

Original Article

Cerulenin induces apoptosis in hepatic cancer HepG2 cells in vitro and in vivo

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Abstract: Cerulenin is believed to play an anti-tumor role in several types of cancers, but its impact on hepatic cancer is not fully clarified. In this study, the growth inhibitory effect of cerulenin on hepatic cancer cell line HepG2 was detected *in vitro* and *in vivo*. Hoechst 33342/PI and Annexin V/PI assay were applied to detect apoptosis and necrosis in HepG2 cells. Expression changes of fatty acid synthase (FASN) and apoptosis related proteins were also investigated. An obvious growth inhibition was found in HepG2 cells after cerulenin treatment. Hoechst 33342/PI and Annexin V/PI assay showed that cerulenin could induce apoptosis *in vitro*. Expression of Bax and Caspase-3, 8, 9 was up-regulated after cerulenin treatment, while Bcl-2 expression was down-regulated. The *in vivo* anti-tumor effect of cerulenin performed as inhibiting the growth of transplanted tumors in nude mice. In conclusion, cerulenin is a pro-apoptotic agent for hepatic cancer cell line HepG2 cells and may be an anti-tumor agent in hepatic cancer treatment.

Keywords: Cerulenin, hepatic cancer cell line, HepG2, apoptosis, transplanted tumor

Introduction

The incidence of liver cancer is increasing yearly. The current clinical treatment of liver cancer bases on surgery, chemotherapy and radiotherapy, while the five year survival rate is still low. Therefore, finding an effective treatment is urgent [1]. Endogenous fatty acids are considered to be important sources of fatty acids which are required for tumor cell growth. Different from normal tissues, the fatty acid metabolism of tumor cells depends on fatty acid synthase (FASN) to synthesize fatty acids to meet the need of cancer cell division and proliferation [2]. Regarded as a cancer antigen gene at present, FASN is highly expressed in a variety of tumors [3-8]. The expression level of FASN is associated with the occurrence and development of tumors, pathological grade, degree of malignancy and prognosis. According to the characteristics of FASN distribution, selective inhibition of FASN has become a new way of tumor treatment.

As a metabolite of fungus *Cephalosporium ceruleans*, cerulenin is reported to inhibit the

activity of FASN [9]. Research has shown that cerulenin can suppress tumor cell growth and induce apoptosis of cancer cells, but there have been few studies on its role in hepatocellular carcinoma. In the present study, the effect of cerulenin on the growth of hepatocellular carcinoma cell line HepG2 was analyzed and to a possible mechanism was uncovered.

Methods

Cell lines and culture

Hepatic carcinoma cell line HepG2 was kindly gifted by the Laboratory of Cellular and Molecular Tumor Immunology of Soochow University, and was cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified incubator at 37°C in 5% CO₂.

Cell viability assay

HepG2 cells were plated in 100 ml medium per well in 96-well plates. One day after seeding,

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cerulenin (0 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml and 40 µg/ml; Sigma-Aldrich, USA) was added in six replicates per concentration. Cell viability was measured after 12, 24, and 48 hour incubation with Cell Counting Kit-8 (Peptide Institute Inc., Osaka, Japan), and the inhibition rate was calculated. Enzyme linked immunosorbent assay (450 nm) was used to measure the absorbance (A) value, and the cell culture medium without cells was adjusted to zero. The cell relative inhibition rate (%) = (1-drug group mean A value/control group mean A value) × 100%.

Hoechst 33342/PI staining

HepG2 cells were inoculated in 6-well plates and cultured overnight. After cell adhesion, cells were cultured by medium containing different concentrations of cerulenin (5 µg/ml, 10 µg/ml and 20 µg/ml). After 48 hour incubation, cells were fixed for 10 minutes, and then washed 2 times by PBS. 5 min after adding Hoechst 33342/PI staining (Sigma-Aldrich, USA), cells were observed by fluorescence microscopy and photographed. Apoptosis rate (%) = (apoptotic cell number/total cell number) × 100%.

Annexin V/PI assay

HepG2 cells were plated in six-well plate. Cells of blank group, normal saline-treated control group and cerulenin-treated (5 µg/ml, 10 µg/ml and 20 µg/ml) group after 48 hour incubation were collected, washed in cold PBS twice and then the cells were mixed in 100 µl of 1 × binding buffer and incubated with an Annexin V/PI double-staining solution (Sigma-Aldrich, USA) at room temperature for 15 minutes. The stained cells were analyzed by flow cytometry and the percentage of apoptotic and necrotic cells were calculated with ModFitLT software (Verity Software House, Topsham, ME, USA). The percentage of apoptotic and necrotic cells were calculated.

Western blot assay

Sample proteins were separated on a 10% polyacrylamide gel electrophoretically and then transferred on to a PVDF membrane. The membranes were blocked with 5% milk solution prepared in PBS for 60 min. The membranes were then incubated with suitable dilutions of

the primary antibodies (FASN, Bax, Bcl-2, Caspase-3, 8 and 9) (Santa Cruz, CA, USA) at 4°C overnight. The membranes were then washed with PBST three times and then incubated with the peroxidase conjugated secondary antibody at a dilution of 1:1000 for 45 minutes. The membranes were washed with PBST three times again, and was developed using the ECL-detection system, quickly dried, and exposed to ECL film. β-actin was used as an internal standard.

Animals

Specific pathogen free female nude mice (athymic, Balb/c nu/nu) aged 5 weeks (15-16 g; Slac, Shanghai, China) were housed in individual ventilated caging system at 23±5°C with 12-hour cycled light and dark environment, being allowed free access to sterilized water and food. The experimental protocol was approved by the guidelines of Animal Care and Use Committee of Soochow University.

In vivo experiment

HepG2 cells were injected subcutaneously into the right anterior armpit of nude mice to establish an animal model of transplanted tumors. HepG2 cells were resuspended in serum-free RPMI-1640. The cell suspension was then injected subcutaneously (5×10^7 cells; total volume 0.5 mL) into the nude mice. Then mice were divided into two groups: normal saline-treated control group, cerulenin-treated group. Five days after the transplantation, cerulenin (80 mg/kg) was administered intraperitoneally daily until the end of the study. Four weeks after the transplantation, mice were sacrificed and the tumor tissues were collected and stored at -80°C for RNA and protein extraction.

Immunohistochemistry

After dewaxing and hydration, paraffin sections were boiled in citrate for antigen retrieval. Endogenous peroxidase activity was blocked by 3% H₂O₂ for 15 minutes at room temperature. The slides were blocked by incubating in 5% bovine serum albumin (BSA) at 37°C for 30 minutes. Sections were then incubated with primary antibodies against FASN (Santa Cruz, CA, USA) and PCNA (Novusbio, CO, USA) overnight at 4°C. The slides subsequently proceeded to the protocol of GTVision™ III Detection

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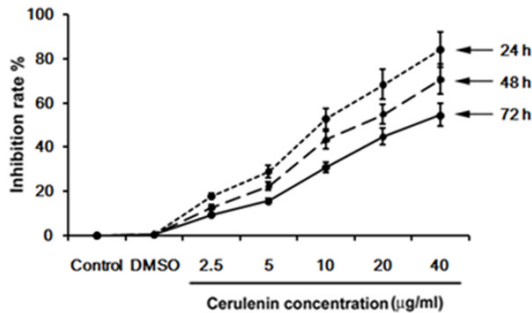


Figure 1. Cerulenin reduces viability of HepG2 cells in a dose- and time-dependent manner, assessed by CCK-8 kit assay. Growth inhibition rates of SW-1990 cells were counted as mentioned in Methods section. The data represent mean \pm SE.

System/Mo&Rb (Genetech, China), followed by counter staining with hematoxylin. Cell death was analyzed using the *in situ* cell death detection kit for TUNEL assay (Beyotime, China). Positive immunohistochemistry signals were shown in brown. For nucleus positive cells, the percentage of positive cells was calculated in each image. For cytoplasm or cytomembrane positive cells, area of positive reactions was analyzed by Image J software. A negative buffer replaced the primary antibody.

Statistical analysis

Data are expressed as mean \pm standard error (SE) and were analyzed using SPSS PC version 18.0 (SPSS Inc, Chicago, Ill, USA). Statistical analysis was performed using one-analysis of variance (ANOVA) followed by SNK tests as post hoc test. Kruskal-Wallis test was used to evaluate the differences of categorical values followed by Mann-Whitney *U* tests as post hoc test. The criterion of significance was a *p* value of less than 0.05.

Results

Survival and growth inhibition of HepG2 cells was measured with or without cerulenin treatment, using the CCK-8 kit assay. The results show that the cell growth was inhibited in a dose- and time-dependent manner (**Figure 1**). Results of Hoechst 33342/PI assay showed that both apoptosis and necrosis of HepG2 cells were observed and increased in a dose-dependent manner, 48 hours after the co-culture of cerulenin, compared with the normal saline treated control (**Figure 2**). A similar phenomenon was found in Annexin V/PI analysis (**Figure 3**). Western blot analysis showed that

the expression of FASN protein was decreased in a dose-dependent manner, 48 hours after the co-culture of cerulenin. The change of Bcl-2 protein expression increased after cerulenin treatment in HepG2 cells, while the changes of Bax, caspase-3, -8 and -9 protein expression had an opposite pattern (**Figure 4**). These results suggest that cerulenin had a cytotoxic effect and promote apoptosis in HepG2 cells. According to the results of the *in vivo* experiment, treatment of cerulenin exerted a beneficial effect to the hepatic cancer in terms of the decreased tumor volume and weight, during the whole observation period (**Figure 5**). Immunohistochemical staining showed that the expression of FASN protein was decreased by cerulenin treatment *in vivo*. PCNA was stained to evaluate cell proliferation, and the result showed that the expression of PCNA was significantly decreased in the cerulenin group compared to the control group. TUNEL assay was applied to evaluate the cell apoptosis in transplanted tumor. Apoptosis was increased after cerulenin, compared with the normal saline treatment (**Figure 6**).

Discussion

Even in a nutrient starvation state, cancer cells have a high potential for proliferation and survival, which may be based on a high potential for cellular fatty acid synthesis [10]. Synthesized from acetyl-CoA and malonyl-CoA, fatty acids are aliphatic acids fundamental to energy production and storage, cellular structure and as intermediates in the biosynthesis of hormones and other biologically important molecules [11]. Endogenous fatty acids are the main source of fatty acids which are required for the growth of many cancer cells. Fatty acid metabolism of tumor cells is different from that in the normal tissue cells, which depends on the fatty acid synthase (FASN)-based synthesis of fatty acids in order to meet the needs of the cancer cells' division and proliferation [12]. Fatty acid synthase (FASN) is a key enzyme in the biosynthesis of fatty acids during the polymerization of long chain fatty acids with small molecular carbon units. The gene that codes for FASN has been investigated as a possible oncogene. In normal tissues, no expression or low expression of FASN was found, except in liver, uterus, lactating breast tissue. However, high expression of FASN was found in many tumors such as breast cancer, prostate cancer, uterine cancer,

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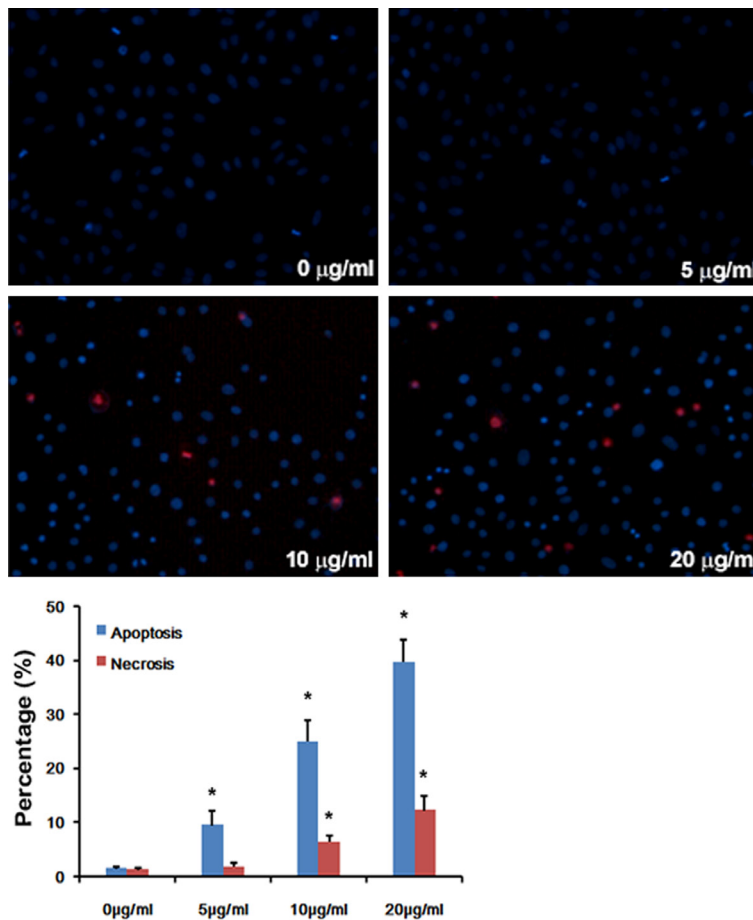


Figure 2. Apoptosis of HepG2 cells were measured by Hoechst33342/PI staining. Apoptosis of HepG2 cells was observed and increased in a dose-dependent manner 48 hours after cerulenin treatment. The data represent mean \pm SE. * $p < 0.05$ versus control.

colorectal cancer, ovarian cancer, melanoma and multiple myeloma [5-8, 13, 14]. High expression of FASN was reported to be related with tumor development, pathological grade, clinical degree of malignancy and poor prognosis. High expression of FASN found in the tumors will contribute to the synthesis of high amounts of fatty acids and high cell viability under nutrient starvation [15]. Recent studies suggest that the inhibition of FASN could be a therapeutic target of some tumors [16]. As an antifungal antibiotics, cerulenin inhibits fatty acid and steroid synthesis. It can bind to β -ketoacyl-ACP synthase in fatty acid synthesis, blocking the interaction of malonyl-CoA, and is also able to stimulate fatty acid oxidation through the activation of CPT1, another enzyme normally inhibited by malonyl-CoA. These two behaviors may increase the availability of energy in the form of ATP. Therefore, cerulenin is often

applied as an inhibitor of FASN in experiments recently [17, 18]. In the present study, the expression of FASN was observed in HepG2 cells, and cerulenin, a FASN inhibitor, could reduce the FASN expression. Then, the effect of cerulenin on HepG2 cells was studied *in vitro* and *in vivo* in this study.

In *in vitro* experiments, cerulenin displayed an obvious inhibitory effect on the proliferation of HepG2 cells in a dose-dependent manner. Hoechst 33342/PI staining revealed the characteristic features of apoptosis in cerulenin-treated HepG2 cells, represented as chromatin condensation and the formation of apoptosis body. Annexin V/PI analysis also showed that cerulenin could induce the apoptosis of HepG2 cells. These results indicate that cerulenin can induce cell apoptosis in a dose-dependent manner, together with an increase of expression of caspase-3, -8 and -9 resulted from cerulenin treatment. Pro-apoptosis is a piv-

otal mechanism for a large number of chemotherapeutic agents, in which process the functions of Bcl-2 (an anti-apoptotic factor) and Bax (a pro-apoptotic factor and natural antagonist of Bcl-2) are well investigated. As a major inhibitor of apoptosis, Bcl-2 can promote the formation of tumor by resisting various forms of cell death, prolonging cell life and increasing the number of cells in tumor [19, 20]. In this study, down-regulation of Bcl-2 and the up-regulation of Bax may be a plausible reason for the pro-apoptotic effect of cerulenin treatment. Therefore, cerulenin may have the anti-proliferative and pro-apoptotic effects on hepatic cancer cells, through its inhibition of FASN. In other words, FASN is essential for the proliferation and survival of hepatic cancer, suggesting that depletion of fatty acids is fatal for hepatic cancer cells. It has been reported that FASN inhibition reduces cell proliferation by blocking

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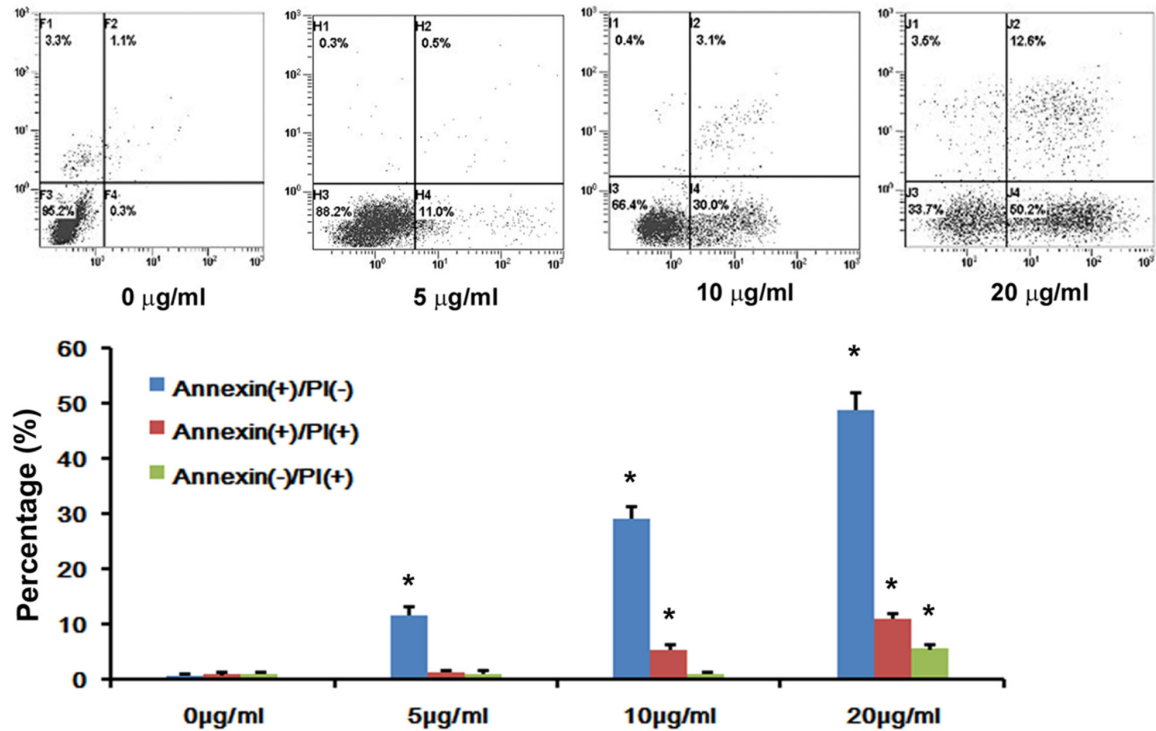


Figure 3. Apoptosis of HepG2 cells were measured by Annexin V/PI assessment. Apoptosis of HepG2 cells was increased in a dose-dependent manner 48 hours after cerulenin treatment. The data represent mean \pm SE. * $p < 0.05$ versus control.

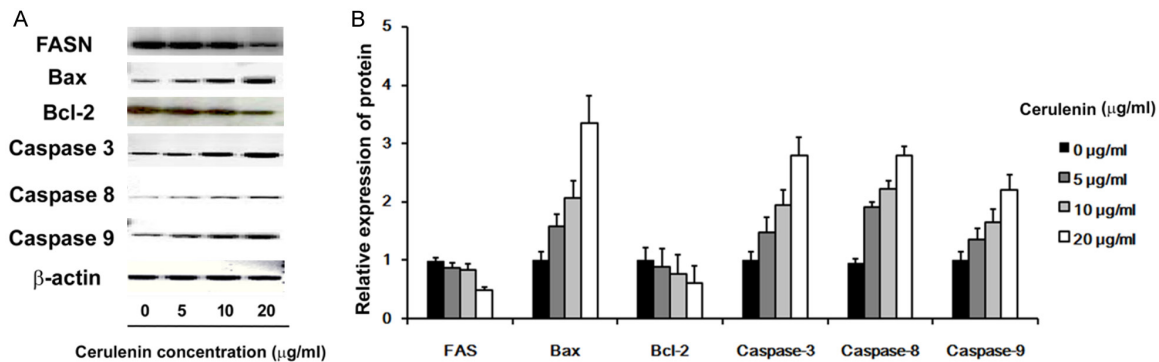


Figure 4. The changes of FASN and apoptosis related proteins expression in HepG2 cells following cerulenin treatment. A, B: Expression of FASN, Bax, Bcl-2, caspase-3, caspase-8, and Caspase-9 proteins in HepG2 cells 48 hours after cerulenin treatment were detected by Western blot analysis. The data represent mean \pm SE. * $p < 0.05$ versus control.

DNA replication during S-phase [21]. However, the biological mechanisms responsible for the FASN inhibition-induced apoptosis are still not clear. The extrinsic pathway of apoptosis triggered by death domains was described in breast cancer cells because of the accumulation of malonyl-CoA and ceramide after FASN silencing with siRNA [22]. Mitochondrial involvement, evidenced by increased levels of the

pro-apoptotic protein Bax and cytochrome c release, was shown in tumor cell lines following pharmacological FASN inhibition [23]. Despite the fact that the expression of a dominant-negative mutant p53 increases sensitivity of colon carcinoma cells to FASN inhibitors, and FASN inhibition-induced apoptosis was described as p53 independent [24]. Intraperitoneal cerulenin treatment of nude mice with xenografts of

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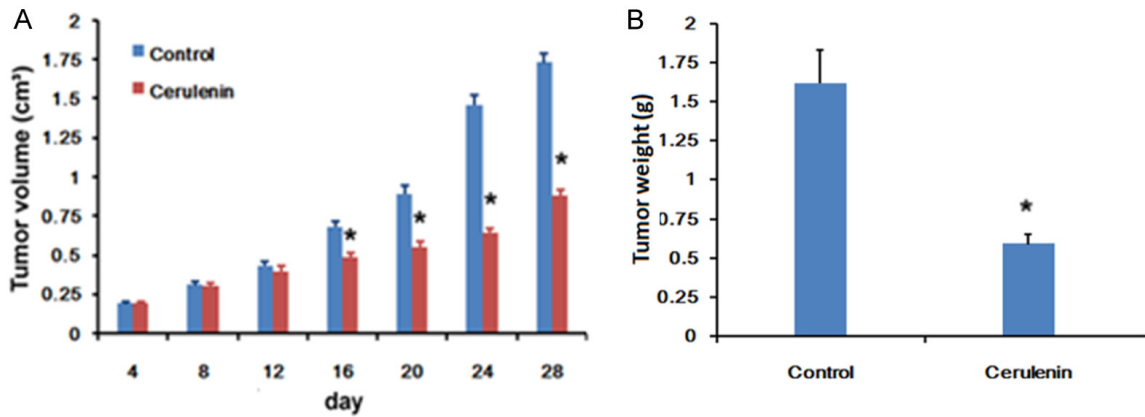


Figure 5. Inhibitory effect of the combined treatment with ceruleinin on the growth of xenografts of hepatic cancer cells in nude mice. A. Tumor volume changes in nude mice after ceruleinin treatment. B. Tumor weights were measured 4 weeks after ceruleinin treatment. Data are shown as mean \pm SE. * $P < 0.05$ versus NS-treated mice.

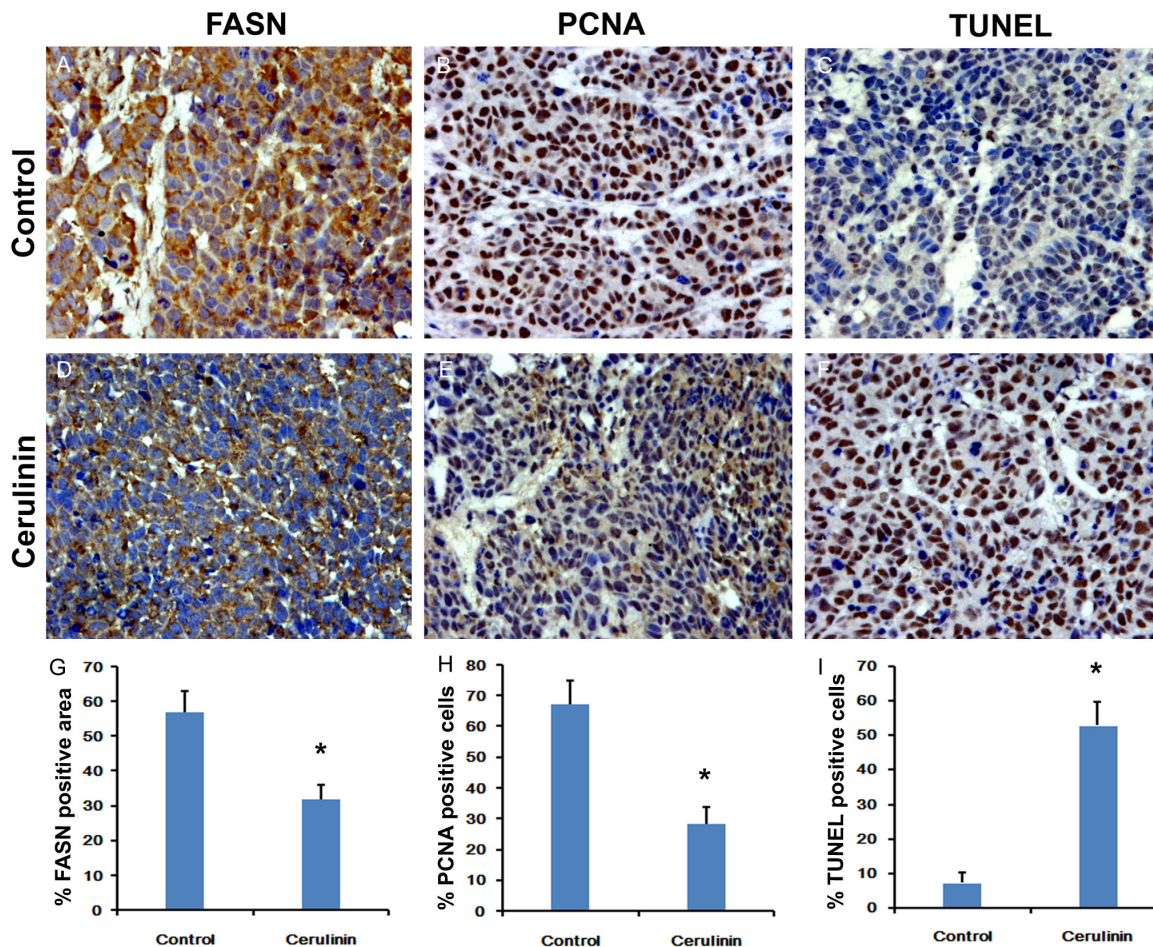


Figure 6. Expression of FASN, apoptosis and acinar regeneration in the transplanted tumors were assessed as described in the Method section 4 weeks after ceruleinin treatment. A, D. Representative histochemical-stained sections of FASN. B, E. Representative immunohistochemical staining of PCNA. C, F. Representative TUNEL-stained sections of apoptotic cells. G-I. Alterations of FASN-positive cells, PCNA-positive cells and TUNEL-positive cells in the transplanted tumors after ceruleinin treatment. Original magnification $\times 400$; data are presented as mean \pm SE; * $p < 0.05$.

hepatic cancer cells was also studied in the present study. The *in vivo* experiment revealed that expression of FASN in tumor tissue was reduced after cerulenin treatment. Furthermore, cerulenin could inhibit growth of the transplanted tumor and could also regulate proliferation and apoptosis, as evidenced by the results of the histochemical staining of PCNA and TUNEL.

In conclusion, cerulenin may be an anti-proliferative and pro-apoptosis agent for hepatic cancer cells *in vitro* and *in vivo* by down-regulating FASN expression and inhibiting intracellular FASN activity. Our results suggest that cerulenin could be applied in treatment of liver cancer. There are a lot of reports of the application of cerulenin in cancer treatment experiments, which may supply useful ideas and new clues in developing target-directed anti-cancer drugs for further studies.

Disclosure of conflict of interest

None.

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