Effect of fumonisin B_1 on the cell cycle of normal human liver cells

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Abstract. Fumonisin B_1 (FB_1) is a well-known liver and kidney carcinogen in rodents and humans. The aim of the present study was to investigate the effect of FB_1 on the proliferation and cell cycle of the normal human liver cell line HL-7702 and to explore the underlying molecular mechanisms of action. The cells were treated with FB_1 (0.0, 0.1, 1.0, 10.0 and 100.0 μmol/l) for 24, 48, 72 and 96 h. Cell proliferation was assessed by colorimetric assay. Cell cycle analysis was performed by flow cytometry. The mRNA and protein expression of cyclin E and P21 were determined by RT-PCR and western blot analysis, respectively. FB_1 was initially demonstrated to significantly inhibit the proliferation of HL-7702 cells; however, cell proliferation increased with increasing treatment time. The percentage of cells in the G0/G1 phase was significantly increased by FB_1; however, significantly decreased with an increasing concentration of FB_1. The mRNA expression of cyclin E was upregulated and then gradually downregulated with increasing treatment time. The mRNA expression of P21 was significantly increased following treatment with 0.1 μmol/l FB_1, and decreased following treatment with 10.0 and 100.0 μmol/l FB_1 for different treatment durations. Western blot analysis showed that FB_1 significantly increased the protein expression of cyclin E and significantly decreased the protein expression of P21 at various concentrations and treatment durations. Our results demonstrated that FB_1 affects the cell cycle of normal human liver cells and that the underlying mechanism of action is associated with alterations in the expression levels of cyclin E and P21 induced by FB_1.

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Introduction

Fumonisins (FBs) are the toxic metabolites produced mainly by *Fusarium verticillioides*, one of the most common molds found on maize and other agricultural products worldwide (1-3). FB_1 is the most abundant and toxic fumonisin. Previous studies in broilers and rats have shown that FB_1 administered in the diet is hepatotoxic, nephrotoxic and hepatocarcinogenic, and induces severe symptoms, including a decrease of body weight, an increase of relative weights of the liver and kidney, and liver necrosis (4). Gelderblom *et al* (5) reported that FB_1 induces the formation of liver tumors in laboratory rodents. FB_1 has also been shown to alter the gene expression and signal transduction pathways in monkey kidney cells (CV-1) (6), increase the mitogenic action of insulin in Swiss 3T3 fibroblasts (7), inhibit the proliferation of IPEC-1 and LLC-PK1 kidney cells (8), induce oxidative damage in primary culture rat hepatocytes (9) and lipid peroxidation in rabbit kidney RK13 cells (10). In addition, studies have demonstrated that FB_1 is able to increase the initial disruption of sphingolipid metabolism and the accumulation of sphinganine in LLC-PK1 kidney cells (11,12), cause DNA damage of apoptotic type in rat astrocytes (13), induce apoptosis, DNA fragmentation and hypermethylation (14), and to alter the collagen secretion pattern in primary human lung fibroblasts and human kidney epithelial cells (15). However, there is inadequate evidence with regard to the carcinogenicity of FB_1 in humans. Several studies have investigated the toxicity of FB_1 in human cell lines to retrieve more information regarding the effects of FB_1 on humans. Exposure to FB_1 has been shown to cause DNA damage in human fibroblasts (16), inhibit clonal expansion of human keratinocytes and human esophageal epithelial cells, and inhibit proliferation of human fibroblasts (15). Furthermore, FB_1 has been demonstrated to induce apoptosis of human proximal tubule-derived cells (17) and cause oxidative stress in the human intestinal cell line Caco-2 (18).

Although the toxic effects of FB_1 on mammalian cells have been extensively investigated, the potential mechanism of action and the involved signaling pathways have not been identified. In recent years, numerous studies have shown that FB_1 affects the cell cycle of certain cells. Cell cycle progression is known to be controlled by cyclin-dependent kinases (CDKs) and cyclins (19,20), while cyclin E is necessary for entry into the S phase (21). CDK inhibitors, including P21, bind

to CDK-cyclin complexes and inhibit CDK activity (22,23). P21 expression has been shown to be upregulated by various types of antiproliferative stimuli and the upregulation of P21 expression results in cell cycle arrest or apoptosis (24,25).

The liver is the critical organ for the metabolism and degradation of chemicals, food contaminants and natural toxins. Therefore, the aim of the present study was to investigate the effect of FB_1 on the cell cycle and the expression of cell cycle-related genes P21 and cyclin E in the normal human liver cell line HL-7702.

Materials and methods

Materials. FB_1, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dimethyl sulfoxide (DMSO), ethidium bromide (EB) and diethyl pyrocarbonate were purchased from Sigma (St. Louis, MO, USA). RPMI-1640 medium and trypsin were purchased from Gibco-BRL (Rockville, MD, USA). A stock solution of FB_1 for cellular assays was prepared in phosphate-buffered saline (PBS) and then diluted in the optimal medium (≤10 μl/ml). Ethylenediaminetetraacetic acid was purchased from Calbiochem (La Jolla, CA, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, Zhejiang, China). RNA TRIzol was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The RevertAid™ First Strand cDNA Synthesis kit was purchased from Fermentas Life Sciences (Glen Burnie, MD, USA). Reagents and membranes used for protein assay, electrophoresis and western blot analysis were obtained from Bio-Rad (Hercules, CA, USA). Antibodies for P21 (mouse monoclonal), cyclin E (mouse monoclonal) and horseradish peroxidase (HRP)-conjugated goat secondary anti-mouse antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody for GAPDH (polyclonal monoclonal) was purchased from KangChen Bio-tech (Shanghai, China).

Cell culture. The normal human liver cell line HL-7702 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 5% FBS at 37˚C, 95% humidity and 5% CO_2 in a humidified incubator. Cell growth was observed using an inverted microscope daily; RPMI-1640 medium was replaced according to its color every 2-3 days. The study was approved by the ethics committee of the School of Public Health, Southeast University, Nanjing, China.

Cell viability assay. HL-7702 cells ($1x10^4$ cells/100 μl/well) were seeded in 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) with 100 μl culture medium containing 5% FBS, and incubated for 24 h to allow cells to attach to the bottom of the plate. The cells were incubated with FB_1 (0.0, 0.1, 1.0, 10.0 and 100.0 μmol/l) for 24, 48, 72 and 96 h. After 100 μl MTT (5 mg/ml in PBS) was added in the culture medium, the cells were incubated for 4 h at 37˚C in a humidified atmosphere with 5% CO_2. The medium was aspirated and the cells were suspended in 150 μl DMSO. The absorption was measured at 490 nm with a Mithras LB 940 Multimode Microplate reader (Berthold Technologies, Bad Wildbad, Germany). The inhibition rate of cell proliferation was calculated as follows: 1- [optical density (OD) of the experimental samples/OD of the control] x 100%. The experiment and assay were repeated at least three times.

Cell harvesting. HL-7702 cells in a logarithmic growth phase were plated at a density of 10^5 cells/ml in 50-cm^2 culture flasks and allowed to grow in 4 ml culture medium. Following cell attachment, the culture medium was discarded. The cells were then treated with FB_1 (0.0, 0.1, 1.0, 10.0 and 100.0 μmol/l) for 24, 48, 72 and 96 h. Then, the cells were trypsinized and collected for cell cycle analysis, or washed twice with ice-cold PBS and removed from the surface of the flask by using a rubber scraper for RT-PCR and western blot analysis.

Cell cycle analysis. The cell cycle phase was examined using a Becton-Dickinson FACSCalibur flow cytometer. The cells were stained with Vindelov's reagent (40 mmol/l Tris, pH 7.6; 100 mmol/l NaCl; 10 mg/ml RNase A; 7.5% PI and 0.1% Nonidet P-40), and data from 10,000 cells were collected for each data file. The experiment and assay were repeated three times.

Semiquantitative RT-PCR. Semiquantitative RT-PCR was used to assess the mRNA expression of cyclin E and P21. Briefly, after the HL-7702 cells were treated with FB_1, total RNA was extracted using TRIzol, followed by chloroform re-extraction and isopropanol precipitation. Purified RNA was dissolved in RNase-free water and quantitated by spectrophotometry. Reverse transcription was performed using the RevertAid First Stand cDNA Synthesis kit with oligo(dT) priming under standard conditions suggested by the supplier. PCR was performed in a 50-μl reaction containing PCR mix SYBR-Green (Takara Bio, Inc., Dalian, China), each primer and cDNA. PCR was performed under the following conditions: 94˚C for 2 min for initial denaturation, followed by 28 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 2 min. The primer sequences used were: 5'-ATACAGACCCA CAGAGACAG-3' and 5'-TGCCATCCACAGAAATACTT-3' for cyclin E; 5'-CAGGGGACAGCA GAGGAAGA-3' and 5'-GGGCGGCCAGGGTATGTAC-3' for P21 and 5'-ACGGATTTGGTCGTATTG-3' and 5'-TGATCT TGAGGCTGTTGTC-3' for GAPDH. The size of the predicted product was visualized by 1.7% agarose gel electrophoresis with EB staining under ultraviolet (UV) illumination. The amounts of PCR products were determined by densitometry analysis using the GelDoc-It™ imaging system (UVP, Upland, CA, USA). Semiquantitative PCR results were generated by grading a ratio between the densitometry results of the target cytokines and GAPDH.

Western blot analysis. The cells were detached by scraping and were centrifuged for 10 min at 16,000 RCF at 4˚C. The cell pellets were lysed in mammalian cell protein extraction reagent (20 mmol/l Tris, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, pH 7.4) and mammalian protease inhibitor mixture. The supernatant was collected after centrifugation for 20 min at 10,000 x g at 4˚C. The protein concentration was determined using Pierce® BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Lysates (30 μl total protein) were separated by SDS-PAGE.

Table 1. Effect of FB_1 on the cell cycle distribution of HL-7702 cells (%, n=3).

Duration of treatment (h)	Cell cycle phase	FB_1 concentration (μmol/l)				
		0	0.1	1.0	10.0	100.0
24	G0/G1	73.3±0.7	76.1±3.2	75.5±3.7	72.6±3.6	71.3±3.6
	S	24.0±0.9	21.1±1.1	22.4±2.9	24.8±1.9	25.5±2.1
	G2/M	2.6±1.2	2.8±2.1	2.1±1.0	2.6±1.9	3.2±1.6
48	G0/G1	71.3±3.3	79.1±3.1	80.3±3.8[a]	82.7±2.6[a]	83.9±2.5[a]
	S	25.3±1.3	16.9±1.8[a]	16.3±2.5[a]	15.9±1.4[a]	14.9±2.4[a]
	G2/M	3.4±2.0	4.0±1.6	3.4±1.4	1.4±1.2	1.3±0.1
72	G0/G1	72.8±6.7	74.7±5.0	70.1±1.4	62.7±5.3[a]	54.6±3.9[a]
	S	23.4±5.3	24.0±4.8	24.9±1.0	26.4±4.6	28.2±2.9
	G2/M	4.0±1.6	1.3±0.6	5.0±1.4	10.9±2.5[a]	17.2±1.4[a]
96	G0/G1	71.9±1.4	72.9±2.1	61.3±3.3[a]	54.5±4.2[a]	43.6±2.1[a]
	S	25.2±1.3	24.1±1.7	32.5±1.1[a]	30.5±0.9[a]	40.5±3.3[a]
	G2/M	2.8±1.4	2.9±0.4	6.3±2.3	15.1±3.3[a]	15.9±1.2[a]

Data are expressed as the means ± SD, from three independent experiments, each performed in triplicate. [a]P<0.05 compared with the control cells. FB_1, fumonisin B_1.

Following electrophoresis, the proteins were transferred onto polyvinylidene fluoride membranes (PVDF). The membranes were blocked in Tris-buffered saline with 0.1% Tween-20 containing 5% non-fat dry milk for 1 h at room temperature, incubated with the corresponding primary antibodies (1:200) at 4˚C overnight. These antibodies included anti-cyclin E, anti-P21 and anti-GAPDH. The membranes were incubated at room temperature with goat anti-mouse IgG-HRP (1:2,000). Following subsequent washing with TBST, incubation with chemiluminescence reagents and detection by Kodak *In Vivo* imaging systems (Carestream Health, Inc., Rochester, NY, USA) allowed for visualization of proteins, which were then quantitated by strip densitometry. GAPDH was used as an internal control.

Statistical analysis. The data are expressed as the means ± SD. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) using the SPSS package (version 13.0). Differences among the groups were evaluated by the parametric Least Significant Difference (LSD) experiment and differences between experimental groups and the negative control group were evaluated by Dunnett's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of FB_1 on the proliferation of HL-7702 cells. As shown in Fig. 1, the inhibition rate of the cells was gradually increased and then significantly decreased with increasing treatment time after the HL-7702 cells were treated with 0.1, 1.0, 10.0 and 100.0 μmol/l FB_1 for 24, 48, 72 and 96 h compared with the control cells. The inhibition rates of HL-7702 cells induced by treatment with 10.0 and 100.0 μmol/l FB_1 for 72 h were significantly decreased by 41.8±2.6 and 44.1±1.2%, respectively, compared with the control cells. The proliferation of HL-7702 cells was significantly inhibited by treatment with FB_1 (0.1, 1.0, 10.0 and 100.0 μmol/l) for 48 h compared with the proliferation of the control cells; however, the proliferation of HL-7702 cells was significantly increased by treatment with FB_1 (0.1, 1.0, 10.0 and 100.0 μmol/l) for 96 h compared with the control cells. The proliferation of HL-7702 cells was significantly increased by ~15% following treatment with 0.1 μmol/l FB_1 for 96 h compared with the control cells.

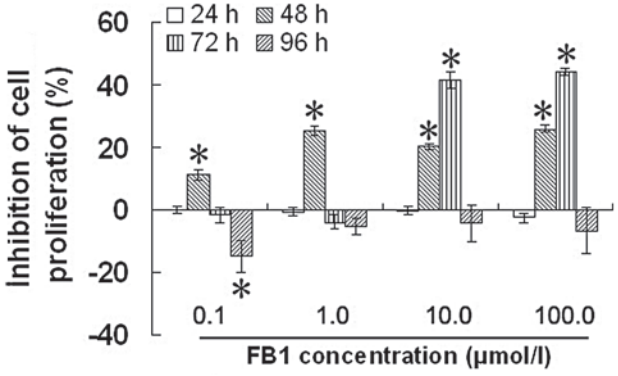

Figure 1. Effect of FB_1 on the growth of HL-7702 cells. HL-7702 cells were treated with various concentrations of FB_1 (0.1, 1.0, 10.0 and 100.0 μmol/l) for 24, 48, 72 and 96 h. Cell growth was measured by MTT assay and the inhibition rate of cells was determined. Data are expressed as the means ± SD from three independent experiments, each performed in triplicate. *P<0.05 compared with the control cells. FB_1, fumonisin B_1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Effect of FB_1 on the cell cycle of HL-7702 cells. In order to investigate the mechanism by which FB_1 affects the proliferation of normal human liver cells, the cell cycle was determined by flow cytometry and the percentages of HL-7702 cells in the different phases of the cell cycle were calculated. As shown in Fig. 2 and Table I, the cell cycle progression of HL-7702

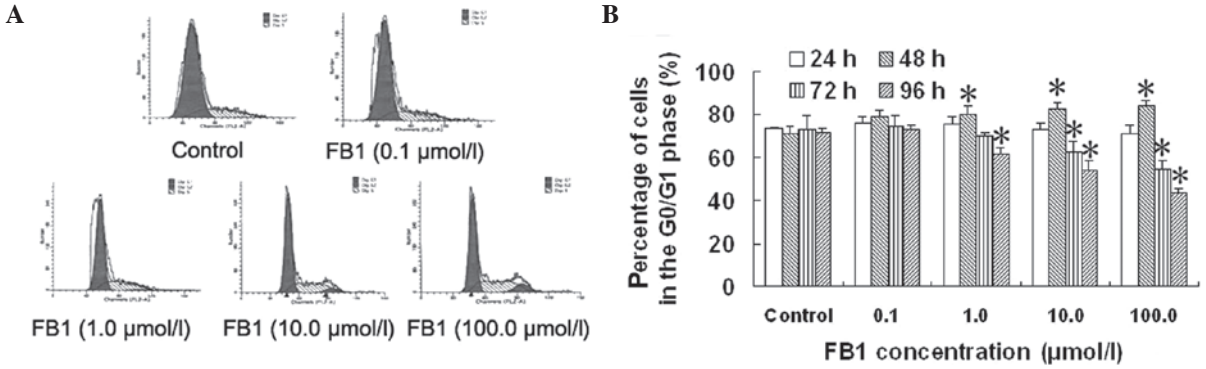

Figure 2. Effect of FB_1 on the cell cycle distribution of HL-7702 cells. The cells were incubated with various concentrations of FB_1 for 24, 48, 72 and 96 h. The distribution of the cell cycle was detected by PI staining and the relative percentages of cells in different cell cycle phases were determined. (A) Representative images showing the cell cycle distribution of HL-7702 cells following treatment with various concentrations of FB_1 for 96 h. (B) The results are expressed as the percentage of cells in the G0/G1 phase of the cell cycle. Data are expressed as the means ± SD from three independent experiments, each performed in triplicate. *P<0.05 compared with the control cells. FB_1, fumonisin B_1; PI, propidium iodide.

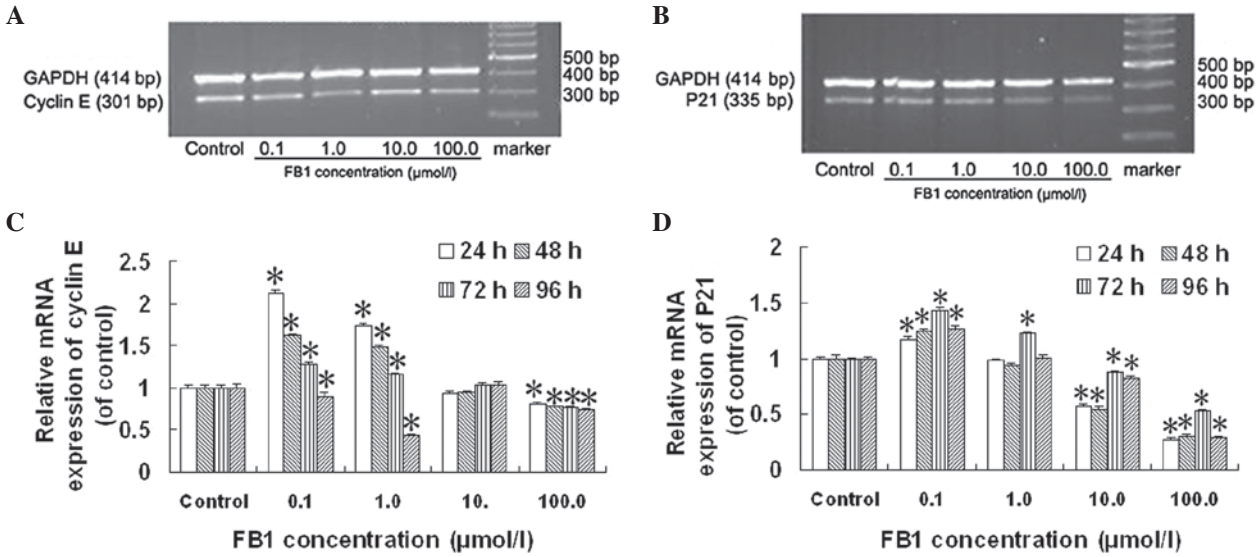

Figure 3. Effect of FB_1 on the mRNA expression of cyclin E and P21 in HL-7702 cells. The cells were treated with the indicated concentrations of FB_1 for 24, 48, 72 and 96 h. The mRNA expression levels of cyclin E and P21 were analyzed by RT-PCR. (A) Cyclin E mRNA expression in HL-7702 cells following treatment with various concentrations of FB_1 for 96 h using semiquantitative PCR. (B) P21 mRNA expression in HL-7702 cells following treatment with various concentrations of FB_1 for 96 h using semiquantitative PCR. (C) FB_1 (0.1 and 1.0 μmol/l) significantly affected the mRNA expression of cyclin E in HL-7702 cells in a time-dependent manner. (D) FB_1 significantly affected the mRNA expression of P21 in HL-7702 cells in a concentration-dependent manner. Data are expressed as the means ± SD from three independent experiments, each performed in triplicate. *P<0.05 compared with the control cells. FB_1, fumonisin B_1.

cells was blocked in the G2/M phase following treatment with FB_1 for 72 and 96 h. Cell progression was also blocked in the G0/G1 phase following treatment with FB_1 for 48 h. The percentages of HL-7702 cells in the G0/G1 phase were 54.5 and 43.6%, respectively, following treatment with 10.0 and 100.0 μmol/l FB_1 for 96 h.

Effect of FB_1 on the mRNA expression of cyclin E and P21 in HL-7702 cells. The mRNA expression of cyclin E in HL-7702 cells was significantly increased following treatment with 0.1 and 1.0 μmol/l FB_1 compared with the control cells (Fig. 3C). The mRNA expression of cyclin E was initially upregulated and then gradually downregulated with increasing treatment time following treatment with 0.1 and 1.0 μmol/l FB_1 compared with the control cells; the level of cyclin E mRNA expression was the highest following treatment for 24 h. The mRNA expression of cyclin E in HL-7702 cells was significantly decreased following treatment with 100.0 μmol/l FB_1 for various treatment durations compared with the control cells (Fig. 3C).

The mRNA expression of P21 in HL-7702 cells was significantly altered following treatment with FB_1 for 24, 48, 72 and 96 h in a concentration-dependent manner compared with the control cells (Fig. 3D). The mRNA expression of P21 in HL-7702 cells was significantly increased following treatment with 0.1 μmol/l FB_1 for various treatment durations compared with the control cells; however, the mRNA expression of P21 in HL-7702 cells was significantly decreased following treatment with 10.0 and 100.0 μmol/l FB_1 for the various treatment durations compared with the control cells (Fig. 3D).

Effect of FB_1 on the protein expression of P21 and cyclin E in HL-7702 cells. The protein expression of cyclin E and P21 in

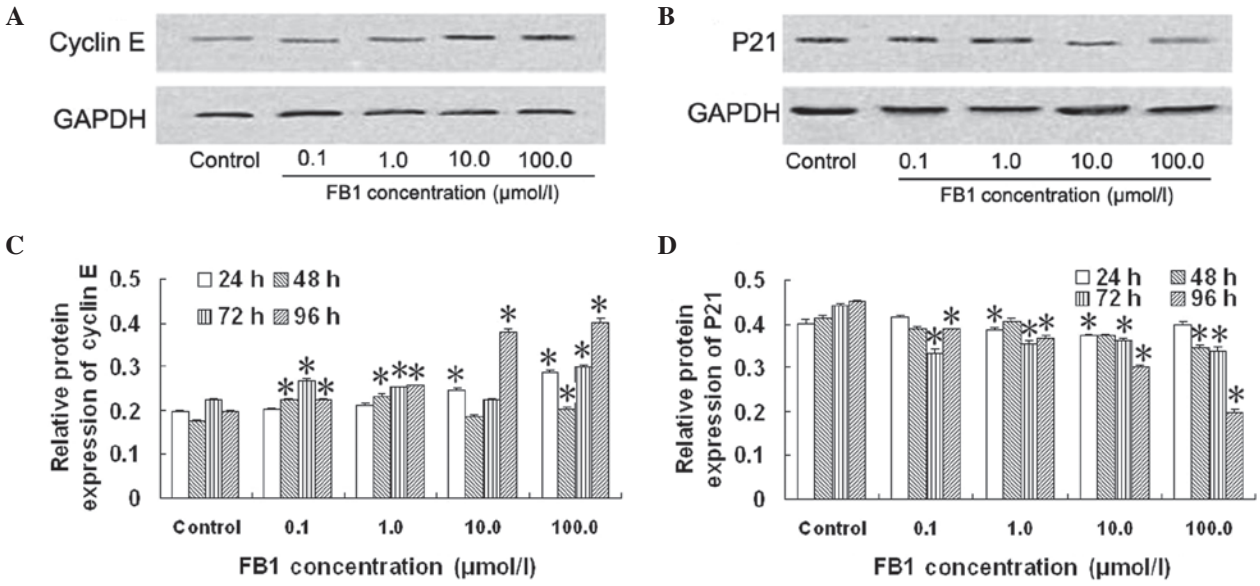

Figure 4. Effect of FB_1 on the protein expression of cyclin E and P21 in HL-7702 cells. The cells were treated with the indicated concentrations of FB_1 for 24, 48, 72 and 96 h. The protein expression of cyclin E and P21 in HL-7702 cells was investigated using western blot analysis. (A) Representative western blots showing cyclin E protein expression in HL-7702 cells following treatment with various concentrations of FB_1 for 96 h. (B) Representative western blots showing P21 protein expression in HL-7702 cells following treatment with various concentrations of FB_1 for 96 h. (C) FB_1 significantly upregulated cyclin E protein expression in HL-7702 cells. (D) FB_1 significantly downregulated P21 protein expression in HL-7702 cells. Data are expressed as the means ± SD from three independent experiments, each performed in triplicate. *P<0.05 compared with the control cells. FB_1, fumonisin B_1.

HL-7702 cells was significantly affected following treatment with FB_1 for 96 h compared with the control cells (Fig. 4). The protein expression of cyclin E was increased on average 14.3±2.4, 30.8±1.2, 93.7±3.2 and 105.0±2.2% following treatment with 0.1, 1.0, 10.0 and 100.0 μmol/l FB_1 for 96 h, respectively, compared with the control cells (Fig. 4C). The protein expression of P21 was decreased on average 14.2±0.2, 19.0±0.3, 33.6±0.2 and 56.6±0.5% following treatment with 0.1, 1.0, 10.0 and 100.0 μmol/l FB_1 for 96 h, respectively, compared with the control cells (Fig. 4D). These results demonstrated that FB_1 significantly increased the protein expression of cyclin E (Fig. 4C) and significantly decreased the protein expression of P21 (Fig. 4D) in HL-7702 cells.

Discussion

Fumonisins are a family of cytotoxic and carcinogenic mycotoxins. The International Agency for Research on Cancer (IARC) has clarified that FB_1 is a class 2B carcinogen and a potential human carcinogen. In order to investigate the effect of FB_1 on the human liver, a liver cell line derived from the normal human liver was used in this study.

In the present study, the proliferative or anti-proliferative effects of treatment with various concentrations of FB_1 for various treatment durations were observed using the cell viability assay in HL-7702 cells. It has been suggested that the mechanism of FB_1 action is associated with the biphasic dose responses, which is also known as 'hormesis', meaning that FB_1 can stimulate the proliferation of human normal liver cell at low doses and exhibit effects of inhibition at high doses (26). Similarly, anti-proliferative effects of FB_1 have been observed in human hepatoma (27) and swine peripheral blood mononuclear cells (28). The results of the present study demonstrated that the maximum inhibition rate of HL-7702 cells was induced following treatment with 100.0 μmol/l FB_1 for 72 h. Although various concentrations of FB_1 inhibited the proliferation of HL-7702 cells for treatment durations up to 48 h, the maximal inhibition rate was still <26%. These results were consistent with those reported by Fornelli *et al* (29), who demonstrated that the inhibition rate of cell proliferation induced by the same concentration of FB_1 was ~20% in SF-9 cells. CD_{50} of FB_1 was 476.2±16.7 μg/ml (~680 μmol/l) in BEAS-2B cells, which indicated that low doses of FB_1 may have no evident cytotoxicity (30).

Treatment with various concentrations of FB_1 for 48 h blocked G0/G1 phase arrest, while cell proliferation was inhibited compared with the control cells. The ratio of the cell population in the G0/G1 phase in all the concentrations of FB_1 used, was initially increased and then gradually decreased with increasing treatment time, which was similar with the results obtained from the MTT assay. In animal experiments, FB_1 affected the check point of the G1/S phase, leading to alterations in the cell cycle (31). The percentage of cells blocked in the G0/G1 phase of the cell cycle has been shown to be significantly increased by FB_1 in swine peripheral blood mononuclear cells (28). A similar result was also found in CV-1 cells *in vitro* where FB_1 blocked the cells in the G1 phase and resulted in cell cycle arrest. However, FB_1 did not exert the same effects on COS-7 cells (32). Consequently, different effects on the cell cycle have been observed in different types of cells following treatment with FB_1.

In eukaryotes, the cell cycle is tightly regulated by several protein kinases composed of cyclin-dependent kinases, corresponding regulatory cyclins and cyclin-dependent kinase inhibitors. The microinjection of anti-cyclin E antibody in cells in the G1 phase inhibited normal fibroblasts to enter the S phase (33). Additional studies in *Drosophila* cyclin E mutant embryos indicated that cyclin E is required for the progres-

sion through the S phase of the mitotic cycle (34,35). These results suggest that cyclin E and its associated kinase in the majority of eukaryotic cells are needed for cell entry into the S phase. With the decrease of cyclin E protein expression and the activation of CDK2 in the cell cycle, the G1/S phase transition is promoted, and the cells proliferate abnormally or tumor formation is stimulated. The decrease of cyclin E mRNA expression may lead to the reduction of cyclin E protein expression, interference of the normal physiological function, transition of G1 to S phase and inhibition of normal cell proliferation. Conversely, the increase of cyclin E expression promotes cell proliferation.

The results of the present study showed that the mRNA and protein expression of cyclin E in HL-7702 cells was significantly increased following treatment with FB_1 (0.1 and 1.0 mol/l) for 48 h compared with the control cells, and that the mRNA and protein expression of cyclin E in HL-7702 cells was not significantly altered following treatment with 10.0 μmol/l FB_1 for 48 h compared with the control cells. However, the mRNA expression of cyclin E in HL-7702 cells was significantly decreased and the protein expression of cyclin E was significantly increased following treatment with 100.0 μmol/l FB_1 for 48 h compared with the control cells. These results indicated that FB_1 affected the genetic transcription of cyclin E and also the protein degradation of cyclin E in HL-7702 cells. The cell cycle is regulated by the level of cyclin E protein expression; however, not the level of cyclin E mRNA expression. According to cell cycle analysis, treatment of HL-7702 cells with FB_1 (1.0, 10.0 and 100.0 μmol/l) for 48 h blocked G0/G1 phase arrest. These results were contradictory with the protein expression of cyclin E. Therefore, these results demonstrated that FB_1 affected the cell cycle of HL-7702 cells through other cell cycle-related pathways than the cyclin E pathway.

The results showed that the protein expression of cyclin E in HL-7702 cells was significantly increased following treatment with FB_1 (0.1, 1.0 and 100.0 μmol/l) for 72 h compared with the control cells, and that the protein expression of cyclin E in HL-7702 cells was significantly increased following treatment with FB_1 (0.1, 1.0, 10.0 and 100.0 μmol/l) for 96 h compared with the control cells. According to cell cycle analysis, the ratio of cells in the G0/G1 phase treated with 1.0 and 100.0 μmol/l FB_1 for 72 h was significantly increased compared with the control cells. The ratio of HL-7702 cells in the G0/G1 phase treated with 1.0, 10.0 and 100.0 μmol/l FB_1 for 96 h was significantly increased. Similar results were obtained following the assessment of cyclin E protein expression. These results indicated that the protein expression of cyclin E was increased following treatment with FB_1 for 72 and 96 h and that FB_1 promoted HL-7702 cells to enter the S phase and increased cell proliferation.

P21 is a member of the CKI family and has a direct role on cyclin E/CDK2. As an inducible growth inhibitor, the high expression of P21 combined with cyclin E/CDK2 arrests the cell cycle at the G1 phase and thus inhibits DNA replication, resulting in the dysregulation of the cell cycle and the normal cell differentiation process. Following western blot analysis, a decrease of P21 protein expression was observed in the normal human liver cells following treatment with various concentrations of FB_1. The mRNA expression of P21 in HL-7702 cells was significantly decreased following treatment with 10.0 and 100.0 μmol/l FB_1 for various treatment durations compared with the control cells, and the mRNA expression of P21 in HL-7702 cells was significantly increased following treatment with 0.1 μmol/l FB_1 for various treatment durations compared with the control cells. These results showed that the protein expression was decreased by treatment with FB_1 for 72 and 96 h, and that FB_1 induced HL-7702 cells to enter the S phase and increased cell proliferation.

According to the study by Zhang *et al* (36), the two Sp1 binding sites within -124 to -101 were necessary and sufficient for FB_1-induced P21 transcription in CV-1 cells (36). However, the concentration of FB_1 used in this study was 5 mmol/l, notably higher than the dose used in our experiment. Therefore, it is suggested that the different doses of FB_1 lead to different effects on P21 expression.

In conclusion, the effect of FB_1 on the proliferation, cell cycle and expression of cyclin E and P21 in the normal human liver cell line HL-7702 indicates that FB_1 is most likely to affect other cell cycle-related factors. Meanwhile, the inconsistency of the mRNA and protein expression of genes indicates different regulatory mechanisms (such as synthesis and degradation rates) acting on the synthesized mRNA and protein, which differentially affect the amount of the two molecules. Therefore, further studies are needed to elucidate the underlying mechanism of action.

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