International Journal of Sciences

Research Article Volume 8 – April 2019 (04)

Study on the Effect of Glutamine on Proliferation and Survival of Bladder Cancer T24 Cells

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Abstract: Objective To observe the effect of glutamine (Gln) on the proliferation and survival of bladder cancer T24 cells and explore its mechanism. Methods MTT was used to detect the proliferation of T24 cells in Gln(+) and Gln(-) groups under different time gradients. The best time was selected and the Gln(+) and Gln(-) groups were analyzed at the this time. Between the Gln(+)+Don gradients groups, T24 cells were tested for cell proliferation. Survival ratio and reactive oxygen species (ROS) content of T24 bladder cancer cells in Gln(+), Gln(-) and Gln(+)+Don groups were detected by Annexin V-FITC/PI double staining and ROS kits, respectively. The Gln(-) group was used as the control group, and the ROS scavenger N-acetyl-L-cysteine (NAC) was added to the experimental group to observe the cell proliferation level and survival ratio. Results Compared with Gln(+) group, the proliferation level of T24 cells in Gln(-) group decreased at 24, 48, and 72h, and it was most obvisously at 72h. Compared with the Gln(+) group, the cell proliferation levels of the Gln(-) group and the Gln(+)+Don group were significantly decreased, the ROS level were increased, and the survival rate were decreased significiently. Versus the Gln(-) group, the levels of ROS in the Gln(-)+NAC group decreased, and the proliferation level and survival rate increased. Conclusion Gln deficiency can inhibit the proliferation and survival of T24 cells by increasing ROS levels.

Keywords: Glutamine, Bladder Cancer, Reactive Oxygen Species, Cell Proliferation, Cell Survival

Introduction

As one of the common tumors in the urinary system, the incidence of bladder cancer has increased year by year in recent years. Research on reprogramming of tumor energy metabolism has been a hot topic in related researches such as tumor molecular biology. As the most abundant amino acid in the microenvironment, glutamine (Gln) can affect the proliferation and survival of tumors by participating in the process of supplementing the Krebs cycle and maintaining the redox state [1, 2]. Inhibition of glutamine metabolism has been shown to be effective in inhibiting cancer cell growth, whereas glutamine supplementation can induce or inhibit cell death depending on cell type [3].

As a second intracellular messenger, ROS can activate apoptosis. In fact, glutamine can act as an inhibitor of apoptosis [4]. Glutamic acid, the product of glutaminolysis, can be used to synthesize the antioxidant, glutathione [5]. Most glutamine-derived carbons can be used to generate NADPH for redox balance [6]. Therefore, glutamine becomes an essential amino acid in glutamine-addicted cancer cells, related to their viability.

However, it is unclear that whether and how glutamine affects the proliferation and survival of bladder cancer. In this study, we investigated the mechanism of glutamine affecting the proliferation and survival of bladder cancer T24 bladder cancer cells from the perspective of reactive oxygen species (ROS), and provided a direction for the precise targeted therapy of bladder cancer in clinical.

2 Materials and Methods

2.1 Cells and reagents

The bladder cancer cell line T24 was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China), routinely cultured in BI's 1640 medium containing 2 g/L of glucose and 300 mg/L of glutamine, and supplemented with 10% fetal bovine serum. The assay medium was BI's modified Eagle's medium without glucose and glutamine, reconstituted with 2 g/L D-(+)-glucose, 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were grown at 37 °C in a humidified 5% CO2 atmosphere. Cells were treated with L-glutamine (Sigma- Aldrich), D-(+)-glucose (Sigma-Aldrich), 0-100 μM of 6-Diazo-5-oxo-L-norleucine (Don) (Sigma-Aldrich), 0-10 mM Acetylcysteine (NAC) (MCE). Reagents were dissolved either in ultrapure water or directly in BI's modified Eagle's medium according to manufacturer's indications. All drugs and reagents were administered to adherent cells in fresh assay

Published at: http://www.ijsciences.com/pub/issue/2019-04/
DOI: 10.18483/ijSci.2009; Online ISSN: 2305-3925; Print ISSN: 2410-4477

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medium.

2.2 Cell culture
T24 cells were cultured in BI medium containing 10% fetal bovine serum, penicillin 100 U/mL, streptomycin 100 mg/L, and placed in a 37 ° C, 5% CO 2 incubator. After adding to the experimental group for 24 h culture, the T24 bladder cancer cells were starved for 8 h and then treated with different experimental mediums.

2.3 Cell proliferation
According to the MTT procedure, the logarithmic growth phase T24 cells were inoculated into a 96-well plate at 5×10 4 cells/mL, and the edges were filled with sterile PBS solution. The cells were divided into 2 groups and added respectively. Gln (Gln+) and Gln-free (Gln-) medium were added to 10 μL of MTT solution at 0, 24, 48, and 72 h, and incubated at 37 ℃ for 4 h, then added to DMSO at 490 nm wavelength. The optical density (OD) value is measured to select the best time. The Gln(-) group was used as the control group. Under the condition of Gln(-), the NAC (ROS scavenger) gradient was established in the experimental group. At the same time, the Gln(+) group was used as the control group, and the Gln(+) condition was used. Next, the experimental group was added with DON (glutaminase inhibitor), and the OD value was measured by a microplate reader according to the procedure of MTT at 72 h, and the optimal concentration of NAC and DON was screened separately.

2.4 ROS detection
Logarithmic growth phase T24 cells were seeded in 96-well plates at 5×10^4 cells/mL. Cells were set to Gln(-), Gln(+), Gln(-)+NAC, Gln(+)+DON processing. After 72 h of optimal culture time, fluorescein was diluted with serum-free medium at a ratio of 1:1 000 to a final concentration of 10 μmol/L, 1 mL of diluted fluorescein was added to each well, and 1 mL of the negative control well was added. Serum-free medium for the probe. Incubate in a 37 °C incubator for 30 min, mix once every 5 min, allow the probe to fully act with the cells, wash it 3 times with PBS, and measure the fluorescence intensity by fluorescence microplate reader.

2.5 Apoptosis assay
T24 cells in logarithmic growth phase were inoculated into 12-well plates at 10×10 4 cells/mL. The experimental group was the same as 1.2.2. After 72 hours of culture, the cells were collected into a centrifuge tube and washed twice with PBS. Centrifuge at 1000 r/min for 5 min, discard the supernatant, resuspend the cells by adding 500 μL of binding buffer, and add 5 μL of fluorescein isothiocyanate (Annexin V-FITC) and 5 μL of propidium iodide (PI). The mixture was mixed and protected from light for 15 min at room temperature. Apoptosis was detected by flow cytometry within 1 h, and the survival ratio was calculated.

2.6 Statistical methods
The values were analyzed by SPSS 13.0 statistical software. The measurement data in accordance with the normal distribution were expressed as mean±standard deviation (x±s). The mean between the two groups was compared by independent sample t test. One-way analysis of variance was used for comparison. Multiple comparisons were performed by LSD-t method. All experiments were repeated at least 3 times independently. P<0.05 was considered statistically significant.

3 Results
3.1 Glutamine affects the proliferation of bladder cancer T24 cells
Compared with the Gln(+) group, the proliferation level of T24 cells in Gln(-) group was decreased at 24, 48, and 72h (P<0.05), and the cell proliferation level of Gln(-) group decreased by 47.4% at 72h which is the most significant. Therefore, 72 h is chosen as the best time (Fig. 1-A). at 72h, in the presence of Gln(+), when the concentration of Don (glutamine analogue) is more than 25 μM, the T24 cells proliferation was lower than that of the Gln(-) group, so 25 μM Don was selected as the optimal concentration (Fig. 1-B). At the concentration of Don 25 ng/ml, the proliferation levels of the Gln (+) group, the Gln (-) group and the Gln (+) + Don group were compared at 72 h. Compared with the Gln(+) group, the cell proliferation levels of the Gln(-) group and the Gln(+)+Don group were significantly decreased, P<0.001 (Fig. 1-C).

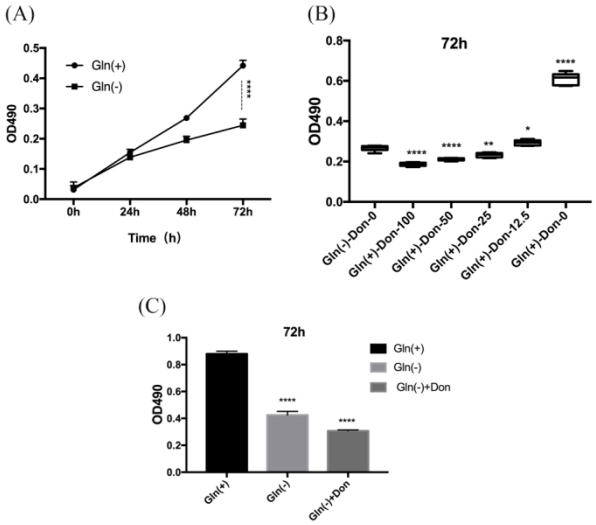

Figure 1. Glutamine affects the proliferation of bladder cancer T24 cells. (A) The growth curve of T24 with glutamine 300 mg/L, Gln(+) and 0 mg/L, Gln(-) for 24, 48 and 72 h, n=6. (B) Analysis of the difference of varying Don concentration gradients , Gln(+) and Gln(-) on cell proliferation at 72 h. (C) The proliferation of Gln(+), Gln(-) and Gln(+)+Don. n=3. Values are expressed as the m ± s of experiments performed in triplicate. *P < 0.05, **P < 0.01, ****P<0.0001. Two-way ANOVA is used in A . One-way ANOVA with a test for homogeneity of variance was used to compare

between groups in (C-D).

3.2 Glutamine affects the survival of bladder cancer T24 cells

At 72 h, compared with Gln(+), the survival levels of Gln(-) and Gln(+)+Don were reduced by 22% and 34%, and the proportion of late apoptosis was significantly higher than that of early apoptosis. Namely, the early apoptosis proportions of Gln(-) and Gln(+)+Don were increased by 1.79% and 7.13% respectively, compared with Gln(+). At the same time, the proportions of late apoptosis were 20.42% and 26.62%.

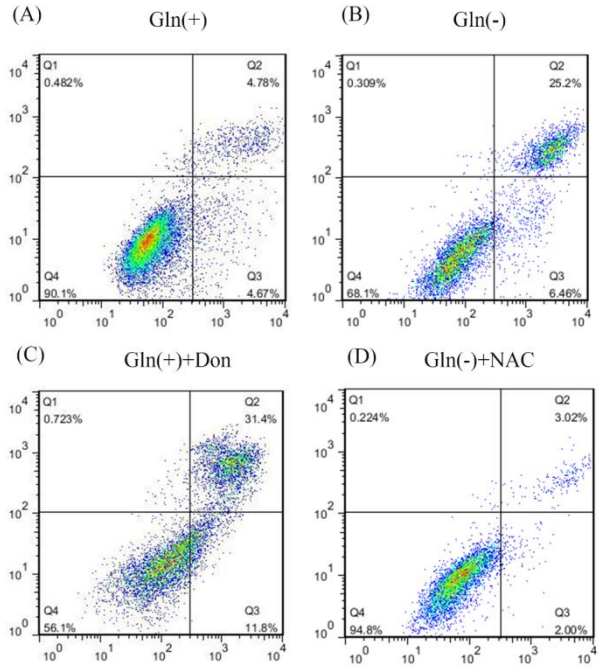

Figure 2 Glutamine affects the survival of bladder cancer T24 cells. (A) The apoptosis effects of different conditions of Gln(-), Gln(+), Gln(+)+Don, Gln(-)+NAC with flow cytometry at 48 h.

3.3 Glutamine affects ROS production in bladder cancer T24 cells

Cultured with lacking of glutamine for 72h, the ROS levels of the tumor cells were significantly increased higher than Gln(+). Bsides that, the Gln (+)+Don group shows more ROS content than Gln(+) too. After the addition of the NAC (Ros scavenger), the ROS levels in the tumor cells , cultured with lacking Gln, were significantly reduced.(Figure 3)

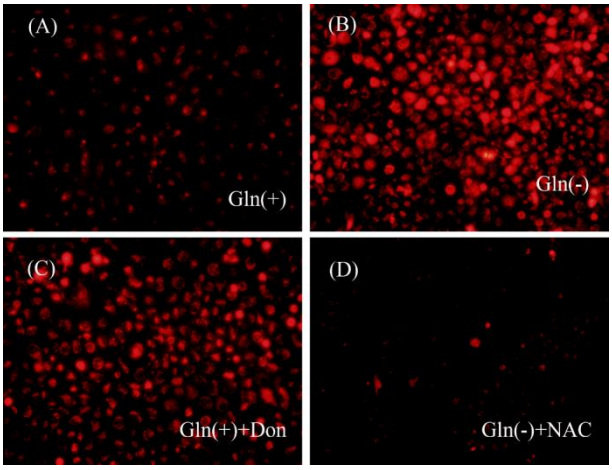

Figure 3 Glutamine affects ROS production in bladder cancer T24 cells. The fluorescence intensity in different treatment groups, namely, Gln(+), Gln(-),Gln(+)+Don, Gln(-)+NAC, by intracellular ROS fluorescence staining. Staff gauges were 100 μm in the figure.

3.4 Glutamine promotes proliferation and survival of bladder cancer T24 cells by neutralizing ROS production

Compared with the Gln(-) group, after the addition of the active oxygen scavenger, NAC, the proliferation level of the tumor cells in the Gln(-)+NAC groups increased at concentrations of 1.25, 2.5, 5, 10mM. And the rate of proliferation is most at 10mM concentrations, which is similar to the Gln+ group (Figure 4-A). Therefore, 10mM was selected as the optimal concentration. At this concentration of NAC, the proliferation levels of Gln(+) group, Gln(-) group and Gln(+)+NAC group at 72h were compared. Compared with the Gln(-) group, the cell proliferation levels of the Gln(+) group and the Gln(+)+NAC group were significantly increased (Figure 4-B). Besides that, as what showed in Figure 2, with the absence of glutamine, the level of apoptosis decreased after the addition of NAC versus Gln(+).

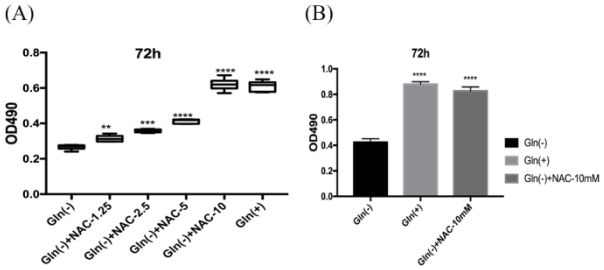

Figure 4. Glutamine promotes proliferation and survival of bladder cancer T24 cells by neutralizing ROS production. (A) Analysis of the difference of varying NAC concentration gradients with absence glutamine, Gln(+) and Gln(-) on cell proliferation at 72 h. (B) The proliferation of Gln(+), Gln(-) and Gln(-)+NAC. n=3. Values are expressed as the m ± s of experiments performed in triplicate. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001. One-way ANOVA

with a test for homogeneity of variance was used to compare between groups in (A-B). using Gln(-) as the control.

Discuss

Tumor microenvironment plays an important role in tumorigenesis and development. As one of the most abundant amino acids in the tumor microenvironment, glutamine (Gln) is mainly involved in important physiological processes such as energy synthesis, biosynthesis, antioxidant defense and cell signal regulation [2, 3]. At present, relevant studies have confirmed that the expression level of glutamine metabolizing enzyme Gls is higher than that in normal tissues in tumors such as small cell lung cancer [7], thyroid cancer [8] and hepatocellular carcinoma [9].

The results of this experiment show that Gln deficiency inhibits the proliferation and survival of bladder cancer T24 cells. Intracellular ROS levels increased in the absence of Gln relative to the Gln(+) group. In addition, in any case, extracellular glutamine levels affect the susceptibility of cancer cells to different apoptosis-inducing factors. Glutamine deprivation has been reported to make Hela cells susceptible to Fas (CD95) ligand, TNF-α (tumor necrosis factor-α) and heat shock-mediated apoptosis [10]. Glutamine deprivation also promotes apoptosis by increasing mitochondrial ROS and decreasing glutathione. It has been confirmed that glutamine deprivation can induce apoptosis of liver cancer, hybridoma, leukemia, myeloma and fibroblasts [11]. Therefore, we further confirmed that there is a correlation between glutamine metabolism and tumor cell apoptosis in bladder cancer cells. For the glutamine-dependent tumor cell line T24, the proportion of apoptosis was significantly increased in the absence of glutamine.

Glutamine metabolism has been shown to be effective in inhibiting tumor growth by inducing apoptosis [12]. Determining the glutamine dependence of myometrial invasive bladder cancer has a guiding role in our clinical treatment. Increased glutamine metabolism utilization not only provides energy and substrate for cancer cell growth and proliferation, but also makes glutamine a powerful candidate for cancer treatment. Tumors such as liver cancer [8], melanoma [13], and breast cancer [14] were found to knock out glutaminase or use glutaminase inhibitors to significantly inhibit tumor growth. Several glutaminase inhibitors have been developed to find more selective glutamine catabolic inhibitors and show tumor-inhibiting activity in preclinical models, including 968, BPTES and CB-389 [14-18].

In summary, glutamine inhibits the proliferation and survival of bladder cancer cell line T24 by neutralizing ROS production. But more specific and other related mechanisms are for further study.

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