Synergistic anticancer effects of curcumin and resveratrol in Hepa1-6 hepatocellular carcinoma cells

QIN DU[1], BING HU[1], HONG-MEI AN[2], KE-PING SHEN[1], LING XU[1], SHAN DENG[1] and MENG-MENG WEI[1]

[1]Department of Oncology and the Institute of Traditional Chinese Medicine in Oncology, and [2]Department of Science and Technology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, P.R. China

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Abstract. Hepatocellular carcinoma remains one of the most prevalent malignancies worldwide. *Curcuma aromatica* and *Polygonum cuspidatum* are one of the commonly used paired-herbs for liver cancer treatment. Curcumin and resveratrol are the major anticancer constituents of *Curcuma aromatica* and *Polygonum cuspidatum*, respectively. Curcumin and resveratrol have been found to exhibit a synergistic anticancer effect in colon cancer. However, the combined effect of curcumin and resveratrol against hepatocellular carcinoma remains unknown. In the present study, we evaluated the combined effects of curcumin and resveratrol in hepatocellular carcinoma Hepa1-6 cells. The results showed that curcumin and resveratrol significantly inhibited the proliferation of Hepa1-6 cells in a dose- and time-dependent manner. The combination treatment of curcumin and resveratrol elicited a synergistic antiproliferative effect in Hepa1-6 cells. The apoptosis of Hepa1-6 cells induced by the combination treatment with curcumin and resveratrol was accompanied by caspase-3, -8 and -9 activation, which was completely abrogated by a pan caspase inhibitor, Z-VAD-FMK. Combination of curcumin and resveratrol upregulated intracellular reactive oxygen species (ROS) levels in Hepa1-6 cells. The ROS scavenger, NAC, partially attenuated the apoptosis and caspase activation induced by the combination treatment of curcumin and resveratrol. In addition, the combination of curcumin and resveratrol downregulated XIAP and survivin expression. These data suggest that the combination treatment of curcumin and resveratrol is a promising novel anticancer strategy for liver cancer. The present study also provides new insights into the effective mechanism of paired-herbs in Traditional Chinese Medicine.

Correspondence to: Dr Bing Hu, Department of Oncology and the Institute of Traditional Chinese Medicine in Oncology, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, P.R. China
E-mail: beearhu@hotmail.com

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Introduction

Liver cancer is the fifth most prevalent malignancy in men and the seventh in women worldwide (1). The incidence of liver cancer is increasing possibly due to the epidemic of obesity and the rise in hepatitis C virus infection (1). Current treatment for liver cancer includes surgical resection, liver transplantation, local ablation, transarterial chemoembolization and radioembolization, and targeted molecular therapy (2,3). Despite improved therapeutic methods, the curative outcome of advanced liver cancer patients remains elusive. Liver cancer remains the second leading cause of cancer-related death in men and the sixth in women (1). The overall 5-year survival rate of liver cancer patients has remained below 12% in the United States (3). Thus, there is a great need to develop novel agents or alternative strategy to treat liver cancer.

In China, Traditional Chinese Medicine (TCM) has played a positive role in liver cancer treatment (4). Based on varied syndromes, different therapeutic methods and Chinese herbs can be employed to ameliorate clinical symptoms and local disease focus. Yujin (*Curcuma aromatica*) is a commonly used Chinese herb for treating liver cancer with blood-stasis and depressed liver-Qi with or without jaundice. *Curcuma aromatica* possesses anticancer potential against liver cancer and colon cancer (5,6). Huzhang (*Polygonum cuspidatum*) is a well-tolerated Chinese herb used for treating liver diseases with damp-heat and blood-stasis syndrome, and has been regarded as an anticancer herb. It is frequently used in liver cancer treatment. *Polygonum cuspidatum* was found to display anticancer effects in liver cancer, oral cancer and lung cancer cells (7-9). *Curcuma aromatica* and *Polygonum cuspidatum* are commonly used as a paired-herbal medication for liver cancer treatment.

Curcumin is an important ingredient of *Curcuma aromatica*, and has been used as a quality control standard for *Curcuma aromatica* (10). Curcumin inhibits proliferation and induces apoptosis in numerous types of cancer cells including liver cancer (11). One of the major components of *Polygonum cuspidatum* is resveratrol (12). In addition to chemo-preventive activity against tumorigenesis, resveratrol exhibits anticancer effects against various types of cancer cells such as lung carcinoma, pancreatic cancer and hepatocellular carcinoma cells and inhibits cancer cell invasion and metastasis (13-18). Both curcumin and resveratrol have displayed synergistic effects

with current cancer therapeutics (19,20). The combination of curcumin and resveratrol was found to demonstrate a synergistic anticancer effect in colon cancer (21). However, as constituents from paired-herbs, the combined effect of curcumin and resveratrol against hepatocarcinoma cells remains unknown. In the present study, we evaluated the anticancer effect of curcumin combined with resveratrol in hepatocarcinoma cells.

Materials and methods

Chemicals and reagents. Curcumin, resveratrol and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and 0.25% (w/v) Trypsin-EDTA were obtained from Gibco-BRL (Gaithersburg, MD, USA). The MTT cell proliferation and cytotoxicity assay kit, 2',7'-dichlorofluorescein diacetate (DCFH-DA), N-acetylcysteine (NAC), caspase-3, -8 and -9 colorimetric assay kits, and Hoechst 33258 were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Z-VAD-FMK was obtained from R&D Systems (Minneapolis, MN, USA). Propidium iodide (PI) and Annexin V-FITC were purchased from BD Pharmingen (Minneapolis, MN, USA). Antibodies against XIAP and survivin were purchased from Bioworld Technology Co., Ltd. (St. Louis Park, MN, USA). Antibodies against β-actin were from Cell Signaling Technology (Danvers, MA, USA).

Cell culture and treatment. Murine hepatocarcinoma cell line Hepa1-6 was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and was grown in DMEM with 10% FBS and 1% penicillin/streptomycin, and maintained at 37˚C in a humidified incubator with a 5% CO_2 atmosphere. Exponentially growing Hepa1-6 cells were seeded into desired plates and allowed to attach for 24 h before treatment. Cells were treated with various doses of curcumin and resveratrol dissolved in DMSO. The identical volume of DMSO was used as a control.

Cell proliferation assay. The effects of curcumin and resveratrol on cell proliferation were detected by MTT assay. Briefly, Hepa1-6 cells were seeded into 96-well plates ($3.5x10^3$ cells/well) and allowed to attach for 24 h before treatment. The cells were exposed to various doses of curcumin and/or resveratrol for 48 h, and cell viability was evaluated every 24 h by MTT assay according to the manufacturer's instructions. The cell survival rate was calculated as follows: Cell survival rate (%) = experimental OD value/control OD value x 100.

Observation of apoptotic morphology. Morphological changes characteristic of apoptosis were detected by Hoechst 33258 staining. Briefly, $6x10^4$ Hepa1-6 cells were seeded into 6-well plates and incubated for 24 h. Hepa1-6 cells were treated with curcumin and/or resveratrol for 48 h, and stained with Hoechst 33258 for 5 min at room temperature. The cells were observed and photographed using an inverted fluorescence microscope (AFM010-2, Nikon, Japan).

Quantification of apoptosis. Hepa1-6 cells were treated as indicated, collected, and stained with Annexin V-FITC and PI as recommended by the manufacturer. Apoptotic cells were detected by flow cytometry, and the extent of apoptosis was calculated with FlowJo software (version 7.6.1).

Detection of reactive oxygen species (ROS). Intracellular ROS production was detected by DCFH-DA staining. DCFH-DA is cleaved intracellularly by non-specific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound DCF (22). Briefly, Hepa1-6 cells ($1x10^5$) were placed in 60-mm culture dishes. On the second day, cells were exposed to the indicated concentrations of agents for a 48 h treatment, and stained with DCFH-DA (10 μM) at 37˚C for 20 min. The stained cells were collected and added to black 96-well plates at a density of $2x10^5$ cells/well. The presence of DCF fluorescence was quantified with a fluorescence microplate reader (Thermo Scientific Varioskan Flash; Thermo Fischer Scientific, Waltham, MA, USA) at wavelengths of 488 nm for excitation and 525 nm for emission. For ROS inhibition, cells were pretreated with NAC (2.5 mmol/l for 2 h), followed by curcumin and/or resveratrol treatment.

Caspase activity assay. After treatment with curcumin and/or resveratrol, caspase-3, -8 and -9 activities were measured by the cleavage of the specific chromogenic substrate according to the manufacturer's instructions. For inhibiton of caspase activity, Hepa1-6 cells were pretreated with Z-VAD-FMK (50 μmol/l for 2 h) and further treated with curcumin and/or resveratrol.

Western blot analysis. Western blot analysis was performed as previously described (23,24). Briefly, after drug treatment, Hepa1-6 cells were collected, lysed and subjected to 6-12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel, and transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). The transferred membranes were blocked with 5% non-fat milk, washed and probed with the indicated antibodies. The specific antigen-antibody complex was visualized using an enhanced chemiluminescence detection method (Amersham, Buckinghamshire, UK).

Statistical analysis. Data are expressed as the means ± standard deviation of at least two independent experiments, each conducted in triplicate. Statistical significances between the control and drug treatment were determined by one-way ANOVA. A value of $P<0.05$ was considered to indicate a statistically significant result.

Results

Effects of curcumin and resveratrol on Hepa1-6 cell proliferation. The antiproliferative effects of curcumin and resveratrol on hepatocarcinoma Hepa1-6 cells were assessed with the MTT assay. As shown in Fig. 1, curcumin (5-40 μM) and resveratrol (10-160 μM) significantly inhibited the proliferation of Hepa1-6 cells in a dose- and time-dependent manner ($P<0.05$).

Synergistic antiproliferative effect of curcumin and resveratrol on Hepa1-6 cells. To assess the combined effects of

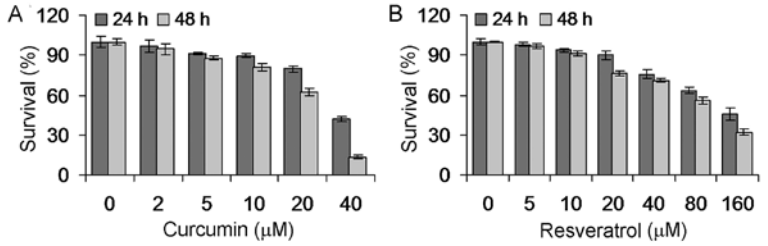

Figure 1. Effects of curcumin and resveratrol on Hepa1-6 cell proliferation. Hepatocarcinoma Hepa1-6 cells were treated with different concentrations of (A) curcumin or (B) resveratrol for 24 or 48 h, and cell viability was evaluated by MTT assay. Data shown are representative of three independent experiments.

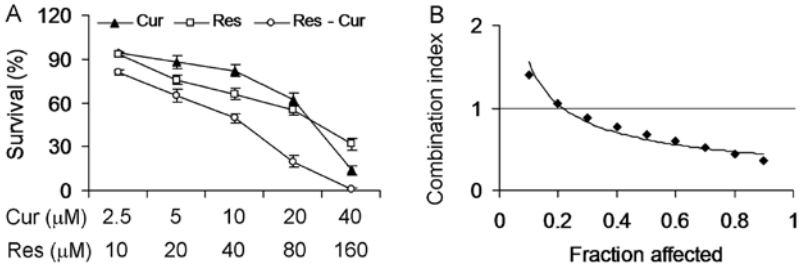

Figure 2. Combined effects of curcumin and resveratrol on Hepa1-6 cell proliferation. (A) Hepa1-6 cells were treated with curcumin (Cur) or resveratrol (Res) or both in a fixed ratio (1:4) for 48 h. Cell viability was measured by MTT assay. (B) CI was calculated by isobologram analysis using the Chou-Talalay method (25). CI =1, additive effect; CI <1, synergistic effect; CI, >1, antagonistic effect. Data represented are from three independent experiments.

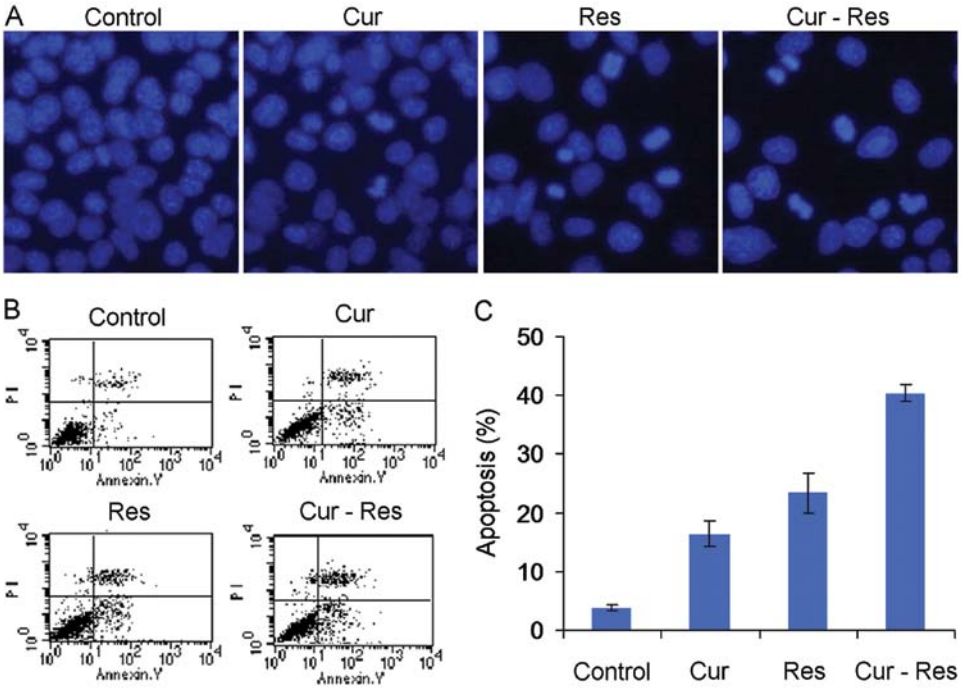

Figure 3. Combination of curcumin and resveratrol induces apoptosis in Hepa1-6 cells. Hepa1-6 cells were treated with curcumin (10 μM) (Cur) and/or resveratrol (40 μM) (Res) for 48 h. (A) Apoptotic cell morphology was assessed by Hoechst 33258 staining, and observed by fluorescence microscopy (magnification, x200). (B) Apoptosis was further confirmed by Annexin V/PI staining and by flow cytometric analysis. (C) Percentage of apoptosis expressed as means ± SD.

curcumin and resveratrol on Hep1-6 cells, we treated Hepa1-6 cells with curcumin, resveratrol or both agents in a constant ratio to one another. As shown in Fig. 2A, the combination treatment of curcumin and resveratrol was more effective in inhibiting the proliferation of Hepa1-6 cells when compared with the treatment of either agents alone (P<0.01), which indicated an interaction between the two drugs. The precise nature of this interaction was further analyzed by the median-effect method (25), where the combination indices (CI) of less than, equal to, and more than 1 indicate synergistic, additive and antagonistic effects, respectively. The CI value was <1 for the combination treatment when the proliferation inhibition was >20%, indicating a synergistic effect between curcumin and resveratrol on Hepa1-6 cell proliferation (Fig. 2B). Based on

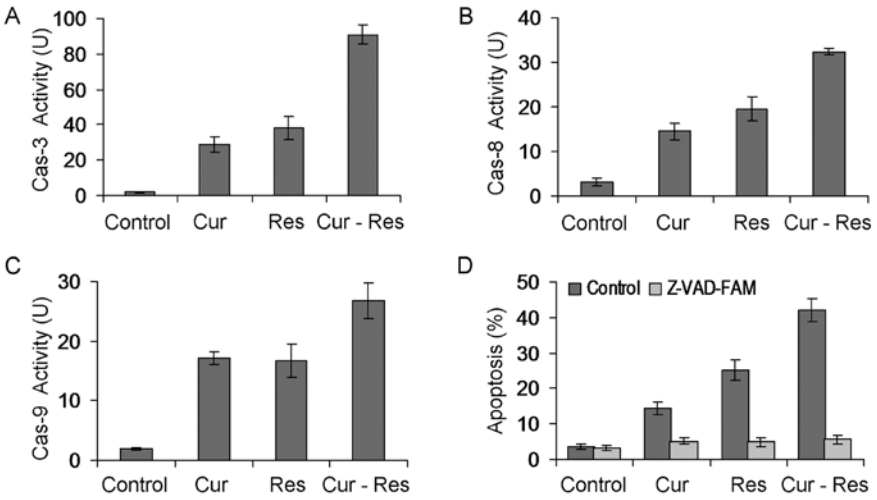

Figure 4. Combination of curcumin and resveratrol activates caspases in Hepa1-6 cells. Hepa1-6 cells were treated with curcumin (10 μM) (Cur) and/or resveratrol (40 μM) (Res) for 48 h. (A) Caspase-3, (B) caspase-8, (C) caspase-9 activity was detected as described in Materials and methods. For inhibition of caspase activity, Hepa1-6 cells were pretreated with Z-VAD-FMK (50 μmol/l) for 2 h, followed by curcumin (10 μM) (Cur) and/or resveratrol (40 μM) (Res) treatment for 48 h, and subjected to Annexin V-FITC/PI staining and flow cytometric analysis (D). Data illustrated are from three separate experiments.

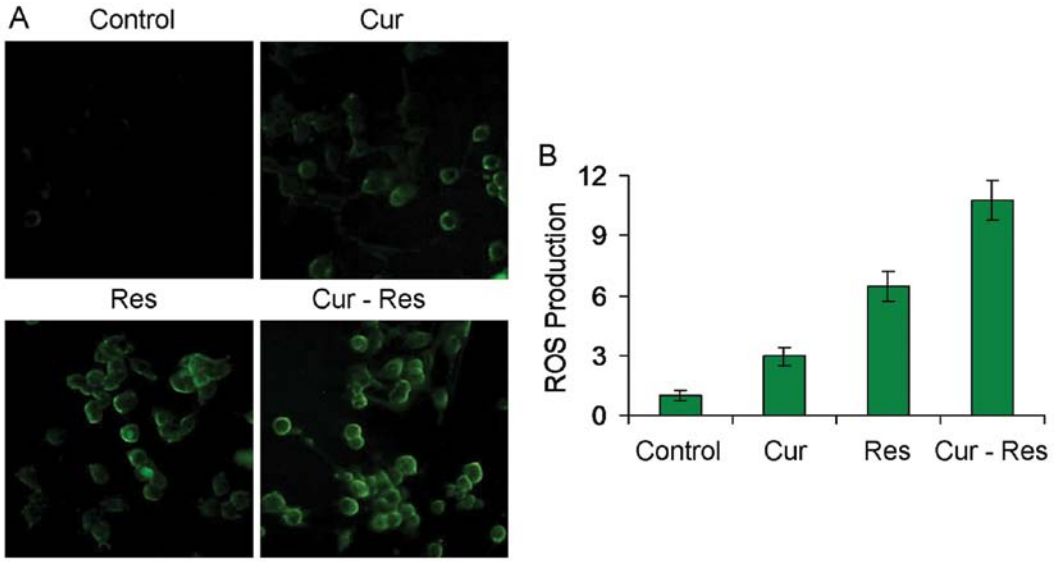

Figure 5. Combination of curcumin and resveratrol induces ROS generation in Hepa1-6 cells. Hepa1-6 cells were treated with curcumin (10 μM) (Cur) and/or resveratrol (40 μM) (Res) for 48 h. (A) The production of intracellular ROS was detected by DCFH-DA staining and observed by fluorescence microscopy (magnification, x40). (B) The intensity of DCF fluorescence was quantified using a fluorescence microplate reader at wavelengths of 488 nm for excitation and 525 nm for emission. ROS production was expressed as fold generation over the control.

these observations, we selected 10 μM of curcumin and 40 μM of resveratrol (~50% inhibitory effect for the combination treatment) to carry out the subsequent studies.

Combination of curcumin and resveratrol induces apoptosis in Hepa1-6 cells. To assess the effects of curcumin and resveratrol on apoptosis, Hepa1-6 cells were treated with curcumin and/or resveratrol and subjected to Hoechst 33258 staining to visualize the apoptotic morphological alterations. As shown in Fig. 3A, following treatment with curcumin and/or resveratrol for 48 h, a portion of Hepa1-6 cells exhibited nuclear shrinkage or fragmentation, indicating the occurrence of apoptotic processes. Flow cytometric analysis was used to quantify the extent of apoptosis. As shown in Fig. 3B and C, curcumin and resveratrol significantly induced apoptosis of the Hepa1-6 cells ($P<0.01$). Combination treatment of curcumin and resveratrol was found more effective in inducing apoptosis than either agent alone ($P<0.01$).

Combination of curcumin and resveratrol activates caspases in Hepa1-6 cells. Cell apoptosis is executed by a caspase (cysteine aspartate-specific proteinase) cascade, and activation of caspases has been recognized as a hallmark of apoptosis (26). We further observed the effects of curcumin and/or resveratrol on caspase activation in Hepa1-6 cells. As shown in Fig. 4A-C, curcumin and/or resveratrol treatment

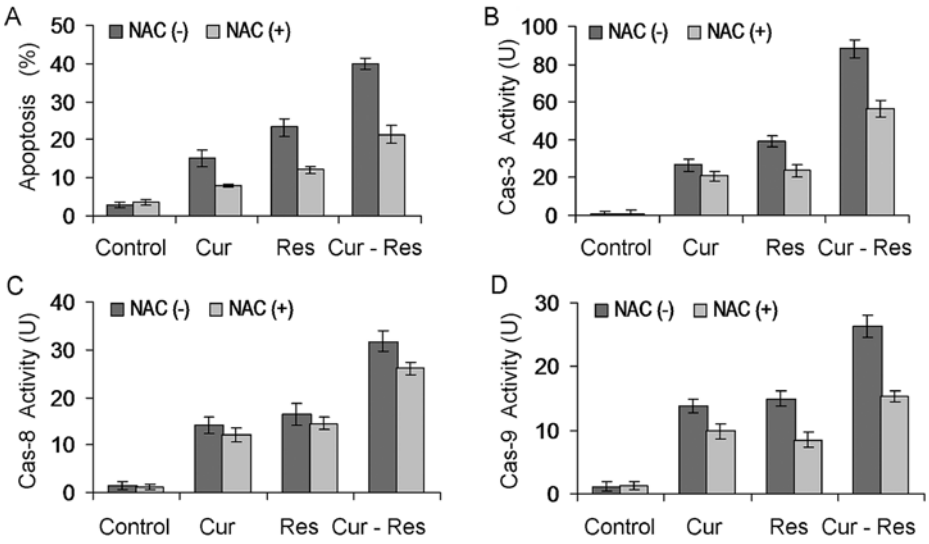

Figure 6. Role of ROS in curcumin and resveratrol-induced apoptosis. Hepa1-6 cells pretreated or untreated with NAC were further treated with curcumin (10 μM) (Cur) and/or resveratrol (40 μM) (Res) for 48 h, and subjected to assays for (A) apoptosis, (B) caspase-3, (C) caspase-8 and (D) caspase-9 activity. Data shown are representative of three independent experiments.

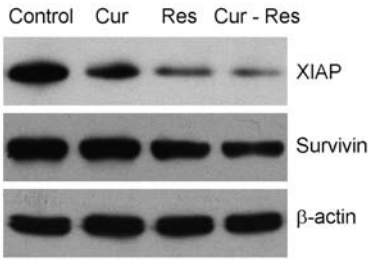

Figure 7. Combined effects of resveratrol and curcumin on expression of the inhibitors of apoptosis proteins. Hepa1-6 cells were treated with curcumin (10 μM) (Cur) and/or resveratrol (40 μM) (Res) for 48 h, and subjected to western blot analysis using antibodies against XIAP and survivin. β-actin was used as a loading control.

significantly activated caspase-3, -8 and -9 in Hepa1-6 cells (P<0.01). Combination treatment of curcumin and resveratrol significantly enhanced caspase-3, -8 and -9 activation when compared with that following treatment with either agent alone (P<0.01). In addition, Z-VAD-FMK, a pan caspase inhibitor, completely inhibited curcumin and resveratrol-induced apoptosis in Hepa1-6 cells (P<0.01) (Fig. 4D). These results suggest that caspase-3, -8 and -9 activation may be an important mechanism involved in the apoptosis of Hepa1-6 cells induced by the combination treatment of curcumin and resveratrol.

Combination of curcumin and resveratrol induces ROS generation in Hepa1-6 cells. It has been confirmed that excess ROS promotes cell death, and both curcumin and resveratrol upregulate ROS to induce apoptosis in cancer cells (27-29). Thus, we further examined the effects of the combination treatment of curcumin and resveratrol on ROS production in Hepa1-6 cells by DCFH-DA staining. As shown in Fig. 5, curcumin and/or resveratrol treatment resulted in significant ROS generation as indicated by brightly green fluorescence in the Hepa1-6 cells (P<0.01). Combination of curcumin and resveratrol significantly enhanced the ROS generation when compared to that following treatment with either agent alone (P<0.01).

Role of ROS in curcumin and resveratrol-induced apoptosis. To elucidate the effects of ROS on curcumin and resveratrol-induced apoptosis, we further evaluated cell apoptosis after NAC (ROS scavenger) pretreatment by flow cytometric analysis. When compared without the blocking of NAC, apoptosis induced by curcumin and/or resveratrol was partially but significantly abrogated by NAC pretreatment (P<0.05) (Fig. 6A). Particularly in the combination treatment group, the percentage of apoptosis was reduced to almost half of the apoptosis noted in the absence of NAC. In addition, curcumin and/or resveratrol-induced activation of caspase-3, -8 and -9 was also reduced by NAC pretreatment, particularly in the combination treatment group (P<0.05) (Fig. 6B-D). These observations suggest that the combination treatment of curcumin and resveratrol induced apoptosis, and caspase activation was associated with ROS production.

Combined effects of resveratrol and curcumin on expression of the inhibitors of apoptosis proteins. It has been reported that the inhibitors of apoptosis proteins, such as XIAP and survivin, are abnormally expressed in hepatocarcinoma cells and they have been confirmed as therapeutic targets for natural products (30-32). We further evaluated the effects of the combination treatment of curcumin and resveratrol on XIAP and survivin expression in Hepa1-6 cells. As shown in Fig. 7, combination treatment with curcumin and resveratrol significantly inhibited XIAP and survivin expression in the Hepa1-6 cells, when compared to the expression in the control or following treatment with either agent alone.

Discussion

In Chinese herbalism, every herb has its own characteristics, and TCM physicians believe that illness can be effectively

treated by combining herbs based on their various features. Combinations of multiple herbs guided by the theories of TCM, called Chinese herbal formula, are the major methods for the application of Chinese herbs. Due to the lack of appropriate ancient Chinese herbal formulas for cancer, most TCM physicians combine multiple herbs for a formula or prescription based on the illness and body condition of patients, TCM principles, progress in pharmacological research and personal experience. However, the combination of different anticancer herbs may elicit additive, synergisic or antagonistic effects (33). Thus, there is a great need to explore the combination effects of anticancer herbs, and establish effective herbal formulas for various situations in cancer treatment (33,34).

Paired-herbs, consisting of two fixed Chinese herbs that play a complementary role, are another simplified form of herbal combinations to strengthen treatment efficacy. Paired-herbs usually originate from clinical experience or an ancient Chinese herbal formula. It is rational to explore the combination rule of anticancer herbs based on paired-herbs. *Curcuma aromatica* and *Polygonum cuspidatum* have been frequently used as a paired-herbal medication for liver cancer treatment. Curcumin and resveratrol are the major anticancer constituents from *Curcuma aromatica* and *Polygonum cuspidatum*, respectively. Combination treatment of curcumin and resveratrol has been found to demonstrate a synergistic anticancer effect in colon cancer (21). Recent research found that resveratrol enhanced curcumin-induced apoptosis in osteosarcoma cells (35). In the present study, we observed that curcumin and resveratrol significantly inhibited Hepa1-6 cell proliferation. Combination treatment with curcumin and resveratrol significantly enhanced the antiproliferative effects when compared with either agent alone. The CI value was <1 for most of the concentrations tested, indicating a synergistic effect between curcumin and resveratrol in Hepa1-6 cells.

Apoptosis, first proposed by Kerr *et al* in 1972, is a normal physiological process that maintains cell homeostasis (36). Apoptosis plays a key role in the pathogenesis of diseases, such as degenerative diseases and cancer (37). Apoptosis has been recognized as a major anticancer therapeutic response (37,38). Manipulation of apoptosis has become one of the popular strategies of cancer treatment. For example, doxorubicin and cisplatin induce apoptosis in HepG2 hepatoma cells via different signaling pathways (39,40). Our data demonstrated that the combination of curcumin and resveratrol induced apoptotic cell death as indicated by nuclear morphological alterations and positive Annexin/PI staining. This suggests a similar mechanism as other anticancer therapy. Therefore, induction of apoptosis is an important mechanism in the elicited anticancer effects of the combination treatment of curcumin and resveratrol.

The death receptor pathway and mitochondrial pathway are the primary apoptotic pathways (37,38). Caspase-8 is the initiator in the death receptor pathway, activated by conformational change. It is reported that curcumin induces apoptosis via activation of the Fas/caspase-8 pathway (41). Caspase-9, an important initial factor in mitochondrial pathways, could be activated by the complex consisting of cytochrome c, procaspase-9 and apoptotic protease activating factor-1 (Apaf-1). Resveratrol markedly promotes caspase-9 activation, which is associated with provoking the release of CytC (42). Once activated, the two pathways converge to a common execution phase and stimulation of caspase-3 activation (43). In the present study, the activities of caspase-8, -9 and -3 were dramatically enhanced by the combination treatment of curcumin and resveratrol. In addition, the apoptosis of Hepa1-6 cells induced by the combination treatment of curcumin and resveratrol was completely abrogated by the pan caspase inhibitor, Z-VAD-FMK. These results suggest that both extrinsic and intrinsic apoptotic signaling pathways are involved in the apoptosis induced by the combination treatment of curcumin and resveratrol.

ROS are natural products and are generated by metabolism and xenobiotic exposure. High levels of ROS promote cell death via the mitochondrial pathway and/or death receptor pathway (27,44). Natural products, such as wogonin, emodin and cordycepin may induce ROS generation to mediate their proapoptosis effects (45-47). In the present study, the combination treatment of curcumin and resveratrol resulted in significant ROS generation in Hepa1-6 cells. Moreover, NAC, a scavenger of free radicals, significantly abrogated curcumin and resveratrol-induced apoptosis. Furthermore, the caspase activation induced by the combination of curcumin and resveratrol was also reduced by NAC pretreatment, suggesting that ROS contributed to the increased apoptosis caused by the combination treatment of curcumin and resveratrol.

XIAP and survivin proteins are members of the inhibitors of the apoptotic protein (IAP) family. Both inhibit caspase activation to protect cells from apoptosis. XIAP can inhibit caspase-3 by a linker region between the first two BIR domains, and suppress caspase-9 via its third BIR domains to protect cells from apoptosis (48). It has been reported that the activity of caspase-9 was significantly elevated in lung cancer A549 cells transfected with siRNA against survivin (49). Our data showed that the combination of curcumin and resveratrol downregulated the expression of XIAP and survivin, suggested that the downregulation of XIAP and survivin may contribute to the anticancer effects induced by the combination treatment of curcumin and resveratrol.

In conclusion, our results demonstrated that combination treatment of curcumin and resveratrol elicits a synergistic anticancer effect in hepatocarcinoma cells via extrinsic and intrinsic apoptosis, and is associated with ROS generation and downregulation of XIAP and survivin. The present study suggests that a combination of curcumin and resveratrol is a promising novel anticancer treatment strategy for liver cancer. The present study also provides new insights into the effective mechanism of paired-herbs in Traditional Chinese Medicine.

Acknowledgements

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