Original Article
Curcumin regulates the proliferation and apoptosis of human oligodendroglioma cells through both the NF-κB and AKT pathways

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Abstract: Curcumin (CUR), a natural and non-toxic polyphenol of turmeric with multiple potential therapeutic properties, is widely used to treat cancer. A number of studies have described the molecular mechanisms at play in the uncontrolled proliferation of cancer cells. However, little work has focused on whether and how CUR resists human oligodendroglioma (OL) cells *in vitro*. We investigated the proliferation and apoptosis of OL cells after CUR treatment, and the levels of target molecule protein, containing p-p65, p65, p-AKT and AKT detected by western blot. We also explored whether the biological activity of CUR could be enhanced by lipidosomes. Results show that CUR could regulate the proliferation and apoptosis of OL cells through both in NF-κB and AKT signal pathways, and that these properties could be enhanced effectively by liposomes containing curcumin (CNLPs). This result could enrich the anticancer activity of CUR and provide new perspectives on the available evidence for the therapeutic target with OL cells.

Keywords: Curcumin, proliferation, apoptosis, NF-κB, AKT, human oligodendroglioma

Introduction

Cancer, caused by cells increasing in an uncontrolled way, affects millions of people every year [1, 2]. Glioma is the most common type among primary tumors, accounting for 15.4% of all primary brain tumors in the central nervous system and distinguished by rapid growth and poor prognosis [3]. The current multimodal standard of care has been in practice for many years without substantial improvements, including surgical resection, phototherapy, adjuvant chemotherapy and radiation [4]. New therapies are required to improve survival and the quality of life for patients with malignant gliomas.

The natural ingredients of plants, with rich sources of bioactive natural compounds are widely used for their therapeutic value and healing effects [5, 6]. Curcumin (CUR), (E, E)-1, 7-bis (4-hydroxy-3-methoxyphenyl) -1, 6-heptadiene-3, and 5-dione, are extracted from a member of ginger family known as turmeric. The major chemical ingredient of turmeric fraction is polyphenol which contains curcuminoids with a yellow color. CUR has been used in Asia, including China and India for more than 2000 years [7]. Various beneficial properties, including anti-inflammatory, antioxidant and the chemotherapeutic potential of CUR were shown in experimental studies on cell- and animal-based models [8, 9], as well as various clinical trials of CUR that point to its therapeutic benefits and efficacy [10]. CUR has been promising in the treatment and prevention of human pathological diseases such as Alzheimer's disease, Parkinson's disease and brain tumors [11, 12].

Increasingly, research focuses on the molecular mechanisms of the uncontrolled proliferation of cancer cells and the alterations to genetic expression in the cell cycle [2]. Over the past two decades, studies on the anticancer effects of CUR demonstrated it can modulate tumor cell death, proliferation, the signaling pathways

and angiogenesis [13]. The anti-proliferative effects of CUR are reported to be related with protein kinases such as Akt/PKB, Raf kinase, MAPK, MLK3, mTOR and AMPK, transcription factors such as NF-κB and AP-1, the early growth response gene and β-Catenin, angiogenesis such as HIF-1/VEGF/bFGF signal pathway [14], and cell cycle regulators such as cyclin B1. Current data show CUR reduced tumorigenesis by activating apoptotic pathways including the expression of Caspase, Bcl-2/Bcl-xL/BAX/BAK, NF-κB, RAS/ERK/EGR-1and JNK [4]. These effects suggest curcumin may be a promising nutraceutical compound for the effective treatment of tumors via anti-proliferative and anti-apoptotic properties.

There is the possibility that CUR could show different properties on various tumor cells. In a previous study, CUR inhibited proliferation and induced apoptosis of human gastric carcinoma AGS cells through the activation of the Ras/ERK signaling pathway and downstream caspase cascade [15]. In H-ras-transformed MCF 10A human breast epithelial cells, CUR treatment induced upregulation of Bax and caspase-3 by oxygen species-mediated and downregulation of matrix metalloproteinase-2 and B cell lymphoma-2 [16].

OL cells are precursors to nerve cells, which play an important role in maintaining neurological function as they comprise the central nervous system white matter. However, the mechanism(s) of CUR effects on OL cells has been scarcely reported. In our study, we focused on the proliferation, cell cycle, and apoptosis of OL cells after CUR treatment.

Materials and methods

Cell culture and treatment

Human OL cells were derived from fetal human oligodendrocytes purchased from ATCC (American Type Culture Collection, Rockville, USA). Cells were grown in cell medium containing: DMEM (DMEM Dulbecco's Modified Eagle's Medium, Gibco BRL Co. Ltd., USA), 10% FBS (fetal calf serum, Gibco BRL Co. Ltd., USA), 1% glutamine (Gibco BRL Co. Ltd., USA), 0.1% penicillin (10 U/ml, Gibco BRL Co. Ltd., USA) and 0.1% streptomycin (10 μg/ml, Gibco BRL Co. Ltd., USA) at 37°C in a 5% CO_2 environment. The OL cell were harvested when cells reached

80% confluence. Briefly, the complete growing medium was removed and 5.0 ml of phosphate buffer saline (PBS, HyClone, Logan, UT) was added to rinse. 1 ml 0.25% trypsin (Gibco BRL Co. Ltd., USA) was used to detach the adherent cells in the flask at 37°C for 1 min. Then, cells were suspended with growth medium after inactivating the trypsin. The suspension cells were split into equal portions and cultured in same condition for further use. Cells were seeded for 24 h and then treated with PBS as control. Investigators also used CUR (Sigma, MO, USA) and CNLPs (contain CUR) to treat OL cells for 48 h according to a previous study [17].

Proliferation assay

CCK8 (Beyotime, Shanghai, China) was performed to investigate the proliferation of the OL cells according to the manufacturer's instructions. First, the suspension cells were counted and seeded at 1×10^4 cells per well in 96-well plates with each well containing 100 μL volume of medium. A total of 10 μL CCK8 solution was added into each well after treatment for 48 h. Then, the cells of 96-well plates were cultured for approximately 2 h. The orange soluble products were formed, and their absorbance was calculated by an enzyme-linked immunosorbent assay reader at 450 nm. All assays were measured in triplicate and constructed in growth curves.

Examination of cell cycle

The cells were detached and digested using 0.25% trypsin to make individual cell suspension with an appropriate concentration of (1-5) $\times 10^6$ cells/mL after pre-cooling PBS and washed 3 times. Cells were washed with pre-cooling PBS again and placed in cold 75% ethanol in the freezer at -20°C for overnight. Thereafter, the fixed samples were centrifuged to remove the supernatant at 1000 r/min for 10 min. Cells were suspended again and washed with pre-cooling PBS, after discarding the supernatant from centrifuging at 1000 r/min for 5 min. To remove the RNA of each samples, the cells were suspended by adding 200 μL RNase A (300 g/mL) and incubated for 30 min. In addition, 150 μL propidium iodide staining was performed in every sample and stained in a dark place at 4°C for 20 min. The measure of
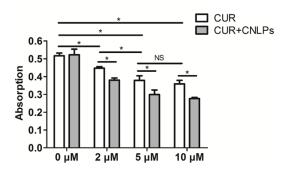
Figure 1. Cellular proliferation rates of the control, CUR and CNLPs at different concentrations (n = 3). $^{*}P < 0.05$, compared with the CUR group. NS means P > 0.05, compared with the CUR. Data are presented as mean ± SD.

cell cycle was determined using flow cytometry (BD, FACS, Calibur) after washing.

Apoptosis detected by annexin V and PI stain

The apotosis of OL cells were detected using the annexinV FITC apoptosis detection kit (Beyotime, Shanghai, China) according to the instructions. First, the suspended cells were collected at a number of approximately 5×10^5 in a volume of 1 ml. After 1000 r/min centrifugation for 10 min, the supernatant was discarded. The cells were re-suspended gently with PBS and the supernatant was removed after 1000 r/min centrifugation for 5 min. Meanwhile, Annexin V-FITC mixed solution was prepared on ice. A 150 mL buffer was added to suspend the cells in each sample. The mixed solution, containing 45 µL buffer and 5 µL Annexin V-FITC, was added to each sample and dyed in a dark place for 20 min on ice. The 10 µL PI solution was added to each sample and incubated in the dark for 15 min at room temperature. Finally, the cell was added to the 200 µL buffer and apoptosis was measured by flowcytometry. All assays were measured in triplicate.

Western blot assay

Total protein of the cells was extracted by a Protein Extraction Kit (KGP9100, Key Gene, Nanjing, China) containing both phosphatase inhibitors and protease inhibitors according to the instructions. After testing the protein concentration by the Bradford method, immunoblotting assay were performed using western

blotting. Briefly, 10-20 µg lysates of cell protein were separated on 10-12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with a constant voltage electrophoresis of 90 V for 15 min and 120 V for approximate 1 h. Then, the protein was transferred to the PVDF (polyvinylidene fluoride) membranes at 200 mA for 2 h. Five percent nonfat milk was added to the membrane for blocking for about 2 h at room temperature. Then, all membranes were incubated with primary antibodies at 4°C overnight. All membranes were washed again following incubating with HRR secondary antibody for 2 h at room temperature. After extensive washing, the signals of antibody-detected protein bands were visualized using Luminata™ Crescendo Western HRP Substrate (Millipore, MD, USA). The antibodies contain anti-p-p65^{ser276}, anti-p65, anti-p-AKT^{Ser473}, anti-AKT and β-actin for normalization (Cell Signaling Technology, MA, USA).

Statistical analysis

Data were presented as mean ± standard deviation (Mean ± SD) and were statistically analyzed with SPSS 19.0 statistical software (SPSS Inc., Chicago, USA). The results of cell proliferation were compared in two groups using the Student's t-test. Statistical significance was determined among control, CUR and CNLPs groups with one way-analysis of variance (ANOVA). A *P* value of less than 0.05 was considered a significant difference in statistics.

Results

The effects of OL cell proliferation after CUR treatment

CCK8 was used to detect the cell proliferation after the cells were treated with different doses of curcumin (0 µM, 2 µM, 5 µM and 10 µM) for 48 h. The data showed that the proliferation of OL cells both in CUR and CNLPs groups decreased significantly in a dose-dependent manner. Moreover, CNLPs could attenuate the OL cells proliferation (**Figure 1**). Treatment with curcumin (5 µM) resulted in an obvious decrease in cell proliferation, while a higher dose of curcumin (10 µM) led to a further inhibition of cell proliferation, which was not statistically significant compared with the 5 µM curcumin group. Thus, 5 µM curcumin was chosen for further studies.

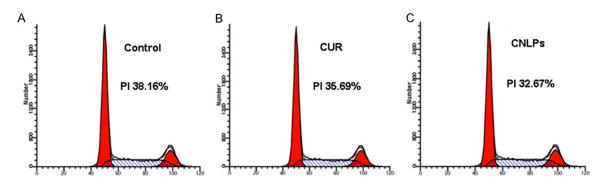**Figure 2.** Cell cycle analysis of three group cells (n = 3). A: Control; B: CUR; C: CNLPs.

Table 1. Percentages of the cells at different phases of cell cycle (n = 3)

Group	Cell cycle phase			
	G0/G1 (%)	G2/M (%)	S (%)	PI (%)
Control	58.66 ± 1.39	7.70 ± 0.20	33.64 ± 1.59	38.16 ± 1.00
CUR	65.82 ± 0.43*	4.08 ± 0.28*	30.10 ± 0.44*	35.69 ± 0.81*
CNLPs	71.47 ± 1.72*,#	3.51 ± 0.28*,#	25.01 ± 1.90*,#	32.67 ± 1.11*,#

Notes: Statistical analysis of the cell proliferation index (PI) by independent sample t-test, *$P < 0.05$ compared with control group; #$P < 0.05$ compared with curcumin group.

The effects of OL cells cell cycle after CUR treatment

In order to confirm the correlation of the cell cycle and the inhibition of proliferation, the cells were incubated with propidium iodide staining and measured by flow cytometry analysis. The data suggested that the number of cells after *CUR* treatment increased significantly during the G0/G1 phase and decreased both in G2/M phase and S phase compared with the control ($P < 0.05$), while these capabilities were enhanced significantly in CNLPs (**Figure 2**; **Table 1**). This study showed CUR could inhibit cell cycle effectively.

The effects of OL cell apoptosis after CUR treatment

Cell apoptosis of each group was analyzed with Annexin V and PI staining using flow cytometry (**Figure 3**). CUR and CNLPs increased apoptotic cells significantly compared with the control. In addition, CNLPs appeared at a higher level than CUR in cell apoptosis assay.

CUR regulated the expression of AKT/NF-κB signaling

Western blot was used to measure the expression of protein of target molecules containing p-p65, p65, p-AKT and AKT after cells treated with *CUR* for 48 h. In this study, the levels of p65, p-p65, AKT and p-AKT decreased significantly compared with the control. Meanwhile, the down-regulated expression of targeted proteins was more apparent in CNLPs (**Figure 4**).

Discussion

Current studies report numerous molecular mechanisms related to the uncontrolled growth of tumor cells. Down-regulated expression of p53 and up-regulated expression of cyclin-dependent kinases (CDK) has been found in the tissues of cancers [18]. Targeting the key molecules in tumor cells is a potentially effective approach in tumor therapy, affecting proliferation, cell cycle, and apoptosis [19]. Over the last few years, natural anticancer agents have been researched widely because of their negligible adverse effects and high potential for therapeutic use [20, 21]. CUR has been studied due to its previously reported antitumoral properties in different types of cancers such as breast, leukemia, lymphoma, neuroblastoma, ovarian, pancreatic, and prostate [22, 23]. CUR can induce cellular death and inhibit uncontrolled proliferation of cancer cells based on different mechanisms *in vitro* and *in vivo*. Also, CUR is reported to act against the uncontrolled growth of human colon cancer cell by regulating EGR-1 [24]. Our study showed that CUR inhibited the proliferation of human OL cells and decreased both in the G2/M phase and S phase using cell cycle assay. In addition, we proved CUR could promote cellular apoptosis in human OL cells. We further found the levels of protein p65 and AKT, as well as their phosphor-
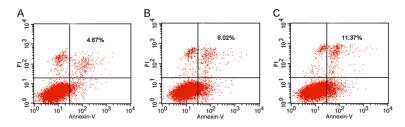
Figure 3. Apoptotic cell counts of three group cells (n = 3). A: Control; B: CUR; C: CNLPs.
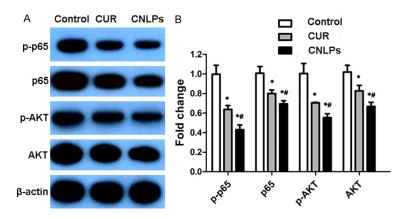

Figure 4. Western blotting validation of AKT/NF--κB Signaling (n = 3). A: Western blotting of p65, p-p65, p-AKT and AKT with β-actin for normalization. B: The levels of targeted proteins were detected in OL cells treated with curcumin for 48 h. *$P < 0.05$ compared with the control, #$P < 0.05$ compared with the CUR. Data are presented as mean ± SD.

types. As a critical cell signaling pathway, serine/threonine protein kinase (AKT) plays a major role in many malignant tumors due to regulating various biological processes containing cell growth, differentiation, proliferation and apoptosis [26]. NF-κB is a transcription factor for which p65 is the subunit of the NF-κB regulating 200 gene expressions [27], activating protein-1 (AP-1) transcription factor which induces cell proliferation and is associated with many types of cancers when it dysfunctions [28]. AKT/NF-κB signaling was detected with western blot to determine the potential mechanism of drug interactions. After CUR treatment for 48 h, the action of the AKT/NF-κB pathway reduced significantly. We found CUR not only reduced the expression of p-p65 and p-AKT levels but also the total amount of p65 and AKT proteins in the nucleus. This suggests that the decreased action of AKT/NF-κB pathway is due to both dephosphorylation and downregulation of total protein.

However, some chemical properties of CUR display poor performance such as a high oil/water partition coefficient (log P = 3.1) and the lack of water solubility (< 0.005%, w/v), which limits its drug efficacy as a therapeutic agent. Moreover, the compound of CUR has a low half-life period within a range for only a few minutes due to rapid decomposition in alkaline media [29]. Thus, for years studies have attempted many strategies to overcome this limitation and attempt to improve the bioavailability of CUR, such as complexation with metal ions, co-administration with piperine and pharmaceutical technologies. In our study, the bioavailability of CUR was improved when OL cells were treated with CNLPs which are used widely and approved by US Food and Drug Administration as spherical lipid bilayer drug delivery vehicles. It seems that liposomes could increase the effect of CUR, by increasing water solubility and

ylated counterpart, were inhibited significantly by CUR. Moreover, the multiple properties of CUR were strengthened in CNLPs. To our knowledge, there is no report about the effect of curcumin both in the proliferation and apoptosis of human OL cells *in vitro.*

In our study, we found CUR could inhibit the cancer cell's growth, induce G2/M cell cycle arrest and apoptosis in human OL cells. Previous studies have indicated that CUR is able to inhibit growth of cell lines and induce G2/M cell cycle arrest and apoptosis. For example, CUR showed a potent anti-proliferative effect on melanoma cell lines. CUR could cause DNA damage which induces the cell apoptosis by triggering the over expression of BAX and down regulating Bcl-2 [25]. In addition, CUR demonstrated a different effect on the inhibition growth of human cell lines (A172, KNS60, U251MG and ONS76), and only KNS-60 and ONS76 were arrested at G2/M. Thus, CUR may play different effects on different cell

inhibiting decomposition of CUR. Clarifying the pathway may ultimately help to find a therapeutic target.

In conclusion, in this study CUR inhibited the proliferation and induced the apoptosis of human OL cells. Treatment with CUR decreased the action of the AKT/NF-κB pathway due to both dephosphorylation and total protein. In addition, these properties were enhanced by liposome.

Acknowledgements

Disclosure of conflict of interest

None.

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