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. AEG-1 is an oncogene overexpressed in multiple types of human cancers including >90% of brain tumors. AEG-1 also promotes gliomagenesis particularly in the context of tumor growth and invasion, two 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 glioma patient samples 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 glioma patient samples demonstrating 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.
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 20-39 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 80-85% of all gliomas and are staged as low grade (grade I) to high grade (grade IV) according to nuclear atypia, mitotic activity, endothelial hyperplasia and necrosis (4). Glioblastoma multiforme (grade IV astrocytoma; GBM) is an extremely aggressive, invasive and destructive malignancy with 2-5 times faster proliferation rate 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, GBM is largely resistant to current treatments 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, even though 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 transporter 1 and 2 (EAAT1 and 2) 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). A number of studies also found that glioma cells release high levels of glutamate, which cause neuronal cell death and promote malignant glioma progression (17-20).

Astrocyte Elevated Gene-1 (AEG-1) is a multifunctional oncogene overexpressed in a variety of human cancers, even though it was originally isolated as a novel HIV-1- and TNF-α-induced transcript from primary human fetal astrocytes (PHFA) (21, 22). As a target of Ras, AEG-1 activates multiple oncogenic signaling pathways including PI3K-Akt, MAPK, Wnt and NF-κB involved in regulation of proliferation, invasion, chemoresistance, angiogenesis, and metastasis (21, 23-30). Especially in tumors of the CNS, such as neuroblastoma, GBM and oligodendrogioma, AEG-1 showed higher expression compared to that in normal brain tissues (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). Additionally, we observed an interesting inverse correlation between expression levels of AEG-1 and EAAT2. AEG-1 expression is elevated following HIV-1 and TNF-α treatment of astrocytes, whereas EAAT2 expression is downregulated (22, 33-36). In the setting of glioma 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. Based on 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) from Tissue Array Networks were performed as previously described (28). Anti-AEG-1, anti-EAAT1 and anti-EAAT2 antibodies were described (34, 38) and anti-NeuN antibody was purchased from Millipore. Images were captured with a confocal laser scanning microscope LSM multiphoton 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 performed with anti-HA antibody (Covance) as described (27).

Cell lines

Normal PHFA (primary human fetal astrocytes), 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 ATCC, and cultured in DMEM with 5% FBS and 10% heat inactivated horse serum at 37°C. The NCsh (normal control sh), AEG-1sh-2 and AEG-1sh-4 cell lines were established by transfection with control shRNA, AEG-1 shRNA #2 and AEG-1 shRNA #4 expression plasmids (SA Biosciences: KH18459H) in T98G cells, respectively, and selected with Hygromycin.

Recombinant adenovirus, siRNA and plasmids

Ad.vec and Ad.AEG-1 were previously described (26). Control and YY1 siRNAs were purchased from Santa Cruz Biotech. The expression plasmids of AEG-1 and AEG-1 deletion mutants
tagged with HA were described (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 1-289, respectively. 5’ deletion mutants of the human EAAT2 promoter reporter (EAAT2Pro) constructs and NF-κB-Luc were previously described (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’-TCGGAGCCCCCGGAGCTCCCCGCCAAGCATTATCCCCGCG-3’, and EAAT2Prom-954mNFκB1, 5’-TCGGA GCCCCGGAGCTCAAAGCCAAGCGCCATCCCCGCG -3’. The mutated sequences are underlined.

Western blotting and immunoprecipitation (IP) assays

Whole cell lysates were prepared, and co-immunoprecipitation and Western blotting was performed as described (25, 38). The antibodies for YY1 (Santa Cruz Biotech), CBP (Abcam) and EF1α (Upstate) were purchased. Whole cell lysates from human tissue samples were previously described (28).

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

Total RNA was extracted using the RNeasy mini kit (Qiagen). RT-PCR was performed using 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 performed (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 nM of control or YY1 siRNA using LipofecAMINE 2000 (Invitrogen), and 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 (EMSA)

EMSA was performed as described (38). The sequences of oligonucleotide used as probe include: 5′-CGCCAAGCGCCATCCCCCG-3′. The YY1 binding site is underlined. The mutant oligonucleotide: 5′-CGCCAAGCATTTATCCCCCGC-3′. The mutated sequences are in bold type.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using 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 performed as described (38).

Cell viability assays

Cells were treated with 1% heat inactivated horse serum containing 100 μM of glutamate for ≤ 10 min, and the conditioned media were collected. PC-12 cells (1×10^4 cells/well) were seeded
in 24-well plates, and treated with neuronal differentiation media (DMEM with 1% heat inactivated horse serum supplemented 1 mM di-butyryl cAMP: Sigma and 50 ng/ml of NGF: Promega) for 3-6 days. Then the PC-12-derived neuron cells were treated with conditioned media for 1 day. Cell viability was measured by 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 two 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 performed immunofluorescence staining of AEG-1 and EAAT2 in glioma patient samples using a Tissue Array containing 35 cases of glioma and 5 normal brain tissues in duplicate (Fig. 1A-C). Specificity of anti-AEG-1 and anti-EAAT2 antibodies for immunostaining was confirmed by competition with each immunogen (Supplementary Fig. 1). While expression of AEG-1 greatly increased in glioma patient samples compared to that in normal brains, EAAT2 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, analyzing 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. 2A-C). These results were also confirmed in GBM patient samples compared to normal brain tissues by Western blotting 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 to 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 glioma patient samples with 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 if 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 Fig. 2B (left panel), overexpression of AEG-1 significantly reduced EAAT2 expression in PHFA. In addition, EAAT2 mRNA was strongly reduced by AEG-1 (Fig. 2B, center panel). This reduction in EAAT2 mRNA expression resulted from decreased transcription as
confirmed using nuclear run-on assays (Fig. 2B, right panel). 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). Real-time PCR and Western blotting 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. 3), confirming that AEG-1 is a negative regulator of EAAT2. In addition, to 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-κB activity as previously shown (25) (Supplementary Fig. 4), whereas it repressed EAAT2 promoter activity (Fig. 2D). Taken together these results demonstrate 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 two putative transcription factor binding sites, YY1 and NF-κB1, 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). In order to determine which transcription factor was responsible for EAAT2 promoter repression, we engineered two mutant EAAT2 promoter-reporter plasmids containing a site-directed mutation of the putative YY1 or NF-κB binding site in the -954 EAAT2 promoter construct (mYY1 and m NF-κB). 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 Fig. 3B, mutation in the YY1 binding site abolished the EAAT2 promoter response to AEG-1, while mutation in the NF-κB 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 performed EMSA using a 20-bp double-stranded probe containing the YY1 binding site of the EAAT2 promoter. As shown in Fig. 3C (lane 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 performed using an unlabeled probe or a mutant probe containing mutations in the YY1 binding site. As shown in Fig. 3C the cold probe (lane 4) completely competed the DNA-protein complex, whereas the mutant probe (lane 5) had little effect on complex formation. In addition, supershift experiments with an anti-YY1 antibody resulted in a retarded mobility of the complex, while a control IgG did not (Fig. 3C, lane 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 one mechanism by which YY1 negatively or positively regulates gene expression (25, 39). Based on 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 if 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 Fig. 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 if these associations occur on the *EAAT2* promoter, PHFA cells were infected with Ad. *vec* or Ad.AEG-1, and ChIP assays were performed using either control IgG or anti-HA, anti-YY1, or anti-CBP antibodies. While only CBP associated with the *EAAT2* promoter in Ad. *vec*-infected PHFA cells, all three 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 highly expressing AEG-1. AEG-1, YY1 and CBP in NCsh cells 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 using AEG-1 deletion constructs. As shown in Fig. 4C, all C’ deletion mutants associated with both YY1 and CBP as did 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 each region, amino acids 1-70 and 71-100 of AEG-1 is responsible for interaction with CBP and YY1, respectively. We next examined if these interactions would be crucial for AEG-1-mediated EAAT2 repression. As shown in Fig. 4D, as expected only wild type AEG-1 repressed the EAAT2 promoter activity while none of N’ deletion constructs 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) (41), which is missing in all of the C’ deletion mutants we analyzed. We also confirmed that AEG-1 and the N1 mutant were located in the nucleus, but the C1 mutant that did not contain the nuclear localization signal was in the cytoplasm (Fig. 4E). Specificity of anti-HA antibody for immunostaining was confirmed by competition with HA peptide (Supplementary Fig. 5). Taken together, these results indicate that interactions among AEG-1, YY1 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 if 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 Fig. 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 blotting (Supplementary Fig. 6). These results indicate that AEG-1 causes glutamate excitotoxicity.

To examine if 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. 7), indicating that this glutamate excitotoxicity causes cell death of neurons, but not astrocytes. Considered with results obtained with patient samples shown in Fig. 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 Fig. 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 combined with previous studies suggest that AEG-1 is involved in the majority of features of glioma progression, i.e., 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 pharmacological agents and/or small molecule drugs that target AEG-1 for extinction would be predicted to delimit the pathogenesis and toxicity of glioma. Additionally, 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 now demonstrate that AEG-1 employs YY1 to repress EAAT2 expression. TNF-α-mediated NF-κB repression is not a general phenomenon in all contexts, since 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, suggesting 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 show 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, resulting in 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), and also 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), suggesting its possible role in HIV-associated dementia. A recent genome-wide association study implicated AEG-1 in migraine (43). In addition, here 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), suggesting its role in ischemia related with EAAT2 and glutamate 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. Considering the assortment of effects of AEG-1 in the context of brain tumors, such as GBM, 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 GBM. Further studies are warranted and are currently in progress to test these possibilities and to develop improved therapies for GBM and methods for ameliorating its pathogenesis.

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References

Figure legends

**Figure 1.** Correlation among expression of AEG-1, EAAT2 and NeuN in glioma patient samples. A, Comparison of AEG-1 and EAAT2 expression in glioma patient samples compared to NC. B, Correlation between AEG-1 and EAAT2 expression. C, Expression of AEG-1, EAAT2 and NeuN. Scale bar: 100 μm. D, Expression of AEG-1, EAAT1 and EAAT2 in 3 NB and 6 GBM patient samples. E, Quantification and comparison of NeuN expression in glioma patient samples compared to NC. F and G, Correlation between NeuN and AEG-1 (F) or EAAT2 (G) expression analyzed using a scatter plot. Data in graphs present mean ± S.D. *, P < 0.01 vs NC. NC, normal cerebrum tissues; NB, normal brain tissues; GBM, glioblastoma multiforme.

**Figure 2.** AEG-1 represses EAAT2 expression. A, AEG-1 and EAAT2 expression in various cell lines. B, PHFA cells were infected with 20 moi of Ad.vec or Ad.AEG-1 and Western blot was performed 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 (center). Nuclear Run-on assays with nuclei from infected cells were performed, and the transcription rate of GAPDH was used as a control (right). C, Real time-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 vs 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 performed at least three times in triplicate. Data: fold-normalized activity relative to that of Ad.vec-infected PHFA taken as 1. *, P < 0.01 vs Ad.vec.
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, mNFκB1 or mYY1. Data: fold-normalized activity relative to that of the -954 EAAT2Pro-Luc in Ad.vec-infected PHFA 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-YY-1 antibody; 7, Ad.AEG-1 + control IgG. D, The infected cells were cotransfected with EAAT2-Luc and control or YY1 siRNA. Data: fold normalized activity relative to that of Ad.vec-infected and control siRNA-transfected PHFA taken as 1. E, The infected and transfected PHFA cell lysates were immunoblotted with the indicated antibodies. All transient transfection and luciferase assays were performed at least three times in triplicate. Data in graphs present mean ± SEM. *, P < 0.01 vs Ad.vec.

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 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-1sh-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 -) taken as 1. *, $P < 0.01$ vs pcDNA. E, PHFA cells were transfected as indicated. The AEG-1 (HA) and DAPI staining were evaluated by confocal microscopy. Scale bar: 20 μm. V: Ad. vec; A: Ad.AEG-1.

**Figure 5.** AEG-1 induces neuronal cell death via inhibiting glutamate uptake in glial cells. A, Glutamate uptake levels (pmol/mg protein/min) were measured in Ad.vec- or Ad.AEG-1-infected PHFA cells. *, $P < 0.01$ vs 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 μM glutamate. One day later MTT assays were performed. C. Glutamate uptake levels (pmol/mg protein/min) were measured in NCsh and AEG-1sh cells. *, $P < 0.01$ vs NCsh. D, The differentiated PC-12 neuron cells were treated with conditioned media prepared from NCsh and AEG-1sh cells following treatment with 100 μM glutamate. One day later MTT assays were performed. *, $P < 0.01$ in MTT assays (B and D) vs Mock-treated cells.

**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.
A

neuron

Glutamate

CBP

EAAT2

BTM

EAAT2 Promoter

B

neuron

Neurotoxicity

Glutamate

AEG-1

CBP

YY1

BTM

EAAT2

Promoter
AEG-1 promotes glioma-induced neurodegeneration by increasing glutamate excitotoxicity

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