

· Basic Research ·

Effect of celecoxib on proliferation, apoptosis, and survivin expression in human glioma cell line U251

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[Abstract] Background and Objective: Celecoxib, one of the new generation of non-steroidal anti-inflammatory drugs (NSAIDs), has a specific inhibitory effect on COX-2. Studies have shown that celecoxib can inhibit the proliferation of tumor cells and induce cell apoptosis, which has been confirmed in colorectal tumors and familial adenomatous polyposis. This study explored the effect of celecoxib on the proliferation and apoptosis of human glioma cell line U251 and elucidated the correlation between the effect of celecoxib and the expression of survivin. **Methods:** U251 cells were treated with different concentrations of celecoxib. Cell morphologic changes were observed by optical microscopy. MTT assay was used to detect the absorbance value and to calculate inhibition and survival rates. The rates of apoptosis of U251 cells after 48 h of treatment with celecoxib were assessed by flow cytometry. The expression of survivin was analyzed by immunocytochemistry (ICC) and Western blot analysis. The expression of survivin mRNA was determined by reverse transcription-polymerase chain reaction (RT-PCR). **Results:** Significant morphologic changes were shown in U251 cells after treatment with celecoxib. The MTT assay results revealed that celecoxib inhibited the proliferation of U251 cells and the inhibitory rates significantly increased in a dose- and time-dependent manner. After 48 h of treatment with celecoxib, the apoptotic cells could be obviously observed, and the apoptosis rate significantly increased with increases in concentrations of celecoxib. The expression of survivin was observed in the control group, however, the expression of survivin was significantly down-regulated as the concentration of celecoxib increased. The level of survivin mRNA expression in U251 cells was significantly down-regulated after treatment with different concentrations of celecoxib ($P < 0.05$). **Conclusion:** The inhibition of proliferation and apoptosis in U251 cells could be induced by celecoxib in a dose- and time-dependent manner, and its mechanism might be the downregulation of the expression of survivin.

Key words: Celecoxib, glioma, cell line, U251, survivin

Glioma is the most common brain tumor that originates from the neuroectoderm, the incidence of which ranks first in primary brain tumors, accounting for 50%–55% of all brain tumors. According to the statistics, the mean survival time of patients with surgical treatment alone was 17 weeks, the mean survival time of patients with surgery plus postoperative radiotherapy was 37.5 weeks, and the mean survival time of patients with surgery, radiotherapy plus chemotherapy was up to 51 weeks^[1]. In sum,

the curative effect of glioma is not yet ideal.

For years people have been developing new anticancer drugs while treating patients with tumors with traditional surgery, drug chemotherapy, and radiotherapy as well as new gene therapies. A large number of epidemiologic and experimental studies^[2,3] have found that long-term use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) can not only significantly reduce the incidence of colorectal cancer, but also abate existing colon polyps or adenoma. A similar effect was also found in many other cancers such as lung, prostate, breast, stomach, and bladder. Celecoxib, approved by United States Food and Drug Administration (FDA) in 1999, is clinically used as a selective inhibitor of COX-2. Survivin, discovered by Ambrosini et al.^[4] in 1997, is a new member of the inhibitor of apoptosis protein (IAP) family. This study explored the non-COX-2 mechanisms of celecoxib in inhibiting glioma by analyzing the effect of celecoxib on the proliferation, apoptosis, and survivin expression of human glioma cell line U251, providing a theoretical basis for the treatment of glioma with celecoxib.

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Materials and Methods

Materials

Human glioma cell line U251 was purchased from the Cell Engineering Research Center, Fourth Military Medical University. The RPMI-1640 medium was purchased from Sigma-Aldrich, USA. Celecoxib was purchased from Searle pharmaceutical factory (USA). Newborn calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Dimethyl sulfoxide (DMSO), tetrazolium salt MTT, trypsin, Annexin V-FITC reagents, and agarose were purchased from Sigma-Aldrich, USA. Penicillin and streptomycin were purchased from Shandong LuKang Pharmaceutical Group Co., Ltd. Rabbit anti-human survivin monoclonal antibodies were purchased from Abgent, Inc., USA. TRIZolTM reagent was purchased from Nanjing KeyGey Biotech Co., Ltd. Primers were synthesized by Shanghai Bio-Engineering Co., Ltd. MMVL, RNasin, 5 × buffer, dNTP, MgCl₂, and Taq enzymes were purchased from the Promega Co., Ltd. USA.

Methods

U251 cells were maintained in RPMI-1640 medium containing 10% newborn calf serum (pH = 7.2–7.4) and incubated at 37°C with 5% CO₂ and saturated humidity. Observed with an inverted microscope, cells were digested with 0.25% trypsin and processed when grown to 80% confluence. Cells were then frozen in liquid nitrogen. Cells in the logarithmic growth phase were used in this experiment.

MTT assay Exponentially growing cells were digested with 0.25% trypsin and the supernatant fluid was decanted after centrifugation at 1000 rpm for 5 min. Culture medium was added to convert to a single cell suspension, and then cells were counted and adjusted to 5×10^4 /mL. A total volume of 100 μ L of cell suspension was added to 96-well plates and then the culture medium was supplemented to reach 200 μ L in each well. Afterwards, the culture plates were placed back into the incubator. After 24 hours, adherent cells were observed and the old culture medium was decanted, followed by adding culture medium with celecoxib. Adjusting the dose so that the final concentrations in the experimental groups rose to 10, 30, 50, 70, and 100 μ mol/L, respectively. The control group did not contain celecoxib. In addition, a blank group (only the culture medium without cells) was set up. There were 6 wells in each group. The incubations were terminated at 24, 48, and 72 h after the drug was administered. After 20 μ L, 5mg/mL of MTT was added to each well, the cells were incubated for another 4 h, and the incubations were terminated. Then, 150 μ L of DMSO was added to each well. After agitation, the optical absorbance (A) at 570 nm was measured and inhibitory rate (IR) was calculated as follows: $IR = (A \text{ of control group} - A \text{ of experimental group}) / A \text{ of control group} \times 100\%$.

Detecting apoptosis by flow cytometry Until cells grew to the logarithmic phase, the old medium was decanted. A total of 4 groups, including control and experimental groups, were set up, with 6 bottles of cells in each group. Celecoxib was added to the

experimental groups to final concentrations of 30, 50, and 70 μ mol/L, respectively, while the control did not contain the drug. Incubated for 48 h, cells were digested with 0.25% trypsin to turn into single cell suspensions. After the cell density was adjusted to 5×10^5 /mL, 1 mL of cells from each bottle were centrifuged and suspended with PBS. Afterwards, cells were centrifuged again and resuspended in 200 μ L of a binding buffer, followed by adding 10 μ L Annexin V-FITC and 5 μ L of propidium iodide, gently mixing and incubating in the dark at room temperature for 15 min. A volume of 300 μ L binding buffer was added and machine detection was performed immediately. The excitation light source was an argon ion laser operating on the 488 nm line. The rate of apoptosis was analyzed by CELLQuest software. The lower left quadrant identified normal cells; early apoptotic cells were present in the lower right quadrant, and late apoptotic and necrotic cells migrated to the upper quadrants. The upper left quadrant represented damaged cells impaired during the collection process.

Detecting survivin expression with immunocytochemistry Cells in the logarithmic growth phase were digested with 0.25% trypsin to single-cell suspension. A volume of 1 mL of cell suspension at a density of 5.0×10^4 cells/mL was seeded to 6-well plates with cover slips inside. Four groups, including control and experimental groups, were set up, with 6 duplications of wells in each group. After the tumor cells adhered to the cover slips, culture media with celecoxib at concentrations of 70, 50, and 30 μ mol/L were added to the experimental groups, while the control group was without the drug. After a 48 h exposure to the drug, avidin-biotin complex (ABC) immunoenzyme staining was performed and positive and negative controls were set. Results were observed under the microscope. Tumor cells with membranes or cytoplasm showing brownish yellow or brown were defined to be positive cells. Three high power fields were selected randomly to analyze the grey value of survivin expression with pathologic image analysis and the mean grey value was calculated.

Detecting survivin expression with Western blot analysis Cells in the control and experimental groups treated with 30, 50, and 70 μ mol/L of celecoxib for 48 h were collected. A lysis buffer was used to lyse cells to extract total protein. After measuring the protein concentration with the bicinchoninic acid (BCA) method, 40 μ g of protein was used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then the protein was transferred to a polyvinylidene fluoride (PVDF) membrane. Blocked with 5% nonfat dried milk for 1 h, the membrane was incubated with the survivin antibody at a concentration of 1:1000 overnight. The next day, after rinsing with tris-buffered saline tween-20 (TBS-T), a horseradish peroxidase (HRP)-labeled second antibody was added and the membrane was incubated for another 1 h followed by washing with TBS-T 3 times. An electrochemical luminescence (ECL) reagent was used for X-ray imaging and the results were then analyzed. The internal standard was β -actin.

Semiquantitative detection of survivin mRNA with reverse transcription-polymerase chain reaction (RT-PCR) All six bottles of cells that were treated by celecoxib at concentrations of

30, 50, and 70 $\mu\text{mol/L}$ for 48 h were placed on ice, from which RNA was extracted. A small amount of RNA was reverse transcribed into cDNA. The primer sequences of survivin were as follows: 5'-TTCAATCCATCAAAGCTACTACA-3' for the forward primer and 5'-CAGAGAAAGTTGAACTCTGTTGGATTC-3' for the reverse primer, giving a 481 bp amplified fragment. The primer sequences of β -actin were as follows: 5'-CGCTGCGCTGGTCTGTCGACA-3' for the forward primer and 5'-GTCACGCACGATTTCCCGCT-3' for the reverse primer, giving a 370 bp amplified fragment. The reaction system of PCR was as follows: 4 μL of 10 \times PCR buffer, 2.4 μL of 2.5 mmol/L MgCl_2 , 0.8 μL of 10 mmol/L dNTPs, 2 μL of *Taq* DNA polymerase, 1 μL (500 nmol/L) of forward primers and reverse primers of survivin and β -actin each, and 8 μL of the cDNA template. Finally, triple-distilled water was supplemented to a total volume of 40 μL . After immediate centrifugation, the reaction system was put into a PCR thermocycler for amplification. The amplification conditions were as follows: predenaturing for 5 min at 95 $^{\circ}\text{C}$; followed by 30 cycles of denaturing for 30 s at 95 $^{\circ}\text{C}$, annealing for 30 s at 58 $^{\circ}\text{C}$, and extension for 45 s at 72 $^{\circ}\text{C}$; after extension for 10 min at 72 $^{\circ}\text{C}$, the amplification was terminated at 4 $^{\circ}\text{C}$. After identification by agarose gel electrophoresis, the PCR products were observed and photographed under ultraviolet (UV) light. The *A* value of the product was read. The relative value of the expected mRNA = *A* value of the expected product/*A* value of β -actin. When the relative value ≥ 0.10 , it was defined as positive.

Statistical analysis

The experimental data was measured by mean \pm standard deviation. SPSS13.0 was used for the statistical analysis. One-way ANOVA was used to analyze the differences among the different groups. Statistical significance was assumed when $P < 0.05$.

Results

Effects of celecoxib on the proliferation

Observations under the microscope After observations under an inverted phase contrast microscope, numerous cells were found in the culture flask of the control group without celecoxib. Cells adhered to the wall and displayed a pleomorphic or spindle morphology with many protrusions on the cell surface. The lucency and refractivity of the cells was high and the proliferation of tumor cells was rapid. It took only 72 h for the cells to fully cover the bottom of the culture flask. By contrast, the sizes of cell bodies in the experimental groups treated by celecoxib reduced significantly and became round, and the lucency and refractivity of the cells weakened, with granules and vacuoles appearing in the cytoplasm. As the concentration increased and the exposure time prolonged, cells began to shrink and rupture, the morphology of cells became irregular with surrounding debris, and the culture medium became turbid. Eventually, a large number of cells collapsed, floated, and finally resulted in death.

MTT assay The *A* value of U251 cells in the groups treated with celecoxib for 24, 48, and 72 h reduced when compared with the control group. The *A* value reduced more obviously in the group treated with a high concentration of celecoxib. The rate of

inhibition increased with the increase of concentration and the prolongation of exposure time (Figure 1). Differences of the rates of inhibition among cells in groups with different concentrations and the same exposure time were significantly different ($P < 0.05$). According to the efficiency equation, the 50% inhibition concentration (IC_{50}) of U251 cells treated with celecoxib for 24, 48, and 72 h were 78.31, 48.64, and 43.75 $\mu\text{mol/L}$, respectively.

Effect of celecoxib on apoptosis

The use of Annexin-V/PI double-staining assay detected apoptosis. Results of flow cytometric analysis revealed the apoptosis of U251 cells observed after 48-h exposure with 30, 50, and 70 $\mu\text{mol/L}$ of celecoxib. The apoptotic rates of the experimental groups were $(10.78 \pm 1.23)\%$, $(16.24 \pm 2.34)\%$, and $(23.46 \pm 3.21)\%$, respectively, with significant differences ($P < 0.05$). The differences of the rates of apoptosis between groups treated with different concentrations of celecoxib were also significant ($P < 0.05$). The rate of apoptosis of U251 cells increased along with the increase of celecoxib concentrations (Figure 1).

Effect of celecoxib on survivin expression

Results of immunocytochemical staining showed that survivin was mainly located in the cytoplasm and could also be seen within the nucleus. A large number of brown-yellow granules were observed in the control group, which suggested strong survivin expression. As the concentration of celecoxib increased, the number of positive cells reduced and the color intensity of the brown-yellow granules decreased (Figure 2). Pathologic image analysis showed that the grey value of the control and experimental groups with 30, 50, and 70 $\mu\text{mol/L}$ of celecoxib were 142.02 ± 2.49 , 154.32 ± 2.65 , 165.96 ± 2.45 , and 184.43 ± 3.47 , respectively. With increasing concentrations of celecoxib, the grey value of survivin expression increased gradually. Variance analysis showed that the difference between the groups was statistically significant ($F = 248.127$, $P < 0.05$). Western blot analysis showed that the ratio of the *A* value of the control and experimental groups with 30, 50, and 70 $\mu\text{mol/L}$ of celecoxib were 0.81 ± 0.03 , 0.72 ± 0.01 , 0.50 ± 0.02 , and 0.39 ± 0.02 , respectively. Compared with the control group, the survivin expression of experimental groups significantly decreased ($P < 0.05$). The differences between groups treated with different concentrations of celecoxib were also statistically significant (Figure 3).

Effect of celecoxib on survivin mRNA expression

The results of RT-PCR and electrophoresis showed that survivin mRNA was highly expressed in U251 cells. After a 48-h exposure to the 3 concentrations of celecoxib, survivin mRNA expression in U251 cells decreased by various extents. The ratios of survivin/ β -actin were as follows: 0.67 ± 0.19 , 0.57 ± 0.26 , and 0.27 ± 0.19 , respectively. When compared with that of the control group (0.87 ± 0.02), the difference was statistically significant ($F = 833.919$, $P < 0.05$) (Figure 4).

Discussion

Glioma is the most common primary intracranial malignant

tumor, with high malignancy, rapid proliferation, short course, poor prognosis, and so on. Studies have found that the average cell cycle of glioma is only 3.5 days [5]. The pathology of this kind of tumor is complex, showing invasive growth. It can easily

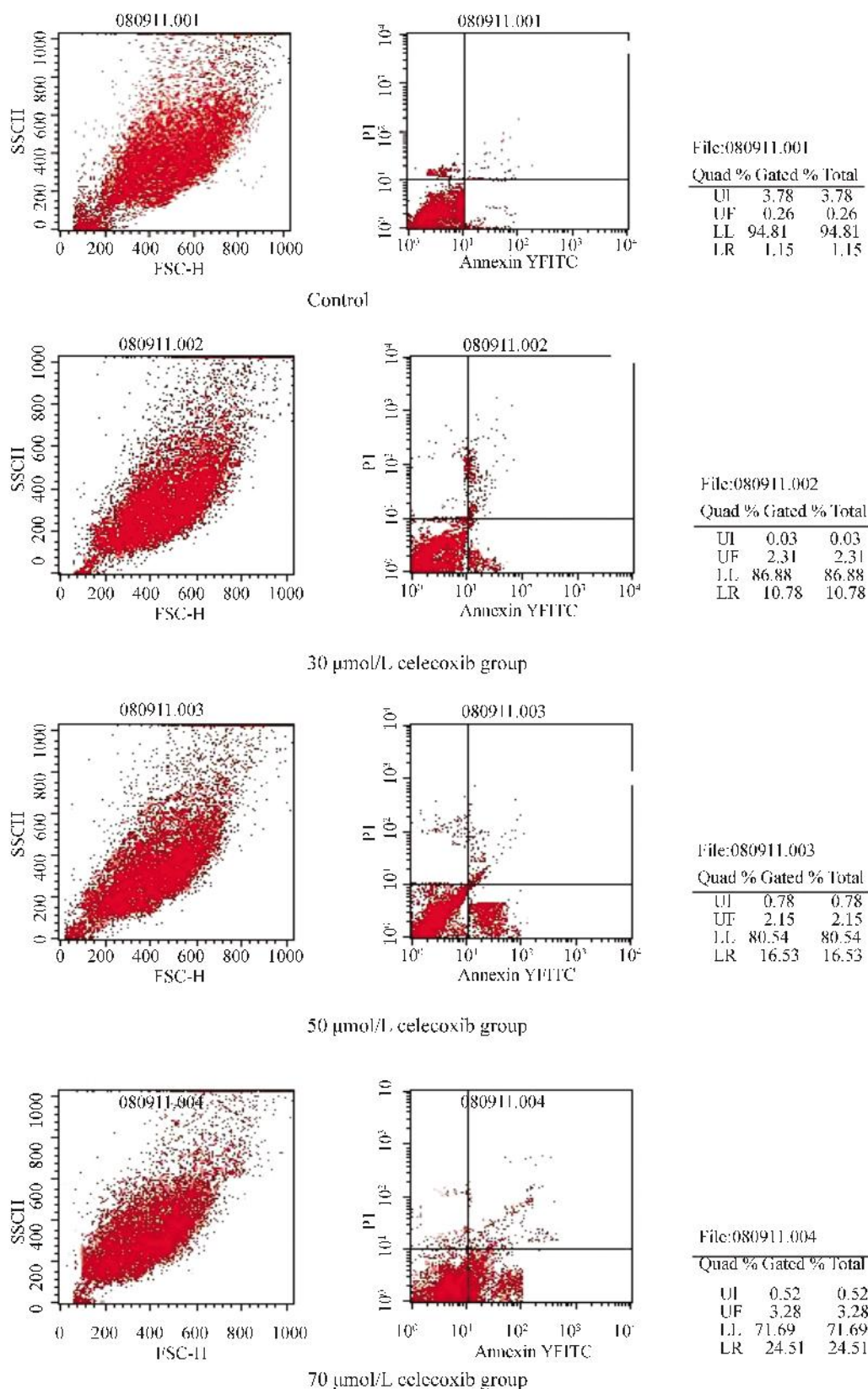


Figure 1 The apoptosis induced by celecoxib detected by flow cytometry

Table 1 Inhibition rate of U251 cell proliferation after treatment with celecoxib

Concentration of celecoxib ($\mu\text{mol/L}$)	Cell proliferation inhibition rate (%)		
	24 h	48 h	72 h
10	7.65 ± 1.24	12.41 ± 0.97	15.02 ± 1.06
30	25.50 ± 2.13	30.51 ± 1.56	34.91 ± 0.97
50	36.16 ± 0.44	51.21 ± 1.17	53.63 ± 0.67
70	44.96 ± 0.38	61.41 ± 1.35	58.14 ± 1.21
100	54.47 ± 0.36	68.06 ± 0.29	71.79 ± 0.33

All values are presented as mean \pm SD of 6 experiments. $P < 0.05$, between various concentrations of celecoxib groups at the same time.

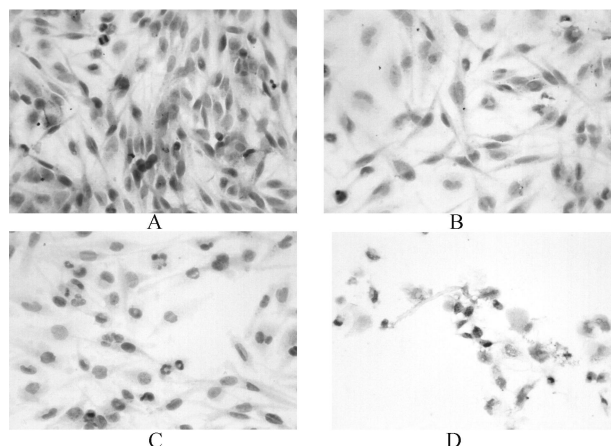


Figure 2 Expression of survivin in U251 cells after treatment with celecoxib (immunocytochemistry $\times 400$)

A, control; B, 30 $\mu\text{mol/L}$ celecoxib; C, 50 $\mu\text{mol/L}$ celecoxib; D, 70 $\mu\text{mol/L}$ celecoxib.



Figure 3 Expression of survivin in U251 cells after treatment with celecoxib detected by Western blot analysis

Lane 1, control; lanes 2–4, U251 cells treated for 48 h with 30, 50, and 70 $\mu\text{mol/L}$ celecoxib, respectively.

protrude into the surrounding interstitial spaces and result in diffusion. With wide infiltration and no clear boundary with normal tissue, it is difficult to remove glioma surgically. Even if it is completely resected, the recurrence rate is very high.

Celecoxib is a COX-2 inhibitor that emerged in the 1990s. Harris *et al.*^[6] discovered that celecoxib could prevent breast cancer. Subsequently, studies have found that celecoxib plays an important role in inhibiting growth and promoting apoptosis of tumor cells in digestive system tumors such as gastric cancer, colon cancer, and cholangiocarcinoma, as well as non-digestive system tumors such as leukemia and bladder cancer^[7,8]. According to current opinions, the antitumor effect is correlated

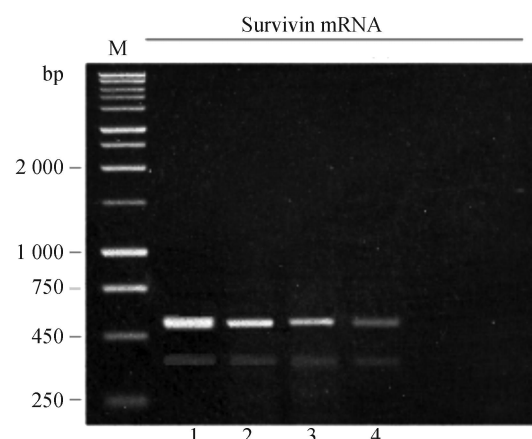


Figure 4 Expression of survivin in U251 cells treated with different concentrations of celecoxib detected by reverse transcription polymerase chain reaction

Lane M, marker; lane 1, control; lane 2, 30 $\mu\text{mol/L}$ celecoxib; lane 3, 50 $\mu\text{mol/L}$ celecoxib; lane 4, 70 $\mu\text{mol/L}$ celecoxib.

with the inhibition of COX-2.

For the present time, it has been clarified that IAP and bcl-2 are the two major gene families that regulate apoptosis. The survivin gene is located on chromosome 17q25, and its gene expression product, the survivin protein, is a new member of the IAP family discovered in the 20th century. The C' terminal of survivin has a unique helix, rich in hydrophobic motifs, allowing it to combine with microtubule proteins of spindle microtubules, thereby playing a role in inhibiting apoptosis. Survivin can also affect the mitosis of cells by interacting with cyclins and cyclin-dependent kinases (CDKs). Because it contains the baculovirus IAP repeat (BIR) motif, which consists of approximately 70 amino acids, the survivin protein can combine with active caspase 3 and caspase 7 to inhibit apoptosis.

At present, there are a lot of studies of celecoxib and survivin at home and abroad. However, studies on the role of celecoxib in inhibiting growth and promoting apoptosis in glioma through interactions with survivin are rare. In this experiment, based on the reports that celecoxib could inhibit proliferation and promote apoptosis in tumor cells, we began with finding whether celecoxib could inhibit proliferation and promote apoptosis of human U251 glioma cells in vitro. Through observing the effect of celecoxib on the proliferation, apoptosis, and survivin expression in human U251 glioma cells, we investigated the possible mechanisms of prevention and treatment of glioma by the NSAID celecoxib.

In this experiment, we treated U251 cells with different concentrations of celecoxib and established a control group with DMSO to exclude the effect of cytotoxicity caused by DMSO to ensure comparability within the experiment. The results showed that different concentrations of celecoxib could inhibit proliferation of U251 cells in a time- and concentration-dependent manner. Based on this conclusion, we used flow cytometry to detect the inducing effects of celecoxib on the apoptosis of tumor cells. After a 48-hour exposure to different concentrations of celecoxib, evident apoptosis emerged in U251 cells. These results were

consistent the relevant research at home and abroad.

Chen *et al.*^[9] treated nasopharyngeal carcinoma cell lines (CNEI, CNEZ, SLJNE) with celecoxib, and the results showed that celecoxib could inhibit proliferation and promote apoptosis of all three cell lines in a time- and dose-dependent manner. Studies of malignant mesothelioma by Catalano *et al.*^[10] have shown that celecoxib could inhibit proliferation and promote apoptosis of malignant mesothelioma in a time- and dose-dependent manner. Researchers have speculated that the mechanism may be associated with the reduction of Akt phosphorylation, expression of bcl-2, and expression of survivin, as well as the inhibition of caspase 3 activation.

There is abnormal apoptosis of almost all of the malignant tumors in tumor pathogenesis. These abnormalities are considered to have broken the balance between the inhibiting genes and the activating genes of apoptosis. Survivin is the most efficient inhibitor of apoptosis ever discovered, with dual efficacy in inhibiting apoptosis and regulating mitosis. Using technologies such as in situ hybridization, RT-PCR, and molecular probes, domestic and foreign studies have found that there is a high expression of survivin in the vast majority of human tumors, including gliomas^[11-13], suggesting that survivin plays a vital role in the origination and the development of tumors.

This experiment found that celecoxib can inhibit proliferation and induce apoptosis of glioma cells. At the same time, future studies of the molecular mechanism of antitumor effects were involved. We used ICC, Western blot analysis, and semiquantitative RT-PCR to detect the effect of celecoxib on survivin expression. The results all showed that celecoxib can downregulate the expression of survivin, an inhibitor of apoptotic proteins. Shankar *et al.*^[14] used antisense RNA directed at different targets of survivin to transfect glioblastoma cells (MSN) and oligodendrocyte cells (TC620) and resulted in proliferation inhibition and the promotion of the rate of apoptosis. Temme *et al.*^[15] used retroviral vectors carrying the survivin mutant gene survivin T34A to transfect glioblastoma tumor cell lines and resulted in a significant decrease in the proliferation of U373 and H4 cells with a large number of multinucleated cells. Considering the results from previous reports and this experiment, we can predict that celecoxib may inhibit proliferation and promote apoptosis of tumor cells through downregulating survivin expression.

In this study, celecoxib, which is the most commonly used NSAID in clinical practice, was proved to inhibit proliferation and promote apoptosis in human glioma U251 cells. Its mechanism of

the action may be associated with the suppression of survivin, besides the inhibition of the activity of COX-2. The discovery provides a new idea for the clinical treatment of gliomas and a theoretical basis for the clinical application of celecoxib as an antitumor drug. However, the exact mechanisms of inhibition for celecoxib on glioma U251 cells are to be further studied.

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