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Original Article

Mechanism of the promotion of steatotic HepG2 cell apoptosis by cholesterol

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Abstract: The role of cholesterol in the pathogenesis of non-alcoholic steatohepatitis (NASH) remains unclear. It is known that apoptosis of hepatocytes is an important characteristics of NASH. The objective of this study was to investigate the effects of cholesterol on steatotic HepG2 cell apoptosis and the possible mechanism *in vitro*. In this study, HepG2 cells were divided into three groups: (1) normal group, (2) steatosis group and (3) cholesterol group. HepG2 cells were treated with oleic acid to establish a steatosis study model. Steatosis was assessed by Oil Red O staining and triglyceride content assay. Cell apoptosis was measured using an apoptosis kit. The expression levels of apoptosis-related proteins (P53, Bcl-2, Bax, caspase-3, cyclin A, cyclin B1 and cyclin E) were determined by western blot analyses. We found that a hepatocyte steatosis model was successfully established by oleic acid (200 μmol/L) induction. The cholesterol (50 mg/L) group had similar amount of lipid droplets and triglyceride content as steatosis group ($P > 0.5$). However, the apoptosis rate ($P < 0.01$) of the cholesterol group was significantly higher than that of the normal group or the steatosis group, and the protein expressions of Bax and caspase-3, but not P53, Bcl-2, cyclin A, cyclin B1 and cyclin E, were also increased in the cholesterol group. Those results suggested that cholesterol markedly promoted apoptosis of steatosis HepG2 cells *in vitro*, likely through the up-regulation of Bax and caspase-3 expression. This study contributes to explain the effect of cholesterol on NASH pathogenesis.

Keywords: Cholesterol, HepG 2 cells, apoptosis, steatosis

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common form of chronic liver disease in the world [1, 2]. Its incidence will increase. In the US, the prevalence of NAFLD is expected to increase by 50% by 2030 [3]. NAFLD encompasses a wide spectrum of conditions associated with the over accumulation of lipids in the liver, ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH) characterized by the accumulation of fat in the liver, along with liver cell damage, inflammation, and various degrees of scarring or fibrosis [4]. But the pathogenesis of NAFLD has not been completely clarified. The most widely accepted model to explain the development of NAFLD and the progression from simple steatosis to NASH is the "two-hit hypothesis". The "first hit" is the accumulation of lipids in the hepatocytes, and insulin resistance is the key pathogenic factor for the development of hepatic steatosis. The "second hit" leads to hepatocyte injury, inflammation and fibrosis. Factors initiating the second hit are oxidative stress and subsequent lipid peroxidation, proinflammatory cytokines, adipokines and mitochondrial dysfunction [5].

Recently, Puri et al. have found a stepwise increase in hepatic cholesterol as disease progresses from steatosis to steatohepatitis [6]. Animal studies have also shown that high-fat cholesterol diet can lead to NASH [7-10]. These data suggest that cholesterol maybe one of the factors initiating the "second hit". But the mechanism of cholesterol-induced NASH is not clear.

A growing body of evidence suggests that hepatocyte apoptosis is a critical mechanism in NASH pathogenesis [11, 12]. Increase in hepat-

ocyte apoptosis is typical in humans and animal models of NASH, but absent in those with hepatic steatosis [13]. In this study, first, we investigated the effect of cholesterol on steatotic HepG2 cell apoptosis. Second, to identify the possible mechanism of the effect of cholesterol on NASH, we measured the expression of apoptosis-related proteins in steatotic HepG2 cells.

Materials and methods

Reagents and antibodies

Cholesterol, oleic acid and Oil Red O were purchased from Sigma (St. Louis, MO, USA). The TG assay kit was obtained from Bioassay Systems (Hayward, CA, USA). The apoptosis kit was purchased from Beijing Bao Sai company (Beijing, China). Anti-P53 antibody was purchased from Calbiochem (Beijing, China). Anti-Bax antibody was purchased from Neomarkers (Beijing, China). Anti-Bcl-2 antibody was purchased from Zymed (Beijing, China). Anti-caspase-3 antibody was purchased from Santa Cruz (Beijing, China). Anti-cyclin (A, B1 and E) antibody was purchased from Life Technologies (Shanghai, China). Anti-GAPDH antibody was purchased from Marine Biological Laboratory (Beijing, China). All other reagents were of analytical grade.

Cell culture and treatments

HepG2 cells were preserved in the State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, and cultured in Dulbecco's Modified Eagle's medium (DMEM) (Hyclone, Boston, MA, USA) supplemented with 10% fetal bovine serum, penicillin (50 U/mL) and streptomycin (50 μg/mL) (Hyclone, Boston, MA, USA) at 37°C in an atmosphere of 5% CO_2. The cells were divided into three groups: the normal group, the steatosis group and the cholesterol group. For the normal group, HepG2 cells were cultured in DMEM for 48 h. For the steatosis group, HepG2 cells were cultured in DMEM plus 200 μmol/L oleic acid for 24 h, and then cultured in DMEM for 24 h. For the cholesterol group, HepG2 cells were cultured in DMEM plus 200 μmol/L oleic acid for 24 h, and then cultured in DMEM plus 50 mg/L cholesterol for 24 h.

Oil Red O staining

HepG2 cells were grown on a cover slip, washed with phosphate-buffered saline (PBS) and then fixed with 10% formalin solution for 5 min at room temperature. After fixation, cells were washed gently with 60% isopropanol and stained with the working solution of 0.5 g Oil Red O in 60% isopropanol for 30 min. The stained hepatocytes were washed with distilled water several times to remove unincorporated dye. Then, the samples were counterstained with hematoxylin for 1 min. Slides were examined using a light microscope.

Intracellular lipid content assessment

HepG2 cells were trypsinized and transferred into an Eppendorf tube (1.5 mL) and centrifuged at 3,000 rpm for 5 min. Cell pellets were washed with PBS once, resuspended in 400 μL PBS buffer and transferred to a micro smashing tube for ultrasonication. After ultrasonication, the concentration of cellular TG was determined using an EnzyChrom™ triglyceride assay kit and normalized with protein concentration according to the protocol provided by the manufacturer.

Cell apoptosis assessment

HepG2 cell apoptosis was measured using an apoptosis kit. Briefly, HepG2 cells were trypsinized and centrifuged at 3,000 rpm for 5 minutes. The cell pellets were resuspended in 250 μl binding buffer (0.01 M HEPES, pH 7.4 containing 140 mM NaCl and 25 mM $CaCl_2$) supplemented with 2.5 μl Annexin V-FITC (5 mg/ml) and 0.5 μl PI (6 mg/ml). The cells were incubated at room temperature for 15 minutes in dark, and then analyzed using a flow cytometer within 1 h.

Western blot analysis

Western blot ananlyses were performed to detect protein expression in HepG2 cells. Briefly, cells were collected, washed and lysed in lysis buffer and heated in denaturing lysis buffer for 10 min. The lysate from each sample was separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. Then the membranes were blocked for 1 h at room temperature with nonfat dry milk, and subsequently probed with primary antibodies against P53, Bax, Bcl-2, caspase-3, cyclin (A, B1 and E) and GAPDH for 24 h at 4°C. After three washes in TBST, the blots were incubated with secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Blots were development with enhanced chemilumines-

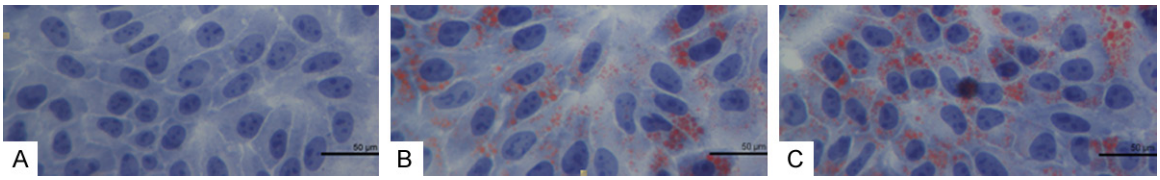

Figure 1. Intracellular lipid droplets in HepG2 cells stained with Oil Red O. No lipid droplets were observed in HepG2 cells in the normal group (A); Lipid droplets were present in HepG2 cells in the steatosis group (B) and the cholesterol group (C). There was no difference in lipid drops between the steatosis group and the cholesterol group.

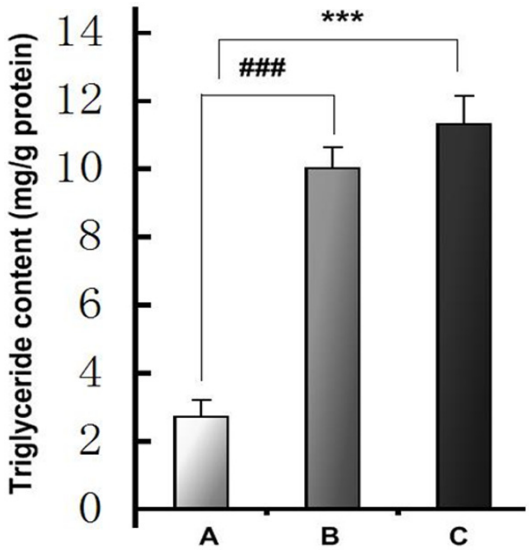

Figure 2. The effect of cholesterol on TG accumulation in steatotic HepG2 cells in normal group (A), steatosis group (B) and cholesterol group (C). TG concentration was normalized with protein content. ###P < 0.01 (steatosis group compared with normal group), ***P < 0.01 (cholesterol group compared with normal group). Experimental procedures are described in the Materials and Methods section. Data are expressed as means ± SD (n = 6).

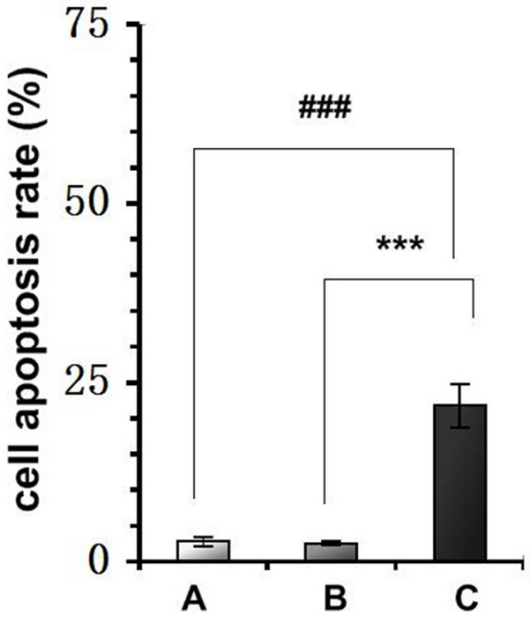

Figure 3. The effect of cholesterol on cell apoptosis rates in HepG2 cells in the normal group (A), the steatosis group (B) and the cholesterol group (C). ###P < 0.05 (cholesterol group compared with normal group), ***P < 0.05 (cholesterol group compared with steatosis group). Experimental procedures are described in the Materials and Methods section. Data are expressed as means ± SD (n = 6).

cence reagents. GAPDH was used as an internal control.

Statistical analysis

Data are shown as mean ± SD. The significance of differences was determined by t-test using the SPSS 17.0 software (SPSS, Chicago, IL, USA). A value of P < 0.05 was considered statistically significant.

Results

Establishment of the steatotic hepatocyte model and the effect of cholesterol on hepatocyte steatosis

To evaluate the effect of cholesterol on steatotic hepatocyte apoptosis, we first used oleic acid to establish a steatotic hepatocyte model. Oil Red O staining showed that 200 μmol/L oleic acid treatment for 24 h induced the accumulation of lipid droplets in almost all HepG2 cells (**Figure 1B**). There were no visible lipid droplets in HepG2 cells in the normal group (**Figure 1A**). Triglyceride assay showed that the triglyceride contents in HepG2 cells treated with 200 μmol/L oleic acid for 24 h were significantly increased compare with that in the normal group (P < 0.05, **Figure 2**). These data suggested that 200 μmol/L oleic acid successfully induced steatosis in HepG2 cells. Additionally, we found that 50 mg/L cholesterol neither enhanced oleic acid-induced lipid droplet accumulation in HepG2 cells (**Figure 1C**), nor increased oleic acid-induced triglyceride con-

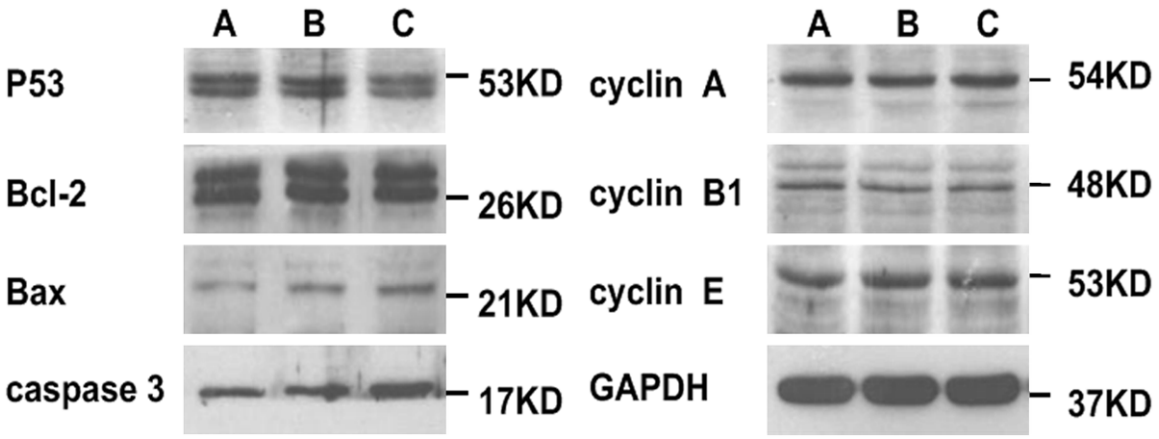

Figure 4. The effect of cholesterol on the expressions of proteins (p53, Bcl-2, Bax, caspase 3, cyclin A, cyclin B1 and cyclin E) involved in cell apoptosis in the normal group (A), the steatosis group (B) and the cholesterol group (C). Experimental procedures are described in the Materials and Methods section. Data are expressed as means ± SD of six independent experiments.

tent in HepG2 cells ($P < 0.05$, **Figure 2**), which suggested that cholesterol failed to aggravate the degree of steatosis.

Cholesterol increase steatotic HepG2 cell apoptosis

Cell apoptosis assessment showed that the percentage of apoptotic cells in normal group, steatosis group and cholesterol group was 2.8%, 2.5% and 21.8%, respectively. The percentage of apoptotic cells in the cholesterol group was significantly higher than that of the normal group or the steatosis group ($P < 0.01$, **Figure 3**). These data suggested that cholesterol promoted apoptosis of steatotic HepG2 cells.

Regulation of apoptosis by cholesterol

To elucidate the possible mechanism of the effect of cholesterol on steatotic hepatocyte apoptosis, we next examined the expressions of proteins involved in cell apoptosis. Western blot analysis showed that the protein expressions of Bax and caspase-3 in HepG2 cells in the cholesterol group were increased compared with those in the normal group or in the steatosis group, but the protein expressions of P53, Bcl-2, cyclin A, cyclin B1 and cyclin E were not different among the three groups (**Figure 4**). This suggested that the up-regulation of Bax and caspase-3 played an important role in cholesterol-induced steatotic HepG2 cell apoptosis.

Discussion

NAFLD mainly encompasses simple steatosis and NASH. Although simple steatosis is characterized by a relatively favorable clinical course, NASH progresses much more frequently to cirrhosis and hepatocellular carcinoma [14]. Exploring the risk factors and the mechanism of NASH has important clinic significance in the prevention of NASH-related cirrhosis. Recent studies have shown that the accumulation of cholesterol resulted from hepatic cholesterol homeostasis is central to the pathogenesis of NASH in mice and in human [15, 16]. Animal studies have confirmed that dietary cholesterol can increase hepatocyte apoptosis in NAFLD [7]. In the present study, we first established a steatotic hepatocyte model using oleic acid, and then investigated the effect of cholesterol on steatotic hepatocyte apoptosis. We found that cholesterol increased steatotic hepatocyte apoptosis. These data suggested that cholesterol-induced apoptosis of steatotic hepatocytes might be one of the important mechanisms of NASH pathogenesis.

Apoptosis is a physiological suicide mechanism that occurs during normal tissue turnover [17], and plays an important role in tumor formation and progression. Apoptosis is a complex process involving multiple genes, and the most important genes are the tumor-suppressor gene p53 and the B-cell lymphoma leukemia-2 (bcl-2) gene family [18]. The TP53 gene is located at chromosome 17p13.1. It induces cell apoptosis in response to DNA damage, and its

inactivation leads to uncontrolled cellular proliferation [19]. P53 is a crucial transcription factor that controls the cell cycle and apoptosis of cells under genotoxic stresses. It is capable of activating the transcription of hundreds of genes by binding to specific sequences at their promoters [20]. The Bcl-2 gene is at a breakpoint of a chromosomal translocation event (t14: 18) that occurs in human B-cell lymphomas. The overproduction of bcl-2 prolongs cell survival upon classical apoptotic stimuli, and the protein is considered to be a suppressor of apoptosis [21]. Bax belongs to the Bcl-2 family of proteins, and is a key player in apoptosis. It accelerates cell death after an apoptotic stimulus. Caspase proteins are cysteine proteases, including initiator caspases and effector caspases. Initiator caspases serve to activate effector caspases. Among the effector caspases, caspase-3 is most frequently involved in neuronal apoptosis. Acting upstream of Bax, P53 may promote cell death [22]. P53 may also induce apoptotic cell death by down-regulating bcl-2. Acting downstream of Bcl-2, caspases can initiate cellular breakdown during apoptosis [23]. Recent studies have shown that a high-fat-cholesterol diet increases hepatic Bax but decreases Bcl-2 in rats with NASH [24]. Caspase-3 is markedly increased in the livers of patients with severe NASH but not in those with simple steatosis [25, 26]. It has also been shown in an animal study that caspase-3 inhibitors protect cholesterol-loaded hepatocytes [27]. In the present study, we found that cholesterol increased the expressions of Bax and caspase-3 proteins, but not p53 and Bcl-2 proteins in steatotic HepG2 cells. These data suggested that the cholesterol-induced Bax upregulation and the possible secondary caspase-3 upregulation may be important mechanisms of cholesterol-induced steatotic hepatocyte apoptosis. Cholesterol-induced Bax increase in steatotic hepatocytes is not likely dependent on the p53 pathway. Studies have shown that some of the upstream molecules, including the BH3-only proteins [28], the glycogen synthase kinase 3β [29] and the hypoxia-inducible factor-1 [30], affect the expression and function of the Bax protein. Further studies are needed to understand the roles of the upstream molecules of Bax in cholesterol-induced hepatocyte apoptosis.

Recent studies have suggested that apoptosis is frequently associated with proliferating cells, implying that the activities of factors in late G1 and S phases facilitate the apoptotic process. Once the cells are committed to cell death, apoptogenic factors, including cytochrome c, are released from mitochondria to initiate a caspase cascade [31]. It has been confirmed that cyclin A, B1 and E play important roles in cell apoptosis [31-34]. Our study showed that cholesterol had no effect on the protein expression of cyclin A, B1 and E in hepatocytes. These results suggested that cholesterol-induced apoptosis in hepatocytes is not depend on cyclin A, B1 or E.

In conclusion, in this study, we provide direct evidence for cholesterol induced apoptosis of seatotic HepG2 cells *in vitro*, which contributes to explain the effect of cholesterol in NASH pathogenesis. This study also demonstrates that the up-regulation of Bax and caspase-3 play important roles in the mechanism of cholesterol-induced apoptosis in seatotic HepG2 cells. However, the upstream molecules affecting Bax need to be further elucidated.

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Disclosure of conflict of interest

None.

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