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Sann-Joong-Kuey-Jian-Tang up-regulates the protein expression of Fas and TNF-α in colo 205 cells *in vivo* and *in vitro*

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Abstract. Sann-Joong-Kuey-Jian-Tang (SJKJT), a traditional Chinese medicine prescription, has been used to treat lymph node diseases and tumors. However, the molecular mechanisms of SJKJT in human colon cancer *in vivo* and *in vitro* have not been clearly elucidated. In the present study, we investigated the molecular mechanisms of SJKJT in human colon cancer colo 205 cells *in vitro* and *in vivo*. In the *in vitro* study, colo 205 cells were treated with various concentrations (0.5, 1 and 2 mg/ml) of SJKJT. The protein expression of TNF-α, Caspase-8 and Caspase-3 in colo 205 cells was measured by Western blotting. The results demonstrate that SJKJT up-regulated Fas, TNF-α, Caspase-8 and Caspase-3 protein expression. In the *in vivo* study, human colon cancer colo 205 cells ($3x10^6$/0.2 ml) were injected subcutaneously into the flank area of nude SCID mice (n=32) randomly divided into four groups. SJKJT was dissolved in saline and then administered orally to the mice at concentrations of 0.01, 0.1 and 0.3 g/kg/day for 30 days. The control group was treated with an equal volume of saline. SCID mice were sacrified by CO_2 inhalation and the xenograft tumors were dissected. Subsequently, the protein expression of Fas, TNF-α, Caspase-8 and Caspase-3 in the tumors was measured by Western blotting. The results demonstrate that SJKJT up-regulated Fas, TNF-α, Caspase-8 and Caspase-3 protein expression, both *in vitro* and *in vivo*. These observations suggest that SJKJT has therapeutic potential in colon cancer.

Introduction

Colon cancer is ranked third as a cause of cancer-related mortality in Taiwan (1). Sann-Joong-Kuey-Jian-Tang (SJKJT), a traditional Chinese medicine, has been prescribed to treat tumors or lymphadenopathy diseases, and as a complementary treatment for colon cancer in Taiwan. In our previous studies, we demonstrated that SJKJT inhibited the proliferation of colo 205 cells *in vitro*, possibly as a result of the observed up-regulation of MAP-LC3-II protein expression, resulting in autophagy (2). However, the molecular mechanisms of SJKJT in human colon cancer *in vivo* and *in vitro* have yet to be clearly elucidated, and the effectiveness of SJKJT in inducing cell growth reduction and apoptosis in human colon cancer cells has not been established. It has been reported that the activation of death-receptor pathways by external stimuli that bind membrane receptors such as Fas ultimately activates caspases, leading to the cleavage of certain metabolic substrates and in turn to apoptosis. When the Fas receptor binds to an appropriate activator, the receptor forms a death-inducing signaling complex, resulting in the activation of the caspase cascade and apoptosis (3). In the present study, we evaluated the protein expression of Fas, tumor necrosis factor (TNF)-α, Caspase-8 and Caspase-3 in human colon cancer colo 205 cells treated with SJKJT *in vivo* and *in vitro.*

Materials and methods

Chemicals and reagents. Fetal bovine serum (FBS), sodium pyruvate, HEPES, dimethyl sulfoxide (DMSO), RPMI-1640, MTT and the antibodies to Fas, TNF-α, Caspase-8, Caspase-3 and β-actin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). 10X Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer, Tris, Tween-20, SDS and 5X TBE buffer were obtained from Amresco (St. Louis, MO, USA). BioMax Film was obtained from Kodak.

SJKJT consists of 17 species of medicinal herbs: *Coptis chinensis* Franch, *Cimicifuga heracleifolia* Komar, *Scutellaria baicalensis* Georgi, *Gentiana scabra* Bunge, *Trichosanthes cucumeroides* Maxim, *Phellodendron amurense* Rupr, *Anemarrhena asphodeloides* Bunge, *Platycodon grandiflorum*, *Laminaria japonica* Aresch, *Bupleurum scorzoneri folium* Willd (Bupleurum chinese DC), *Glycyrrhiza uralensis* Fisch, *Sparganium toloniferum* Buch, *Curcuma aeruginos*a Roxb, *Forsythia suspense* Vahl, *Pueraria lobata* Ohwi, *Paeonia lactiflora* Pall and

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Angelica sinensis Diels (4). The crude extract of SJKJT was obtained from Chuang Song Zong Pharmaceutical Co., Ltd. (Ligang Plant, Taiwan, R.O.C.).

Cell culture. The human colon adenocarcinoma colo 205 cell line was obtained from the Food Industry Research and Development Institute (Hsin-chu, Taiwan, R.O.C.). Cells were grown in 75 cm^3 tissue culture flasks at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI-1640 medium containing 10% heat-inactivated FBS, 2% penicillin-streptomycin (10,000 U/ml penicillin; 10 mg/ml streptomycin), 1% HEPES, 1% sodium pyruvate and 1% glutamine.

Cell proliferation assay. The colo 205 cells were plated in 6-well plates at a density of $1x10^5$ cells/well and allowed to adhere and grow for 24 h. The medium was then replaced with 2 ml/well of fresh medium, to which various concentrations (0, 50, 100, 250, 500, 1000 and 2000 μg/ml) of SJKJT were added. Cells were allowed to grow for another 24 h, then 200 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added, and the cells were incubated at 37˚C in the dark for 2 h. Subsequently, the medium was removed and 1 ml DMSO was added to the wells. Absorbance was measured using an ELISA plate reader at 590 nm. Data were calculated as the percentage of proliferation using the following formula: Proliferation (%) = $(OD_{test} - OD_{blank}) \times 100$, where OD_{test} and OD_{blank} are the optical density of the test substances and the blank controls, respectively.

Effect of SJKJT on Fas, TNF-α, Caspase-8, Caspase-3 and β-actin protein expression in colo 205 cell xenograft tumors. Three-week-old male nude SCID mice were xenografted with colon cancer colo 205 cells ($3x10^6$/0.2 ml), divided randomly into four groups and maintained in a pathogen-free environment (Laboratory Animal Center of Tzu Chi University, Hualien, Taiwan, R.O.C.). From day 10, SJKJT (dissolved in normal saline) was administered orally in respective concentrations of 0.01, 0.1 and 0.3 g/kg/day for 30 days. As a control, mice bearing xenograft tumors were treated separately with normal saline (0.1 ml/10-g body weight). The mice were sacrificed by CO_2 inhalation, the xenograft tumors were dissected, and the proteins were extracted for Western blot analysis.

Protein preparation. Proteins were extracted as previously described (5). Briefly, the xenograft tumors were dissected and thick liquid was resuspended in modified Pro-prep™ buffer (Intron Biotechnology) for 40 min at 4˚C. Lysates were immediately centrifuged at 13,000 x g for 20 min at 4˚C, and the supernatant was collected, aliquated (20 μl/tube) and stored at -80˚C until assay. The extracted protein concentrations were measured using the Bradford method (6).

Western blotting. The protein expression of Fas, TNF-α, Caspase-8, Caspase-3 and β-actin was measured by Western blotting. All protein samples were separated by 10% (Caspase-8 and β-actin) and 15% (Fas, TNF-α and Caspase-3) SDS-PAGE as previously described (7). The SDS separated proteins were equilibrated in transfer buffer (25 mM Tris, pH 8.5, 0.2 M glycine and 20% methanol) and transferred onto PVDF membranes (Millipore Corp., Bedford, MA, USA). The membranes were incubated with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h, then washed and incubated at 4˚C overnight with appropriate dilutions of specific antibodies: Fas (1:500), TNF-α (1:500), Caspase-8 (1:200), Caspase-3 (1:1000) (all from R&D Systems Inc.) and β-actin (1:15000) (Sigma-Aldrich, St. Louis, MO, USA). After incubation with anti-mouse peroxidase-conjugated antibody (1:15000) (Sigma-Aldrich), the immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) (Millipore Corporation) detection kit. β-actin was used as an internal control for Western blotting analysis. Immunoreactive bands were scanned (GS-800; Bio-Rad Life Sciences, Hercules, CA, USA) and analyzed using a digital scanning densitometer (Quantity One, v4.4.0; Bio-Rad Life Sciences).

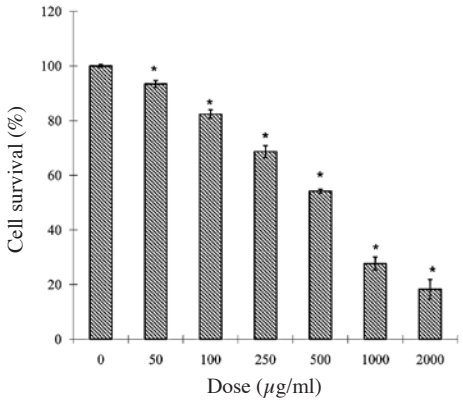

Figure 1. Percentage of cell survival in colo 205 cells ($1x10^5$ cells/well) treated for 24 h with various concentrations of SJKJT (0, 50, 100, 250, 500, 1000 and 2000 μg/ml). The cell survival percentage was determined using the MTT assay. The cytotoxicity of SJKJT in colo 205 cells was dose-dependent. Each point is the mean ± SD of three experiments. *P<0.05.

Effects of SJKJT on Fas, TNF-α, Caspase-8, Caspase-3 and β-actin protein expression in colo 205 cells in vitro. Colo 205 cells ($5x10^6$/10-cm dish) were cultured overnight in medium. The medium was then removed and cells were treated with various concentrations of SJKJT (0, 0.5, 1.0 and 2.0 mg/ml) for 24 h before being harvested by centrifugation. Fas, TNF-α, Caspase-8, Caspase-3 and β-actin proteins were extracted and examined by SDS-PAGE and Western blotting.

Statistical analysis. Values are presented as the mean ± SD. The Student's t-test was used to analyze statistical significance. A P-value <0.05 was considered statistically significant for all tests.

Results

Cytotoxicity of SJKJT in colo 205 cells. MTT assay analysis of the colo 205 cells treated for 24 h with SJKJT concentrations of 50, 100, 250, 500, 1000 and 2000 μg/ml revealed the percentages of viable cells relative to the control to be 93.43±1.31, 82.37±1.55, 68.63±2.15, 54.11±0.73, 27.66±2.36 and 18.32±3.56%, respectively, with an IC_{50} of 619.63 μg/ml. This indicates that the cytotoxicity of SJKJT in colo 205 cells was dose-dependent (Fig. 1).

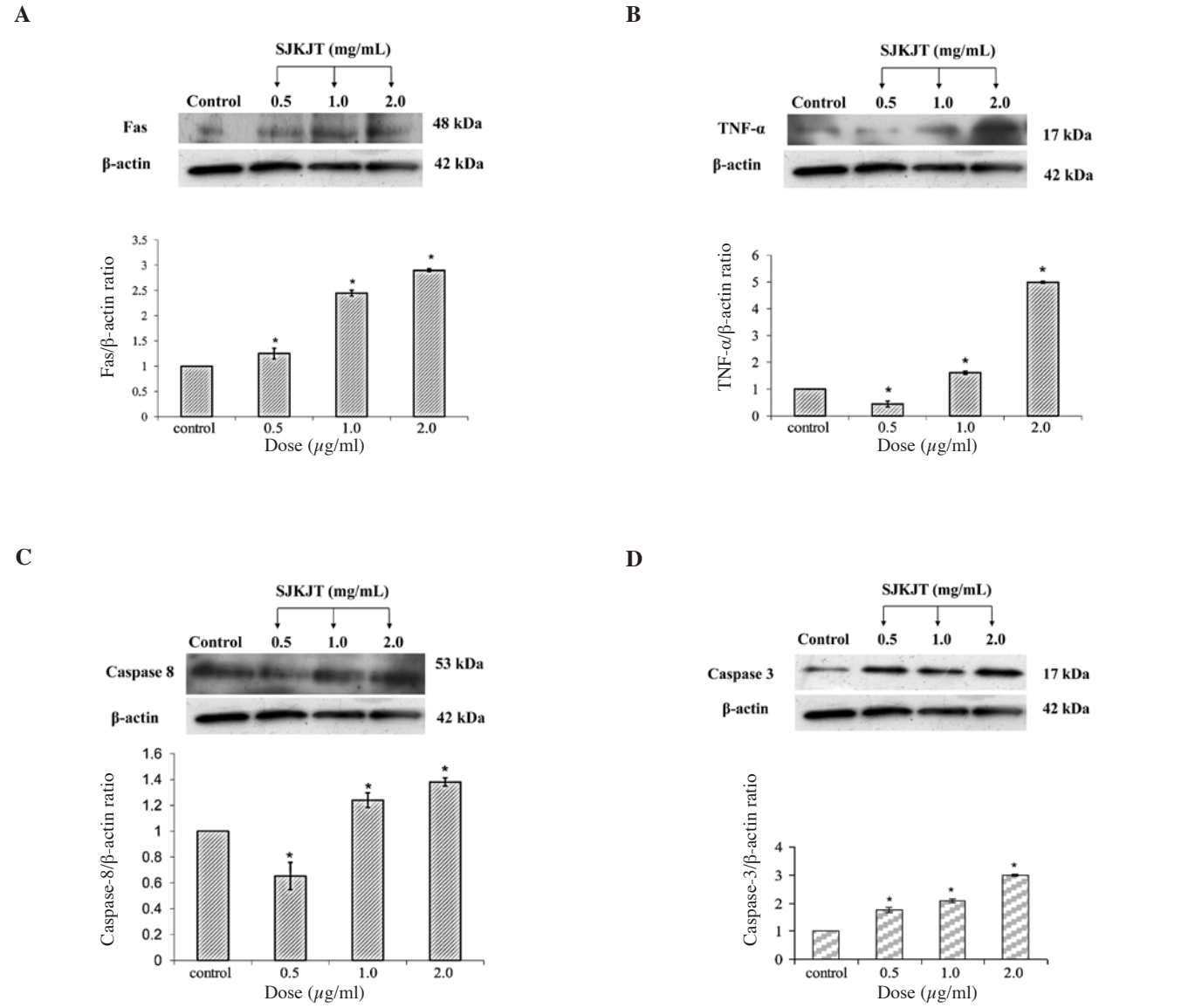

Figure 2. Protein expression of Fas, TNF-α, Caspase-8 and Caspase-3 in colo 205 cells (5x10^6/10-cm dish) treated for 24 h with Sann-Joong-Kuey-Jian-Tang (SJKJT) concentrations of 0, 0.5, 1.0 and 2.0 mg/ml. The protein expression of Fas, TNF-α, Caspase-8 and Caspase-3 was analyzed using Western blotting. SJKJT in colo 205 cells up-regulated the protein expression of Fas (A), TNF-α (B), Caspase-8 (C) and Caspase-3 (D) as compared to the controls. Each point is the mean ± SD of three experiments. *P<0.05, significant difference compared to the controls.

Effect of SJKJT on Fas, TNF-α, Caspase-8, Caspase-3 and β-actin protein expression in colo 205 cells in vitro. In colo 205 cells treated for 24 h with SJKJT concentrations of 0, 0.5, 1.0 and 2.0 mg/ml, the protein expression of Fas (Fig. 2A), TNF-α (Fig. 2B), Caspase-8 (Fig. 2C) and Caspase-3 (Fig. 2D) was up-regulated as compared to the controls.

Effect of SJKJT on Fas, TNF-α, Caspase-8, Caspase-3 and β-actin protein expression in colo 205 cell xenograft tumors. The colo 205 cell xenograft tumors from the SCID mice treated for 30 days with normal saline or SJKJT concentrations of 0.01, 0.1, 0.3 g/kg/day were dissected and individually weighed. The weight (mean ± SD) of the tumors was 9.5±4.41, 5.66±2.89, 8.73±5.52 and 7.3±2.59 g, respectively (Fig. 3). Tumors in SCID mice treated with various concentrations of SJKJT (0.01, 0.1 and 0.3 g/kg/day) were smaller (40.42, 8.11

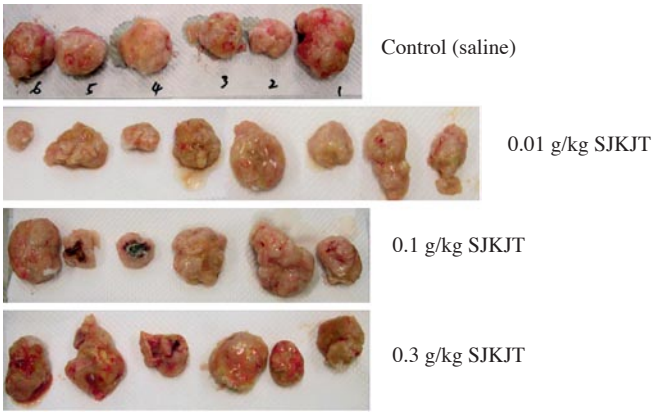

Figure 3. Dissected colo 205 cell xenograft tumors from SCID mice. Mice were treated for 30 days with normal saline only (control) or with Sann-Joong-Kuey-Jian-Tang (SJKJT) concentrations of 0.01, 0.1 and 0.3 g/kg/day, then sacrificed by CO_2 inhalation.

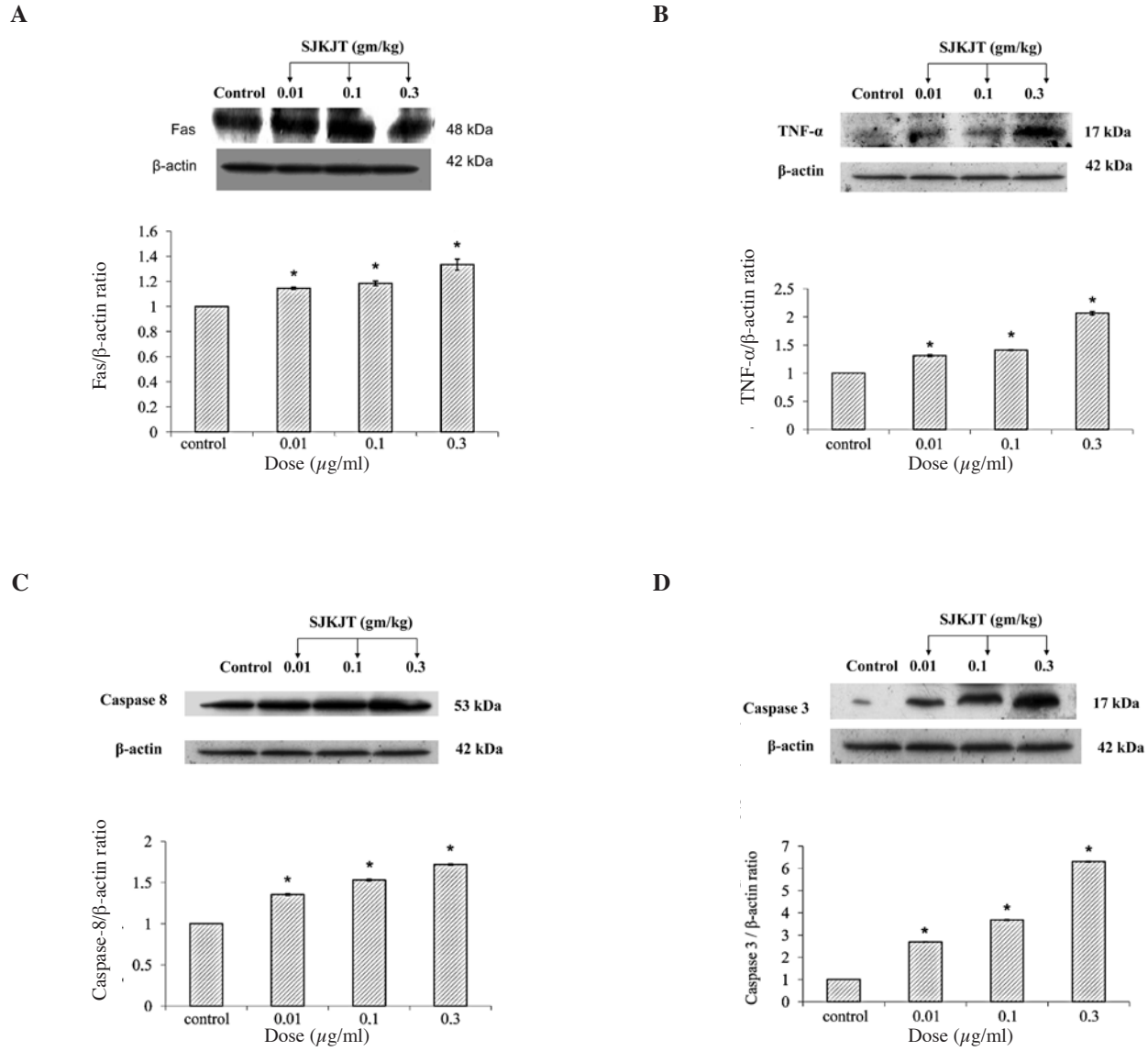

Figure 4. Protein expression of Fas, TNF-α, Caspase-8 and Caspase-3 in SCID mice with colo 205 cell xenograft tumors treated with Sann-Joong-Kuey-Jian-Tang (SJKJT) concentrations of 0.01, 0.1, 0.3 g/kg/day. Mice were sacrificed by CO_2 inhalation, then the tumors were dissected and the proteins extracted for Western blot analysis. SJKJT up-regulated the protein expression of Fas (A), TNF-α (B), Caspase-8 (C) and Caspase-3 (D) as compared to the controls. Values are expressed as the mean ± SD. * P<0.05, significant difference compared to the controls.

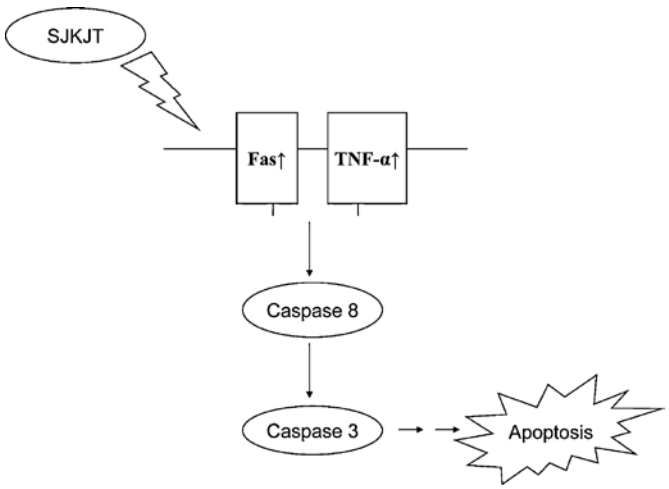

Figure 5. The proposed signalling pathways of SJKJT-induced apoptosis in human colon cancer colo 205 cell *in vitro* and *in vivo*.

and 23.16%, respectively) than those of the control group. In the SCID mice with colo 205 cell xenograft tumors, SJKJT inhibited tumor growth and up-regulated the protein expression of Fas (Fig. 4A), TNF-α (Fig. 4B), Caspase-8 (Fig. 4C) and Caspase-3 (Fig. 4D) as compared to the controls.

Discussion

The present study demonstrated that SJKJT inhibits the proliferation of human colon cancer colo 205 cells in a dose-dependent manner *in vitro* and the growth of colo 205 cell xenograft tumors *in vivo*. It is well documented that the active form of Caspase-8 relays the apoptotic signal from Fas, then cleaves and activates Caspase-3, leading to apoptosis (8). TNF receptor type-1 binds to TNF-α, forming a death-inducing signaling complex, and results in the recruitment of caspases. The induction of its autoproteolytic activation and the subse-

quent cleaving of effector Caspase-3 leading to cell apoptosis has been thoroughly reported (9). Our results show that SJKJT enhances the expression of Fas and TNF-α protein, consistent with an elevation in the active forms of Caspase-8 and -3 in colo 205 cells *in vitro* and *in vivo*. One of the molecular mechanisms responsible for the cell survival inhibition of colo 205 cells *in vitro* and the regression of colo 205 cell xenograft tumors by SJKJT may be related to the up-regulation of Fas, TNF-α, Caspase-8 and Caspase-3 protein expression, which may induce apoptosis (Fig. 5). These observations indicate that SJKJT-induced apoptosis in colon cancer cells may involve additional extrinsic cell death pathways. This is the first study to demonstrate that SJKJT up-regulates Fas, TNF-α, Caspase-8 and Caspase-3 protein expression in human colon cancer cells *in vitro* and *in vivo*.

Acknowledgements

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