Original paper

Effects of transforming growth factor-β inhibitor on the proliferation of glioma stem/progenitor cell

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Transforming growth factor-β (TGF-β) signaling pathways play an important role in inhibition and promotion of cell proliferation in neural stem cells (NSCs) and glioma stem/progenitor cells (GSPCs), respectively. However, the mechanisms underlying these processes remain unknown. We presumed that there may be functional inhibition at the receptor downstream of TGF-β signaling pathway leading to the activation of non- TGF-β/Smad signaling pathway, which stimulates the proliferation of GSPCs. In this study, GSPCs, from glioma cell lines SHG44, were cultivated with TGF-β receptor inhibitors (LY2157299 and LY2109761), and then the proliferative capability of GSPCs was measured; as well, the synthesis of TGF-β ligands, and the mRNA expression level of TGF-β and some key molecules of non-Smad signaling pathways were also detected. Our results showed that inhibitors against TGF-β receptors could promote the proliferation of GSPCs, and the synthesis of TGF-β ligands was enhanced. Furthermore, the inhibition of TGF-β receptor may lead to the activation of non-Smad signaling pathways (mTOR and NF-κB). In conclusion, the down-regulation of TGF-β receptor capability by TGF-β receptor inhibitors can increase TGF-β ligands synthesis and secretion, which then promote GSPCs proliferation by activating non-Smad signaling pathways.

Key words: transforming growth factor-β (TGF-β), TGF-β receptor inhibitor; Non-Smad signaling pathways, glioma stem/progenitor cells.

Introduction

Malignant glioma is one of the most lethal and aggressive forms of brain tumors in adults. Despite treatments advance combining maximal surgical resection with radiotherapy and adjuvant chemotherapy, patient outcome remains disappointing and survival is limited to 14.6 months with few cases of long-term survivors [1]. Recently, as the cancer stem cell theory was put forward [2], the researches of glioma stem cells (GSCs) have been an area of ongoing investigation [3]. GSCs possess characteristics of self-renewal, infinite proliferation, and inhibitory differentiation [4]. Moreover, GSCs are also regarded as the source of glioma recurrence. Therefore, therapeutic targeting at GSCs may effectively block tumor progression, promote tumor differentiation and improve patients' prognosis [5].

Transforming growth factor-β (TGF-β) is one of the most abundant cytokines in the tumor microenvironments. It can inhibit proliferation and induce differentiation in neural stem cells (NSCs) [6]. However, TGF-β acts differently in GSCs as it maintains tumorigenicity, promotes proliferation and inhibits differentiation to normal neuroglial cells [7, 8, 9]. The mechanisms underlying these processes need further research.

It was reported that the levels of all the three kinds of TGF-β ligands (including TGF-β1/2/3) in glioma tissues were significantly higher than that of normal brain tissues [10]. Moreover, the peripheral blood levels of TGF-β1 and TGF-β2 in glioblastoma patients were even higher than that of normal healthy persons [11]. Thus TGF-β receptor (TβR) signaling, but not TGF-β ligands, seems to distinguish NSCs from GSCs. Smads are the intracellular effectors molecule of TGF-β signaling pathway. It was reported that the levels of Smad2, Smad3, and Smad4 mRNA decreased significantly in glioblastoma tissues comparing with normal brain tissues, astrocytoma, or anaplastic astrocytoma tissues [12]. Based on these studies, we reasoned that there may be functional inhibition at the receptor downstream of TGF-β classical signal pathway, and this leads to the activation of non- TGF-β/Smad signaling pathway, which stimulates the proliferation of GSPCs.

In the current study, we used specific inhibitors to block TβR downstream signaling in glioma stem/progenitor cells (GSPCs) from glioma cell lines of SHG44. Our results showed that the inhibition of TβRs could induce the increased synthesis and secretion of TGF-β ligands. Consequently, the elevated TGF-β ligands could activate non-Smad signaling pathways to promote GSPCs proliferation.

Material and methods

Cells and groups

The human brain glioma cell line SHG44 was purchased from the Chinese Academy of Sciences. The SHG44-GSPCs were separated and proliferated from SHG44 cell line as our previous report [13] Tumor sphere cells were cultivated in serum-free culture medium which contained 2% B27, 20ng/ml EGF and bFGF in a humidified atmosphere with 5% CO_2 at 37°. GSPCs were kept into a control group (without receptor inhibitor), and experimental groups (treated with receptor inhibitors LY2157299 or LY2109761 purchased from American selleck biotechnology Co.).

Cell proliferation assay

The *in vitro* cell growth effect of receptor inhibitor on GSPCs was evaluated by CCK-8 assay. Briefly, the GSPCs of each group were dispensed into 96-well culture plates at 2×10^3 cells/well. On the next day, receptor inhibitors (LY2157299 or LY2109761) were added into each well of experiment groups. According to the final concentrations (0.1 μM or 2 μM) of receptor inhibitors, the experimental groups were divided into two groups, i.e. a low dose group and a high dose group. Then, after 72 or 96 hours' incubation, the viability of GSPCs was analyzed using CCK-8 kit according to company's protocol. And these absorbancy (O.D.) were detected to represent cell proliferative capability.

Cell cycle was detected to compare the cell proliferation phase ratio before and after LY2157299/LY2109761 treatment. Briefly, 2×10^5 cells/well with 5 replicates in each group were seeded in 6-well plates. They were then cultured in a humidified atmosphere with 5% CO2 at 37°C. Three days later, cells were harvested, re-suspended and rinsed with PBS. Then, 250 μl Solution A (trypsin buffer) were added to the cells suspension for incubation for 10 min, followed with the addition of 200 μl Solution B (trypsin inhibitor and RNase buffer) for 10 min at room temperature, and 200 μl cold Solution C (propidium iodide stain buffer) for another 10 min in ice (avoiding light). Finally, cells were collected and detected by a flow cytometry (FCM).

ELISA

The expressions of TGF-β1, TGF-β2, and TGF-β3 in the supernatant of both groups were detected by ELISA analysis. Briefly, cells were spread equably at 2×10^4 cells/ml and 1ml/ well in 12-well culture plates with 3 repetitions each group. After 72 hours' treatment, the culture supernatants in both groups were collected, and the concentration of the TGF-β1, TGF-β2, and TGF-β3 was analyzed by ELISA according to manufacturer's instruction (from American Rapidbio co.).

Real time-PCR

These key molecules in TGF-β-Smad pathway and non-Smad pathway were determined by Real time-PCR analysis. Cells in each group were cultivated for 72 hours and collected, and then total RNA was extracted with the RNAiso Reagent kit (Takara, Dalian, China), and cDNA was generated by reverse transcription of 2 mg of total RNA using random primers and Primescript™ RT Reagent Kit (Takara, Dalian, China) in a 20 μl of reaction volume according to the manufacturer's instructions. The PCR was carried out using cDNA as templates and primers as following: 5'-CCA GAG TGG TTA TCT TTT GAT GTC A-3' and 5'-GAA CCC GTT GAT GTC CAC TTG-3' for TGF-β1; 5'-AAG ACC CCA CAT CTC CTG CTA A-3' and 5'-AGC AAT AGG CCG CAT CCA-3' for TGF-β2; 5'-TCA CCA CAA CCC TCA TCT AAT CC-3' and 5'-TCC AAG TTG CGG AAG CAG TA-3' for TGF-β3; 5'- TTC TGT GGC TGT GAG GTC TG -3' and 5'- TTG CCT TCT GCC TCT TAT GG-3' for mTOR; 5'- TCT ATG GCG CTG AGA TTG TG -3' and 5'- GTC CTT GTC CAG CAT GAG GT-3' for AKT1; 5'- CGT TTC TGC TTT GGG ACA AC -3' and 5'- CCT GAT GAT GGT CGT GGA G-3' for PI3KCA; 5'- GCA ATC ATC CAC CTT CAT TCT-3' and 5'- CTC CAC CAC ATC TTC CTG CT-3' for NF-

κB; 5'-TGA TGA CAT CAA GAA GGT GGT GAA-3 and 5'-TCC TTG GAG GCC ATG TGG GCC-3' for human GAPDH. Real time quantitative PCR was performed in an iCycler 5 (Bio-Rad). A 20-fold dilution of each cDNA was amplified in a 20 µl volume, using the Fast Start DNA MasterPLUS SYBR Green I master mix (Roche Applied Science), with 200 nM final concentrations of each primer. PCR cycles duration temperature was 10 min at 95°C, then 95°C for 10 s, and 58°C for 30, 72°C for 10 s for 40 cycles. The amplification specificity was evaluated with melting curve analysis. Threshold cycle Ct, which correlated inversely with the target mRNA levels, was calculated using the second derivative maximum algorithm provided by the iCycler software. For each cDNA, the mRNA levels were normalized to GAPDH mRNA levels. Statistical analysis was carried out by one-way analysis of variance (ANOVA). Differences were considered significant when $p < 0.05$.

Statistical analysis

All data were analyzed by Statistical Package Social Science SPSS19.0 and expressed as the mean ± standard deviation. Differences between groups were determined by t-test and considered statistically significant at $p < 0.05$.

Results

Proliferation promotion by receptor inhibitors

To detect *in vitro* cell growth effect of TβR inhibitors on GSPCs, SHG44-GSPCs were respectively treated with either LY2157299 or LY2109761 for 72/96 hours at concentrations of 0.1 µM or 2 µM. The results showed that both inhibitors could promote the proliferation of GSPCs when the concentration of TβR inhibitor was 2µM, however, when at a low dose, the TβR inhibitors seem to inhibit the proliferation of GSPCs (Fig. 1).

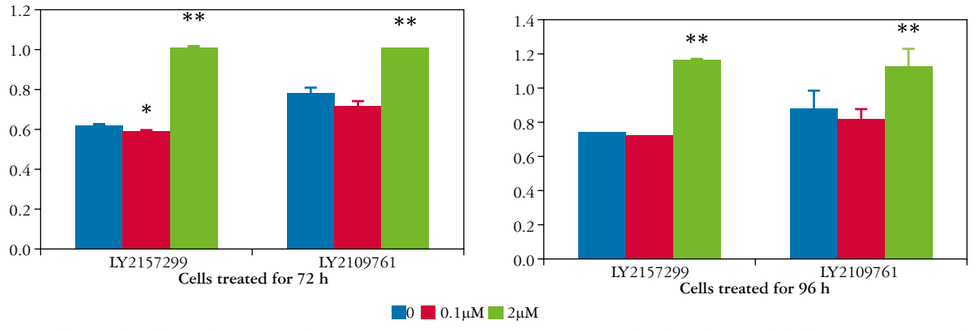

Fig. 1. Cell growth effects of LY2109761 or LY2157299 on SHG44-GSPCs. The absorbance of different groups was detected by CCK-8 assay. A, SHG44-GSPCs were treated with LY2109761 or LY2157299 at 0, 0.1 µM, or 2 µM for 72 h; B, SHG44-GSPCs were treated with LY2109761 or LY2157299 at 0, 0.1 µM, or 2 µM for 96h. The variations between experimental group and control one had statistically significant (*$p < 0.05$, ** $p < 0.025$)

Cell cycle stimulation by LY2157299 and LY2109761

To explore the potential mechanism by which receptor inhibitor at a high dose promotes GSPCs growth, the cell cycle was further analyzed by flow cytometry. As shown in Fig. 2A and B, the G0/G1 phase in SHG44-GSPCs counted 83.78%, and 36.49% in the control group and LY2157299 treated group, respectively. Meanwhile, that of G2/M phase was 5.42%, and 40.51%, respectively. For LY2109761, the results were similar (Figs. 2C and D), and the G0/G1 phase counted 86.95%, and 62.04% in the control group and experimental group while that of the G2/M phase were 12.17%, and 25.57%.

Effect of receptor inhibitor on transcription and expression of TGF-β ligands

To understand the effect of TβR inhibition (when at high dose), the transcription of TGF-β1, TGF-β2, and TGF-β3 in SHG44-GSPCs were detected. The results showed that: when GSPCs were treated with 2 µM LY2157299, both TGF-β2, and TGF-β3 showed marked increase. However, when GSPCs were treated with 2 µM LY2109761, all TGF-β subtypes' expression level went up significantly (Fig. 3A). Similar results were confirmed by ELISA. As shown in Figure 3B, it suggested that the secretory proteins of TGF-β1 and TGF-β2 by SHG44-GSPCs generally elevated, and the variations were statistically significant ($p < 0.05$), but TGF-β3 had no significant change.

Probable molecular mechanism

In order to understand the relationship between receptor inhibitors and SHG44-GSPCs proliferation, we detected the transcriptional changes of the four key molecules involving in cell proliferation, i.e. mTOR, AKT1, PI3KCA and NF-κB, with Real time-PCR. The data showed that the expressions of all four molecules were significantly elevated com-

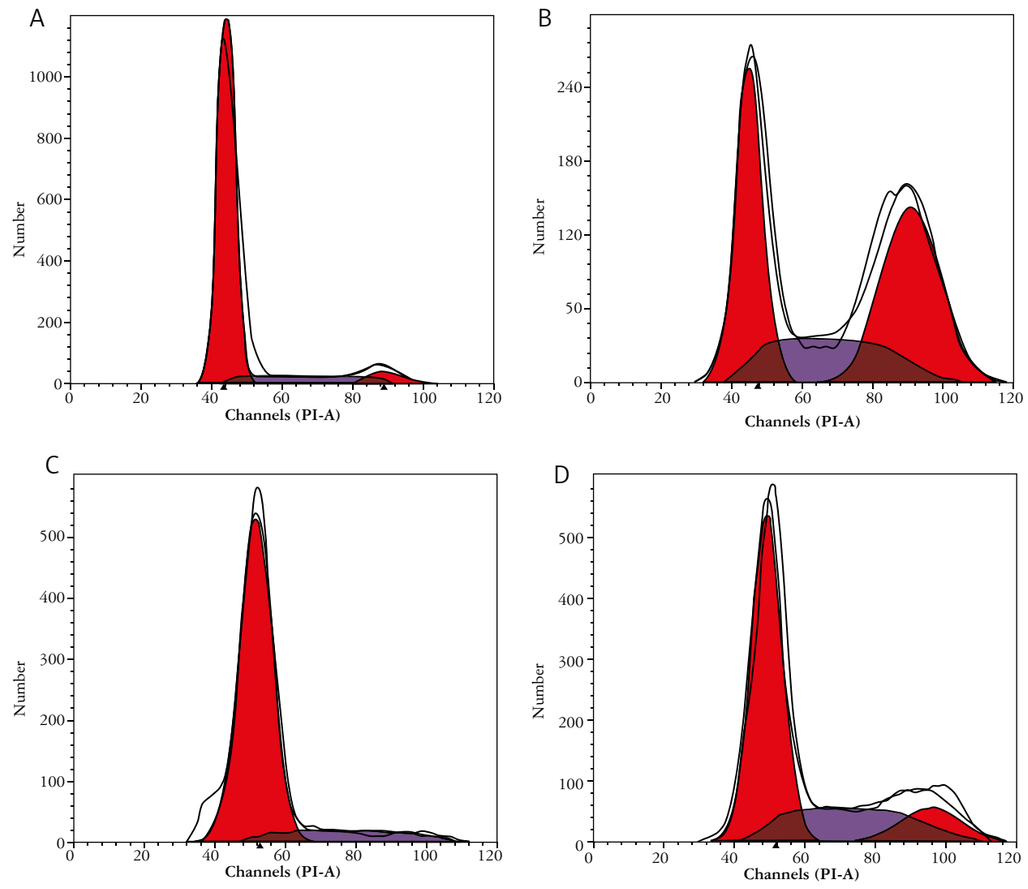

Fig. 2. Cell-cycle distribution effects of inhibitorLY2157299 and LY2109761 on SHG44-GSPCs were detected by FCM: A and B respectively showed the results ofLY2157299 on SHG44-GSPCs of control group and experimental group. C and D respectively showed the results of LY2109761 on control group and experimental group

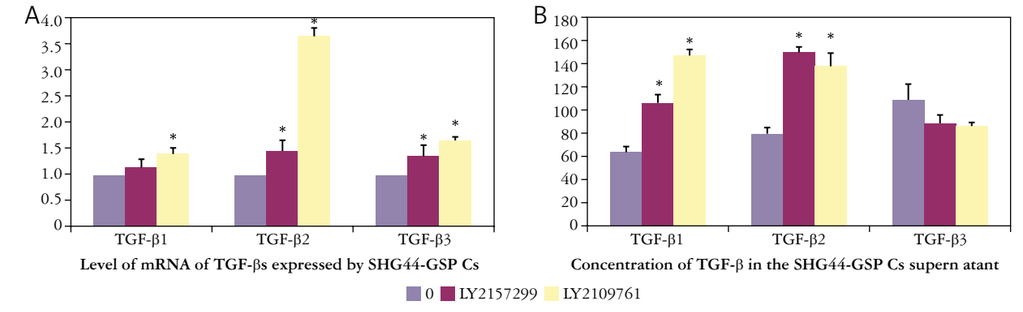

Fig. 3. The effect of receptor inhibitor on TGF-β expressions. **A)** the transcriptional level of TGF-β1/2/3 in SHG44-GSPCs treated by inhibitors LY2157299 and LY2109761 were detected by Q-PCR. *$P < 0.05$ compared with the control group, one-way repeated measures ANOVA and multiple comparisons, $n = 3$ replicates/condition. **B)** ELISA were used to detect the TGF-β1, TGF-β2, and TGF-β3 concentration in the cell culture supernatant, and the results showed that both receptor inhibitors could enhance the synthesis and secretion of TGF-β1 and TGF-β2, but not that of TGF-β3. *$P < 0.05$ compared with the control group, one-way repeated measures ANOVA and multiple comparisons, $n = 3$ replicates/condition

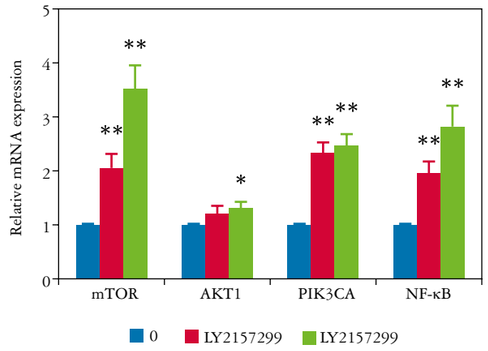

Fig. 4. SHG44-GSPCs were treated with TβR inhibitor, either LY2157299 or LY 2109761 at a concentration of 2μM. By Q-PCR, both kinds of receptor inhibitor could enhance the transcription of mTOR, AKT1, PI3KCA, and NF-κB. *p < 0.05, **p < 0.025 compared with the control group, one-way repeated measures ANOVA and multiple comparisons, n = 3 replicates/condition

pared to the control, while that of the LY2109761 was even more promoted that that of the LY2157299 (Fig. 4).

Discussion

Transforming growth factor-β is a pleiotropic cytokine which presents extensively in all cells to perform a series of biological effects, including cell proliferation, differentiation, apoptosis, angiogenesis and epithelial-mesenchymal transition. However, recent reports revealed that TGF-β showed a bilateral natures during cancer development: TGF-β acted as a tumor suppressor via inhibition of cell proliferation, cell differentiation promotion, and cancer cell apoptosis at the early stage of cancers, while it transformed into a tumor promoter by accelerating tumor development, inhibiting their differentiation, stimulating blood vessel growth and suppressing immunoreactions in the late period [14, 15]. The mechanism underlying these processes remains unknown.

As the cancer stem cell theory being put forward, it was gradually recognized that the occurrence and development of tumors were derived from the proliferation and differentiation of cancer stem cells which were only a small part of cancer. Even if most of the tumor cells were destroyed, cancer stem cells were still capable of growing and lead to tumor recurrence. For glioma stem cells, they have characteristics of unlimited growth, multipotential differentiation, tumorigenic ability and resistance to traditional treatment. All of these brought new challenges to the treatment of glioma.

Transforming growth factor-β signaling pathways played a crucial role of unlimited growth, differential inhibition and tumorigenic ability of GSPCs. Therefore, in theory, blockage or inhibition of TGF-β signaling pathways with TβR inhibitor can suppress GSPCs growth. However, our results confirmed that down-regulation of TGF-β signaling pathways by receptor inhibitors could promote the growth of GSPCs, as shown in Figs. 1 and 2. In the current study, the receptor inhibitors used in our research are TβR-I inhibitor LY2157299 and TβR-I/II dual inhibitor LY2109761, both of which can block the TGF-β/Smad classic signaling pathways. Why did the inhibition of TGF-β signal pathway promote, but not suppress, the proliferation of SHG44-GSPCs? We presumed there existed a concentration-depended biochemical reaction. At a low concentration, the TβR inhibitors do suppress the proliferation of GSPCs (Fig. 1); however, when the concentration became high enough, a deep blockage of TGF-β/Smad signaling pathways may lead to a feedback up-regulation of TGF-β ligands' secretion. Then, with the blockage of TGF-β/Smad classic receptor, the accumulated TGF-β ligands may act on the receptors of other signaling pathways, i.e. non-Smad signal pathways, e.g. mTOR, NF-κB etc. Furthermore, the activation of those non-Smad signal pathways may result in an enhanced proliferation of GSPCs.

To support this hypothesis, we first detected the expression levels of TGF-β ligands after either LY2157299 or LY2109761 treatment. The results indicated that both transcriptional and translational expressions of TGF-β ligands were significantly enhanced. Meanwhile, we detected the transcription of the key molecules of two non-Smad signal pathway, including mTOR and NF-κB. In addition, our results showed an increased expression of both molecules.

mTOR is a serine/threonine protein kinase which is centrally involved in the control of cell growth, proliferation, differentiation and cell cycle regulation, through the PI3K/Akt/mTOR pathway. The activation of PI3K/Akt/mTOR pathway has a close relationship with tumor genesis, and may stimulate cell cycle, decrease cell apoptosis, and promote tumor cells migration, which has been reported in glioma [16], breast cancer cells [17], etc. Moreover, TGF-β is also found to regulate apoptosis by PI3K/AKT/mTOR pathway [18, 19]. NF-κB is another crucial cytokine, which regulates tumor cells' proliferation, differentiation, apoptosis, invasion and metastasis [20]. It was recently observed that TGF-β-induced growth arrest response is attenuated, in association with aberrant activation of NF-κB, which indicated a crosstalk between TGF-β and NF-κB [21]. These data indicated that TGF-β may promote cell proliferation by the activation of NF-κB [22].

According to all listed literatures, we hypothesized that TGF-β ligands may act on some other signal pathways, but not the classic Smad pathway, to promote cells' proliferation. Factually, it also has been reported that TGF-β expression level of GSPCs was significantly higher than ordinary glioma cells, when cultivated *in vitro* [23]. Moreover, expect mTOR and NF-κB, TGF-β can maintain the tumorigenicity of GSPCs through Sox2/4 signaling pathways [7]. Therefore, the mechanism in which inhibition of TGF-β signal pathway promotes the proliferation of SHG44-GSPCs, we believe, is the activation of some non-Smad signal pathways, e.g. mTOR, NF-KB by the accumulation of TGF-β ligands.

Now, come to the question raised at the beginning, why does TGF-β act as a tumor promoter to normal neuroglial cells, in which it maintains the tumorigenicity of GSCs promotes their proliferation and inhibits their differentiation [7-9], but act as a suppressor to neural stem cells (NSCs) by inhibiting proliferation and inducing differentiation [6]? We believe that the functional inhibition at the receptor downstream of TGF-β classical signal pathway leads to the increase of synthesis and secretion of TGF-β ligands, which triggers a non-Smad signal pathway and results in the promotion of GSPCs proliferation.

Is there any conclusion with some therapeutic implications against glioma from our research? Factually, here we only detected the proliferation of GSPCs *in vitro* and no other aspects of tumorgenesis, e.g, angiogenesis, local immunosuppression, local hypoxia acidic environment, endothelial mesenchymal transition and so on. Therefore, researches *in vivo* seem to be necessary first.

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The authors declare no conflict of interest.

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