Original Paper

Juniperus Communis Extract Exerts Antitumor Effects in Human Glioblastomas Through Blood-Brain Barrier

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Key Words

Juniperus • Glioma • Cytotoxicity • Apoptosis • Neovascularization

Abstract

Background/Aims: Herbal materials derived from *Juniperus communis* (JCo) possess anticancer activity. In this study, we evaluated the efficacy of a JCo berry extract in suppressing glioblastoma growth. **Methods:** The effects of JCo extract on the viability of normal and glioma cell lines was analyzed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The synergistic therapeutic effect of JCo extract and temozolomide (TMZ) on glioma cells was examined by MTT analysis. Flow cytometry analysis, the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) test, and western blotting were performed to identify the apoptotic pathway. To determine the *in vivo* efficacy of the JCo extract, rats were injected with 5×10^4 rat glioma RG2 cells in the back skin and brain hemisphere and then received a subcutaneous injection in the back skin that contained either JCo extract or vehicle. Finally, blood and histologic examinations were performed to evaluate JCo toxicity. **Results:** The IC_{50} values of JCo extract were 57–69 $\mu\text{g/mL}$ and 49–67 $\mu\text{g/mL}$ in the glioblastoma cell lines after 24 and 48 h, respectively. However, in non-tumor cell lines, the respective IC_{50} values of JCo extract were 76–105 $\mu\text{g/mL}$ and 77–108 $\mu\text{g/mL}$. The JCo extract had a stronger cytotoxicity and a larger range of IC_{50} values in glioma than in normal cells as compared to those effects caused by temozolomide (TMZ). In addition, the results of flow cytometry analysis, TUNEL test, and western blotting revealed that the JCo extract induced glioma cell cycle arrest through intrinsic and extrinsic apoptotic pathways. In the *in vivo* studies, a significant reduction of tumor size in JCo-treated rats, as measured by animal MRI, demonstrated that the JCo extract effectively inhibited glioma cell growth and successfully penetrated the blood-brain barrier. The immunohistochemical (IHC) staining detected positive signals of PCNA, VEGFR-1, and VEGFR-2 in 44.49%, 5.88%, and 5.85% of JCo-treated glioma cells, respectively. However, positive signals of PCNA, VEGFR-1, and VEGFR-2 were W.-C. Tsai and N.-M. Tsai contributed equally to this work.

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detected in 73.08%, 9.67%, and 11.70% of vehicle-treated glioma cells, respectively. The IHC examination of PCNA and VEGFR-1 and -2 indicated that JCo extract significantly decreased the degree of neovascularization. However, no significant differences in serum levels of blood cell count and hepatic enzymes, renal function index, and the histologic appearance of vital organs were detected between the JCo and vehicle-treated rats. **Conclusion:** The JCo extract penetrated the blood-brain barrier and significantly induced glioma cell apoptosis by reducing neovascularization via suppression of the PI3K/AKT/mTOR pathway. Furthermore, JCo extract was less cytotoxic to non-neoplastic vital organs than TMZ.

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Introduction

Between 2008 and 2012, the number of primary central nervous system (CNS) tumor incidences was approximately 28.57 per 100,000 people in the United States [1]. In children and adolescents, primary CNS tumors have the highest incidence and mortality rates. Although meningioma is the most common type and accounts for more than half of primary brain tumors (PBTs) [1], approximately 80% of malignant PBTs are gliomas. Glioblastoma accounts for 46.3% of all malignant gliomas and has the poorest prognosis. Surgical intervention combined with radiotherapy and chemotherapy are the standard treatments for glioblastoma [2]. The complications of radiotherapy and chemotherapy include neurotoxicity, cognitive disturbance, radionecrosis, and secondary malignancy [3, 4]. A recently developed therapeutic, temozolomide (TMZ), acts as an alkylating agent which causes DNA damage and induces tumor apoptosis [5]. The combination of irradiation and chemotherapy has become the principally therapeutic strategy for high-grade gliomas. Recent reports indicated that O₆-methylguanine-DNA methyltransferase (MGMT) promoter unmethylation and long non-coding RNA MALAT1 expression may result in a reduced efficacy of the TMZ treatment [6, 7]. In addition, TMZ has side effects that induce ineffective marrow functions, including neutropenia-related syndrome, thrombocytopenia, and lymphopenia [8]. Recently, immune checkpoint inhibitors, such as anti-PD-1/PD-L1 and anti-CTLA-4, have demonstrated the ability to block the progression of glioma cells [9]. A potential risk of immunotherapy might be the induction of immune cell dysregulation, which increases the possibility of encephalitis [9]. Several studies have proposed to use traditional Chinese medicine for the treatment of PBTs [10-13].

Medicinal herbs provide alternative therapies for several human diseases. The pharmaceutical effects of medicinal herbs include anti-inflammatory [14], antibiotic [15], and immunoregulatory activities [16]. In recent studies, extracts from several types of plants, such as lichens, *Juniperus*, and *Angelica*, were proven to be effective against brain tumor cell progression and angiogenesis [17-19]. More than half of the pharmaceutical herbal therapeutics are extracted from natural dietary plants, which have fewer side effects than other chemotherapeutic agents [20]. However, the applications of herbal medicine in cancer treatment remain limited by certain disadvantages [21]. Firstly, plant extracts contain many compounds that are often difficult to purify. The interactions between these compounds may lead to unpredictable herbal drug effects. Furthermore, as the underlying mechanisms of some herbal therapeutics are still unknown, discrepancies in cytotoxic effects observed during *in vitro* and *in vivo* studies also restricts the extended use of medicinal herbs. Finally, it is difficult to use herbal therapeutics for suppressing brain tumor growth because of the blood-brain barrier (BBB). Therefore, the development of herbal therapeutics with improved penetration through the BBB and strong cytotoxicity to brain tumors might offer an effective therapeutic option for patients with glioblastoma multiforme (GBM).

Juniperus communis (JCo) is a high-altitude plant that grows in south-western Asia and North America [22]. Some alkaloids, flavonoids, glycosides, tannins, phenolic compounds, and steroids from JCo berries and leaves can be extracted using chloroform and methanol [23]. The pharmacological activities of these chemical constituents include anti-inflammatory [24, 25], antifungal [26], analgesic [27], hepatoprotective [23], antimicrobial, anti-mycobacterial

[28, 29], and neuroprotective [22] effects. In a recent study, Benzina et al. demonstrated that deoxypodophyllotoxin extracted from JCo induced apoptosis in breast cancer cells [30]. In addition, Lantto et al. showed that JCo berry extract potentially induced p53-related apoptosis in neuroblastoma cells through inhibition of the PI3K/AKT/mTOR pathway [18]. Therefore, we analyzed the effect of JCo extract on glioblastoma cell proliferation.

In this study, we applied *in vitro* and *in vivo* assays to examine the effects of JCo extract on glioblastoma cells. Our results demonstrated that the JCo extract constituents penetrated the BBB to inhibit tumor proliferation and induce cell apoptosis in human glioblastomas.

Materials and Methods

Preparation of plant extract by chloroform and acetone extraction

The roots of *Juniperus communis* (JCo) were supplied by Chung-Yuan Co. (Taipei, Taiwan). A voucher herbarium specimen was deposited at the School of Pharmacy at the National Defense Medical Center (Taipei, Taiwan). JCo extraction was performed as described previously [19]. A total of 12 kg dried and powdered rhizomes of JCo were extracted three times with acetone (24 L per extraction), three times with chloroform (24 L per extraction), and, then, again with acetone to obtain the plant material. The extract was dissolved in DMSO, incubated with shaking at 25 °C for 1 h and stored at 4 °C before proceeding to further studies.

Cell lines and culture conditions

DBTRG-05MG (human GBM), RG2 (rat GBM), SVEC (mouse vascular endothelial cells), and MDCK (mouse kidney cells) were obtained from the American Type Culture Collection (Rockville, MD). In addition, GBM8401, GBM8901, G5T/VGH, and N18 cells were obtained from the laboratory collection of Chung-Shan Medical University. The DBTRG-05MG, GBM8401, GBM8901, G5T/VGH, and N18 cells were cultured in RPMI 1640 at 37 °C in a humidified atmosphere containing 5% CO₂. The RG2, SVEC, and MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) at 37 °C in a humidified atmosphere containing 5% CO₂. The media were supplemented with 10% fetal bovine serum (GIBCO, Mexico), 1% sodium pyruvate (GIBCO, USA), 1% HEPES buffer solution (GIBCO, USA), and 1% penicillin/streptomycin (GIBCO, USA).

Analysis of cytotoxicity

To determine the cytotoxicity of the JCo extract, we used a modified 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay to measure cell viability. First, 5×10^3 of glioma or normal cells in 100 µL of growth medium were incubated in 96-well plates and grown for 20 h before treatment. Subsequently, 100 µL JCo extract and TMZ were dissolved in medium (500 µg/mL) and added to the cells. After incubation for 24–48 h, the drug-containing medium was replaced with 50 µL fresh medium containing MTT (500 µg/mL) for incubation of 8–12 h. The MTT medium was then removed and 50 µL DMSO was added to each well. The absorbance of the dissolved solutions at 550 nm was detected using an MRX Microtiter Plate Luminometer (DYNEX, Sunnyvale, CA). The absorbance value of untreated control cells was defined as 100% and the IC₅₀ was defined as the concentration that resulted in a 50% decrease in absorbance in drug-treated cells as compared with that in untreated cells.

Synergistic effects of JCo and TMZ

First, 5×10^3 cells/100 µL were placed in each well of a 96-well plate and cultured for 12–18 h until the cells reached 70%–80% confluence. Two types of treatments were performed: group I was treated with 50 µL of TMZ at 0, 20, 40, 80, 120, and 160 µg/mL; and group II was treated with either 50 µL of JCo extract (40 µg/mL) or 50 µL of JCo extract at 0, 10, 20, 40, 60, and 80 µg/mL combined with 50 µL TMZ (80 µg/mL). After incubation for 24 and 48 h, the MTT assay was performed to analyze cell viability. The synergistic effect was determined from the calculation of the combination index (CI) = (IC₅₀ of drug combined/ IC₅₀ of drug only of group I) + (IC₅₀ of drug combined/ IC₅₀ of drug only of group II). According to the Chou-Talalay method, the following criteria of drug-drug interactions were considered by CI: synergistic effect, CI < 0.9; additive effect, CI = 0.9–1.1; antagonist effect of drugs, CI > 1.1 [31].

Cell cycle analysis

DBTRG-05MG and RG2 (2×10^6 cells) were incubated for 18 h and divided into a time course and a drug dosage group. In the time course group, the cell cycle was analyzed at 0, 6, 12, 24, and 48 h after adding 60 $\mu\text{g/mL}$ JCo extract; in the drug dosage group, various concentrations of JCo extract, containing 0, 30, 60, and 90 $\mu\text{g/mL}$, were examined. The cell cycle status was detected by flow cytometry until 24 h after administration of the JCo extract. Both groups of glioma cells were trypsinized, collected, and washed twice with PBS. The cells were centrifuged at 1000 rpm for 5 min, resuspended in 0.5 mL PBS, fixed in 4.5 mL 70% ice-cold ethanol, and stored at 4 °C. On the day of analysis, the cells were collected by centrifugation and the pellets were resuspended in 0.2 mg/mL propidium iodide containing 0.1% Triton-100 (Sigma) and 1 mg/mL RNase A (Sigma). The cell suspension was incubated in the dark for 30 min at 20 °C $\pm$ 2 °C and subsequently analyzed for DNA content by using a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA). The percentage of cells in each phase of the cell cycle was calculated using a BD Cell quest computer program. Data were collected from three experiments performed in triplicate.

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay to detect cell apoptosis

To evaluate the induction of apoptosis in glioma cells by JCo extract, we used the TUNEL assay (In Situ Cell Death Detection Kit, TUNEL kit, Roche, Germany). Initially, 2×10^6 cells were cultured and analyzed at discrete time points (0, 6, 12, 24, and 48 h after 50 $\mu\text{g/mL}$ JCo extract treatment). In the JCo-treated cell group, the suspended cells were collected. In the control group, the adherent and floating cells were collected. The cells were fixed with 3.7% formaldehyde at room temperature for 15 min on silane-coated glass slides. All procedures for the TUNEL assay followed a previously published protocol [19]. After fluorescent staining, we used a fluorescence microscope (Nikon, Kawasaki, Japan) to review the slides and quantify the extent of apoptosis. Finally, we examined the image representative for 48 h JCo extract treatment.

Western blotting analysis

To detect the cell cycle and the apoptosis-related protein expression after JCo extract treatment, we used western blotting. First, 2×10^6 DBTRG-05MG and RG2 cells were incubated in a culture dish for 18 h. The cells were collected after the application of 60 $\mu\text{g/mL}$ JCo extract for 0, 6, 12, 24, and 48 h in the time-course group. To analyze the dose-dependency of the treatment, the cells were collected at 24 h after the addition of 0, 30, 60, and 90 $\mu\text{g/mL}$ JCo extract. All cells were lysed by incubation in RIPA buffer (100 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, and 1% Triton X-100) at 4 °C for 10 min. The crude cell lysates centrifuged at 15,000 rpm for 10 min to obtain cleared supernatants. Cell lysates (30 μg) from each group were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred onto polyvinylidene difluoride (Millipore, MA, USA) and blocked with 5% skim milk in TBST for 1 h at room temperature. After blocking with 5% nonfat milk in TBST (12.5 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20), the blots were incubated overnight at 4 °C with the primary antibodies followed by an incubation with the secondary antibody. In this study, the primary antibodies recognized the cell cycle-related proteins cyclin A, cyclin B1, cyclin D1, Cdk2, Cdk4, RB, p-RB, p53, p-p53, p21, and p-p21 (1/200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and the apoptosis-related protein Fas, Fas ligand, caspase-8, Bax, Bcl-2, AIF, caspase-9, and caspase-3 (1/200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The blots were incubated with an appropriate HRP-labeled secondary antibody for 30 min at room temperature, washed extensively, and developed using a T-Pro LumiFast Plus Chemiluminescence Detection Kit (T-Pro BIOTECHNOLOGY, USA) in accordance with the manufacturer's instructions. A monoclonal mouse anti- β -actin antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as the internal control.

Caspase inhibitor assay for detecting apoptotic pathways

To identify the apoptotic pathway of glioma cells treated with JCo extract, the relative protein expression of various subtypes of pro-caspases was detected by western blotting analysis. Initially, 5×10^5 DBTRG-05MG and RG2 cells were incubated for 18 h. These cells were divided into four groups: caspase-inhibitor (-)/JCo (-); caspase-inhibitor (+)/JCo (-); caspase-inhibitor (-)/JCo (+); and caspase-inhibitor (+)/JCo (+). The primary antibodies were pro-caspase 8, 9, and 3, and β -actin was used as the internal control.

Therapeutic effects of JCo in an animal model of subcutaneous tumors

To check the therapeutic effects of JCo extract in an *in vivo* study, the DBTRG-05MG human GBM cells were used in animal experiments to monitor the antitumor activity. Subcutaneous implantation of 1×10^7 cells/100 μ L was performed in male Foxn1 nu/nu mice (10–12 weeks old) obtained from the National Laboratory Animal Center (Taipei, Taiwan). The tumor-bearing group and vehicle group of animals were treated with JCo by subcutaneous injection (sc. injection) of 200 mg/kg JCo extract and 5% DMSO, respectively, every 2 days between 5 and 23 days after tumor implantation. The distance between tumor implantation and the JCo sc. injection sites was 2 cm. The animals were sacrificed when the tumor volume exceeded 1,500 mm³ or 100 days after inoculation. The tumor volume was calculated as $L \times H \times W \times 0.5236$ (20).

Therapeutic effect of JCo in an in-situ glioma model

To detect the therapeutic effects of JCo in animals with brain tumors, the difference in tumor size between the JCo-treated and vehicle-treated rats was examined by MRI. Eight F334 female rats (8–10 weeks old, 150–180 g in weight) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Initially, 5×10^4 RG2 (rat GBM) cells were inoculated in the brain hemisphere of all rats by stereoscope under proper anesthesia. Subsequently, the rats were divided into two equal groups, for administration of either JCo extract or the vehicle. The JCo extract (200 mg/kg with 5% DMSO) was sc. administered daily between day 3 to day 7 after tumor inoculation. In the vehicle group, 5% DMSO was injected into the subcutaneous area at the same time points as the JCo treatment. The tumor volume was measured and calculated by 7-T magnetic resonance imaging (7 Tesla, Bruker BioSpec 70/30 MRI) at National Taiwan University (Taipei, Taiwan) on days 11 and 13 after inoculation of the tumor cells. Finally, the animals were sacrificed when the tumor volume exceeded 1,500 mm³ or 100 days after tumor inoculation. Tumor components and some vital organs, including the heart, liver, spleen, lung, kidney, and intestine, were subjected to hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining.

H&E staining

For the histological H&E staining of GBM of tumor and normal tissues in the presence or absence of JCo extract, the tissues were fixed in 10% neutral formalin. After the tissues were dehydrated and embedded in paraffin wax, tissue sections (4 μ m/section) were placed on clean glass slides and dehydrated in an oven for 30 min at 60 °C. Prior to staining, the tissue slides were deparaffinized, rehydrated, and then stained with Mayer's hematoxylin and eosin Y solution for 3 min.

Immunohistochemistry

To examine the possible mechanism underlying the effect of JCo extract in an *in vivo* study, we performed IHC staining of angiogenic factors. All tissue sections were de-waxed in xylene, rehydrated in alcohol, and immersed in 3% hydrogen peroxide for 5 min to suppress endogenous peroxidase activity. Antigen retrieval was performed by heating (100 °C) each section for 30 min in 0.01 mol/L sodium citrate buffer (pH 6.0). After three rinses for 5 min each in phosphate-buffered saline [PBS], the sections were incubated for 1 h at room temperature with primary antibodies for anti-PCNA, anti-VEGFR1, anti-VEGFR2, and anti-cleaved caspase-3 (1/200 dilution; Santa Cruz Biotechnology Inc., CA, USA) diluted in PBS. After three washes of 5 min each in PBS, the sections were incubated with biotin-labeled secondary immunoglobulin (1:100, DAKO, Glostrup, Denmark) for 1 h at room temperature. After three additional washes, peroxidase activity was developed by using the 3-amino-9-ethylcarbazole substrate chromogen system (DAKO, Glostrup, Denmark) at room temperature.

Results

Cytotoxic effects of JCo extract and TMZ on brain tumor and other cell lines

To compare the cytotoxicity of JCo extract and TMZ in tumor and non-tumor cells, we evaluated the IC_{50} values in DBTRG-05MG, G5T/VGH, GBM8401, GBM8901, RG2 (glioblastoma cell lines), N18 (neuroblastoma cell line), CTX TNA2 (rat astrocyte cell line), SVEC (vascular endothelial cell line), and MDCK (kidney endothelial cell line). The treatment of the glioblastoma and neuroblastoma cells with JCo extract resulted in various degrees of inhibition of cell viability based on the drug concentration between 24 and 48 h of observation (Fig. 1). The IC_{50} values of JCo extract were 57–69 $\mu\text{g/mL}$ and 49–67 $\mu\text{g/mL}$ in the glioblastoma cell lines after 24 and 48 h, respectively. The respective IC_{50} values of JCo extract were 76–105 $\mu\text{g/mL}$ and 77–108 $\mu\text{g/mL}$ in non-tumor cell lines (Table 1). The difference in IC_{50} values between the tumor and non-tumor cell lines confirmed the safety and efficacy of the drug administration. In contrast, the IC_{50} values of TMZ were significantly higher than those of JCo extract at 24 and 48 h, but differences between tumor and non-neoplastic cell lines were not significant.

Arrest of cell cycle by JCo extract

To detect the cell cycle interruption of glioblastomas, we used flow cytometry to analyze DBTRG-05MG and RG2 glioma lines after the addition of JCo extract at various concentrations and times. The administration of 60 $\mu\text{g/mL}$ JCo extract to GBM cells resulted in a significant but gradual arrest of the cell cycle in the S phase, 12.61%, 5.51%, 7.49%, 5.57%, and 6.26% and the G2/M phase, 36.86%, 36.93%, 32.04%, 33.00%, and 35.93% at 0, 6, 12, 24, and 48 h, respectively (Fig. 2A). In addition, compared to that of the control group (G0/G1: 50.54%; S: 12.61; G2/M: 36.86%), a higher proportion of GBM cells were in the G0/G1 phase (57.48%, 61.29%, 57.58%), and a lower percentage of tumor cells were in the S (7.24%, 6.35%, 8.38%) and G2/M

Fig. 1. The growth inhibition of GBM cells by JCo extract. The tumor cells, which included DBTRG-05MG, RG2, GBM8401, GBM8901, G5T/VGH, and N18, were incubated in 96-well culture plates overnight and treated with JCo extract (0–200 $\mu\text{g/mL}$) for 24 and 48 h. The cell viability was determined using the MTT assay. Untreated wells were used as control group.

Table 1. The IC₅₀ of JCo extract in GBM cell lines. Note: Values are mean ± SD (μg/ml) at 24h and 48h. a: It was significant difference between tumor and normal cells. (p < 0.05). b: Significant difference between JCo treatment group and TMZ group (p < 0.05)

Cell line	Tumor type	JCo		TMZ	
		24h	48h	24h	48h
Brain tumor					
DBTRG-05MG	Hu GBM cell	67.04 ± 1.41 ^{a,b}	49.46 ± 1.1 ^{a,b}	127.06 ± 4.33	96.48 ± 5.29
G5T/VGH	Hu GBM cell	63.3 ± 5.5 ^{a,b}	67.85 ± 4.83 ^a	150.29 ± 10.23	N.D.
GBM8401	Hu GBM cell	57.14 ± 1.68 ^b	46.68 ± 0.85 ^a	148.56 ± 2.27	N.D.
GBM8901	Hu GBM cell	58.45 ± 3.53 ^{a,b}	55.49 ± 4.02 ^a	> 150	N.D.
RG2	Rat GBM cell	69.97 ± 1.47 ^{a,b}	53.8 ± 0.96 ^{a,b}	118.82 ± 23.96	71.52 ± 1.81
N18	Mo neuroblastoma	61.11 ± 5.09 ^{a,b}	68.94 ± 1.77 ^a	94.84 ± 12.83	N.D.
Normal cell					
CTX TNA2	Rat astrocytes	105.46 ± 2.08	108.05 ± 4.72	128.68 ± 8.09	84.49 ± 19.35
SVEC	Mo vascular endothelial cell	76.89 ± 0.73	77.13 ± 0.35	136.06 ± 2.86	111 ± 15.56
MDCK	Canine kidney endothelial cell	80.73 ± 4.69	80.56 ± 0.55	> 150	141.15 ± 9.75

(35.28%, 32.36%, 34.04%) phases after the application of 30, 60, and 90 μg/mL JCo extract, respectively (Fig. 2B). The degree of cell cycle arrest depended on the level of JCo reaction time and dosage. The western blotting analysis revealed that the JCo extract not only enhanced the expression of tumor suppressor genes, such as p53, phosphorylated p53, and p21, but also inhibited the expression of cell cycle checkpoint proteins, including Rb, CDK2, CDK4, cyclin A, cyclin B1, and cyclin D1 (Fig. 2C). Therefore, the inhibition of glioblastoma cell proliferation was effectively arrested by administration of JCo extract via the inhibition of the G1-to-S phase checkpoint.

JCo extract triggers GBM cell apoptosis by interacting with intrinsic and extrinsic pathways

The cell cycle analysis indicated that JCo-treated DBTRG-05MG and RG2 glioblastoma cell samples contained a higher percentage of the sub-G1 phase tumor cells (14.03%, 25.93%, 42.06%, 45.60% in DBTRG-05MG cells; 16.22%, 29.36%, 40.39%, 59.30% in RG2 cells) than the control group (2.04%, 1.98%, 1.45%, 2.05% in DBTRG-05MG cells; 6.64%, 6.57%, 6.64%, 6.57% in RG2 cells) at 6, 12, 24, and 48 h (Fig. 3A and 3B). In the TUNEL test, most of the JCo-treated glioblastoma cells showed a DNA condensation that was detectable using fluorescence microscopy (Fig. 3C). For the investigation of the apoptotic pathway in glioblastoma cells induced by the JCo extract, the Fas and Fas-L proteins were detected in DBTRG-05MG and RG2 glioblastoma cell lines before and after the JCo treatment. Relative to vehicle-treated glioblastoma cells, JCo-treated tumor cells had higher Fas levels, but lower Fas-L levels after 6 h (Fig. 3D). The transition from procaspase-8 to activated caspase-8 is a crucial step of the extrinsic apoptotic pathway. The lower expression of procaspase-8 in glioblastoma cells treated with JCo extract suggested the activation of the extrinsic apoptotic pathway. The factors of the intrinsic apoptotic pathway include Bax, Bcl-2, AIF, caspase-3, and caspase-9. Therefore, we performed an immunoblotting experiment to determine if JCo extract activated the extrinsic apoptotic pathway. The protein expression of Bax, AIF, C-caspase-3, and C-caspase-9 was observed in JCo-treated DBTRG-05MG and RG2 was higher in tumor cells than that in non-treated tumor cells. In addition, a reduced expression of Bcl-2 and procaspase 8 and 9 was observed in JCo-treated cells. Moreover, the addition of inhibitors of caspase-3, -8, and -9 in these two glioblastoma cell lines significantly increased procaspase-3, -8, and -9 protein expression. However, the combination of JCo extract and caspase inhibitors might induce the expression of the active forms of caspase-3, -8, and -9 (Fig. 4A and 4B). Based on the expression of those factors, we demonstrated that the JCo extract induced glioblastoma apoptosis through both the intrinsic and extrinsic apoptotic pathways.

Fig. 2. The cell cycle distribution and related proteins expression of JCo extract-treated cells. DBTRG-05MG and RG2 cells treated with 60 µg/ml JCo extract for 0-48 h as a time course analysis; 30, 60 and 90 µg/ml JCo for 24 h as a dosage analysis. After JCo extract treatment, cell cycle analysis for FL2 intensity was performed by flow cytometry. JCo extract induced cell cycle arrest at G0/G1 phase during (A) a time course and using (B) a concentration series in DBTRG-05MG and RG2. Each column shows the mean ± SD (*: compared with control was significantly increased; #: compared with control was significantly decreased; $p < 0.05$). (C) The cell cycle associated proteins were detected during time course and by dosage analysis using western blotting. The protein expression indexes = (sample intensity/sample β-actin intensity) / (control intensity/control β-actin intensity). N.D. means not detectable.

Fig. 3. JCo extract triggers GBM cell apoptosis through an intrinsic and extrinsic pathway. The GBM cells were treated with JCo extract for time course (60 µg/ml for 0-48 h) and dosage (30, 60 and 90 µg/ml for 24 h) analysis. After treatment, the increase in the percentage of sub-G1 phase cells was (A) time- and (B) dosage-dependent. The results show the mean ± SD (*p<0.05). (C) GBM cells showed apoptotic formation after 60 µg/ml JCo extract treatment in TUNEL assay and PI counterstaining. (a) Apoptotic body, (b) DNA fragmentation and (c) DNA condensation were observed under fluorescence microscopy (400X). (D) The detection of apoptosis associated proteins by time course and dosage analysis using western blotting. The protein expression indexes = (sample intensity/sample β-actin intensity) / (control intensity/control β-actin intensity).

Synergistic effects of JCo extract and TMZ in glioblastomas

The JCo extract treatment in the range of 0–45 µg/mL did not inhibit the progression of DBTRG-05MG and RG2 cells. A cytotoxic effect of JCo extract was measured at 60 µg/mL. Higher concentrations of JCo extract caused greater cytotoxicity (Fig. 6A). TMZ is one of the most common chemotherapeutic agents for glioblastomas. The use of TMZ alone significantly decreased DBTRG-05MG and RG2 tumor cell viability at concentrations above 20 µg/mL. The combination of various concentrations of JCo extract (0–75 µg/mL) with 60 µg/mL TMZ or various doses of TMZ (0–100 µg/mL) with 40 µg/mL JCo extract resulted in a higher percentage of cell apoptosis than those administered with the equivalent concentrations of TMZ or JCo extract alone in DBTRG-05MG and RG2 cells (Fig. 5A and 5B, CI=0.8 and 0.68, respectively). Therefore, the combination of JCo extract with TMZ exerted positive synergistic effects on the inhibition of glioblastoma growth. To identify the pathway through which the combination of JCo extract and TMZ functioned, western blotting analysis of cell proliferative and apoptotic factors was performed. The combination treatment of JCo extract and TMZ downregulated the expression of AKT, mTOR, P70S6K, and their phosphorylated forms, but upregulated that of c-caspase-3, as compared with that of the treatment with either JCo extract or TMZ (Fig. 5C).

JCo extract effectively inhibits subcutaneous glioblastomas in BALB/c nude mice

To evaluate the *in vivo* antitumor effects of JCo extract, we injected JCo extract into BALB/c nude mice bearing subcutaneous glioblastomas. The tumor volume was significantly smaller after day 16 in JCo-treated mice than that in vehicle-treated mice. In addition, the tumor size in the vehicle-treated mice was above 1500 mm³ on day 33, but the volume of all tumors in the JCo-treated mice was below 1300 mm³ between day 1 and 48 (Fig. 6A). All mice that were not treated with JCo were sacrificed on day 31 because of behavioral dysfunction and large tumor volume. In contrast, more than half of the JCo-treated mice survived until day 60 (Fig. 6B). Therefore, the survival time of JCo-treated nude was longer than that of the vehicle group.

JCo extract significantly inhibits in situ glioblastomas in F344 rats

To detect the ability of the JCo extract to suppress in situ tumor growth, sc. injection of the JCo extract into F344 rats bearing in-situ brain glioblastomas was performed. From the animal MRI images, the tumor sizes of JCo-treated rats measured 6.69 ± 2.48 mm³ and 12.22 ± 2.43 mm³ on day 11 and 13, respectively. In contrast, the tumor volumes of vehicle-treated rats measured 58.34 ± 7.35 mm³ and 96.42 ± 10.74 mm³ on day 11 and 13, respectively. Therefore, the in situ glioblastomas of JCo-treated rats were significantly smaller than those of the vehicle-treated rats ($P < 0.05$, Fig. 6C). Moreover, the survival rate of JCo-treated rats was significantly higher than that of the vehicle-treated rats (Fig. 6D). The in situ histology analysis identified more apoptotic bodies in the glioblastomas from JCo-treated rats than those in the vehicle-treated rats after animal sacrifice (Fig. 6E).

Mechanism of suppression of glioblastoma proliferation by JCo extract derived from in vivo analysis

To verify the mechanism of tumor growth inhibition by JCo extract, we performed IHC staining and a TUNEL assay on the in situ inoculated glioblastomas. In all rats with in situ glioblastomas, the labeling index of PCNA, VEGFR1, VEGFR2, c-caspase 3 were 73.08%, 9.67%, 11.70%, 2.43% in vehicle group of tumors respectively. However, in the JCo-treated glioblastomas, 44.49%, 5.88%, 5.85% and 6.53% of tumor cells had increased levels of PCNA, VEGFR1, VEGFR2 and c-caspase 3. Therefore, the JCo-treated glioblastomas had a significantly lower expression of PCNA, VEGFR1, and VEGFR2, but a higher expression of c-caspase-3 as compared to those of the vehicle-treated glioblastomas (Fig. 7A). In addition, the TUNEL test showed that the group with JCo-treated intracranial glioblastomas had a higher percentage of apoptotic cells than those of the vehicle-treated glioblastomas (Fig. 7B).

Fig. 4. (A) Before treated caspase-3, 8 or 9 inhibitor, GBM cells treated with 60 μ g/ml JCo (24 h), and analyzed relative protein activation by western blotting. C-Caspase-3: Cleaved Caspase-3. (B). The results show the mean $\pm$ SD (* compared with control, $p < 0.05$; # compared with JCo, $p < 0.05$).

Fig. 5. (A) JCo extract (0, 15, 30, 45, 60 and 75 µg/ml) combined with TMZ (60 µg/ml) for 48 h on DBTRG-05MG and RG2 cells. TMZ (0, 20, 40, 60, 80 and 100 µg/ml) combined with JCo extract (40 µg/ml) for 48 h on DBTRG-05MG and RG2 cells. The synergistic effect was confirmed by JCo extract combined with TMZ treatment of GBM tumor cells. *: Significant difference between combination group and single drug ($P < 0.05$). (B) Cells were treated with TMZ (60 µg/ml), JCo extract (40 µg/ml) or drug combination (T+J) for 48 h and analyzed for expression of cell proliferation and apoptosis proteins detected for synergistic effect analysis by western blotting. The protein expression indexes = (sample intensity/sample β-actin intensity) / (control intensity/control β-actin intensity).

Fig. 6. JCo extract suppress tumor growth and induce apoptosis in a subcutaneous and an orthotopic therapeutic model. (A) Nude mice bearing DBTRG-05MG tumors were treated with vehicle (n=5) and 200 mg/kg JCo extract (n=10) by injection (s.c.) every 2 days with a total of 10 doses. Tumor volume was measured every 2 days and calculated as L x H x W. (B) Mice were sacrificed when tumor size exceeded 1500 mm³, the survival time was detected. RG2 cells were implanted in intracranial region (striatum) of F344 rats. The rat bearing tumors were treated with vehicle (n=4) or 200 mg/kg JCo extract (n=4) by s.c. injection at day 3, 4, 5, 6 and 7 for a total of 5 times. (C) 7-T MRI imaging serial sections (1 mm per section) of tumor volume were captured. V1 to V6 were from vehicle control rats; J1 to J6 were from JCo treatment rats (yellow arrow as tumor area). Tumor volume was calculated (tumor area x thick) by Image J software. Columns showed mean $\pm$ SE (*P<0.05). (D) The survival rate was monitored daily. (E) The tumor mass was analyzed by H&E stain (400x).

Fig. 7. The in vivo anti-tumor mechanism of JCo extract. The rat bearing RG2 tumors were treated with vehicle (n=4) and 200 mg/kg JCo extract (n=4) by subcutaneous therapy at days 3 to 7 for 5 times. The tumor mass was collected and analyzed by immunohistochemistry (IHC) and TUNEL assay. The primary antibodies were anti-PCNA, anti-cleaved-Caspase-3, anti-VEGFR1, and anti-VEGFR2. The preparations were analyzed under a light microscope at 400× magnification. *significantly difference between vehicle and JCo extract group (P<0.05).

*Physiologic and
pathologic toxicity of
JCo extract in the in vivo
analysis*

To verify the toxicity of the JCo extract in other non-neoplastic organs, the data from the blood analysis and the internal organs of the experimental nude mice and rats were collected. Neither inflammatory cell aggregation nor cellular apoptosis in the heart, liver, spleen, lung, kidney, and intestine was identified by histological analysis of tissues from JCo extract-treated and vehicle-treated animals (Fig. 8). In addition, the serum levels of red blood cells (RBC), platelets (PLT), hemoglobin (HGB), hematocrit (HCT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (CRE) also showed no significant difference between tissues from JCo-treated and vehicle-treated animals (Fig. 9). In addition, we also checked the platelet function and other immune cell kinetics at 12 and 24 h because of the limited number of blood samples. In all test for coagulative functions, PT (prothrombin time) and APTT (activated partial thromboplastin time) were analyzed. The average PT in the JCo-treated glioma rats was 15.6 ± 1.9 s and 15.8 ± 2.1 s at 12 and 24 h, respectively. The PT in the vehicle-treated glioma rats was 15.7 ± 2.2 s and 15.9 ± 2.0 s at 12 and 24 h, respectively. In addition, the averages of APTT in the JCo-treated and the vehicle-treated glioma rats were 22.2 ± 1.7 s and 22.8 ± 2.2 s at 12 h and 22.7 ± 2.4 s and 23.0 ± 2.1 s at 24 h, respectively. Therefore, analysis of the coagulative functions showed no differences between the two treatment groups of rats. In addition, the CD4/CD8 ratio of T cells and the levels of interleukin-1 beta (IL-1 β) and tumor necrotic factor-alpha (TNF- α) were evaluated in the JCo-treated and vehicle-treated animals at 12 and 24 h. However, there was no statistically significant difference between the two treatment groups.

Fig. 8. JCo extract showed low or no physiological and pathological toxicity in vivo. Mice, at 10-12 weeks of age, were treated with vehicle (n=5) and 200 mg/kg JCo extract (n=5) by sc. injection every 2 days for 10 times. After sacrificing the animals, the organs (heart, liver, spleen, lung, kidney, intestine and stomach) were collected and analyzed for damage by H&E staining.

Fig. 9. The 6-8 weeks rats were treated with a single dose of vehicle (n=4) and 200 mg/kg JCo (n=4) by sc. injection. The analysis of blood cells (white blood cell, hemoglobin, hematocrit and platelet) and serum biochemistry (ALT, AST, blood urea nitrogen, and creatinine) were analyzed after JCo extract treatment for 0, 3, 6, 12 and 24 h. No significant differences were observed between vehicle and JCo treated group. The results show the mean $\pm$ SE.

Discussion

The underlying mechanism for the suppression of glioma by the JCo extract is not clearly understood. Lantto et al. proved that the JCo extract effectively enhanced p53 to induce apoptosis in neuroblastoma cells *in vitro* [18]. In human breast, prostate, and colon cancer cell lines, the JCo extract had a lower LC_{50} than that of doxorubicin; but in some human cancer

cell lines, the cytotoxic effect of JCo extract was stronger than that of other chemotherapeutic agents [32]. Unfortunately, the hypothesis of the suppression of in situ brain glioma by JCo extract still lacks compelling evidence. This study is the first to demonstrate that the JCo extract can penetrate the BBB to induce brain glioma cell apoptosis by sc. injection of JCo extract.

In our *in vitro* studies, the IC_{50} values of the JCo extract in all GBM cell lines were significantly lower than those in normal astrocytes and endothelial cell lines after both 24 and 48 h. However, the IC_{50} values of TMZ indicated an overlap of cytotoxic concentrations in some GBM cell lines and normal astrocytes and endothelial cells. In addition, the IC_{50} values of the JCo extract were also lower than those of TMZ in GBM cell lines. Compared with TMZ, the JCo extract induced stronger cytotoxic effects with greater specificity for GBM cells. To explore the mechanism of inhibition of GBM cells by the JCo extract, we used flow cytometric analysis to demonstrate that cell cycle arrest might play an important role in the cytotoxicity of JCo-treated GBM cells. The overexpression of tumor suppressor genes, such as p53, and the inhibition of cell-proliferation related proteins, such as Rb, supported a possible antitumor activity of the JCo extract. Interestingly, the cyclin/CDK family is known to be essential for cell replication, especially CDK2, CDK4, and cyclin-A, -B, -D, and -E [33]. In contrast, p21 acts as an inhibitor for CDK proteins [33]. The JCo extract effectively blocked GBM cells in the G0/G1 phase from entering the S and G2/M phase by exerting control over the cell cycle checkpoints, causing an inhibition or delay in GBM cell replication.

The degree of tumor apoptosis may be an important factor for selecting new anticancer drugs for development [34]. The higher percentage of sub-G1 phase tumor cells after drug treatment could be an indicator for a greater degree of cellular apoptosis. Using fluorescence microscopy, the TUNEL test highlighted DNA fragmentation and the condensation of tumor cells [35, 36]. The results from our two examinations demonstrated that JCo extract significantly induced GBM cell apoptosis. Furthermore, a higher concentration of JCo extract appeared to more effective in inducing cytotoxicity in GBM cells. The mechanism of cellular apoptosis can be divided into the intrinsic and the extrinsic pathway [37]. The indicative factors of the extrinsic apoptotic pathway include Fas ligand (Fas-L), Fas receptor, cleaved caspase-8, and cleaved caspase-3 [38]. The BAX/BAK system and apoptotic protease activating factor-1 (APAF-1) are the main factors of the intrinsic apoptotic pathway [34]. Bcl2/Bcl-XL acts as an antagonist for BAX [39]. Our study is the first to demonstrate that the treatment with JCo extract caused DNA damage in GBM cells and induced apoptosis via the intrinsic and the extrinsic pathway.

TMZ is a widely used chemotherapeutic drug for patients with recurrent and highly aggressive astrocytoma, especially for patients with GBM [40]. A possible serious complication of TMZ is the suppression of hematopoiesis, which can induce aplastic anemia, bleeding, and immunodeficiency [41]. In this study, the therapeutic effect observed with the combination of the JCo extract with TMZ was a more effective treatment than the effect observed using either of these two therapeutics as single treatment. The synergistic effect of JCo extract and TMZ could allow a reduced the dosage of TMZ, which would reduce the incidence of serious complications, such as bone marrow suppression. In addition, the interaction of the mTOR and PI3K pathways is a central regulator of the cellular proliferative signal transduction that induces tumor cell survival and angiogenesis [42, 43]. Akt, mTOR, and p70S6 expression could activate those pathways and induce a phosphorylation cascade that stimulates cell growth. In this study, we successfully demonstrated that the synergistic effects of a combination of the JCo extract and TMZ inhibited GBM proliferation by blocking Akt, mTOR, and p70S6 expression.

In our *in vivo* studies, the subcutaneous tumor size was significantly smaller in JCo-treated mice than that in vehicle-treated mice. A larger proportion of tumor apoptosis was also observed in the JCo-treated mice than in the vehicle-treated mice. In addition, because the BBB can cause the dysfunction of a chemotherapeutic drug, we established an in situ GBM animal model and sc. injected JCo extract to examine if the JCo extract could penetrate the BBB. Importantly, the MRI and histological analysis indicated that the JCo extract

could effectively inhibit in situ brain tumor proliferation and induce cell apoptosis. To our knowledge, we have presented the first study published in English that the JCo extract could penetrate the intratumoral BBB.

GBM-induced angiogenesis is essential for tumor cell proliferation and invasion [44]. The apoptosis of GBM cells results from radiotherapy and chemotherapy, which induces hypoxia in the tumor microenvironment that enhances platelet-derived growth factor subunit B (PDGF-B) and/or epidermal growth factor (EGF) receptor expression [45]. In contrast, some antivasular therapeutic drugs may induce perivascular GBM cell invasion, which may result in the resistance to second-line therapies [46, 47]. In our study, a lower expression of VEGFR-1 and -2 and a higher expression of cleaved caspase-3 were observed in the JCo-treated mice with in situ GBM tumors than in the vehicle-treated groups, which indicated that the JCo extract not only inhibited intratumoral angiogenesis, but also induced tumor cell apoptosis. In contrast, low functional and histological damage to non-neoplastic vital organs should be the most important requirement for the development of new drugs. The histological analysis demonstrated that most of the vital organs in JCo-treated rats, including the heart, liver, kidney, lung, intestine, and spleen, had a similar microscopic appearance as those in vehicle-treated animals. In addition, no significant differences in blood cell count, the serum levels of hepatic enzyme, and renal function index were identified between JCo-treated and vehicle-treated animals. Therefore, the pharmacological effects of the JCo extract appeared to be specific for GBM cells and did not affect other non-neoplastic vital organs, which confirmed the safety of this new herbal therapy.

Conclusion

In this study, we successfully demonstrated that the JCo extract appears to be a safe herbal therapeutic that effectively suppressed glioma cell proliferation by inhibiting angiogenesis. In addition, a combination of JCo extract and TMZ exerted synergistic effects to induce tumor apoptosis. Therefore, JCo extract may represent a beneficial therapeutic choice for high-grade gliomas.

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Disclosure Statement

The authors declare that there was no conflict of interests.

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