negative regulator of EAAT2 expression by inhibiting CBP. To examine this possibility, we first established whether these proteins directly physically interact with each other by immunoprecipitation assays using Ad.vec- or Ad.AEG-1–infected PHFA cell lysates. As shown in Figure 4A, anti-HA, anti-YY1, and anti-CBP antibodies effectively immunoprecipitated YY1 and CBP, AEG-1 and CBP, and AEG-1 and YY1, respectively. To clarify whether these associations occur on the EAAT2 promoter, PHFA cells were infected with Ad.vec or Ad.AEG-1, and ChIP assays were carried out using either control IgG or anti-HA, anti-YY1, or anti-CBP antibodies. Although only CBP was associated with the EAAT2 promoter in Ad.vec–infected PHFA cells, all 3 of the proteins, AEG-1, YY1, and CBP as a complex, bound to the EAAT2 promoter in Ad.AEG-1–infected PHFA cells (Fig. 4B). These results were further confirmed in T98G glioma cells that were highly expressing AEG-1. YY1, and CBP in NCsh cells were bound to the EAAT2 promoter as a complex, but AEG-1 knockdown in these cells abolished AEG-1 as well as YY1 association with the EAAT2 promoter (Fig. 4B). These data indicate that AEG-1 increases YY1 binding to the EAAT2 promoter and suggest that AEG-1 might function as a bridge molecule between YY1 and CBP and the basal transcription machinery, thus facilitating YY1 inhibition of CBP function as a co-activator on the EAAT2 promoter.

To further clarify these interactions, we analyzed which domain of AEG-1 was responsible for the interactions by using AEG-1 deletion constructs. As shown in Figure 4C, all C'-deletion mutants were associated with both YY1 and CBP as were wild-type AEG-1. However, only the N1 construct among the N'-deletion mutants interacted with YY1, and none of the N'-deletion constructs physically interacted with CBP (Fig. 4C), suggesting that, in each region, amino acids 1–70 and 71–100 of AEG-1 are responsible for interaction with CBP and YY1, respectively. We next examined whether these interactions would be crucial for AEG-1–mediated EAAT2 repression. As shown in Figure 4D, as expected only wild-type AEG-1 repressed the EAAT2 promoter activity whereas none of N'-deletion mutants induced repression. Intriguingly, all of the C'-deletion mutants failed to modify EAAT2 promoter activity (Fig. 4D). A recent study suggested that the predominant nuclear localization signal of AEG-1 is located at the end region of the C terminus (amino acids 546–582; ref. 41), which is missing in all of the C'-deletion mutants we analyzed. In addition, we confirmed that AEG-1

Figure 4. AEG-1 interacts with CBP and YY-1 on the EAAT2 promoter. A, the Ad.vec (V)- or Ad.AEG-1 (A)-infected PHFA cell lysates were used for immunoprecipitation (IP) analysis using anti-HA, anti-YY1, or anti-CBP antibody and the same antibodies for immunoblotting. B, the nuclear pellet containing chromatin isolated from the infected PHFA cells, NCsh, and AEG-1-hsh-2 cells were immunoprecipitated with control IgG, anti-AEG-1, anti-YY1, or anti-CBP antibody, and then the eluted DNAs were subjected to PCR. C, PHFA cells were transfected with control vector (pcDNA), full-length AEG-1, or each AEG-1 deletion-expression construct as indicated. The cell lysates were immunoprecipitated and immunoblotted as indicated. D, PHFA cells were cotransfected with EAAT2-Luc and each AEG-1 deletion mutant. Data (mean ± SEM; fold-normalized activity relative to that of PHFA cells transfected with pcDNA denoted as ‘1’.) was taken as 1.* P < 0.01 versus pcDNA. E, PHFA cells were transfected as indicated. The AEG-1 (HA) and 4’,6-diamidino-2-phenylindole staining were evaluated by confocal microscopy. Scale bar, 20 μm. V, Ad.vec; A, Ad.AEG-1.
and the N1 mutant were located in the nucleus, but the C1 mutant that did not contain the nuclear localization signal was present in the cytoplasm (Fig. 4E). Specificity of anti-HA antibody for immunostaining was confirmed by competition with HA peptide (Supplementary Fig. S5). Taken together, these results indicate that interactions among AEG-1, Y11, and CBP are crucial for AEG-1–mediated EAAT2 repression.

AEG-1 promotes glioma-induced neurodegeneration by blocking EAAT2 function

EAAT2 is the predominant glial glutamate transporter in the brain and it functions to remove glutamate from the synapse to prevent excitotoxicity. Dysregulation of EAAT2 causes glutamate excitotoxicity, which is implicated in various types of neurodegenerative diseases and glioma-induced neurodegeneration (10–14, 17–20, 37). In addition, we found a negative correlation between AEG-1 expression and both EAAT2 and NeuN expression in glioma patients (Fig. 1). For these reasons, we hypothesized and investigated whether AEG-1–mediated EAAT2 repression is essential for glioma-induced neurodegeneration. We first measured glutamate uptake in PHFA cells infected with Ad.vec or Ad.AEG-1 to determine the functional significance of AEG-1–mediated EAAT2 repression in astrocytes. As shown in Figure 5A, AEG-1 decreased glutamate uptake in PHFA cells, suggesting that the AEG-1–mediated EAAT2 repression impairs glutamate uptake of astrocytes. In addition, AEG-1 knockdown in T98G cells increased the glutamate uptake in these cells (Fig. 5C). Expression of AEG-1 and EAAT2 in each gain-of-function and loss-of-function study was confirmed by Western blot analysis (Supplementary Fig. S6). These results indicate that AEG-1 causes glutamate excitotoxicity. To examine whether decreased uptake of glutamate causes neuronal cell death, we cultured PC-12–differentiated rat neuronal cells in conditioned media prepared from Ad.vec or Ad.AEG-1–infected PHFA cells treated with glutamate. The conditioned media from Ad.vec–infected PHFA cells containing intact glial glutamate transporters did not cause neuronal cell death. In contrast, the conditioned media from Ad.AEG-1–infected cells induced severe neuronal cell death (Fig. 5B). Furthermore, AEG-1 knockdown in glioma cells inhibited glutamate-induced neuronal cell death (Fig. 5D). However, these conditioned media from glioma cells and AEG-1–overexpressing PHFA cells had no cytotoxic effect on PHFA cells (Supplementary Fig. S7), indicating that this glutamate excitotoxicity causes cell death of neurons, but not astrocytes. Considered together with results obtained from patient samples shown in Figure 1, these observations indicate that AEG-1 represses EAAT2 expression and glutamate uptake, thereby causing neuronal cell death in glioma patients. These provocative findings highlight a novel mechanism by which gliomas induce neurodegeneration as summarized in Figure 6.

Discussion

Brain tumors induce pathogenic changes by rapidly proliferating and invading surrounding normal tissues and by promoting neuronal cell death through glutamate excitotoxicity (10). Consequently, a primary therapeutic focus to limit brain tumor-induced damage is by inhibiting cancer cell proliferation and invasion and potentially altering defects in glutamate homeostasis. To achieve these objectives requires an enhanced understanding of the genetic and epigenetic changes that promote development, progression, and pathogenesis of brain cancers. Previous studies have documented that AEG-1 plays crucial roles in malignant glioma progression, and its expression level significantly correlates with clinicopathologic stages of glioma (28, 31). In the present study, we report that AEG-1 expression also significantly correlates with reduction of EAAT2 expression and neuronal cells in glioma patient samples. In addition, AEG-1 overexpression in glioma impairs glutamate uptake by reducing EAAT2 expression, a primary glutamate transporter, culminating in glioma-induced neurodegeneration. These newer data when combined with previous studies suggest that AEG-1 is involved in the majority of features of glioma progression, that is, rapid tumor growth, destructive invasion of surrounding normal brain tissue, and glioma-induced neurodegeneration. Accordingly, we hypothesize that AEG-1 could be a primary regulator of glioma progression and thus could be a potential therapeutic target for this fatal disease. Developing pharmacologic agents and/or small molecule drugs that target AEG-1 for extinction would be predicted.

Figure 5. AEG-1 induces neuronal cell death via inhibiting glutamate uptake in glial cells. A, glutamate uptake levels (μmol/mg protein/min) were measured in Ad.vec– or Ad.AEG-1–infected PHFA cells. *, P < 0.01 versus Ad.vec. B, the differentiated PC-12 rat neuron cells were treated with conditioned media prepared from Ad.vec– or Ad.AEG-1–infected PHFA cells following treatment with 100 μmol/L glutamate. One day later, MTT assays were conducted. C, glutamate uptake levels (μmol/mg protein/min) were measured in NCsh and AEG-1sh cells. *, P < 0.01 versus NCsh. D, the differentiated PC-12 neuron cells were treated with conditioned media prepared from NCsh and AEG-1sh cells following treatment with 100 μmol/L glutamate. One day later, MTT assays were conducted. *, P < 0.01 in MTT assays (B and D) versus Mock-treated cells.
to delimit the pathogenesis and toxicity of glioma. In addition, restoring glutamate transporter function in glioma might also provide a means of reducing neuronal damage in patients with malignant glioma, particularly when combined with inhibition of AEG-1.

Gliomas release glutamate at levels that are neurotoxic (10, 11, 17, 20). Clearance of extracellular glutamate is markedly impaired in glioma cells compared with that in normal astrocytes mainly due to a loss of the predominant astroglial glutamate transporter EAAT2 (10, 16). This glutamate release also promotes growth of malignant gliomas (20). We previously reported that TNF-α induces AEG-1 expression, and AEG-1 functions as a coactivator for NF-κB by its direct interaction with p65 (22, 25, 33). However, TNF-α reduces EAAT2 expression in an NF-κB–dependent manner (35, 42). These results suggest that NF-κB might be a signaling pathway in AEG-1–mediated EAAT2 repression. However, we have now showed that AEG-1 employs YY1 to repress EAAT2 expression. TNF-α–mediated NF-κB repression is not a general phenomenon in all contexts, because it can also function as a positive regulator of NF-κB expression, and this process is poorly understood. We previously observed that TNF-α preferentially recruits N-myc to the EAAT2 promoter resulting in a repression of NF-κB–mediated EAAT2 promoter activation, indicating a mechanism by which TNF-α overcomes intrinsic NF-κB–mediated activation through a pathway not involving the direct inhibition of NF-κB (42). Instead, we now showed that AEG-1 recruits YY1 to the EAAT2 promoter resulting in reduction of EAAT2 expression. A previous report showed that AEG-1 acts as a bridge molecule facilitating interaction among NF-κB, CBP, and the basal transcriptional machinery, and therefore, functions as a coactivator facilitating the induction of NF-κB–dependent genes (25). In the present study, we document that AEG-1 plays a critical role as a link between YY1 and CBP on the EAAT2 promoter, causing YY1 to function as a negative regulator of EAAT2 expression by inhibiting CBP. These results indicate that interactions among AEG-1, YY1, and CBP are crucial for AEG-1–mediated EAAT2 repression, and also suggest that AEG-1 functions in the nucleus as a bona fide transcriptional cofactor.

Excitotoxicity caused by impaired glutamate uptake by glial cells has been implicated in various neurodegenerative conditions such as ischemia, stroke, epilepsy, amyotrophic lateral sclerosis, and HIV-associated dementia (41), in psychiatric disorders like depression and schizophrenia, as well as in certain forms of pain (12, 14–16). Although most studies of AEG-1 have focused on its functions in tumor progression, AEG-1 was originally isolated as a HIV-1–inducible gene (22, 33), implying a possible role for it in HIV-associated dementia. A recent genome-wide association study implicated AEG-1 in migraine (43). In addition, in this article we document that AEG-1 reduces expression of EAAT2 in astrocytes, causing neuronal cell death, suggesting that AEG-1 might also play crucial roles in neurodegenerative diseases, not only in glioma-induced neurodegeneration. We have also observed that AEG-1 expression is negatively correlated with EAAT2 expression and neuronal cell survival in a transient focal ischemia animal model (unpublished data), indicating its role in ischemia associated with EAAT2 and glutamate

Figure 6. Potential molecular mechanism by which EAAT2 reduction mediated by AEG-1 promotes neurodegeneration in human glioma. A, in normal astrocytes, EAAT2 functions as a primary glutamate transporter. B, in glioma cells, increased AEG-1 negatively regulates EAAT2 expression and induces glutamate excitotoxicity and neuronal cell death.
excitotoxicity. In this context, our present studies are focused on developing a conditional transgenic animal model to express AEG-1 specifically in astrocytes using the GFAP and nestin promoters, which would be beneficial in more precisely defining AEG-1 functions in astrocytes for examining in vivo brain tumor development/progression as well as for neurodegeneration/glioma-induced neurodegeneration.

In view of the assortment of effects of AEG-1 in the context of brain tumors, such as glioblastoma multiforme, this gene provides a viable target not only for delimiting the direct pathogenesis of brain tumors but also for reducing the indirect toxicity to neurons promoted by defects in glutamate transport observed in glioblastoma multiforme. Further studies are warranted and some are currently in progress to test these possibilities and to develop improved therapies for glioblastoma multiforme and methods for ameliorating its pathogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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