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Transient axonal glycoprotein-1 induces apoptosis-related gene expression without triggering apoptosis in U251 glioma cells

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Abstract

Previous studies show that transient axonal glycoprotein-1, a ligand of amyloid precursor protein, increases the secretion of amyloid precursor protein intracellular domain and is involved in apoptosis in Alzheimer's disease. In this study, we examined the effects of transient axonal glycoprotein-1 on U251 glioma cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that transient axonal glycoprotein-1 did not inhibit the proliferation of U251 cells, but promoted cell viability. The terminal deoxynucleotidyl transferase dUTP nick end labeling assay showed that transient axonal glycoprotein-1 did not induce U251 cell apoptosis. Real-time PCR revealed that transient axonal glycoprotein-1 substantially upregulated levels of amyloid precursor protein intracellular C-terminal domain, and p53 and epidermal growth factor receptor mRNA expression. Thus, transient axonal glycoprotein-1 increased apoptosis-related gene expression in U251 cells without inducing apoptosis. Instead, transient axonal glycoprotein-1 promoted the proliferation of these glioma cells.

Key Words: *nerve regeneration; brain injury; glioma cells; transient axonal glycoprotein-1; APP intracellular domain; p53; epidermal growth factor receptor; NSFC grant; neural regeneration*

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Introduction

Glioma is the most common (Jacobs et al., 2011) and malignant human brain tumor, accounting for about 51% of all central nervous system tumors (Hess et al., 2004). Traditional treatments, including surgery, chemotherapy and radiotherapy, have advanced greatly over the past few decades; however, therapy for malignant glioma remains mostly palliative (Hutterer et al., 2006) and prognosis is still poor (Gilbert, 2011), with a 6-month survival rate of 42.4%, a 1-year survival rate of 17.7% and a 2-year survival rate of only 3.3% (Ohgaki et al., 2004).

New treatment strategies, including molecular and gene therapy, have renewed hope for patients with glioma. Recently there has been an intense interest in signaling pathways associated with glioma pathogenesis. There is a clinically noticeable phenomenon that among patients with Alzheimer's disease, brain tumor is a very rare complication. This may indicate that signaling pathways associated with Alzheimer's disease may inhibit the development of gliomas. Increased p53 expression in sporadic Alzheimer's disease has been reported in several studies using immunohistochemical staining, especially in a subpopulation of cortical neurons undergoing degeneration (de la Monte et al., 1997; Kitamura et al., 1997; García-Ospina et al., 2003; Ohyagi et al., 2005).

Extracellular amyloid-β (Aβ) deposits (Haass et al., 1993) are the main histopathological hallmark of Alzheimer's disease. Aβ is generated from the amyloid precursor protein (APP), which is a type I transmembrane glycoprotein with a large extracellular domain, a single hydrophobic transmembrane region, and a short cytoplasmic tail (Kang et al., 1987; Goldgaber et al., 1987). Aβ is generated through the enzymatic hydrolysis of APP, and concomitantly, the APP intracellular C-terminal domain (AICD) is released as well (Pardossi-Piquard et al., 2012). AICD was described in Alzheimer's disease long after the identification of APP (Passer et al., 2000). Many AICD isoforms, varying in the number of amino acids, have been identified, including AICD57,

AICD59, AICD50 (Sastre et al., 2001), AICD48 (Yu et al., 2001) and AICD31 (Lu et al., 2000). Recent reports show that AICD59 and AICD50 trigger p53-dependent cell death and increase p53 activity and mRNA levels (Alves et al., 2006). Apoptosis induced by AICD in human H4 glioma cells was also demonstrated in a previous study (Kinoshita et al., 2002). Moreover, AICD activates p53 and enhances its transcriptional and pro-apoptotic functions in H4 cells (Ozaki et al., 2006). Zhang et al. (2007) reported that APP/AICD was involved in regulation of the epidermal growth factor receptor (EGFR), and that over-expression of AICD suppressed the elevation in EGFR levels. Apart from p53 and EGFR, some other factors, such as KAI1 and GSK3β, have been found to be regulated by AICD-dependent mechanisms (Muller et al., 2008). Previous studies showed that AICD can induce apoptosis and inhibit tumorigenesis through the APP/AICD/p53 (Gilbert, 2011) or APP/AICD/EGFR (Beckett et al., 2012) signaling pathways.

Recent findings indicate that transient axonal glycoprotein-1 (TAG-1), a ligand of APP, can increase the release of AICD and impact the onset and development of Alzheimer's disease (Tachi et al., 2010). TAG-1 interacts with APP and initiates an intracellular signaling cascade that regulates disease-related genes. This raises the intriguing question of whether there is a connection between TAG-1 signaling pathways (*e.g.*, TAG-1/APP/AICD/p53 or TAG-1/APP/AICD/EGFR) and the genesis and development of gliomas. TAG-1 is a member of the contactin family of proteins, which in turn belongs to the immunoglobulin superfamily, and is expressed exclusively in the nervous system (Shimoda et al., 2009). There are two forms of TAG-1-cell surface and released.

In this study, we used recombinant human contactin-2/TAG-1 in place of released TAG-1, and we examined the effects of the protein on U251 cells. We evaluated the impact of the APP ligand on AICD levels and p53 and EGFR expression in U251 cells, with the aim of advancing treatment strategies for glioma.

Materials and Methods

Cell culture

Human U251 glioma cells were donated by Affiliated Tumor Hospital of Sun Yat-sen University in China, and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone Inc., Logan, UT, USA) and cultured at 37°C in an incubator containing 5% CO_2 and 95% air.

Immunocytochemistry

Rabbit anti-APP polyclonal antibody, which recognizes several isoforms of both mature and immature Aβ (A4) precursor protein, including APP695, APP770 and APP751, was purchased from Cell Signaling Technology (Boston, MA, USA) and diluted 1:200. Donkey anti-rabbit antibody (Alexa Fluor 488, FITC; Invitrogen Inc., Carlsbad, CA, USA), diluted 1:400, was the secondary antibody. For immunofluorescence labeling, cells were washed with 0.01 mol/L protein block solution and fixed with 3.7% formaldehyde solution for 15 minutes at room temperature, followed by three washes (5 minutes each) with protein block solution. 5% donkey serum mixed with protein block solution/Triton (30 μL Triton into 10 mL protein block solution) was used for 1 hour at room temperature to block non-specific binding. Subsequently, cells were incubated with 30 μL/well of primary antibody, diluted 1:200 with protein block solution/Triton at 4°C overnight. Cells used for the staining control were incubated with protein block solution only. Cells were then washed with protein block solution three times (5 minutes each), then incubated with 50 μL labeled secondary antibody, diluted 1:400 with protein block solution/Triton, at room temperature for 90 minutes in the darkroom. After washes with protein block solution, the samples were examined and photographed with a fluorescence microscope (Leica, Solms, Germany) at a wavelength of 488 nm.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay for cell viability

The effects of TAG-1 on cell viability and cell proliferation were assessed with the MTT assay. U251 cells were seeded onto 96-well plates at a density of 5×10^4 cells per well in 100 μL RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C for 12 hours. The original medium was replaced with fresh serum-free RPMI-1640 medium supplemented with TAG-1 and incubated for another 1 hour. The final concentration of fetal bovine serum was 10%, while that of TAG-1 was 0, 5, 10 or 20 μg/mL. The effect of each TAG-1 concentration was tested three times with a negative control of 0 μg/mL TAG-1 and a blank control of pure culture solution. After 48 hours, the MTT assay was applied following the manufacturer's protocol (Chemicon Inc., Billerica, MA, USA). A 0.01 mL aliquot of AB solution was added, mixed well, and incubated for 4 hours. After adding 0.1 mL solution C, the absorbance values were measured at 570 nm using a microplate reader (Bio-Tec Instruments Inc., USA). The percentage of cell survival, for each TAG-1 concentration, was calculated using the following equation: cell survival (%) = $A_t/A_c \times 100\%$, where A_t is the absorbance of the test well and A_c is the absorbance of the negative control. For the alamarBlue assay (Invitrogen), 0.01 mL was added directly to cells in culture medium and incubated at 37°C for 4 hours in a cell culture incubator. Absorbance was then monitored at 570 and 630 nm. Cell viability using alamarBlue was assessed using the following equation:

$$\text{Percent difference in reduction} = \frac{A_{LW} - (A_{HW} \times R_O) \text{ for tested well}}{A_{LW} - (A_{HW} \times R_O) \text{ positive growth control}} \times 100\%$$

$R_O = A_O570/A_O630$

A_O570 = absorbance of oxidized form at 570 nm.

A_O630 = absorbance of oxidized form at 630 nm.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay

The TUNEL assay (TACSTMTdT Kit, Catalog#TA4625, R&D systems, MN, USA) was used to examine the effect of TAG-1 on apoptosis. Three different final concentrations of TAG-1 in the culture medium were evaluated: 0, 10 and 20 μg/mL. Cells were incubated with TAG-1 at 37°C for 24 hours, washed with 0.01 mol/L protein block solution, and fixed with 3.7% formaldehyde solution at room temperature for 10 minutes. The slides were put into 100%, 95%, and 70% ethanol solutions sequentially for 5 minutes each and then into protein block solution at room temperature for 10 minutes. 100 μL Cytonin solution was added to each slide, and incubated at room temperature for 20 minutes. Deionized

water was used to wash the cells twice (2 minutes each). U251 cells treated with freshly prepared TACS-Nuclease (1 μL TACS-Nuclease into 50 μL TACS-Nuclease Buffer) at room temperature for 20 minutes were used as the positive control.

All the samples were incubated in endogenous enzyme inactivation solution (45 mL methanol and 30% H_2O_2) for 5 minutes. Followed by washes with protein block solution, the samples were immersed in TdT labeling solution for 5 minutes. 50 μL labeling reaction mixture (prepared according to the manufacturer's instruction) was then added to each slide, coverslipped, and incubated at 37°C for 1 hour. The samples were immersed in TdT termination solution (prepared according to the manufacturer's instruction) for 5 minutes and washed twice (2 minutes each) with protein block solution. Thereafter, 50 μL detection solution, containing biotinylated horseradish peroxidase (1 μL streptavidin/50 μL protein block solution) was added to each sample, coverslipped, and incubated at room temperature for 10 minutes. The samples were washed twice with protein block solution for 2 minutes each and then transferred into 3,3′-diaminobenzidine working solution at room temperature for 5 minutes. Samples were then washed with distilled water and transferred into methyl green solution at room temperature for 2 minutes. The slides were immersed sequentially into distilled water, 70% ethanol, 95% ethanol, 100% ethanol and 100% xylene. The slides were sealed, and photographs were taken under a microscope (Olympus, Tokyo, Japan). The 0 μg/mL group was taken as the negative control, and the TACS-Nuclease group was used as positive control for permeabilization and the labeling reaction. Apoptotic cells were detected as brown fluorescence signals, the positive control was visible as grey staining, and the cells treated with methyl green were light green.

Real-time polymerase chain reaction (RT-PCR)

To assess whether TAG-1 induced changes in gene expression of AICD, p53 or EGFR in U251 cells, real-time PCR was conducted. U251 cells were maintained in serum-free RPMI-1640 medium containing TAG-1 for 1 hour. TAG-1 was added to the culture at a final concentration of 0 or 20 μg/mL, and 48 hours later, the cells were washed with protein block solution and total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Using M-MLV as the reverse-transcriptase and Oligo-dT as the primer, the first strand was synthesized from 1 μg of total RNA with a cDNA Synthesis Kit (Gene Copoeia Inc., Rockville, MD, USA), followed by RT-PCR amplification on an iQ5 Real-Time PCR Detection System (Bio-Rad Inc., Hercules, CA, USA). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the internal control. The thermal cycling protocol consisted of initial denaturation at 95°C for 10 minutes and 40 cycles of 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 15 seconds. The sequences of primers for AICD, p53, EGFR and GAPDH are listed as follows:

Gene	Sequences of primers (5′–3′)
AICD59	Forward: ATA GCG ACA GTG ATC GTC ATC Reverse: CTC CTC TGG GGT GAC AGC
Tp53	Forward: CAT CAT CAC ACT GGA AGA CTC C Reverse: TTG CGG AGA TTC TCT TCC TC
EGFR	Forward: CAG AGT GAT GTC TGG AGC TAC G Reverse: GGG AGG CGT TCT CCT TTC T
GAPDH	Forward: ACA CCC ACT CCT CCA CCT TT Reverse: TTA CTC CTT GGA GGC CAT GT

The relative changes in gene expression were calculated using the $\triangle\triangle C_T$ equation: $\triangle C_T = C_{T.X} - C_{t.GAPDH}$; $\triangle\triangle C_T = \triangle C_{T.Test\_group} - \triangle C_{T.Control\_group}$; relative expression = $2^{-\triangle\triangle Ct}$.

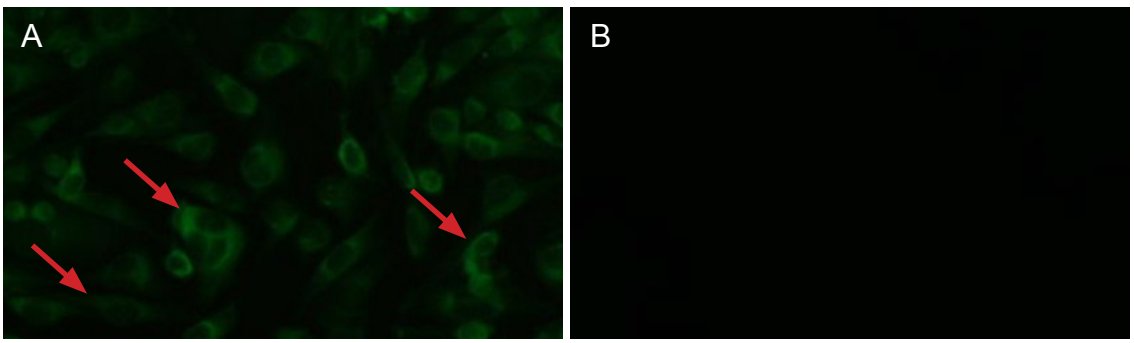
Statistical analysis

Data were expressed as mean ± SD and were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Comparisons among the groups were performed with a two-sample *t*-test and one-way analysis of variance and least significant difference test. A value of $P < 0.05$ was considered statistically significant.

Results

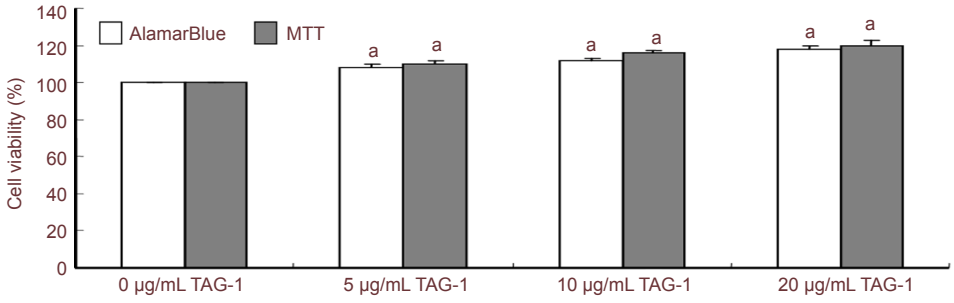
U251 cells strongly expressed APP

TAG-1, the APP ligand, enhances the release of AICD, and their interactions regulate downstream genes. APP has been reported to be present on the surface of neuronal and glial cells (Shivers et al., 1988; Yamazaki et al., 1997). APP expression has also been detected in other glioma cells lines (Gegelashvili et al., 1996; Kumar et al., 1999). Nonetheless, we first confirmed that APP was expressed in U251 cells by immunostaining. As shown in Figure 1, the staining was distributed mainly on the cell membrane and dendrites. Staining was also distributed in the cytoplasm, but was not significant. This result is consistent with previous reports (Gegelashvili et al., 1996; Kumar et al., 1999).



(A) APP immunoreactivity (arrows) is detectable on the membrane of U251 cells by immunofluorescent staining. (B) No significant APP staining was detected in U251 cells in which protein block solution was used in place of primary antibody .

Figure 1 Amyloid precursor protein (APP) is expressed in U251 cells (× 200).



Cells were treated with various concentrations of TAG-1 (0, 5, 10, 20 μg/mL) for 48 hours. 0 μg/mL TAG-1 was used as the control. TAG-1 may have a growth promoting effect in U251 cells. Experiments were performed in triplicate. Data are expressed as mean ± SD for each group. [a]$P < 0.05$, *vs.* 0 μg/mL TAG-1 (one-way analysis of variance and least significant difference test). The experiment was repeated six times.

Figure 2 Effects of transient axonal glycoprotein-1 (TAG-1) on U251 cell viability as measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and alamarBlue assays.

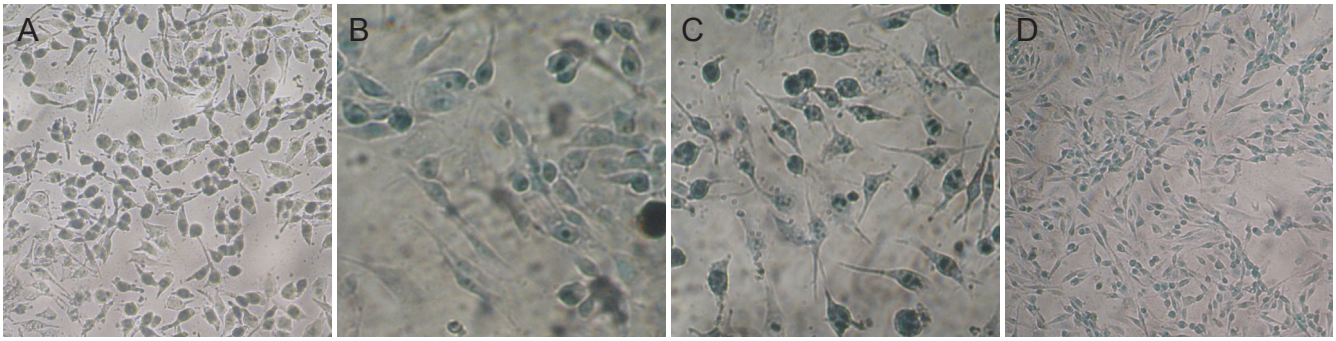


Figure 3 Effects of transient axonal glycoprotein-1 (TAG-1) on U251 cell apoptosis detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay.
U251 cells were fixed with 3.7% formaldehyde and the TUNEL assay was performed. (A) Positive control; (B) negative control; (C) 24 hours after treatment with 10 μg/mL TAG-1; (D) 24 hours after treatment with 20 μg/mL TAG-1. No apoptosis was induced by TAG-1. Cells in Figures B, C and D were counterstained with methyl green following 3,3′-diaminobenzidine coloration and showed no significant apoptosis. (A) × 200; (B, C) × 300; (D) × 100.

TAG-1 did not inhibit U251 cell proliferation

The effects of TAG-1 on cell viability and U251 cell proliferation were examined by MTT assay. In comparison with the negative control (0 μg/mL TAG-1), the absorbance values of U251 cells treated with varied concentrations of TAG-1 were higher ($P < 0.05$; Figure 2). Considering the toxicity of MTT, we used another survival assay, alamarBlue, which interferes less with normal metabolism. TAG-1 did not inhibit U251 cell growth, but increased survival and viability compared with control (Figure 2). Therefore, there was no significant inhibitory effect of TAG-1 on U251 cells.

Effects of TAG-1 on apoptosis in U251 cells

The positive control showed TUNEL staining in all nuclei. The negative control stained with TUNEL did not show any signal. Cells exposed to 10 or 20 μg/mL TAG-1 could not be distinguished from control (0 μg/mL; Figure 3), indicating that no apoptosis was induced by TAG-1.

Effects of TAG-1 on AICD, p53 and EGFR expression in U251 cells

Changes in AICD, p53 and EGFR gene expression in U251 glioma cells were evaluated by RT-PCR. AICD expression was higher in the treatment group (20 μg/mL TAG-1) than in the control group ($P < 0.05$). p53 and EGFR expression levels were significantly higher in the treatment group than in the control group ($P < 0.05$; Table 1).

Table 1 Effect of TAG-1 on expression of AICD, p53 and EGFR genes in U251 cells as determined by RT-PCR 48 hours after treatment with TAG-1

Group	AICD	Tp53	EGFR
0 μg/mL TAG-1 (control)	1.0	1.0	1.0
20 μg/mL TAG-1 (treatment)	2.6[a]	4.1[a]	2.7[a]

The controls were treated with TAG-1-free medium (0 μg/mL TAG-1). Expression levels of AICD, p53 and EGFR in U251 cells treated with 20 μg/mL TAG-1 were significantly increased. Shown are the relative gene expression levels ($2^{-\triangle\triangle Ct}$), *i.e.*, treatment group relative to control group. Data are expressed as mean for each group (two-sample *t*-test; [a]$P < 0.05$, *vs.* 0 μg/mL TAG-1). The experiment was repeated six times. TAG-1: Transient axonal glycoprotein-1; AICD: APP intracellular C-terminal domain; EGFR: epidermal growth factor receptor.

Discussion

Alzheimer's disease, a neurodegenerative disease, is characterized by an imbalance in pro-apoptotic and anti-apoptotic processes (Ruiting et al., 2013). Research on the apoptotic potential of AICD has been explored by many authors using various models. Kinoshita et al. (2002) demonstrated that human H4 neuroglioma cells transfected with AICD58 underwent apoptosis 48–72 hours later, as detected by TUNEL staining. AICD31 (residues 665 to 695) induced apoptosis when transfected into N2A and 293T cells (Kumar et al., 1999). When AICD57 was transfected into p19

cells, it induced neuron-specific apoptosis (Bertrand et al., 2001). Wild-type blastocysts and HEK cells transfected with AICD59 showed increased p53 activity and transactivation of p53 promoters, and AICD59-dependent cell death was abolished by a p53 inhibitor (Alves et al., 2006). Transfection of AICD57 or AICD59 in wild-type p53-bearing U2OS cells increased p53-mediated apoptosis, but no effect was observed in p53-deficient H1099 cells (Ozaki et al., 2006). EGFR gene expression was shown to be regulated by AICD, which may function as a tumor suppressor by altering EGFR signaling (Yu et al., 2001). Extracellular stimuli may increase AICD levels, thereby regulating downstream signaling pathways. Indeed, TAG-1 was shown to increase AICD release from APP in a study published in 2008 (Ma et al., 2008).

APP was strongly expressed in U251 cells in the present study, and APP has been shown to be over-expressed in glioma cells (Miyazaki et al., 1993). Hence, cells should undergo apoptosis through the TAG-1/APP/p53 or TAG-1/APP/EGFR pathway. Ligand-induced apoptosis, triggered by the activation of downstream apoptotic genes, is a potential therapeutic strategy for glioma.

p53 expression in Alzheimer's disease

Stresses such as DNA damage, oncogene activation and anoxia not only bring about p53 activation, but also inhibit cell proliferation by blocking the p53-dependent cell cycle (Taira et al., 2012). p53 plays a critical role in inhibiting the development of human gliomas (Taira et al., 2012). In Alzheimer's disease, a strong increase in p53 expression is found in cortical neuron subpopulations (Fogarty et al., 2010). In addition, its relationship with neuronal apoptosis has been well established.

Connections between AICD, p53 and EGFR

Extracellular Aβ generated from APP is a key factor in the pathogenesis of Alzheimer's disease. APP is a type I transmembrane glycoprotein with a large extracellular domain, hydrophobic transmembrane regions and a short intracellular tail (Zhang et al., 2012). Along with the generation of extracellular Aβ, AICD is released at the same time. Various forms of AICD (Ward et al., 2010), including AICD57, AICD59, AICD50, AICD48 and AICD31, are classified according to the number of amino acids. In this study, we used AICD59. In various cell lines, AICD has been shown to regulate transcription by translocating to the nucleus, where it induces the expression of pro-apoptotic genes. In addition, AICD may also directly mediate apoptosis in human H4 glioma cells, and activate p53 in H4 glioma cells to enhance gene expression and induce apoptosis (Gilbert, 2011). Furthermore, APP/AICD modulates EGFR expression in cells, and AICD overexpression is able to inhibit the effects of EGFR, and thereby suppress tumor development (Ohgaki et al., 2004). Therefore, AICD can enhance pro-apoptotic activity of P53 and downregulate EGFR expression.

Effects of TAG-1 on the intracellular release of AICD

Previous studies focused on direct transfection of AICD into different cell lines to observe the effects of the APP fragment on cells and its connection with p53 and EGFR. However, in this study, we added the APP ligand TAG-1 extracellularly and examined the effects on AICD, p53 and EGFR. Recent studies (Tachi et al., 2010) showed that TAG-1 increases the secretion of AICD, and that APP is abundantly expressed in U251 cells. Thus, TAG-1 might be able to increase the intracellular release of AICD. Apoptosis and tumor inhibition may be mediated by the TAG-1/APP/AICD/p53 or TAG-1/APP/AICD/EGFR signaling pathways.

Analysis of the effects of TAG-1 on U251 cells

We used human recombinant TAG-1 to stimulate APP on the membrane so that apoptosis might be induced by an increase in AICD release. However, our findings were unexpected. The increased levels of AICD observed in this study did not have an inhibitory effect on U251 cell proliferation, nor did it induce apoptosis. Thus, we consider that APP might not be the only receptor for TAG-1, or that the interaction between them is non-specific. However, AICD is the intracellular fragment of APP, and thus, an increase in AICD levels can also result from an increase in APP expression. Indeed, it has already been shown that APP is overexpressed in gliomas (Dehvari et al., 2012).

The increase in EGFR expression in U251 cells treated with TAG-1 indicates that TAG-1 may interact with other receptors on the cell membrane to upregulate EGFR expression, activate downstream genes and promote U251 cell proliferation by activating unknown signaling pathways. We found that TAG-1 did not play an inhibitory role in cell proliferation, nor did it induce glioma cell apoptosis. Yet, real-time PCR results showed that p53 and EGFR expression were increased. p53 gene mutations are found in over 50% of primary gliomas (Paolella et al., 2011; Jha et al., 2010) and in over 60% of secondary gliomas. In almost 60% of gliomas, p53 inactivation is found (Louis et al.,1993). These data indicate that mutations in the p53 gene are common in gliomas.

EGFR also plays an important role in glioma development and proliferation (Dasari et al., 2010). The EGFR gene in over 30% of gliomas is highly amplified, while in about 40–50% of gliomas, it is overexpressed (Dasari et al., 2010). EGFR expression is not only related to tumor proliferation and the erosion of surrounding tissues, but also drug resistance and survival rate. Moreover, EGFR can promote tumor proliferation without dependency on ligand binding (Jha et al., 2010).

Based on past studies, there is a possibility that TAG-1 might upregulate EGFR expression through unknown signaling pathways and induce U251 cell proliferation and activate the p53 gene. However, tumor proliferation was not inhibited, likely due to inactivation of the p53 gene. The increase in p53 gene expression might be a stress response to the high proliferative rate of U251 cells induced by the overexpression of the EGFR gene.

Our study demonstrated that TAG-1 promoted cell proliferation. Expression of AICD, p53 and EGFR in U251 cells

treated with TAG-1 were all increased. Signaling pathways are not isolated and can interact with each other and activate various signaling networks at different levels. In addition, the function of a molecule is influenced by its cell type and cell state; thus, the same signaling pathway can have distinct effects in different cells.

Although apoptosis in U251 cells does not appear to be mediated by the TAG-1/APP/AICD/p53 or TAG-1/APP/ACID/EGFR pathways, the effects of TAG-1 on glioma proliferation are not negligible. Therefore, blocking these signaling pathways may provide a new approach for the treatment of glioma.

We must mention that we tried repeatedly to observe changes in the TAG-1/APP/AICD/p53 and TAG-1/APP/AICD/EGFR pathways in U251 cells. However, under our experimental conditions, we observed no change in these pathways. Possible explanations include low cell density and the low total protein concentration, and the short half-life of related proteins, especially AICD. Follow-up studies are in progress.

In summary, for the first time, we reported the effects of TAG-1 on cell viability and p53, EGFR and AICD expression in U251 glioma cells. Our pilot study showed that the signaling pathways induced by TAG-1, TAG-1/APP/AICD/p53 and TAG-1/APP/AICD/EGFR, did not inhibit glioma development by inhibiting cell proliferation or by inducing apoptosis. Instead, these signaling pathways enhanced proliferation. These findings provide novel insight into the mechanisms of glial tumorigenesis.

AICD can translocate into the nucleus and serve as a transcriptional regulator and play a critical role in cytoskeletal dynamics and apoptosis (Kimberly et al., 2001; Muller et al., 2008). Consequently, increasing levels of intracellular AICD using extracellular stimuli may have therapeutic potential in the treatment of glioma. Because the highest concentration of TAG-1 in our experiment was 20 μg/mL, the effect of higher TAG-1 concentrations on U251 cells remains unclear. Further work is required to identify the receptor for TAG-1 and the ligand for APP in U251 cells. Further study is required to clarify the role of TAG-1 in glioma cells and to identify the changes in gene expression induced by the ligand.

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Conflicts of interest: *None declared.*

Peer review: *This study showed that transient axonal glycoprotein-1 apparently increased the expressions of amyloid precursor protein intracellular domain, p53 and epidermal growth factor receptor mRNA, but did not induce U251 cell apoptosis. Results are significant to understand the biological properties of glioma cells, but still require the evidence at the protein level.*

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