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

Quanbin Zhang1, Wei Guo2, Chong De3, Meiqing Lou4, Haimeng Li5, Yaodong Zhao1,2

1Department of Neurosurgery, Shanghai 10th People’s Hospital, Tongji University School of Medicine, Shanghai, China
2Department of Neurosurgery, Shanghai General Hospital, Shanghai Jiaotong University, Shanghai, China
3Department of Neurosurgery, Shanghai 10th People’s Hospital, Tongji University School of Medicine, Shanghai, China
4Department of Neurosurgery, Shanghai Zhongshan Hospital, Shanghai, China

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 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 treatment advances 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 patient’s prognosis [5].

Transforming growth factor-β (TGF-β) is one of the most abundant cytokines in the tumor microenvironment. It can inhibit proliferation and induce differentiation in neural stem cells (NSCs) [6]. However, TGF-β affects differently in GSCs as it maintains tumorigenicity, promotes proliferation and inhibits differentiation to normal neuralgial 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-B1/2/3) 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 classically signaling 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% CO2 at 37°C. GSPCs were kept into a control group (without receptor inhibitor), and then experiments groups with receptor inhibitors LY2157299 or LY2109761 (treatment with receptor inhibitors LY2157299 or LY2109761 purchased from American selleck biotechnology Co.).

Cell proliferation assay

The in vitro cell growth effect of receptor inhibitors on GSPCs was evaluated by CCK-8 assay Briefly, the GSPCs of each group were dispersed into 96-well culture plates at 2 × 104 cells/well. On the next day, tumor sphere cells were transferred into well of 12-well culture plates with 5 replicates in each group were seeded in 6-well culture plates 5 replicates in 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 measured by ELISA according to manufacturer’s instructions (from Ameri
can R&D Bios). 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 × 104 cells/ml and 1ml/well in 12-well culture plates with 5 replicates 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 following the manufacturer’s instructions (from American R&D Bios). For the real-time PCR, the absorbancy (O.D.) were detected to represent cell proliferative capability.

Cell cycle was detected to compare the cell proliferation phase ratio between LY2157299 and LY2109761 treatment. Briefly, 2 × 104 cells/well with 3 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 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. These key molecules in TGF-β1, TGF-β2, and TGF-β3 were chosen to evaluate the cell growth effect of receptor inhibitors on GSPCs. The ELISA was generated by reverse transcription of 2 mg of total RNA using random primers and Primerset™ RT Reagent Kit (Takara, Dalian, China). The sample solution (including primers and primerset™ RT Reagent Kit) was placed into the sample wells and incubated for 40 min, followed with the addition of 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).

Real-time PCR

These key molecules in TGF-β1, TGF-β2, and TGF-β3 were evaluated by real-time PCR analysis. Cells in each group were collected 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 Primerset™ 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 TGT GAT CTC A-3' and 5'-GAA CCC GGT GAT GTC CAT TTC-3' for TGF-β1; 5'-AAC ACC CCA CAT CTC CTG CTA A-3' and 5'-AGG AAT AGG CGG CAT CCA CTA-3' for TGF-β2; 5'-TCA CCA TTT TTG ACC TCC C-3' and 5'-TAC AAG TTG CAG AAG CAG TA-3' for TGF-β3; 5'-TTG GTG GTG TGT AGC CTC TGC-3' and 5'-TTG CGG TCT GCC TGC CTC TGC-3' for mTOR; 5'-TCT ATG GCG CTT AGA TTG TG-3' and 5'-GCT GCT TTC CAG GAG TGT GA-3' for AKT1; 5'-GCT TGC TTT GGG ACA AC-3' and 5'-GCT GAT GGT GGT GGA G-3' for PI3KA; 5'-GCA ATC AAC CAT TTG CTC TAT-3' and 5'-TTG CAC CAT CCC TGT CTC TG-3' for NF-

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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 compared to the control group and experimental group while that of the G2/M phase were 12.17% and 25.57%.

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 determined 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 96 h. The variations between experimental group and control one had statistically significant (*p < 0.05, **p < 0.025)
tial inhibition and tumorigenic ability of GSPCs. Therefore, in theory, blockage or inhibition of TGF-β signaling pathways with TIR can inhibit GSPC growth. However, we 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 TIR-1/3 inhibitor LY2157299 and TIR-2 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 SHG4-GSPCs? We presumed there existed a concentration-dependent biological reaction. At a low concentration, the TIR 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 level of TGF-β ligands 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 [12, 18]. 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 crossstalk 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 these 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 cultured in vitro [23]. Moreover, we 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. Therefore, the mechanism in which inhibition of TGF-β signal pathway promotes the proliferation of SHG4-GSPCs, we believe, is the activation of some non-Smad signal pathways, e.g. mTOR, NF-κB 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 neural 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, we only detected the proliferation of GSPCs in vitro and non as other aspects of tumorigenesis, e.g. angiogenesis, local immunosuppression, local hypoxic acidic environment, endothelial mesenchymal transdifferentiation and so on. Therefore, researches in vivo seem to be necessary first.

**The current research was supported by National Natural Science Foundation of China (No.811101909). The authors declare no conflict of interest.**

**References**


