Regulation of autophagy inhibition and inflammatory response in glioma by Wnt signaling pathway

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Abstract. The objective of this study was to investigate the mechanism of the function of Wnt signaling pathway in regulating autophagy and inflammatory response in glioma cells. Human brain glioma cells U118 were selected and divided into three groups: i) the Wnt signaling inhibitor IWR-1 group (the observation group); ii) the PBS negative control group (the PBS group) and iii) the blank control group. After 24 h culture, Wnt5a/β-catenin protein, autophagy marker, microtubule-associated-proteins-1A1B-light-chain-3C (LC-3) II and Beclin I, and inflammatory factors IL-6 and TNF-α protein expression levels were evaluated using western blotting. Compared with both control groups, Wnt5a/β-catenin, IL-6 and TNF-α protein expression levels were significantly lower, and LC-3II and Beclin I protein expression levels were significantly higher in the observation group. In conclusion, Wnt5a/β-catenin signaling pathway regulates autophagy and inflammatory response of glioma cells.

Introduction

Glioma, a major malignant tumor of the central nervous system, is characterized by high incidence, recurrence and mortality rates, and low cure rate (1). In-depth analysis of the mechanism of tumorigenesis and the identification of specific molecular markers and intervention targets has become a hot topic in tumor research. Several studies have confirmed that, Wnt signaling pathway, which is closely related to central nervous system diseases, is involved in the proliferation, migration and differentiation processes of neural stem cells (2). The classic Wnt/β-catenin pathway has been extensively studied (2). Autophagy, also known as type II programmed cell death, has been proved to be closely related to the maintenance of cell homeostasis and occurrence of disease. Autophagy can participate in the cell cycle, cell proliferation, cell apoptosis and angiogenesis of tumor cells, thus affecting occurrence and development of tumors (3,4). A variety of chemotherapy drugs can induce autophagic activity of glioma cells, which in turn show a curative effect, and reduce drug resistance (5). Inflammation is also an important factor involved in the development, progression and treatment processes of various tumor cells (6). Based on the above, the aim of this study was to analyze the mechanism of Wnt signaling pathway in the regulation of autophagy and inflammatory response in glioma cells.

Materials and methods

Experimental materials. Human glioma cell line U118 was purchased from Research Science (Shanghai, China); High glucose DMEM medium and fetal bovine serum were purchased from Sigma (St. Louis, MO, USA); Wnt inhibitor IWR-1 was from Beyotime (Jiangsu, China); RIPA lysate and phenylmethylsulfonyl fluoride (PMSF) were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; BCA kits were from Europe B.V. (Venlo, The Netherlands); mouse anti-human Wnt5a, β-catenin, marker microtubule-associated-proteins-1A/1B-light-chain-3C (LC-3) II and Beclin I, IL-6 and TNF-α monoclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) (cat. nos. sc-365370, sc-65480, sc-398822, sc-48341, sc-130326 and sc-130349); β-actin antibody and rabbit anti-mouse polyclonal antibody were purchased from Invitrogen (Carlsbad, CA, USA) (cat. no. MA5-15739, A-11059); TBST and ECL solutions were purchased from General Electric Co. (Fairfield, CT, USA); Cell culture plates and CO2 incubators were from Bio-Rad (Hercules, CA, USA), electrophoresis apparatus tropheosis was from Applied Biosystems (City Foster, CA, USA); Polyvinylidene fluoride film (PVDF) was from R&D Systems (Minneapolis, MN, USA).

Experimental grouping. After resuscitation, U118 cells were cultured with high glucose DMEM medium containing 10% fetal bovine serum in an incubator (37˚C, 5% CO2). Subcultures were prepared and medium was replaced every other day. Cells were collected during the logarithmic growth phase and resuspended in PBS to adjust the density to 2x10⁶/ml. Cells were divided into 3 groups: the Wnt signal pathway inhibitor IWR-1 group (5 µmol/l, observation group), the PBS negative
control group (PBS group) and the blank control group. All groups were incubated for another 24 h.

**Observation indicators and examination methods.** Western blot was used to evaluate Wnt5a/β-catenin protein, autophagy marker microtubule-associated-proteins-1A1B-light-chain-3C (LC-3) II and Beclin I, and inflammatory factors IL-6 and TNF-α protein expression levels. Western blotting: medium containing cells of each group was placed on ice, and 150 µl of RIPA lysate and 1.5 µl of PMSF were added and incubated for 30 min, followed by centrifugation (2,000 x g) at 4°C for 20 min and the supernatant was collected. Protein concentration was measured using BCA method. Protein (15 µg) from each sample was subjected to 8% polyacrylamide gel (SDS-PAGE) electrophoresis, followed by transfer to PDVF membrane. Membrane was blocked with 5% skimmed milk at room temperature for 2 h. Mouse anti-human Wnt5a, β-catenin, LC-3 II, Beclin I, IL-6 and TNF-α monoclonal antibodies (1:2,000) were then added and incubated overnight at 4°C. After washing with TBST, rabbit anti-mouse polyclonal antibody (1:500) was added and incubated at room temperature for 4 h. After washing with TBST, color development with ECL was performed, and results were scanned and recorded. Lab Works 4.5 gel imaging software (Invitrogen) was used for semi-quantitative analysis with results expressed as integrated optical density (IOD). The relative expression levels of targeted proteins were represented by the ratio of IOD to that of β-actin (endogenous control).

**Statistical analysis.** Statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as mean ± standard deviation. Single factor ANOVA analysis was used for comparisons among multiple groups and LSD-t method was used for the comparisons between 2 groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Expression levels of Wnt5a/β-catenin protein.** Wnt5a/β-catenin protein expression levels in the observation group were significantly lower than those in the other two groups (P<0.05) (Fig. 1).

**Expression levels of LC-3 II and Beclin I.** LC-3 II and Beclin I expression levels in the observation group were significantly higher than those in the other two groups (P<0.05) (Fig. 2).

**Expression levels of IL-6 and TNF-α.** IL-6 and TNF-α expression levels in the observation group were significantly lower than those in the other two groups (P<0.05) (Fig. 3).

**Discussion**

Wnt pathway includes classical Wnt/β-catenin, Wnt/PCP (7) and Wnt/Ca²⁺ pathway (8), within which the activation of...
Wnt/β-catenin can lead to the binding of Wnt protein to specific cell surface frizzled receptor proteins and low density lipoprotein receptor-related protein 5/6 (9). This binding can activate the intracellular PDZ domain containing dishevelled protein and Axin protein to inhibit the phosphorylation of glycogen synthase kinase 3 (GSK 3), and consequently lead to the accumulation of β-catenin in cytoplasm (9). β-catenin can enter nucleus and bind to the transcription factor T cytokine/lymphotropic enhancer family protein and initiate the transcription, thereby regulating the expression of downstream related target genes and promoting proliferation, invasion and metastasis of tumor cells (10). Our results showed that Wnt5a/β-catenin, IL-6 and TNF-α protein expression levels were significantly lower and LC-3II and Beclin 1 protein expression levels were significantly higher in observation group. Therefore, we believe that Wnt5a/β-catenin signaling pathway regulates the autophagy and inflammatory response of glioma cells.

Prior studies confirmed (11) that Wnt is a key factor in the self-renewal of neural stem cells, and Wnt also participates in neural formation in adult brain tissue. Mutations of Wnt gene and knockout of Wnt1 gene can lead to the loss of midbrain in mice (12). Wnt can act on mouse neural precursor cells to increase the number of new neurons (13). Wnt pathway can also affect the synaptic transmission and plasticity of hippocampal neurons (14). Wnt/β-catenin pathway in adult brain is closely related to the occurrence of Alzheimer’s disease (15). Wnt5a protein is a member of the Wnt protein family, which can mediate nonclassical pathways to inhibit classical pathways, and the inhibition of classic pathway can in turn trigger Wnt5a pathway to participate in regulating the development of the central nervous system (16). IWR-1 is a small molecule inhibitor of the Wnt pathway that plays a role in stabilizing Axin degradation complex (17). LC3 is a marker protein used for detecting autophagy. LC3-I has the ability to bind to phosphatidylethanolamine after autophagy and form LC3-II. LC3-II is localized in the phagocytic endomembrane and adventitia and can be kept stably in autophagic membrane until the fusion with lysosomes (18). LC3-II level can reflect the number of autophagosomes and the degree of autophagy (18). Beclin 1 is the first identified mammalian autophagy protein, and it has been shown that Beclin 1 gene deletion is directly related to the development of human breast cancer (19), colorectal cancer (20) and prostate cancer (21). The recovery of Beclin 1 expression can induce autophagy and inhibit the occurrence and development of tumors. Mechanism of autophagy in treatment response of cancer is complex. Autophagy can both suppress and protect tumor cells (22) and there are researchers who believe that the regulation of autophagy may be a new target for the treatment of a variety of tumors.

In addition, inflammatory response with IL-6 and TNF-α as the central network factors, is also an important mechanism involved in tumorigenesis. It has been confirmed that, the occurrence, development, treatment and prognosis of lung cancer (23), liver cancer (24), colorectal cancer (25) among other cancers are closely related to inflammation. Prolinflammatory stimulation factors such as IL-6 and TNF-α can mediate the activation of a variety of inflammatory cells such as neutrophils and fibroblasts and inflammatory media tors such as IL-1, IL-8, INF-γ and NF-xB, which in turn cause inflammatory disorders. High levels of IL-6 and TNF-α have been reported in glioma patients, and these levels have been shown to be associated with the chemotherapy outcomes and the prognosis (26,27).

We concluded that the occurrence of glioma is related to Wnt5a/β-catenin signaling pathway activation, cell autophagy and inflammatory response. Our study provided a new direction for bio-targeted therapy.

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