DAPT suppresses the proliferation of human glioma cell line SHG–44

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Objective: To explore the suppressing effect of γ-secretase inhibitor DAPT on proliferation of human glioma cell line SHG–44 in vitro and its mechanism.

Methods: The SHG–44 cell was treated by DAPT with different concentration. The proliferation of cells was detected by MTT assay; cell cycle and TSC of CD133³ were determined by flow cytometry analysis technique; the key factor in Notch signaling pathway (Notch–1, Delta–1, Hes–1) was measured by reverse transcriptase–polymerase chain reaction and western blotting.

Results: DAPT inhibited the growth and proliferation of SHG–44 cells significantly (P<0.05). And the inhibiting effect on SHG–44 cells produced by DAPT showed a dose–dependent manner. DAPT increased the rate of cells in G0/G1 phase of SHG–44 cells, while it decreased the rate of cells in S phase. TSC of CD133³ was significantly reduced after DAPT treated SHG–44 cells. The expression of protein and mRNA of Notch–1, Delta–1 and Hes–1 were gradually downregulated with the increase of DAPT doses.

Conclusions: DAPT can downregulate these key factor in Notch signaling pathway, reduce the TSC of CD133³ and inhibit the proliferation of SHG–44 cells.

1. Introduction

Recent researches show that tumor stem cells (TSCs) account for only a small part in the overall tumor cells, which cause relapse, metastasis and growth of neoplasms. In recent years, foreign and domestic scholars have successfully separated brain tumor stem cells (BTSCs) [1] from glioma tissue and cell lines, which play a pivotal role in glioma initiation, growth and recurrence. A prior study[2] demonstrated that via Notch signaling pathway, the key protein is expressed abnormally during tumor cells proliferation, primarily mediating self–renewal, proliferation and differentiation of normal stem cells and TSCs. It is of great significance to inhibit Notch pathway and the proliferation of TSCs at the same time. γ-secretase, the key enzyme during the Notch signaling activation, plays an important role in cascade reactions. γ-secretase inhibitors may block Notch intracytoplasmic domain (NICD) generation in Notch signaling pathway, thus suppress abnormal cells differentiation and proliferation in NICD. However, there are fewer reports that γ-secretase inhibitors disturbing TSCs survivals due to blocking or inhibiting Notch pathway which will eventually cure tumor.

N–[N–(3,5–difluorophenyl acetyl )–L–alanyl]–S–phenylglycine butyl ester (DAPT), an artificially synthesized γ–secretase inhibitor, is well–known for blocking Notch signaling pathway. Therefore, γ–secretase inhibitor DAPT will be used to treat human glioma cell line SHG–44. Notch pathway and TSCs quantities change will be determined and suppression effect of DAPT on cell line SHG–44 proliferation will be analyzed. We will explore the possible mechanism and provide new ideas and theoretical basis for glioma treatment.

2. Materials and methods

2.1. Cell lines

SHG–44 human glioma cell line was obtained from the Cell
2.2. Reagents and Instruments

L-DMEM medium was purchased from Hyclone, USA. Fetal calf serum was obtained from Hangzhou Sijiqing Bioengineering Material Co., Ltd. Total RNA Kit was purchased from Nanjing KeyGEN Biotech Co., Ltd. One-Step RT–PCR Kit was purchased from Tiangen Biotech (Beijing) Co., Ltd. DAPT was obtained from Alexis Corporation, Switzerland. Propidium iodide (PI) and MTT were purchased from Amresco Corporation, USA. Anti-Notch1, Anti-Delta1 and Anti-Hes1 were products of Chemicon Corporation, USA. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The major devices used are as the following: gel imaging system (Tanon, Beijing) and PCR amplifier (MJ Research, USA).

2.3. Methods

2.3.1. Cell Culture

Frozen cell line SHG-44 was resuscitated. Cells were incubated in L-DMEM containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO2 saturated humidity incubator. Media was changed every 3 d. When cells reached a > 90% confluent monolayer, cells were passaged into new tissue culture flasks.

2.3.2. Randomization

SHG-44 cells were randomized into five groups in which different concentrations of DAPT were applied (Group A: 0 μmol/L; Group B: 0.5 μmol/L; Group C: 1.0 μmol/L; Group D: 5.0 μmol/L; Group E: 10.0 μmol/L). Incubation continued at 37 °C and 5% CO2 in saturated humidity. Culture medium was changed every 3 days.

2.3.3. Suppression effect of DAPT on SHG-44 cells proliferation by MTT

Subcultured SHG-44 cells were obtained after 0.25% trypsin digestion. Cells were seeded onto 96 well plates with a density of 1×10^4 cells/mL and incubated for 24 h. Then DAPT was added according to the groups. One plate was taken out every 24 h during 5 d and 20 μL MTT was added to each well (5 mg/mL). Plates were incubated at 37 °C for 4 h, then the medium was discarded. DMSO 150 μL was added in each well and shook for 10 min. Optical density (OD value) were determined at 570 nm by MTT enzyme-linked immunometric meter. Cell proliferation curves of each group were obtained.

2.3.4. Cell cycle detection by flow cytometry (FCM)

Different concentrations of DAPT treated SHG-44 cells were collected from the above 5 groups after about 48 h. They were digested with 0.25% trypsin, counted and adjusted to 1×10^6/mL. The cells were precipitated by centrifugation (1 000 r/min for 5 min), resuspended with precooled 70% ethanol and 4 °C immobilized overnight. The next day, immobilized cells were centrifuged at 800 r/min for 5 min, 500 μL propidium iodide (PI) and MTT was added (0.05 ° PI, 0.02% RNase) to resuspend. Resuspension solution stand was kept at 4 °C away from light for 30 min and cell cycle was detected.

2.3.5. CD133+ cells detection by FCM

After about 48 h DAPT treated SHG-44 cells (the above 5 groups) were collected, digested with 25% trypsin, counted and adjusted to 1×10^6/mL. They were centrifuged at 1 000 r/min for 5 min, 4 °C. The cells were resuspended and transferred into EP tubes (100 μL/tube), 10 μL FITC labeled CD133 antibody was added and no antibody was added in the control group. Them were kept away from light for 30 min, excess uncombined antibody was washed with PBS, centrifuged at 1 000 r/min 4 °C for 5 min. Then 500 μL PBS was added and percentage of CD133+ cells were detected.

2.3.6. Notch-1, Delta-1 and Hes-1 mRNA expression detection by RT–PCR

48 hs later DAPT treated SHG-44 cells were collected and 1 mL Trizol was added into 2×10^6 cells. Cells were digested to extract total RNA. RNA concentrations and purity were detected by nucleic acid and protein quantitation instrument. Takara RT–PCR kit was used to conduct reverse transcription in accordance with the instructions: 52 °C, 30 min; 99 °C, 5 min; 5 °C, 5 min, save, cDNA synthesized and PCR amplification. After PCR amplification, 1% agarose gel electrophoresis was undertaken to analyze the PCR products. Then electrophoresis pictures were obtained using gel image analysis system. Image analysis software Gene Tools were used to analyze results. PCR amplification primer and annealing temperature details were as follows (Table 1).

2.3.7. Notch-1, Delta-1, Hes-1 protein expression detection by western blotting

48 h later, DAPT treated SHG-44 cells were collected. RIPA lysis buffer was used to extract total proteins and BCA method to determine protein concentrations. They were mixed well, and added with loading buffer to the

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<td>Primer</td>
</tr>
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<td>Notch-1</td>
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precipitations and loaded to the gel. Then proteins were transferred from gel to the membrane, the blot was removed from the transfer apparatus and immediately placed into blocking buffer. They were probed with primary antibody rabbit anti-human Notch-1 (1:1000), Delta-1 (1:800), Hes-1 (1:1000) in TTBS/1% NFDM at 4 °C overnight and was rinsed. Then were combined with HRP labeled second antibody and rinsed again. Finally, color development with HRP-ECL was detected.

3. Results

3.1. Cell morphology

Significant morphology and quantity changes occurred to DAPT treated SHG-44 cells 48 h later. Group A (control group) had the largest quantities of cells, a large part of which was spindle cells, while abnormal cells accounted for a relatively lower proportion. Cells adhesion was not decreased and cells arranged intensively in order on the bottom of plates. The number of cells in group B was slightly decreased and exhibited no change in morphology. The number of DAPT (1.0 µmol/L) treated SHG-44 cells in group C was significantly decreased, in which the percentage of abnormal and large-volume cells increased. Cells distributed onto the bottom of plates sparsely. The numbers of cells in group D and E were significantly decreased, wherein necrotic dead cells were observed. The remaining viable cells were abnormal in morphology, irregular border and suspended in culture medium because they could not adhere to the bottom easily (Figure 1A–E).

3.2. MTT results

Cell viability in each group was detected by MTT. Compared with those in group A, proliferation of SHG-44 cells in group B, C, D and E were inhibited by DAPT. For group B and A, the results were significantly different (P<0.05). However, cell viability of group B was significantly higher than those in group C, D and E (P<0.05). The results showed that increased inhibition effect was related to increased DAPT concentrations. However there was no difference among group C, D and E (P>0.05). It indicated that DAPT is a concentration-dependent inhibitor that may obviously inhibit SHG-44 cells proliferation. As concentration of DAPT higher than 1.0 µmol/L showed no more obvious disparities in cell inhibition, concentration of 1.0 µmol/L was our priority (Figure 2).

3.2. DAPT on SHG-44 cell cycle

Compared with group A, DAPT treated SHG-44 cells in group B, C, D and E were increased in quantities at stage G0/G1, while decreased at stage S (P<0.05). Change of the percentages of cells at each stage in group B was significantly less than those in group C, D and E (P<0.05), while there were no differences among group C, D and E (P>0.05). It indicated that SHG-44 cells may be stopped at stage G1/G0 and percentages of cells at stage S were decreased, especially at the concentration of 1.0 µmol/L group (Table 2).

3.3. CD133+ TSC percentages

48 h after SHG-44 cells in group B, C, D and E were

<table>
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<th>Group</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
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<tr>
<td>A (0 µmol/L)</td>
<td>56.25±1.31</td>
<td>28.47±1.24</td>
<td>15.38±0.87</td>
</tr>
<tr>
<td>B (0.5 µmol/L)</td>
<td>61.97±5.80*</td>
<td>23.89±4.42*</td>
<td>15.25±3.61*</td>
</tr>
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| C (1.0 µmol/L) | 67.19±4.72* | 18.57±2.30* | 14.05±1.53*#
| D (5.0 µmol/L) | 68.73±7.68* | 18.45±2.20*△ | 13.82±1.36*△ |
| E (10.0 µmol/L) | 70.58±9.44* | 16.39±3.35*△ | 13.03±3.41*△ |

*P<0.05 compared with A group; △P>0.05 vs. group A; △P<0.05 post-group vs. pro-group; △P>0.05 post-group vs. pro-group.
treated by DAPT, CD133 cell percentages were decrease, with expression level of (25.75±2.13)%, (19.57±3.04)% (15.91 ±2.98)%, (10.15±2.33)% and (7.02±3.17)%, respectively. Compared with group A, CD133 cell percentages in group B, C, D and E were decreased significantly (P<0.05), which was inversely proportion to drug concentrations (P<0.05). It is demonstrated that DAPT significantly decreased CD133 TSC percentages in a dose-dependent manner.

3.5. RT–PCR results

RT–PCR assay was used to detect changes of expression levels of Notch–1, Delta–1 and Hes–1 mRNA of DAPT treated SHG–44 cells in each group 48 h later. The results showed that similar tendency of different expressions of genes like Notch–1, Delta–1 and target gene Hes–1 acting upstream of the Notch signaling pathway was observed. Compared with those in group A, the expression levels of the three genes in cells of DAPT treated four groups were significantly decreased (P<0.05). The expression level in group B was significantly higher than group C, D and E (P<0.05), while no statistical differences were observed in among group C, D and E (P>0.05). It is demonstrated that, DAPT could down regulate expression levels of Notch–1, Delta–1 and Hes–1 in SHG–44 cells, wherein group C (1.0 μ mol/L DAPT) had the similar gene expression level with group C and D (higher than 1.0 μ mol/L DAPT). Therefore, it was highly suggested to take 1.0 μ mol/L DAPT as the best choice (Figure 3).

3.6. Western blotting results

48 h later, protein levels of Notch–1, Delta–1 and Hes–1 gene expression of DAPT treated SHG–44 cells in each group were measured by Western blotting. The results showed that similar tendency of the three critical protein expression was observed. Compared with those of group A, protein levels of group B, C, D and E significantly decreased (P<0.05), wherein those in group B were higher than group C, D and E (P<0.05). However, there was no statistical differences among group C, D and E (P>0.05). It is suggested that DAPT could down regulate protein level of upstream gene Notch–1, Delta–1 and target gene Hes–1 in Notch signaling pathway of SHG–44 cells and DAPT concentrations higher than 1.0 μ mol/L (group C) had the similar down–regulation effect in group D and E (Figure 4).

4. Discussion

Glioma is the most common intracranial malignant tumor with a relatively high morbidity and mortality, which is often hard to control. The traditional chemotherapy regimens targeting tumor cells with highly proliferative ability could removal or kill tumor tissues and cells to the largest extent. The latest research shows that the presence of TSC is the source of tumor relapse, metastasis and resistance to chemotherapy. Notch signaling pathway plays a vital role in the proliferation and differentiation of stem cells and tumor stem cells. In addition, there are abnormal activations of Notch signaling pathway in several neoplasms[3-5].

Notch signaling pathway comprises Notch receptor, Notch ligand and SCL (a DNA binding protein). The Notch receptor has undergone two proteolyseses successively after the combination of ligand and receptor. The second proteolysis is γ–secretase (presenilin, PS), which is the critical enzyme during Notch signal activation[6,7] resulting in protein incision at the intramembrane locus near the transmembrane domain. NICD with nuclear localization signal is then released, translocated into the nucleus and interacts with DNA binding protein CSL to activate transcription, resulting in activation of downstream Hes, Hey, HERP and b HLH genes and influencing cell proliferation, differentiation and apoptosis[8,9]. A γ–secretase inhibitor could block NICD generation in Notch signaling pathway, thus inhibiting abnormal cell differentiation, proliferation and apoptosis originating from NICD. Furthermore, a γ–secretase inhibitor interferes with Notch signal transduction between tumor cells and vascular endothelial cells to block angiogenesis.

DAPT is a well–known, artificially synthesized γ–secretase inhibitor that effectively blocks Notch signaling pathway and has proven to have great potential in anti–tumor effect[10-13]. In our study, SHG–44 cell line was treated by different concentrations of DAPT to observe cell proliferations and explore its possible mechanisms. MTT method was used to detect DAPT treated SHG–44 cells and results show DAPT significantly inhibit cells growth in a dose–dependent manner. When DAPT concentrations increased to 1.0 μ mol/L, the inhibition effect was most obvious and showed no differences with higher concentration groups, indicating 1.0 μ mol/L is the maximal effective concentration in clinical application. Cell cycle detection by flow cytometry
showed a large percentage of SHG–44 cells stopped at stage G2/M, and the percentages of cells at stage S decreased. The inhibition effect enhanced with DAPT concentrations increased. Western blotting and RT–PCR assay shows different concentrations of DAPT (1.0, 5.0, 10.0 μm/L) may down regulate mRNA and protein expression levels of critical factors (Notch-1, Delta-1, Hes-1) in Notch signaling pathway of SHG–44 cells in a dose–dependent manner. Prior study[14] detected mRNA and protein expression levels of Notch–1 and ligands Delta–1, Jagged–1 in 6 human brain glioma cell lines and primary human brain glioma, indicating their over expression. In addition, immunohistochemical staining results shows moderate and high level of Notch–1 protein expressions in the majority of grade II and III glioma. The results proved the prior study results, showing the severity of tumors is in correlation with Notch signaling activation. Based on MTT and results from cell cycle detection, inhibition of DAPT on glioma cell line SHG–44 proliferation may be related to the down regulation of Notch–1, Delta–1 and Hes–1mRNA. Furthermore, Hes–1 protein could promote cell proliferation by inhibition on Cyclin– dependent kinase inhibitor p27 transcription[15–18] and down regulate Hes–1 expression to inhibit cell proliferation. Besides, our study shows that CD133⁺ decrease to approximate one fourth in SHG–44 cell line by DAPT. Fan et al[9] discovered CDD133⁺ in SHG–44 cells decreased to one fifth the original quantities by blocking Notch signaling in BTSC cultures in vitro. Our experimental results are similar with these findings. CD133⁺ is considered as the characteristic indicator of glioma stem cells where the CD133⁺ cell expression decrease indicating significantly decrease of SHG–44 cell line in TSCs. Inhibiting Notch signaling pathway could obviously suppress TSCs proliferation, the mechanism of which may be related with down-regulation of Notch–1, Delta–1 and Hes–1 mRNA. It is a critical mean to inhibit tumor cell proliferation by decreasing the number of TSCs, thus providing an effective regimen to cure malignant tumor.

We can conclude that DAPT may suppress SHG–44 cell proliferation by blocking Notch signaling pathway wherein the concentration of 1.0 μm/L achieved the most significant effect due to low cytotoxicity and less adverse drug reactions. In addition, the study has proven DAPT may obviously inhibit TSCs proliferation and expected to be the effective drug for glioma treatment or serve as an adjuvant drug to improve radiotherapy and chemotherapy sensitivity. Further studies are still needed to explore the specific mechanism of DAPT anti–tumor effect, especially on TSCs, and may provide guideline to cure glioma.

Conflict of interest statement

We declare that we have no conflict of interest.

References


