RESEARCH ARTICLE

Schedule-Dependent Effects of Kappa-Selenocarrageenan in Combination with Epirubicin on Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) has a relatively higher incidence in many countries of Asia. Globally, HCC has a high fatality rate and short survival. Epirubicin, a doxorubicin analogue, may be administered alone or in combination with other agents to treat primary liver cancer and metastatic diseases. However, the toxic effects of epirubicin to normal tissues and cells have been one of the major obstacles to successful cancer chemotherapy. Here, we investigated the effects of epirubicin in combination with kappa-selenocarrageenan on mice with H22 implanted tumors and HepG-2 cell proliferation, immune organ index, morphology, cell cycle and related protein expressions *in vivo* and *in vitro* with sequential drug exposure. The inhibitory rate of tumor growth *in vivo* was calculated. Drug sensitivity was measured by MTT assay, and the King's principle was used to evaluate the interaction of drug combination. Morphological changes were observed by fluorescent microscopy. Cell cycle changes were analyzed by flow cytometry. Expression of cyclin A, Cdc25A and Cdk2 were detected by Western blotting. *In vivo* results demonstrated that the inhibitory rate of EPI combined with KSC was higher than that of KSC or EPI alone, and the *Q* value indicated an additive effect. In addition, KSC could significantly raise the thymus and spleen indices of mice with H22 implanted tumors. In the drug sensitivity assay *in vitro*, exposure to KSC and EPI simultaneously was more effective than exposure sequentially in HepG-2 cells, while exposure to KSC prior to EPI was more effective than exposure to EPI prior to KSC. *Q* values showed an additive effect in the simultaneous group and antagonistic effects in the sequential groups. Morphological analysis showed similar results to the drug sensitivity assay. Cell cycle analysis revealed that exposure to KSC or EPI alone arrested the cells in S phase in HepG-2 cells, exposure to KSC and EPI simultaneously caused accumulation in the S phase, an effect caused by either KSC or EPI. Expression of cyclin A, Cdc25A and Cdk2 protein was down-regulated following exposure to KSC and EPI alone or in combination, exposure to KSC and EPI simultaneously resulting in the lowest values. Taken together, our findings suggest that KSC in combination with EPI might have potential as a new therapeutic regimen against HCC.

Keywords: Kappa-selenocarrageenan - epirubicin - combination therapy - HCC - cell cycle

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Introduction

Human hepatocellular carcinoma (HCC) is one of the most common malignancies all around the world, especially in Asia (Norsa'adah et al., 2013; Wiangnon et al., 2012). It occurs with great frequency and is becoming more common as a complication of chronic Hepatitis B or Hepatitis C (Zekri et al., 2012; Liang et al., 2013; Yeo et al., 2013). Until now, many therapeutic approaches have been applied clinically such as surgery, interventional or micro-traumatic techniques, physical or chemical methods (Mao et al., 2012). But the high incidence of tumor recurrences, possibly from micrometastasis of tumor cells prior to curative surgery, further reduces patients' 5-year survival (Li et al., 2013; Zhu et al., 2013). And this cancer seems to be stubborn as it is resistant to any chemical agents developed until now and chemical therapies used to treat cancer are highly toxic and often nonspecific (Kong et al., 2013).

Epirubicin (EPI), an anthracycline antitumour antibiotic which is structurally related to doxorubicin, is among the most active single agents used in the management of patients with breast cancer and other human tumour cells. Epirubicin is most active in S and G_2 phases of the cell cycle, although the drug exhibits activity in all phases of the cell cycle (Coukell et al., 1997). Epirubicin may be administered alone or in combination with other agents such as vinorelbine, fluorouracil, cyclophosphamide, docetaxel to patients with early breast cancer and to those with metastatic disease and shows more effective with less toxicity (Findlay et al., 1998; Yan et al., 2010; Roche et al., 2012; Cao et al., 2013; Chen et al., 2013). Comparative and noncomparative clinical trials have demonstrated that regimens containing conventional

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doses of epirubicin achieved equivalent even higher objective response rates and overall median survival as similar doxorubicin-containing regimens in the treatment of advanced and early breast cancer, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), non-Hodgkin's lymphoma, ovarian cancer, gastric cancer and nonresectable primary hepatocellular carcinoma (Nair et al., 1998; Khasraw et al., 2012). Recently, dose-intensive regimens of epirubicin have achieved high response rates in a number of malignancies including early and advanced breast cancer and lung cancer (Unek et al., 2012; Vici et al., 2012). However, improved overall survival has not yet been demonstrated. The major adverse effects of epirubicin are acute dose-limiting haematological toxicity and cumulative dose-related cardiac toxicity. In vitro and clinical studies have shown that epirubicin is less myelotoxic and cardiotoxic than equimolar doses of doxorubicin. Other major adverse effects of epirubicin administration include mucositis, nausea and vomiting, reversible alopecia and local cutaneous and vesicant reactions (Launchbury et al., 1993). Reducing drug side-effects would be a most important approach to improve the success of anticancer chemotherapy.

Selenium is an essential trace element, the deficiency of which is associated with an increased incidence of some human diseases (Alatise et al., 2013; Kryczyk et al., 2013; Lener et al., 2013). Dietary supplementation with selenium has been reported to produce a decrease in the incidence of some human cancers (Wrobel et al., 2013; He et al., 2013). Kappa-selenocarrageenan (KSC) is a new-type Se-containing polysaccharide, which consists of selenium and kappa-carrageenan (Lin et al., 1993). KSC has been demonstrated effective in inhibiting proliferation of hepatocarcinoma, osteosarcoma, breast cancer and multidrug-resistant K562 /ADM cells (Wei et al., 2006; Ling et al., 2009). The possible molecular mechanisms of anti-tumor were through antioxidation, induction of tumor cell apoptosis, blockade of cell cycle, and enhancement of immunity (Zhang et al., 2005). In addition, the combined treatment of KSC with other chemotherapeutic drug can better improve anti-tumor efficacy, enhance immune functions and decrease its toxic effects (Hu et al., 1997; Li et al., 2000; Ling, 2012).

In this study, we investigated the anti-tumor effects of KSC in combination with epirubicin on hepatoma both in vivo and in vitro and the possible mechanisms, and used different sequences to determine the role of cell cycle on cytotoxicity of the combinations, which may help design appropriate treatment schedules for clinical application.

Materials and Methods

Drugs and reagents

Kappa-selenocarrageena (organic selenium containing 1.68% selenium and polysaccharide, solid powder, made by TianCiFu Biological Engineering Co., Ltd); Epirubicin was obtained from Sigma Chemical Co., Ltd. RPMI1640 medium was bought from GIBCO BRL, Life Technologies Inc. (New York, USA). Fetal bovine serum (FBS) was obtained from Hangzhou SiJiQing Life Technologies. 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetra-zolium bromide (MTT), Hoechst 33258, propidium iodide (PI), Triton X-100, and ribonuclease-A were purchased from Sigma (St. Louis, MO, USA). Rabbit anti-human Cyclin A, Cdk2 and Cdc25A polyclonal antibody were purchased from Santa Cruz Biotechnology Inc.

Animals and cell line

Mice with hepatoma H22 were obtained from Chinese Academy of Medical Sciences and Kunming mice (famale and male weighing, 18 g-22 g) from the Experimental Research Center of Changchun high and new Medical Animal. Human hepatoma HepG-2 cells were obtained from the Institute for Cancer Research of Heilongjiang Cancer Hospital and maintained in our laboratory. HepG-2 cells were cultured in RPMI1640 medium with 10% fetal bovine serum and 100U/mL penicillin-streptomycin at 37°C in an atmosphere containing 5% CO_2.

Measurement of anticancer activity in vivo

The mice with hepatocarcinoma H22 were killed and their ascites extracted, adjusted to $1\times10^7\cdot mL^{-1}$ and implanted by subcutaneous injection 200 μL to each mouse. Eighty mice with implanted H22 tumor were randomly divided into control group (saline), and groups of EPI alone (6 $mg\cdot kg^{-1}\cdot d^{-1}$), KSC alone (20 $mg\cdot kg^{-1}\cdot d^{-1}$, 40 $mg\cdot kg^{-1}\cdot d^{-1}$, 80 $mg\cdot kg^{-1}\cdot d^{-1}$), EPI combined with KSC (EPI 6 $mg\cdot kg^{-1}\cdot d^{-1}$+KSC 20, 40, 80 $mg\cdot kg^{-1}\cdot d^{-1}$). EPI and combined groups were injected intravenously and KSC groups intragastric administration 24 h after transplatation once a day for 10 days continuously. The mice were killed on the 11th day after the treatment and the tumor, thymus and spleen isolated and weighed. The inhibitory rate of tumor was calculated as follows: Inhibitory rate of tumor (%)= (1-mean tumor weight in experiments/mean tumor weight in controls)×100%, thymus index=thymus weight/mice weight, spleen index=spleen weight/mice weight.

In vitro drug sensitivity assay

HepG-2 cells in exponential growth were washed with phosphate buffered saline (PBS), trypsinized with 0.25% trypsin for 5 min at 37°C, counted and seeded in 96-well plates at a density of 4×10^3 cells/well in 100 μL RPMI1640 containing 10% FBS. After 24h, various concentrations of KSC and EPI were added to the medium for 24h. After drug exposure, the drug-containing medium was aspirated from the plate, fresh medium was added and the cultures incubated for 24 h. Control wells without anticancer drugs were treated identically. After discarding the media, 100 μL tetrazolium (MTT, 0.5 mg/mL) was pipetted into each well and incubated for 4 h at 37°C. Sucking out the media, added 150 μL dimethyl sulfoxide to dissolve the violet-crystal and measured the absorption at 570 nm. Five wells were used for each drug concentration. Experiments were triplicated. The inhibitory rate was calculated as follows: IR (%)= (1-mean absorption in experiments/mean absorption in controls)×100%. The drug concentrations inhibiting cell growth by 50% (IC_{50}) were obtained by graphical analysis.

Five different protocols were used to investigate the interaction of KSC and EPI: Protocol 1: exposure to KSC for 48 h; Protocol 2: exposure to EPI for 48 h; Protocol 3:

exposure to KSC and EPI simultaneously for 48 h (SIM); Protocol 4: exposure to KSC for 24 h prior to incubation in drug-free medium for 24h and finally exposure to EPI for 24 h (K+E); Protocol 5: exposure to EPI for 24 h prior to incubation in drug-free medium for 24h and finally exposure to KSC for 24 h (E+K)

At the end of treatment, cells were washed with PBS and incubated at 37°C in an atmosphere containing 5% CO_2 for 48 h. Cell inhibition ratios were determined as described above.

Evaluation of drug interaction

The interaction between KSC and EPI was evaluated by Q value, which was calculated as follows: Q=E (AB)/[EA+ (1-EA)×EB]. E (AB) is the inhibiting tumor rate of the combination and EA or EB is that of a drug alone. When Q value was equal to 0.85-1.15, or less than 0.85 or more than 1.15, additive or antagonistic or synergistic interaction was thought to occur (Wang et al., 2001).

Hoechst 33258 staining for morphological analysis

HepG-2 cells at 2×10^5 cells/well were cultured in 6-well plates containing cover slips overnight. After incubation with KSC and EPI alone or in combination for 48 h, the cover slips were washed twice with PBS, fixed in 0.5 mL solution (the ratio of methanol to iced acetic acid was 3 to 1) at 4°C for 30 min, rinsed twice with PBS, and stained with Hoechst 33258 (5 μg/mL) at room temperature for 30 min, rinsed twice with PBS, and wet mounted using 20 μL of the cell suspension. The cover slips were observed under a Leica DMI3000B fluorescence microscope.

Cell cycle analysis by flow cytometry

HepG-2 cells were cultured at 2×10^5 cells per 25 mm dish. The same protocols as described for in vitro cytotoxicity assay were used. After incubation, the cells were harvested by trypsinization, washed twice with PBS, and fixed in 70% ethanol at 4°C for 1h. The cells were stained with propidium iodide (PI) solution (50 mg/L of PI, 100 mg/L RNase, and 0.1% Triton X-100 in PBS) at 4°C for 30 minutes and analyzed for cell cycle distribution by flow cytometry (EPICS XL, BECKMAN COULTER). Data from 10, 000 cells per sample were collected and analyzed with CellQuestTM program.

Western blotting analysis

HepG-2 cells were cultured at 1×10^6 cells per 90 mm dish. The cells were treated using the protocols described above. Cells were collected, washed twice with PBS, lysed in lysis buffer (10 mmol/L Tris, pH 7.4, 5 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100 and 0.1 mmol/L PMSF) and then centrifuged at 12, 000 g for 20 min at 4°C and the amount of protein was determined using a DC protein assay (Bio-Rad). The lysates were boiled for 5 min, separated by 15% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation) in transblotting buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). The membranes were incubated for 2 h at room temperature with a blocking buffer (5% non-fat milk, 0.1% Tween 20 in TBS), and

Table 1. Effect of KSC and/or EPI on H22 Implanted Tumor in Mice (n=10)

Group	Dose (mg/kg·d) EPI	Dose (mg/kg·d) KSC	Tumor weight (g)	Inhibition rate (%)	*Q* value
Control	-	-	1.481±0.0775	-	-
KSC1	-	20	1.2077±0.1745**	18.45%	
KSC2	-	40	0.8106±0.0576**	45.46%	
KSC3	-	80	0.7007±0.0051**	52.68%	
EPI	6	-	0.4432±0.0675**	70.06%	
EPI+KSC1	6	20	0.3059±0.0307**#	79.34%	1.04
EPI+KSC2	6	40	0.1584±0.0265**##	89.51%	1.07
EPI+KSC3	6	80	0.1228±0.0149**##	91.71%	1.07

**$p<0.01$ *vs* control, ##$p<0.01$ *vs* EPI alone

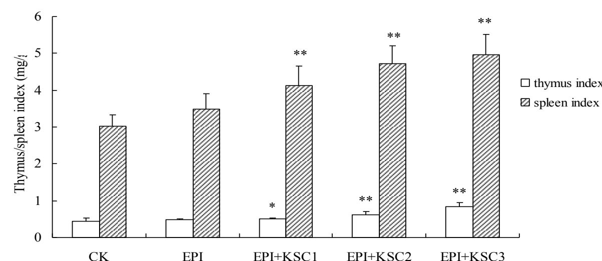

Figure 1. Effect of EPI Combined with KSC on Thymus Index and Spleen Index of Mice with H22 Implanted Tumor. **$p<0.01$ compared with control or EPI alone

then with polyclonal antibodies to rabbit anti-Cyclin A, anti-Cdk2 and anti-Cdc25A (Santa Cruz Biotechnology) diluted 1:1000 in the same buffer for 16 h, washed three times with TBS-T, and incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG (Amersham Biosciences, Piscataway, N.J.; diluted 1:1000 in TBS-T) for 2 h. After the membranes had been washed three times with TBS-T, the signal was visualized by enhanced chemiluminescence with an ECL system (Amersham Biosciences).

Statistical analysis

Data were expressed as mean±standard deviation (SD) of three independent experiments and analyzed using two-way analysis of variance (ANOVA) of SPSS software version 16.0. The differences between two groups were determined using the t-test, and statistical significance was set at $P<0.05$.

Results

The effect of KSC and EPI on H22 implanted tumor

The inhibiting tumor rate of EPI in combination with KSC was higher than that of KSC or EPI alone and Q value was less than 1.15 but more than 0.85, which indicated an additive effect (Table 1). In addition, KSC can raise significantly thymus index and spleen index of mice with H22 implanted tumor as compared with EPI alone ($P<0.01$) (Figure 1).

Cytotoxicity of KSC and EPI on HepG-2 cells

MTT assays were performed to evaluate the potential cytotoxic effects of combining KSC and EPI. As shown in Figure 2, either KSC or EPI alone inhibited HepG-2 activity in a dose-dependent manner, but the effect was greater when the two agents were combined

Table 2. Q value of KSC Combined with EPI on HepG-2 Cells in Different Administration Ways

Groups	EPI (nmol/L)						
	0.5	1	2	4	8	16	32
SIM	0.802	0.862	0.901	0.853	0.868	0.947	0.963
K+E	0.5602	0.653	0.708	0.764	0.811	0.826	0.883
E+K	0.53	0.567	0.595	0.655	0.777	0.811	0.843

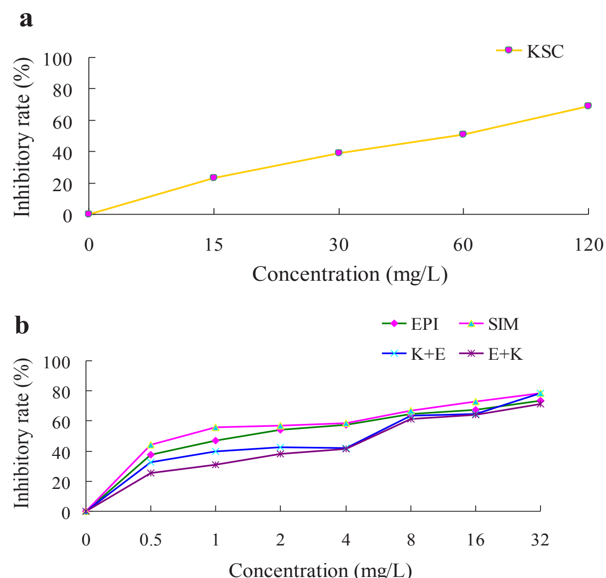

Figure 2. Dose Response Curves for (a) KSC and (b) KSC in Combination with EPI in HepG-2 Cells for 48 h. Drug exposure protocols 1-5 as described in Materials and methods. The points represent the means±SD of three determinations. Isobolograms at IC_{50} were generated based upon these dose-response curves for the combinations

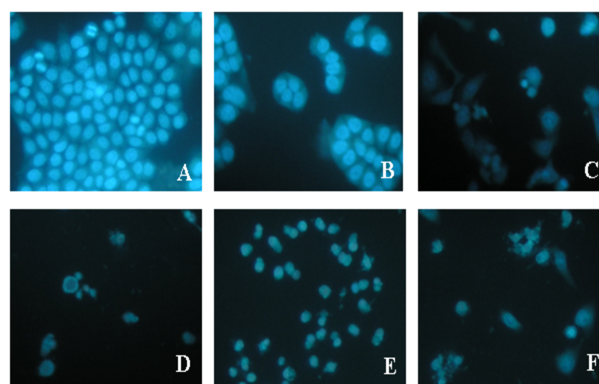

Figure 3. The Morphological Changes of HepG-2 cells. A) Control; **B-F)** Drug exposure protocols 1-5 as described in Materials and methods

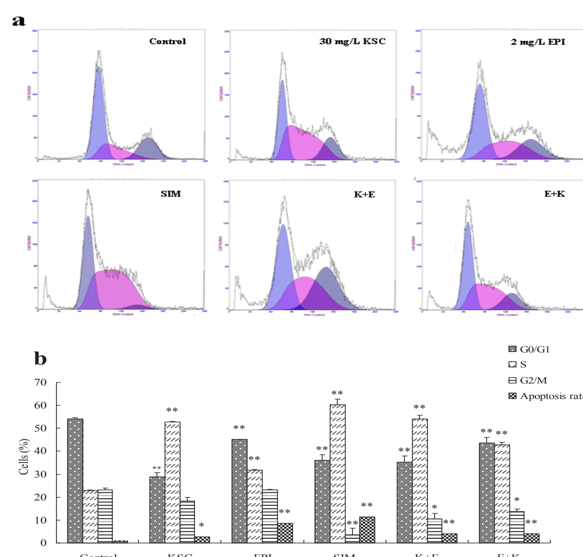

Figure 4. Effects of KSC and EPI on Cell Cycle and Apoptosis Rates of HepG-2 Cells. a) Distribution charts of cell cycle by flow cytometry. **b)** The statistical analysis of FCM results. Data represented as means±SD of three independent experiments. *p<0.05, **p<0.01, significantly different from the control group

simultaneously (SIM). Sequential exposure to KSC and EPI, all the combined data points fell below the SIM group. Protocol 4 (K+E) was a little better than protocol 5 (E+K). The IC_{50} values of KSC or EPI alone at 48h were 53.295 mg/L and 3.612 mg/L. Simultaneous exposure to two agents, that of EPI decreased to 0.807 mg/L, while those of protocol 4 and 5 were 2.776 mg/L and 4.618 mg/L, respectively.

To determine whether the combination of KSC and EPI in HepG-2 cells results in synergistic cytotoxic effects, Q values were performed. In this analysis, there is synergy when q value is over 1.15, additivity when q is 0.85~1.15, and antagonism when the q is less than 0.85. The studies demonstrated that cytotoxic interaction between KSC and EPI in HepG-2 cells was schedule-dependent. Additive effects (0.85<q<1.15) were observed for simultaneous exposure to KSC and EPI and for sequential exposure to KSC first, followed by high concentration EPI (32 mg/L), while antagonistic effects (q<0.85) were observed for sequential exposure to EPI first, followed by KSC, and for sequential exposure to KSC first, followed by low concentration EPI (0.5~16 mg/L) (Table 2).

Morphological changes

Morphological evidence of apoptosis was demonstrated by Hoechst-33258 fluorescence staining. Cells treated with KSC and EPI showed typically apoptotic changes, such as chromatin condensation, membrane blebbing, deformed and fragmented nuclei and apoptosis bodies (Figure 3). In agreement with the MTT results, EPI alone appear to cause a significant increase in the apoptotic process and decrease in cell number. Addition of KSC significantly increased the number of apoptotic cells.

Effect of KSC and EPI on cell cycle

DNA flow cytometry studies were performed to determine the effect of different KSC-EPI combinations on the cell cycle distribution. In general, HepG-2 cells treated with KSC or EPI alone or in combination resulted in a significant decrease in G_1 and G_2/M and an increase in S phase as compared to control, which indicated S phase arrest. Protocol 3 with exposure to KSC and EPI simultaneously arrested cells in S phase more significantly, while cells in G_2/M phase decreased sharply. For the study of sequential applications, exposure to KSC for 24 h prior to EPI (protocol 4) resulted in an increase in S and G_2/M and a decrease in G_1 phase as compared to control. For the reversed sequences, exposure to EPI prior to KSC

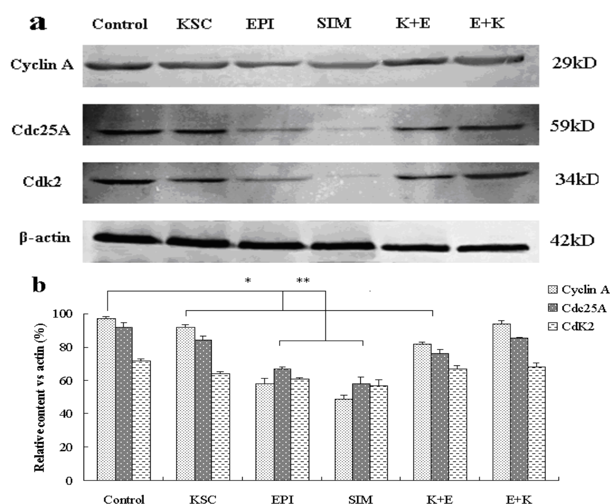

Figure 5. Effect of KSC and EPI on Expressions of Cyclin A, Cdc25A and Cdk2 in HepG-2 Cells (a) and Relative Content *vs* β-actin (b)

(protocol 5) caused cell cycle changes similar to protocol 1 and 2, blocking the cells in S phase after a 48 h drug exposure (Figure 4).

Detection of Cyclin A, Cdk2 and Cdc25A protein

To analyze the underlying biochemical mechanisms involved in the regulation of the S phase arrest, protein expressions of cyclin A, cdc25A and Cdk2 were determined by Western blotting. As shown in Figure 5, treatment of HepG-2 cells for 48h with KSC and EPI resulted in a prominent decrease in the expressions of Cyclin A, Cdc25A and Cdk2 as compared with the untreated control. The expression level of the three proteins was lower in cells exposed to KSC and EPI simultaneously than in those exposed sequentially. These observations suggest that down-regulation of Cyclin A, Cdc25A and Cdk2 proteins in both drugs treated HepG-2 cells is closely related to the induction of S arrest of cell cycle progression.

Discussion

Currently, a variety of cytotoxic and antiproliferative agents have been tested in Hepatocellular carcinoma (HCC) treatment, which are used alone, or in combination with other drugs or other treatment modalities (Zhu, 2006; Boulin et al., 2011; Berk et al., 2013). However, high doses of these drugs lead to severe toxicities, which have a negative effect on patients' survival (Berk et al., 2013). The use of less toxic doses in combination with other anti-proliferative agents would be desirable (Lee et al., 2004; Abou-Alfa et al., 2010). Genistein could reinforce the inhibitory effect of cisplatin on HCC cell proliferation and tumour recurrence and metastasis after curative hepatectomy in nude mice, possibly through mitigation of cisplatin-induced MMP-2 upregulation (Chen et al., 2013).

Modern pharmacological and clinical investigations demonstrated that KSC had anti-tumor and immunoregulatory activities (Zhang et al., 2005). Its anti-tumor and immunoregulatory properties, along with low toxicity, raise the possibility that it could be effective in the cancer patients receiving conventional chemotherapy and/or radiation treatment, to build up immune resistance and decrease toxicity. Previous studies demonstrated that selenium could reduce the nephrotoxicity and bone marrow suppression induced by cisplatin in cancer patients, and no toxicity of seleno-kappacarrageenan was noted (Hu et al., 1997). Synergistic efficacy of selenium in combination with adriamycin and taxol showed that selenium was a very effective growth inhibitor of several breast and other cancer cells, and inhibition was selenium dose-dependent and induced selective apoptotic changes in these cell types (Vadgama et al., 2000).

To further investigate the best therapeutic way of KSC and raise the effect on hepatocarcinoma, we studied KSC and EPI in combination. The experiments in vitro showed that KSC in combination with EPI can increase the effect on HCC HepG-2 cells and the increase extent varies at different concentrations and in different sequential administration, which was greater at lower concentrations. The possible reason is that the anti-tumor activity of an individual drug is saturated at high concentrations and difficult to increase after combination or there was antagonistic action to some extent between two drugs in combination and counteracted part of anti-tumor activity of a drug. Q values showed that in vitro the nature of interaction is addictive between KSC and EPI in the simultaneous group and antagonistic in the sequential groups. On the basis of the experiments in vivo, low dose EPI combined with KSC was applied to treat H22 tumor implanted in mice, and inhibitory rate of tumor evidently increased as compared with that of a drug alone. Q value showed that in vivo markedly addictive interaction between KSC and EPI were thought to occur, which agreed with the results in vitro. Meanwhile, thymus index and spleen index of mice with H22 implanted tumor were raised significantly as compared with EPI alone. These results suggested that low-dose EPI and KSC in combination could increase evidently anti-hepatocarcinoma effect. Epirubicin is the main anti-HCC agent, but its toxicities in kidney, liver or heart restrict its clinical application, as a result patients cannot tolerate the high dose agents whereas low dose is difficult to achieve satisfactory results (Li et al., 2012). Considering selectively inhibitory effect of KSC on HCC in vitro and unobvious toxicity in vivo, the effect may be improved evidently without increased toxicities or keep satisfactory in poorly-tolerated patients with low dose of EPI when KSC and EPI in combination are applied to treat hepatocarcinoma.

KSC induces cell cycle arrest at the S phase (Wu et al., 2004). Our flow cytometry results indicated that exposure to KSC or EPI only increased the proportion of cells in the S phase. In HepG-2 cells exposed to KSC and EPI simultaneously were remarkably arrested at the S phase. Exposure to KSC prior to EPI or exposure to EPI prior to KSC caused similar cell cycle perturbations, blocking the cells in the S phase. These findings indicate that accumulation at the S phase might be an effect of KSC or EPI. We conjecture that different cytotoxicities in the same cell line following different drug exposure sequences were caused by different mechanisms. Besides,

KSC and/or EPI could cause significant decrease in the expression of Cyclin A, Cdc25A and Cdk2 in HepG-2 cells. The possible mechanism of S phase arrest may be that the treatments activate the S phase checkpoint, and Chk2 activity is activated with a catalytic activity of Chk2 by phosphorylation of Cdc25A to promote its ubiquitination degradation and thus Cdk2 activation is suppressed, which will block the formation of Cdk2 and CyclinA complex that lead to S phase arrest and hinder the process of cell-cycle (Weinert, 1998).

In summary, in vitro studies further extend observations on selenium's antitumor actions per se and as a potential synergistic agent with anticancer drugs. The present results can provide new hope for chemotherapy of hepatoma cancer. Furthermore, an improved understanding of the interactions between phytochemicals with the genes that are critical to the regulation of cancer cell growths will provide strong armaments to cancer therapy.

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