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Inhibition of cell growth and induction of G1-phase cell cycle arrest in hepatoma cells by steroid extract from *Meretrix meretrix*

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Abstract

In this study, we report that the steroid extract 5α, 8α-epidioxycholest-6-ene-3β-ol (MME) from *Meretrix meretrix* has the ability to inhibit growth of hepatoma cells and to induce G1-phase cell cycle arrest in two human hepatoma cell lines, HepG2 and Hep3B. HepG2 cells were more sensitive than Hep3B to MME. The extract markedly up-regulated the expression of p53 and p21WAF1/CIP1 in HepG2, suggesting that MME-induced G1 phase cell cycle arrest in HepG2 might be p53-dependent. Therefore, the up-regulation of p27KIP1and p16INK4A in both cell lines indicates that a p53-independent pathway might be involved in the mechanism of MME inducing cell cycle arrest. In conclusion, MME induces G1 phase cell cycle arrest via both p53-dependent and p53-independent pathways.

Keywords: Steroids; *Meretrix meretrix*; Hepatoma; $p21^{Waf1/Cip1}$; p53; p16INK4A

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. The high incidence of the liver cancer has been attributed to factors such as persistent infection with hepatitis virus and contact with hepatocarcinogens like nitrosamines and aflatoxins. Because of the mutlifocal nature of liver carcinoma, most cancer patients are considered non-resectable at the presentation of case. In these patients, chemotherapy is the only choice of treatment. Unfortunately, development of drug resistance in tumor after treatment is always a major obstacle to the successful management of liver cancer. Thus, developing new therapeutic agents that can overcome drug resistance becomes an urgent need for cancer patients.

Antitumor activity of many marine animal tissues has widely reported, and at least two antineoplastic

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agents currently in clinical use are derivatives of marine sponge metabolites [1,2]. *Meretrix meretrix* belongs to *Veneridae of Bivalvia* [3]. Many extracts from clams showed several bioactivities, such as immune bioactivity, pharmacological properties and virus–cell fusion inhibitory activity [4–6].

Some steroid compounds can inhibit the growth of cancer cells, induce apoptosis or cell cycle arrest by p53-dependent or p53-independent pathways [7,8]. In this study, we have demonstrated the antitumor activity of a steroid extract 5α,8α-epidioxycholest-6-ene-3β-ol (MME) from *M. meretrix*. To investigate the effects of MME on the cell cycle regulars and apoptosis, we analyzed cell cycle changes, apoptosis and cell cycle-related proteins including p53, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p16^{INK4A}$ and pro-apoptotic regulators, Bax and Fas in two hepatoma cell lines HepG2 (with wild-type p53) and Hep3B (with p53 deleted) treated with different concentrations of MME for different time intervals.

2. Materials and methods

2.1. Materials

Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), penicillin G, streptomycin and amphotericin B were obtained from GIBCO BRL (Gaithersburg, MD, USA). Steroid extract MME from *M. meretrix* was prepared by our laboratory [9]. Dimethyl sulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were purchased from Sigma Chemical Co. (St Louis, MO, USA). MTT was purchased from Sigma. PVDF membrane was purchased from Millipore (Millipore, Bedford, MA, USA). An enhanced chemiluminescence detection kit was purchased from Amersham (Amersham, Aylesbury, UK). β-actin, p53, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p16^{INK4A}$, and Bax and Fas antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

Human hepatoma cell lines (HepG2 and Hep3B) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured with DMEM supplemented with 10% (v/v) FCS, 100 μg/ml streptomycin and 100 unit/ml penicillin in 75 cm^2 tissue culture flasks in a humidified incubator at 37 °C with 5% CO_2.

2.3. Cytotoxicity assay

Cells were seeded in 96-well microplates (1×10^4 cells/well in 180 μl medium) and routinely cultured in a humidified incubator (37 °C in 5% CO_2) for 24 h. MME extracts were added in serial concentrations and re-incubated for 48 h. Then 20 μl of tetrazolium dye (MTT) solution (5 mg/ml in PBS) was added to every well and the solution was incubated for an additional 4 h. The medium was discarded, 10 μl of DMSO was added to dissolve the formazan crystals formed. The plate was then read on a microplate reader at 540 nm. MTT solution with DMSO (without cells and medium) acted as a blank control in microplate reading [10,11].

2.4. Flow cytometry

Flow cytometry was performed on the MME treated cells to observe the effects of MME on the cell cycle. Thus, the MME treated cells were cultured and harvested at the indicated times, and fixed in 1 ml of 70% ethanol (1×10^6 cell/sample) for 30 min at 4 °C. The cells in each of these ethanol solutions were washed twice with PBS and incubated in the dark in 1 ml of PBS containing 100 μg propidium iodide and 100 μg Rnase A for 30 min at 37 °C. Flow cytometric analysis was performed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The effect on cell cycle was determined by changes in the percentage of cell distribution at each phase of the cell cycle and assessed by histograms generated by the computer program Cell Quest and Mod-Fit.

2.5. Western blot analysis

Cells were harvested and lysed in ice-cold lysis buffer containing 10 mM TRIS–HCl (pH 7.5), 0.1% NP-40, 0.5 sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin. After 30 min of incubation on ice, cells were homogenized with a dance homogenizer, then centrifuged at 100,000×*g*

for 30 min at 4 °C; supernatants were stored at −80 °C before use. Protein concentrations were determined by the Bradford method. Equal protein amounts were loaded onto SDS–polyacrylamide gels and the proteins were electrophoretically transferred to a PVDF membrane. Immunoblots were analyzed using specific primary antibodies (antibodies to p53, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p16^{INK4A}$, Bax, Fas and β-actin that were diluted 500-, 500-, 300-, 300-, 300-, 500- and 2000-fold, respectively), then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution), and proteins were visualized using an enhanced chemiluminescence detection kit. β-actin was used as internal conrol to confirm that the amounts of protein were equal.

2.6. Semi-quantitative PCR of $p21^{WAF1/CIP1}$ and Fas

Total RNA was isolated from the cultured cells using Trizol reagent following the recommendation of the manufacturer (Gibco BRL, NY, USA). One microgram of total RNA was used for RT-PCR analysis. PCR was performed using the following couples of primers: 5′ -CGGAGGATTGCTCAACAAC-3′ and 5′-TTGGTATTCTGGGTCCG-3′ for amplification of Fas; 5′-AAGACCATGTGGACCTGTCA-3′ and 5′-AGGAAGTAGCTGGCATGAAG-3′ for amplification of $p21^{WAF1/CIP1}$ [12,13], 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′ for amplification of β-actin as housekeeping gene. The samples were incubated in an automated heat-block Minicycler (MJ Research, Waltham, MA, USA) using the following parameters: 95 °C for 30 s, 68 °C for 90 s and 72 °C for 120 s using 42 cycles (Fas), 95 °C for 30 s, 55 °C for 90 s and 72 °C for 120 s using 30 cycles ($p21^{WAF1/CIP1}$), and 95 °C for 30 s, 55 °C for 90 s and 72 °C for 120 s using 25 cycles (β-actin). The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. Densitometric analysis was performed using an FXmolecular imager (Bio-Rad, Hercules, CA, USA).

2.7. Statistical analysis

The data are presented as the arithmetic mean for each experimental point ±SEM. Statistical calculations were performed using a one-way ANOVA. Differences among groups were examined using the Bonferroni *t*-test when the *F* value was significant. A *P* value <0.05 was considered significant.

3. Results

3.1. The effect of MME on the growth of human hepatoma cell lines

The effect of MME on the hepatoma cell lines HepG2 and Hep3B was determined by MTT assay. MME treatment markedly inhibited the cell growth of both cell lines. HepG2 cells were slightly more sensitive than Hep3B cells to MME. After 48 h, MME at 7.5 μg/ml resulted in 60% cell growth inhibition in HepG2 cells and 70% inhibiton in Hep3B cells. IC_{50} and IC_{90}, defined as the drug concentrations resulting in 50 and 90% loss of cell viability relative to the untreated cells, respectively, were determined in HepG2 and Hep3B cells (Fig. 1). There was no significant difference between the IC_{50} and IC_{90} concentrations for HepG2 and Hep3B cells.

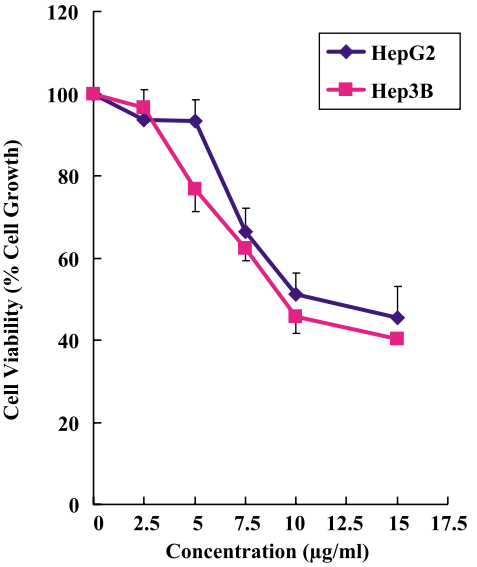

Fig. 1. The effect of MME on the growth of HepG2 and Hep3B cells as determined by MTT assay. Cells were treated with different concentrations of MME for 48 h. Results are expressed as percentages of cell growth relative to DMSO control group (concentration 0).

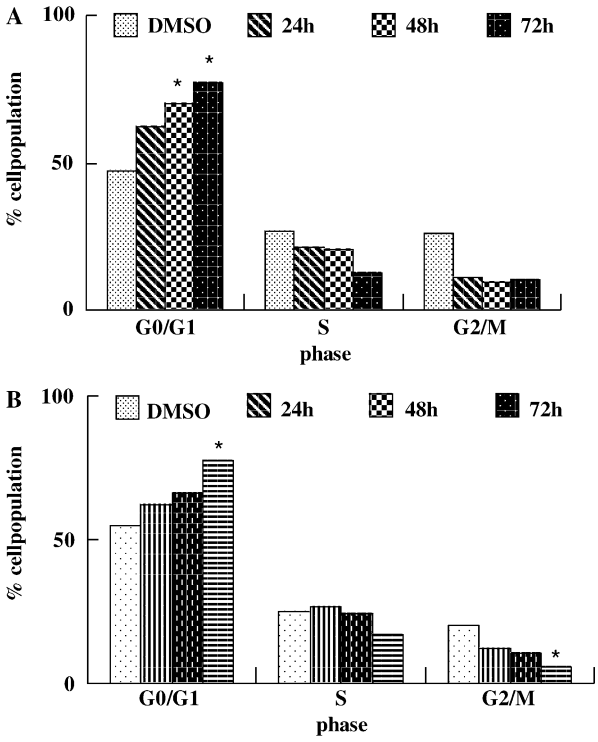

Fig. 2. Effect of MME on cell cycle distribution in HepG2 and Hep3B cells. The distribution of cell cycle in MME-treated HepG2 cells (A) and Hep3B (B). Cells treated with DMSO for 72 h were used as control. Cells were treated with MME for 24, 48 and 72 h, respectively, and the distribution of cell cycle was assessed by flow cytometry. The data shown are the mean ± SD of three independent experiments, each with triplicate wells. The asterisk indicates a significant difference between control and MME-treated cells, $*P < 0.05$.

7.5 μg/ml (higher than IC50) of MME was used in the subsequent experiment.

3.2. Cell cycle changes of hepatoma cells following MME treatment

The effects of MME on the cell cycles of HepG2 and Hep3B were determined by the flow cytometry. As is shown in Fig. 2, MME induced an accumulation of the G0–G1 phase of the cell cycle in a time-dependent pattern. For the HepG2, the populations of G0–G1 phase at 24, 48 and 72 h after the treatment with MME were 62.46, 69.71 and 77.16%, respectively, whereas the control at 72 h was 47.51%. The populations of G0–G1 phase for the Hep3B were 62.04, 65.82, and 77.06%, respectively, at 24, 48 and 72 h after the treatment with MME, whereas the control at 72 h was 54.55%. These results suggested that MME can induce an accumulation of G0–G1 phase cells.

3.3. The effects of MME on mRNA expression of $p21^{WAF1/CIP1}$ and Fas in HepG2 and Hep3B cells

The time course study showed that MME treatment induced the expression of Fas and $p21^{WAF1/CIP1}$

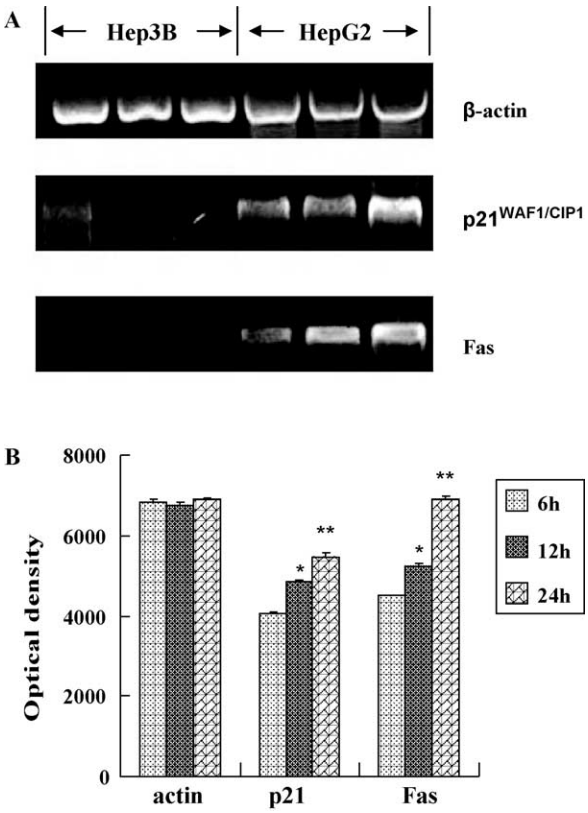

Fig. 3. MME-induced upregulation of Fas and $p21^{WAF1/CIP1}$ on HepG2 cells. Semi-quantitative PCR for Fas and $p21^{WAF1/CIP1}$ mRNA expression in HepG2 and Hep3B cells after treatment with 7.5 μg/ml MME for indicated times (A) and the optical density result of HepG2 cells (B). The PCR product size for Fas, $p21^{WAF1/CIP1}$ and β-actin is 397, 497 and 540 bp, respectively. Fas and $p21^{WAF1/CIP1}$ mRNA expression in Hep3B cell lines were undetectable. The data shown are the mean ± SD of three independent experiments, each with triplicate wells. The asterisk indicates a significant difference between control and MME-treated cells, $*P < 0.05$, $**P < 0.01$.

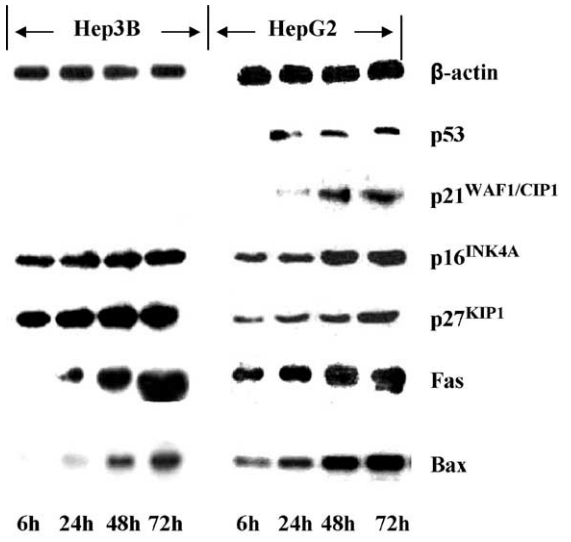

Fig. 4. The expression levels of p53, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p16^{INK4A}$, Bax and Fas in response to MME in HepG2 and Hep3B cells. Both HepG2 and Hep3B cells were exposed to 7.5 μg/ml MME for indicated time. Cells were harvested and subjected to Western blotting analysis with monoclonal antibodies against p53, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p16^{INK4A}$, Bax and Fas. β-actin was used as an internal control to ensure equal loading of each lane.

mRNA at a dose of 7.5 μg/ml, and the Fas mRNA level increased at 6 h and peaked at 12 h on the HepG2 cell line, while no expression was found on the Hep3B cell line (Fig. 3). Similarly, $p21^{WAF1/CIP1}$ mRNA levels increased to a peak at 24 h following MME treatment on HepG2 cells, and no expression occurred in Hep3B cell (Fig. 3). The data suggested that MME induced the expression of Fas and $p21^{WAF1/CIP1}$ at the transcriptional level, and the upregulation of Fas mRNA following MME treatment preceded that of $p21^{WAF1/CIP1}$ in HepG2 cells.

3.4. The effect of MME on the expression of p53, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p16^{INK4A}$, Bax and Fas proteins

To determine the molecular mechanism of MME-mediated cell cycle arrest, we examined the status of G0/G1 regulatory proteins in HepG2 cells and Hep3B cells after MME treatment. Cells were treated with 7.5 μg/ml MME and protein extracts analyzed by immunoblot using anti-p53, $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p16^{INK4A}$ Bax and Fas antibodies. Treatment with MME induced expression of p53 and $p21^{WAF1/CIP1}$ proteins in HepG2, but there was no induction of these two proteins in Hep3B (Fig. 4). In HepG2, the p53 protein level began to rise 6 h after treatment, and reached a maximum level at 48 h. The $p21^{WAF1/CIP1}$ protein level increased at later time points and reached a maximum level at 72 h. $p27^{KIP1}$and $p16^{INK4A}$ levels were slightly increased in both cell lines at a later time point as compared to the untreated cells. Increased levels of Bax and Fas were also found in both cell lines.

4. Discussion

The present study has demonstrated that MME significantly inhibited cell growth and induced G0–G1 phase cell cycle arrest in hepatoma cell lines HepG2 and Hep3B. The effect of MME on cell growth was observed at as low as 5 μg/ml and the maximal effect was seen at 15 μg/ml.

In this study, 7.5 μg/ml MME obviously inhibited cell growth and induced G0–G1 phase cell cycle arrest in both hepatoma cell lines, HepG2 and Hep3B, as evidenced by MTT assay and flow cytometry analysis. HepG2 cells, which have wild-type p53, were more sensitive to MME than Hep3B cells which have p53 deleted, suggesting that wild-type p53 might be one of the factors modulating the sensitivity of hepatoma cells to MME. HepG2 cells, which have wild-type p53, were transiently arrested at the G1 phase following exposure to 7.5 μg/ml MME, whereas there was no evidence of G1 arrest in Hep3B cells which have p53 deleted. An increase in p53 protein level was observed in HepG2 after MME treatment at 7.5 μg/ml MME, and this was followed by an increase in both $p21^{WAF1/CIP1}$mRNA and protein levels. This suggested that the upregulation of $p21^{WAF1/CIP1}$ in HepG2 cells following MME treatment was due to transcriptional activation and might be via p53-dependent pathway(s). In this study, no induction of either p53 or $p21^{WAF1/CIP1}$ protein was observed in Hep3B cells after MME treatment. This data suggested that the presence of wild-type p53 might not be an absolute prerequisite for induction of apoptosis in hepatoma cell lines, and MME was able to induce G0–G1 phase cell cycle arrest via both p53—dependent and—independent pathways.

$p27^{KIP1}$, a cyclin-dependent kinase inhibitor, controls G1/S transition by inhibiting the activity of a wide variety of cyclin/cyclin dependent kinase (CDK) complexes. Unlike $p21^{WAF1/CIP1}$, $p27^{KIP1}$ arrests cells only at the G1 phase [14,15]. In the present study, 7.5 μg/ml MME induced $p27^{KIP1}$ expression in HepG2 cells. Similar expression patterns of $p27^{KIP1}$ were found in Hep3B cells. These observations suggested that the transient G1 arrest induced by MME might be dependent on $p27^{KIP1}$ in both HepG2 and Hep3B.

It has been reported that $p16^{INK4A}$ is a CDK inhibitor [16,17]and controls G1/S transition. Enforced expression of $p16^{INK4A}$ protein has been determined to arrest cells in the G1 phase in an Rb-dependent fashion [18]. In the present study, demonstrable changes of $p16^{INK4A}$ protein levels were observed following MME treatment at 7.5 μg/ml in both cell lines. These indicated that $p16^{INK4A}$ might be involved in MME-induced growth inhibition and cell cycle arrest in both HepG2 and Hep3B cells.

Bax is a member of the rapidly expanding Bcl-2 family of proteins which have been demonstrated to regulate apoptosis in response to chemotherapy both in vivo and in vitro [19]. Enforced overexpression of Bcl-2 or Bax has been demonstrated to inhibit or induce apoptosis [20], and in some cases to provide a true survival advantage after many diverse stimuli, including chemotherapeutic agents, γ-irradiation and growth factor deprivation [21]. In this study, an increased level of Bax was observed in both cell lines following treatment with 7.5 μg/ml MME. The findings suggested that the altered expression pattern of the Bcl-2 protein family caused by MME in hepatoma cells might be involved in chemosensitivity.

The effect of MME on Fas expression was assessed in HepG2 and Hep3B cells. An increased expression of Fas at both mRNA and protein levels was observed in HepG2 cells following treatment with 7.5 μg/ml MME. No Fas expression was found in Hep3B cells. It appeared that induction of Fas expression might be correlated with the appearance of MME induced cell growth inhibiton in HepG2 cells. Although the Fas expression on Hep3B cells was enhanced by MME, no apoptosis was detected in either cell line (data not shown). The upregulated Bcl-2 expression may protect these cells from apoptosis, which needs to be addressed in the future.

In conclusion, our studies have shown that MME could inhibit hepatoma cell growth, which may be related to MME-induced G0–G1 cell cycle arrest both in HepG2 and Hep3B cells. This cell cycle arrest process may be associated with the upregulation of p53 and $p21^{WAF1/CIP1}$ in HepG2 cells. The upregulation of $p27^{KIP1}$ and $p16^{INK4A}$ observed in both cell lines may be involved in the cell cycle arrest induced by MME. Importantly, MME can induce G0–G1 cell cycle arrest in Hep3B cells, which were deficient of p53, as efficiently as in p53 wild-type HepG2. This data strongly suggested that MME-induced cell cycle arrest in Hep3B cells operates through a p53-independent pathway. Interestingly, upregulation of Bax in both cell lines were also observed. These results were in agreement with those of the previous reports [22,23]. Nevetheless, additional studies to determine the molecular mechanisms of MME-mediated cell cycle arrest are required.

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