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LAB INVESTIGATION-HUMAN/ANIMAL TISSUE

ATRA-inhibited proliferation in glioma cells is associated with subcellular redistribution of β-catenin via up-regulation of Axin

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Abstract Retinoic acid (RA) is a major chemopreventive agent which exerts strong anti-tumor activity partly by trans-repressing the Wnt/β-catenin signaling pathway in some tumor cell lines. However, the definite mechanism of RA trans-repression of the Wnt/β-catenin signaling pathway has not been elucidated clearly. In this work, we found that all-trans retinoic acid (ATRA) significantly inhibited proliferation of glioma cells, accompanied by up-regulation of expression of Axin and altered subcellular distribution of β-catenin. Transfecting C6 cells with rAxin further confirmed that increased expression of Axin is obligate for inhibition of proliferation and the increase of the cytoplasmic β-catenin. Our results suggested that Axin might play an important role in RA-mediated anti-proliferative effects of glioma cell lines.

Keywords All-trans retinoic acid · Axin · β-Catenin · Glioma cell lines · Subcellular redistribution

Introduction

Malignant gliomas are the most common primary brain tumors in adults. Despite multimodal treatment that includes surgery, radiation and chemotherapy, tumor recurrence is frequent and the majority of these patients eventually die from progressive tumor. Thus, new modes of treatment are needed to further improve the outcome of patients with these tumors.

Retinoic acid (RA), a potent anti-tumor agent, is an important regulator of cell proliferation and differentiation [1–3]. The effect of retinoic acid in glioma is well documented [4–7]. Many cell lines are used as model systems to study its anti-proliferative effects [8–12]. It is established that the actions of RA are mediated through retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [13]. Interestingly, recent studies revealed that in some tumor cell lines all-trans retinoic acid (ATRA) inhibits proliferation partly by suppressing the activity of the Wnt/β-catenin signaling pathway without affecting the expression level of β-catenin [14–17]. However, the definite mechanism of ATRA repression of the Wnt/β-catenin signaling pathway remains unclear.

β-Catenin exerts its signaling activity only in the nucleus [18–20]. So it is possible that ATRA might regulate the Wnt/β-catenin signaling pathway by changing the subcellular localization of β-catenin. Interestingly, recent experiments with *Drosophila* and mammalian cells suggested that Axin, a key negative regulator of the Wnt/β-catenin signaling pathway [21], promotes the cytoplasmic localization of β-catenin by enhancing nuclear export and cytoplasmic anchorage of β-catenin [22–24]. It is unclear whether ATRA-mediated inhibition of cell proliferation relies on expression of Axin and the subcellular redistribution of β-catenin.

In this work we found that ATRA-mediated growth-inhibition of glioma cells was associated with up-regulation of Axin and an increase of cytoplasmic β-catenin, by which the activity of the Wnt/β-catenin signaling pathway may be inhibited. Our research suggested an important role for Axin in enabling retinoic acid to inhibit the proliferation of glioma cells.

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Materials and methods

Cell lines and treatment

Human glioma U251 and rat glioma C6 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, in humidified 5% CO_2. U251 and C6 cells were treated with 2.5 μmol/l ATRA (Sigma) or with an equivalent amount of ethanol for 24 h.

Stably transfected cell line

To create stable transfectant glioma cell line, rat glioma C6 cells were transfected with $p^{IRES2\text{-}EGFP\text{-}rAxin}$ and $p^{IRES2\text{-}EGFP}$ using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's procedure. Two days after transfection, transfectants were subcultured and selected in culture media supplemented with 1.0 g/l G418. Each selected clone was analyzed by fluorescence microscopy. The detailed methods have been described elsewhere [25].

Cell viability analysis and plate colony formation assay

The effect of ATRA on the proliferation of U251 and C6 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to the manufacturer's instructions (Promega). Briefly, 1×10^3 cells were seeded in 96-well plates, incubated at 37°C for 24 h, and treated with either ethanol or ATRA for 6 days. At 0, 24, 48, 72, 96, 120, and 144 h after treatment, MTT reagent (20 μl/well) was added. After incubation at 37°C for 4 h the absorbance was measured at 490 nm. To determine the effect of ATRA on clonal proliferation, plate colony formation assays were performed. Cells (50, 100, or 200) were seeded in six-well plates and cultured with ATRA or ethanol. After 2 weeks, the plates were stained with Giemsa's stain (0.4 g Giemsa's stain, 25 ml methanol, 25 ml glycerol), and the colonies were counted. Each experiment was repeated in triplicate. The results presented are averages from three independent experiments.

Cell proliferation assay and immunofluorescent staining

The ability of ATRA to inhibit DNA synthesis was determined by estimating the amount of BrdUrd incorporation into DNA by immunocytochemistry staining. Briefly, cells were cultured on 12-mm^2 coverslips with or without ATRA for 24 h. BrdUrd (20 μmol/l, Sigma) was added, and the cells were reincubated for an additional 24 h. Coverslips were fixed with 4% paraformaldehyde and washed in phosphate-buffered saline (PBS), then permeabilized with 1.0% Triton X-100 for 10 min and blocked in 3% H_2O_2 for 20 min. The cells were incubated with 5% normal goat serum (Sigma) for 30 min, and incubated overnight at 4°C with mouse monoclonal anti-BrdUrd antibody (Zhongshan, China). Following three washes with PBS, cells were incubated for 20 min at room temperature with biotinylated goat anti-mouse IgG followed by streptavidin enzyme conjugate (Zhongshan, China) for 30 min at room temperature. The reaction product was visualized with diaminobenzidine tetrahydrochloride (DAB). All slips were counterstained with hematoxylin. Quantification of percentage of cells immunoreactive for BrdUrd was determined by capturing images from random fields. Results were presented as percentage inhibition of BrdUrd incorporation by ATRA compared with controls. For indirect immunofluorescent staining, cells were grown on 12-mm^2 coverslips. After fixing, the cells were incubated overnight at 4°C with rabbit monoclonal anti-β-catenin antibody (Sigma). After rinsing with PBS the cells were incubated with a fluorescein isothiocyanate-conjugated secondary antibody for 30 min (Cy3, Sigma) at room temperature. The nuclei were counterstained with DAPI. Cells were examined using a fluorescence microscope (Nikon).

RT-PCR

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 1 μg total RNA using PrimeScript RT reagent kit (Takala). PCR primers were: human Axin: forward, 5′-CCCTCCCACCTCTTCATCC-3′, reverse, 5′-CAGTCAAACTCGTCGCTCAC-3′; rat Axin: forward, 5′-AGGGTCTGGAACAGGGAA-3′, reverse, 5′-GGATAGC GTGTCAGCATCA-3′; human β-catenin: forward, 5′-ATT CTTGGCTATTACGACA-3′, reverse, 5′-GAGACCTTC CATCCCTTC-3′; rat β-catenin: forward, 5′-ACCTCCCAA GTCCTTTATG-3′, reverse, 5′-TACAACGGGCTGTTTC-TAC-3′; β-actin: forward, 5′-TCACCCACACTGTGC CCATCTA-3′, reverse, 5′-CATCGGAACCGCTCATTGC CGATAG-3′. A template-free negative control was included in each experiment. PCR conditions were: 30 cycles of amplification with 30 s of denaturation at 94°C and 30 s of annealing at 58°C. Band intensity was quantified by use of Bandscan software. The gray values of bands were normalized relative to those of β-actin. The gray values were expressed in relation to that of control and presented as means ± SD from three independent experiments.

Western blot assay

To obtain total protein, cells were lyzed in 2× SDS loading buffer (containing 25 mmol/l Tris-Cl/SDS, pH 6.8, 4 g SDS, 20 ml glycerol, 1.0 g bromophenol blue, and 3.1 g DTT, add H_2O to 100 ml). Total extracts were obtained in the supernatant. To prepare the cytoplasmic fraction, cells were lysed using nuclear and cytoplasmic protein extraction kit (Beyotime) according to the manufacturer's instructions. The lysates were ultracentrifuged at 12,000×*g* for 10 min at 4°C. The clear supernatants were collected as the cytoplasmic fraction. The concentration of protein in the cytoplasmic fraction was measured using Bradford's reagent (Bio-Rad). For Western blot assay, approximately 20 µl of samples were resolved on 10% SDS-PAGE, and transferred to PVDF membranes. Western blot used following antibodies. A rabbit monoclonal antibody for *β*-catenin (Sigma), a rabbit polyclonal antibody for Axin (Santa Cruz) and a rabbit polyclonal antibody for *β*-actin (Sigma) were used to detect the corresponding proteins. Peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma) was used, and then the proteins were detected by using an enhanced chemiluminescence reagent (Pierce). The blots were then stripped at 56°C for 30 min (stripping buffer, 62.5 mmol/l Tris-Cl, pH 7.5, 2% SDS, 1.7% *β*-mercaptoethanol), and blocked in 5% nonfat milk before re-probing. Band intensity was quantified by use of Bandscan software. Protein expression was normalized to the quantity of *β*-actin. The gray values were expressed in relation to that of control and presented as means ± SD from three independent experiments.

Statistical evaluation

SSCP 11.5 for Windows was used for statistical analysis. Data are expressed as mean ± SD. One-way ANOVA was used to assess statistical significance between means. $P < 0.05$ was considered to be statistically significant.

Results

ATRA inhibited the proliferation of glioma cells

The ability of ATRA to inhibit proliferation of glioma cells was examined by cell viability analysis, cell proliferation assay, and plate colony formation assay. To examine the growth-inhibitory effect, the cell viability was measured by MTT assay. The cells were treated with ATRA and compared with controls. Results are shown in Fig. 1. The data revealed that ATRA resulted in an average loss of approximately 58.7% of U251 cells and 54.9% of C6 cells after treatment. To understand whether the decrease in the number of viable cells was due to decreased DNA synthesis leading to decreased cell proliferation, the incorporation of BrdUrd into DNA was determined in the presence of ATRA. As shown in Fig. 2, the results were expressed as the percentage of inhibition of BrdUrd incorporation relative to ethanol-treated control. Results revealed that ATRA treatment resulted in a significant decrease in incorporation of BrdUrd into DNA in U251 cells and C6 cells by 51% and 49%, respectively. To determine the anti-proliferative effect in vitro, the ability of ATRA to suppress colony formation was examined. The number of colonies in controls and treated groups was counted and is summarized in Fig. 3. From these results it was evident that ATRA caused significant inhibition of colony formation in U251 and C6 cells (83%, 78.5%, respectively). Our data suggested that ATRA inhibited proliferation of U251 and C6 cells.

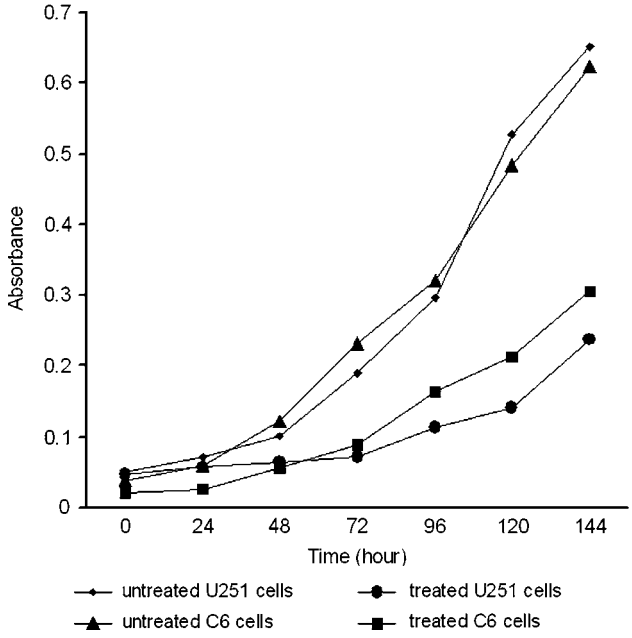

Fig. 1 Effect of ATRA on the survival of U251 cells and C6 cells. Human glioma U251 cells and rat glioma C6 cells were seeded in 96-well plates (1×10^3 cells/well). Next day the cells were treated with either ethanol or ATRA and cultured for six days, and the number of viable cells were determined by MTT assay 0, 24, 48, 72, 96, 120, and 144 h after treatment

ATRA increased the cytoplasmic distribution of *β*-catenin without altering the level of *β*-catenin

Indirect immunofluorescence staining was performed to detect the subcellular distribution of *β*-catenin. In ATRA-treated U251 (Fig. 4a) and C6 cells (Fig. 4b), most of *β*-catenin was detected in the cytoplasm. In contrast,

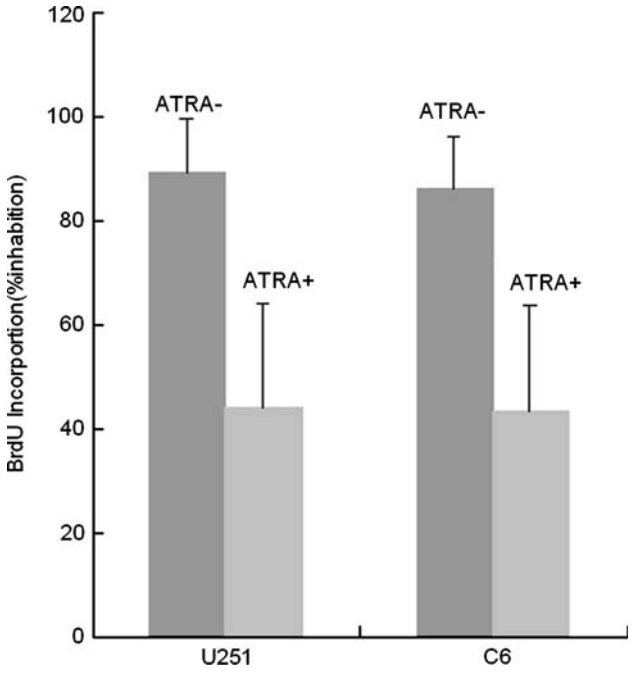

Fig. 2 Effect of ATRA on DNA synthesis in U251 cells and C6 cells. U251 and C6 cells were cultured on coverslips with or without ATRA for 24 h. BrdUrd was added and the cells were reincubated for an additional 24 h. Coverslips were fixed with 4% paraformaldehyde and incubated overnight at 4°C with mouse monoclonal anti-BrdUrd antibody. Then cells were incubated with biotinylated goat anti-mouse IgG followed by streptavidin enzyme conjugate. Results were expressed as the percentage of viable cells over control

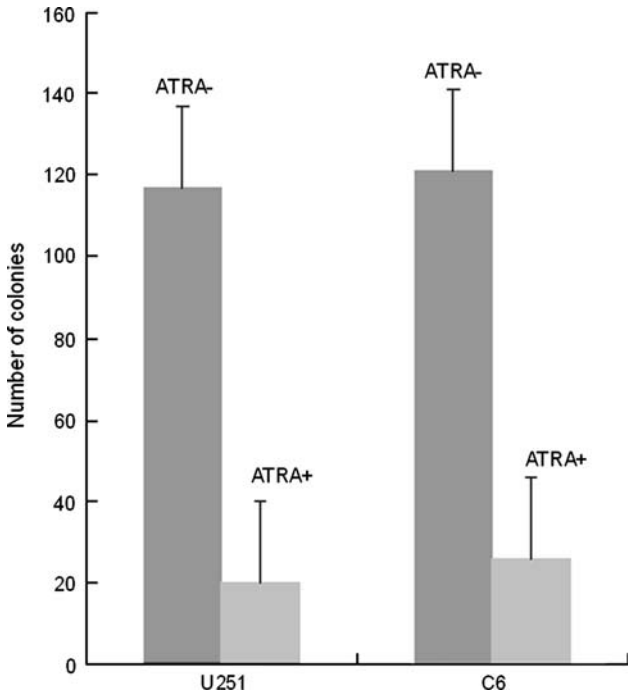

Fig. 3 Effect of ATRA on anchorage-independent growth of U251 cells and C6 cells. 50, 100, or 200 cells were seeded in six-well plates and cultured with or without ATRA for two weeks. The plates were stained with Giemsa's stain and the colonies were counted

β-catenin was found in both the cytoplasm and nucleus in U251 (Fig. 4c) and C6 (Fig. 4d) control groups. Then, taking β-actin as a loading control, the β-catenin mRNA and protein were detected by RT-PCR and Western blot, respectively. There were no changes in the level of β-catenin mRNA (Fig. 5a) and total protein (Fig. 5b), whereas cytoplasmic β-catenin protein was steadily accumulated (Fig. 5b). It was shown that ATRA increased cytoplasmic β-catenin protein without affecting the level of its mRNA and total protein.

ATRA up-regulated the expression of Axin

The levels of Axin mRNA and protein were examined by RT-PCR and Western blot, respectively, in U251 and C6 cells grown with or without ATRA. Compared to β-actin control, the level of Axin protein was significantly elevated (Fig. 5b) in correspondence with an increase in its mRNA after ATRA treatment (Fig. 5a). Our results indicated that the activation of Axin participated in ATRA-inhibited glioma cells proliferation.

Over-expression of Axin promoted cytoplasmic distribution of β-catenin

To identify whether over-expression of Axin could change subcellular localization of β-catenin in glioma cells, rat glioma C6 cells were stably transfected with wild-type Axin. Indirect immunofluorescent staining was performed to detect β-catenin protein. Figure 4e showed that most of β-catenin was detected in the cytoplasm in C6 cells stably transfected with rAxin. In cells stably transfected with empty vector, β-catenin protein was found in the cytoplasm and the nucleus (Fig. 4f). RT-PCR and Western blot were then performed to detect the levels of β-catenin mRNA and protein. As shown in Fig. 5a, the β-catenin mRNA of the cells transfected with Axin was similar to that of the cells transfected with empty vector. The cytoplasmic β-catenin protein was increased in C6 cells transfected with Axin, whereas the total β-catenin level was unchanged (Fig. 5b). These results indicated that the over-expression of Axin increased the cytoplasmic β-catenin without changing the level of its mRNA and protein.

Discussion

This study demonstrated for the first time that ATRA-mediated inhibition of proliferation of glioma cells was associated with nuclear export and cytoplasmic anchorage of β-catenin resulting from the up-regulation of Axin, by

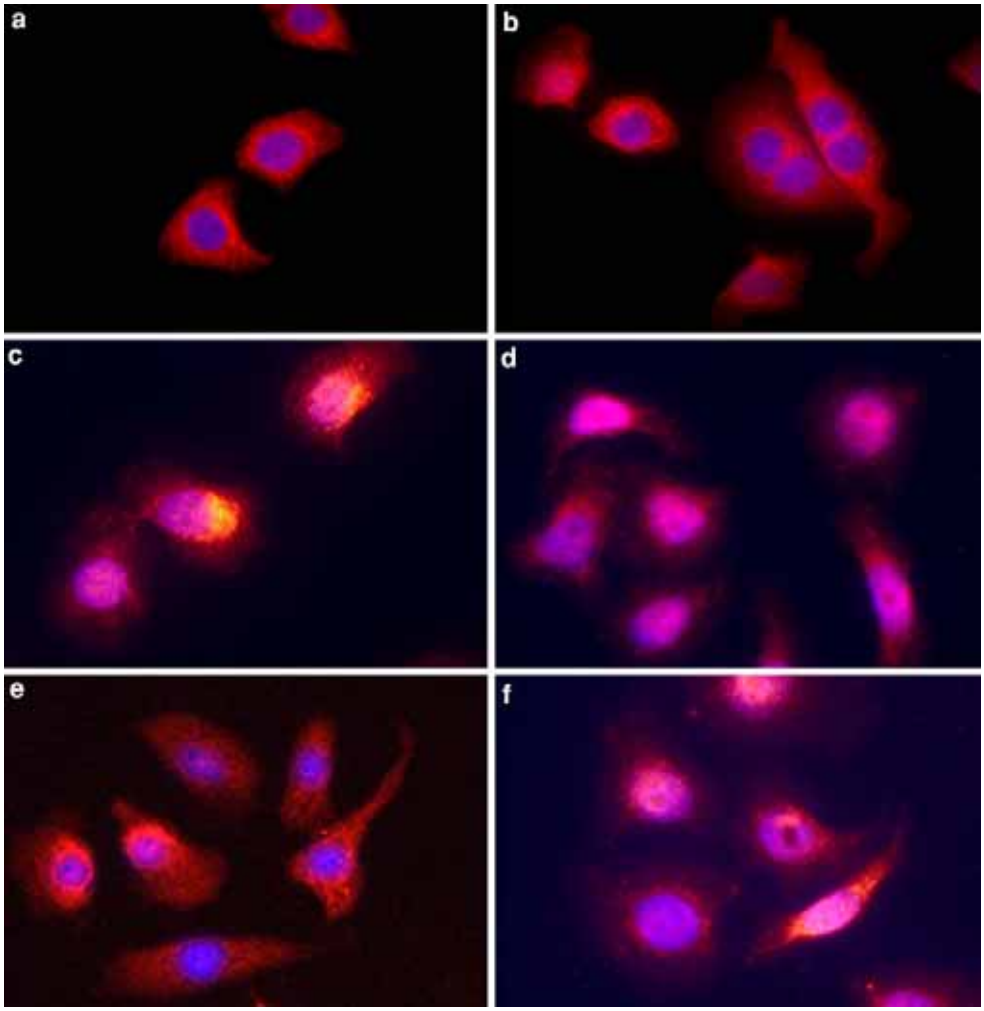

Fig. 4 ATRA and over-expression of Axin changed subcellular distribution of β-catenin (10 × 40). U251 cells and C6 cells were cultured with or without ATRA for 24 h. In treated cells, most of β-catenin was detected in the cytoplasm (**a**, **b**). In contrast, β-catenin was found in both the cytoplasm and nucleus in control groups (**c**, **d**). Similarly to ATRA-treated cells, in C6 cells stably transfected with pIRES2-EGFP-rAxin, most of β-catenin was detected in the cytoplasm (**e**). In C6 cells stably transfected with empty vector pIRES2-EGFP, β-catenin was detected in both the cytoplasm and nucleus (**f**)

which the activity of the Wnt/β-catenin signaling pathway may be repressed.

Previous research has shown that RA can inhibit the proliferation of glioma cells [8, 9]. Consistent with these findings, our results suggested that ATRA had a strong growth-inhibitory effect in glioma cells. It is well known that the biological effect of RA in target cells is mediated by binding to retinoic acid receptors (RAR-α, β, and γ) and retinoid X receptors (RXR-α, β, and γ), which are ligand-dependent DNA-binding transcription factors [26]. ATRA specifically activates RARs [27, 28]. Interestingly, recent studies revealed that ATRA inhibits cell proliferation by the Wnt/β-catenin signaling pathway without altering the level of β-catenin protein in some tumor cells [14–17]. It has been established that nucleo-cytoplasmic transport of β-catenin is important in the regulation of the Wnt pathway [29, 30]. Only in the nucleus can β-catenin activate expression of its target genes [18–20]. Its export is crucial to terminating its signaling activity [31]. So we proposed that ATRA might regulate Wnt/β-catenin signaling by affecting the localization of β-catenin. Here, we determined the subcellular distribution of β-catenin and the level of its mRNA and protein by indirect immunofluorescent staining, RT-PCR, and Western blot, respectively. Our results revealed that the cytoplasmic β-catenin was increased without changes of its mRNA and total protein expression after ATRA treatment. These data indicated that ATRA inhibited the proliferation of U251 and C6 cells partly by promoting the cytoplasmic distribution of β-catenin in glioma cells, which might result in the attenuation of the Wnt/β-catenin signaling activity.

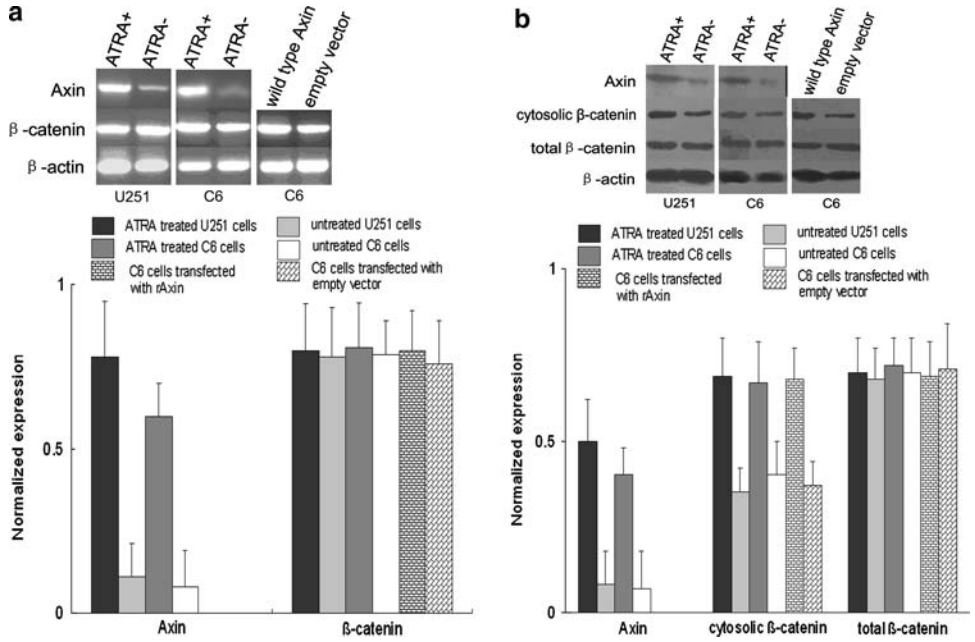

Fig. 5 ATRA up-regulated the mRNA and protein of Axin, and over-expression of Axin resulted in accumulation of cytoplasmic β-catenin without altering the level of β-catenin mRNA and protein. U251 cells and C6 cells were cultured with or without ATRA for 24 h. RT-PCR (**a**) and Western blot (**b**) illustrated that for U251 cells and C6 cells treated with ATRA the level of Axin protein was increased with a corresponding increase in its mRNA. Both the level of β-catenin mRNA and total protein were unchanged while their levels of cytoplasmic protein were increased after treatment. In C6 cells stably transfected with rat wild-type Axin or with empty vector, the cytoplasmic β-catenin was accumulated without apparent change in its mRNA and total protein. Expression of mRNA and protein was normalized to the quantity of β-actin in the bar-graphs

Axin, as a scaffolding protein, negatively regulates the Wnt/β-catenin signaling pathway. Interestingly, recent experiments with some mammalian cells and *Drosophila* strongly supports a role for Axin, independent of its ability to promote β-catenin degradation [21], in controlling the cytoplasmic-nuclear distribution of β-catenin by promoting nuclear export and cytoplasmic anchorage of β-catenin [22–24]. We hypothesized that ATRA might increase the level of cytoplasmic β-catenin by enhanced the expression of Axin. To detect whether ATRA-mediated redistribution of the β-catenin results from up-regulation of Axin, U251 and C6 cells were grown with or without ATRA for 24 h. Levels of Axin mRNA and protein were examined. We found there was up-regulation of Axin protein with a corresponding increase in its mRNA after treatment with ATRA. These data suggested that ATRA enhanced the expression of Axin in U251 and C6 cells. To verify whether up-regulation of Axin is important for proliferative suppression and subcellular re-distribution of β-catenin in glioma cells, we created stable transfectant C6 clones with wild-type Axin expression vector and with empty vector. Indirect immunofluorescent staining of subcellular distribution of β-catenin protein showed that most of the β-catenin localized in the cytoplasm in C6 cells was stably transfected with pIRES2-EGFP-rAxin. In contrast, β-catenin was detected in both the cytoplasm and the nucleus in control groups. Western blot analysis further confirmed that the over-expression of Axin changed subcellular distribution of β-catenin protein and implied that these changes might suppress Wnt/β-catenin signaling pathway. Others have reported that over-expression of Axin caused apoptosis in some cell lines [32]. Our group found that G1/S phase progression through the cell cycle is inhibited in C6 cells transfected with rAxin [33]. These results revealed that over-expression of Axin can also inhibit cell proliferation apart from inducing apoptotic cell death. Biochemical studies showed that intracellular concentrations of Axin are apparently lower than those of other destruction complex components [34], suggesting that fluctuation of Axin protein can efficiently regulate the Wnt/β-catenin signaling pathway. It has been reported for some colon cancer cell lines that over-expression of hAxin promotes the downregulation of β-catenin [35, 36]. However, it should be noted that the level of β-catenin in these experiments were much higher than normal, because of mutations of adenomatous polyposis coli (APC) or abnormal accumulation of β-catenin. In this work, we found there were no apparent changes in either β-catenin mRNA or protein after stably transfecting C6 cells with rAxin. This might result from differences in the cellular context. A possible explanation is that endogenous Axin is relatively highly expressed or/and β-catenin is physiologically

expressed in glioma cells, so that induction of exogenous Axin does not further enhance the down-regulation of β-catenin.

Taking these results together, we have provided evidence for the role of Axin, specifically, in ATRA-mediated anti-cancer effects in glioma cells. Our data implied that ATRA inhibited proliferation of glioma cells by promoting the proportion of cytoplasmic β-catenin resulting from activation of Axin, which might inhibit the Wnt/β-catenin signaling pathway in glioma cell lines.

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