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Glycochenodeoxycholic Acid Induces Cell Death in Primary Cultured Rat Hepatocyte: Apoptosis and Necrosis

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Intracellular accumulation of bile acids in the hepatocytes during cholestasis is thought to be pathogenic in cholestatic liver injury. Due to the detergent-like effect of the hydrophobic bile acids, hepatocellular injury has been attributed to direct membrane damage. However histological findings of cholestatic liver diseases suggest apoptosis can be a mechanism of cell death during cholestatic liver diseases instead of necrosis. To determine the pattern of hepatocellular toxicity induced by bile acid, we incubated primary cultured rat hepatocytes with a hydrophobic bile acid, Glycochenodeoxycholate (GCDC), up to 5 hours. After 5 hours incubation with 400 μM GCDC, lactate dehydrogenase released significantly. Cell viability, quantitated in propidium iodide stained cells concomitant with fluoresceindiacetate was decreased time- and dose-dependently. Most nuclei with condensed chromatin and shrunk cytoplasm were heavily labelled time- and dose-dependently by a positive TUNEL reaction. These findings suggest that both apoptosis and necrosis are involved in hepatocytes injury caused by GCDC.

Key Words: Hepatocyte, GCDC, Apoptosis, Necrosis

INTRODUCTION

Cholestasis, the impairment of bile flow, is the common feature of many liver diseases (Scharschmidit, 1990). Intracellular accumulation of bile acids in the hepatocytes during cholestasis is thought to be pathogenic in cholestatic liver injury.

Most hydrophobic bile acids have detergent properties and are cytotoxic. In cholestasis, hydrophobic bile acids such as chenodeoxycholic acid (CDC), deoxycholic acid are mainly accumulated in hepatocytes whereas the amount of other hydrophilic bile acid is slightly changed (Greim et al, 1972). Hepatic tissue concentration of CDC, increased 20-fold during cholestasis, is much higher than those of other toxic bile acids. 95% of CDC in bile is conjugate to glycine or taurine during cholestasis. Glycine conjugates, glycochenodeoxycholic acid (GCDC), are generally more toxic than taurine conjugates (Scholmerich et al, 1984; Crosignani et al, 1991).

Due to the detergent-like effect of the bile acids, hepatocellular injury has been attributed to direct membrane damage, a feature of necrosis. However necrosis is not a prominent histological feature of cholestatic liver diseases. In contrast, cell dropout with acidophillic bodies, is frequently identified in human cholestatic liver diseases (Searle et al, 1987). These histological findings suggest that apoptosis is a mechanism of cell death during cholestatic liver diseases.

Thus, the purpose of this study was to determine the pattern of hepatocellular cell death induced by GCDC in primary cultured rat hepatocytes.

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METHODS

Isolation and culture of hepatocytes

Freshly isolated hepatocytes were prepared from adult male Sprague-Dawley rats weighing between 200 and 230 g. After animals were anesthetized with ketamine (10 mg/100 g body weight) via intraperi-

toneal injection, hepatocytes were isolated by two-step collagenase perfusion according to Seglen (Seglen, 1976). The liver was perfused with Ca^{2+}. Mg^{2+} free Hank's balanced salt solution (HBSS) and subsequently perfused with HBSS containing 0.05% collagenase (Type II, Gibco, Gaithersburg, MD, USA), 1% BSA, Ca^{2+} and Mg^{2+}. After excision, the liver capsule was removed and cells dispersed by gently agitation. The resulting cell suspension was filtered serially through a 50 mesh sieve (Sigma, St. Louis, MO, USA) and nylon meshes (Falcon, Franklin lakes, NJ, USA). Hepatocytes collected by centrifugation were purified by percoll (Amersham-pharmatiabiotech, NJ, USA) density gradient separation (Kreamer et al, 1986). They were plated at 5×10^5 cells/ml in 35 mm culture dishes (Falcon, Franklin lakes, NJ, USA) or 24 well culture plates (Falcon, Franklin lakes, NJ, USA) coated with collagen (0.1 mg/ml, Gibco, Gaithersburg, MD, USA) in Hepatocyte Attachment Medium (Gibco, Gaithersburg, MD, USA). After 2 hours (hr), unattached cells and medium were removed and replaced with serum free medium (Hepatozyme-SFM, Gibco, Gaithersburg, MD, USA).

Bile acid treatment

Hepatocytes were treated with GCDC(Sigma, St. Louis, MO, USA) up to 400 μM concentration because the intracellular concentration of CDC and its conjugates are elevated up to 800 μM at the state of cholestasis (Spivey et al, 1993). Hepatocytes were used after 2 hr of culture in 5% CO_2/95% air at 37°C. We chose to study hepatocytes after 2 hr in culture, because hepatocytes in culture are known to rapidly dedifferentiate that causes decrease of intrahepatic bile salt uptake in the long term cultures (Kwo et al, 1995).

Determination of cell viability

Simultaneous staining with fluorescein diacetate (FDA)-propidium iodide (PI): Cultured hepatocytes (5×10^5 cells/ml) were stained with PI (10 μg/ml) and FDA (15 μg/ml) for 3 min at a time. Fluorescence was visualized using 450 nm excitation and 520 nm emission filters under a phase contrast inverted fluorescence microscope (DMIRB, Leica, Germany). At least 200 cells were counted each time. Cell viability was expressed as a percentage of FDA stained viable cells over the total cells stained with FDA or PI.

$$\% = \frac{\text{FDA stained cells}}{\text{FDA stained cells} + \text{PI stained cells}} \times 100$$

And these values were expressed as a percentage of the control group.

Measurement of lactate dehydrogenase (LDH) release: Extracellular released LDH activity was measured using a cytotoxicity Detection Kit (Boehringer Mannheim, Mannheim, Germany). At the end of the incubation period, total celluar LDH content was determined using the detergent Triton X-100 (1% in hepatozyme-SFM, 30 min, at room temperature) to induce 100% lysis. Released LDH activity was presented as percent of total cellular LDH content and these values were transformed as a percentage of the control group.

TUNEL assay

To evaluate the extent of nuclear DNA fragmentation, a terminal deoxynucleotidyl transferase-mediated dUTP-nick end-labelling (TUNEL) method was performed. Briefly, cultured hepatocytes are fixed in 4% paraformaldehyde in PBS, pH 7.4. To inactivate endogenous peroxidase, 2% H_2O_2 was added to the cells. The cells were washed and then labelled for 1 hr at 37°C with biotinylated-dUTP in a 50 μl reaction buffer containing 30 mM Trizma base (pH 7.2), 140 mM sodium cacodylate, 1 mM cobalt chloride, 0.05 units of TdT enzyme, and 0.01 nmol biotinylated-dUTP. The reaction was terminated by transferring slides to TB buffer (300 mM NaCl, 30 mM sodium citrate). Follwing washing, the cells were incubated with PBS containing 2% BSA for 10 min. And then incubated with extra-avidin peroxidase (for 30 min at 37°C). The cells were stained with DAB (0.05% DAB, 0.01% H_2O_2) and counterstained with mayer hematoxilin (Merck, Darmstadt, Germany). Following washing, labelled cells are visualized by an inverted fluorescence microscopy.

Statistical analysis

All the data are summerized as mean$\pm$S.E.. To analyze the data statistically, we performed a one-way analysis of variance (ANOVA). We then used Dunnett's multiple range *t*-test to determine which means were significantly different from the mean of the control group. We considered differences significant at $p < 0.05$.

RESULTS

The evidences of necrosis

Cytolysis was evaluated by measurement of LDH release into the culture medium. After 1 hr incubation with ascending concentration of GCDC (0, 50, 100, 200, 400 μM), LDH released up to 103±3.07%, 105.85±3.44%, 104.08±3.29%, 110.12±4.04% respectively comparing with control group. LDH released up to 106.49±2.78%, 108.33±2.90%, 116.84 ±2.71%, 127.75±3.61% after 3 hr incubation with GCDC and up to 113.23±3.14%, 120.56±3.59%, 134.77±3.75%, 147.95±4.27% after 5 hr incubation (Fig. 1). After 1 hr or 3 hr incubation with ascending concentration of GCDC, no significant release of LDH was noted at all doses. Whereas after 5 hr incubation with at 400 μM concentration the enzyme released significantly up to 147.95±4.27% ($p<0.05$).

Cell viability was quantitated in PI stained cells concomitant with FDA after incubation of hepatocytes with GCDC (Fig. 2). Because PI is a membrane impermeable dye, PI stained nuclei were considered necrotic cell death. GCDC induced cell death in hepatocytes was dose- and time- dependent. After 1 hr incubation with GCDC, only at the concentration of 400 μM cell viability was decreased significantly to 76.49±4.39% comparison with control ($p<0.05$). Whereas After 3 hr incubation, at the higher concentrations (100, 200, 400 μM) cell viability was decreased significantly ($P<0.0001$) to 81±2.82%, 73.79 ±2.93%, 72.73±9.72% respectively (Fig. 3). At the end of 5 hr incubation, also cell viability was decreased significantly ($P<0.05$) to 74.99±3.73%, 64.66 ±2.89%, 59.09±3.02% respectively (100, 200, 400 μM).

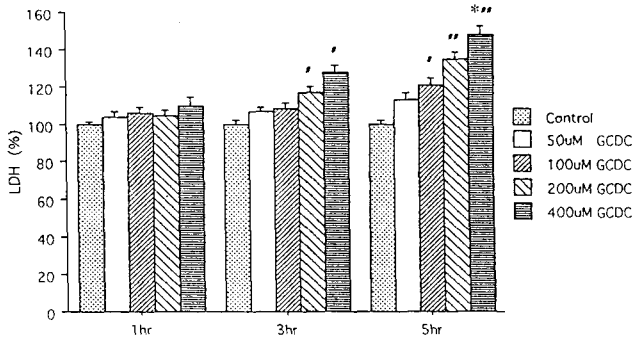

Fig. 1. Release of lactate dehydrogenase (LDH) after incubation with 50 to 400 μM Glycochenodeoxycholic acid (GCDC) for 5 hr. Results are expressed as a percent of the control group (mean±SE). Significant increase of LDH was noted at GCDC 400 μM after 5 hr (*$p<0.05$). LDH released significantly in time- dependent manner compared with 1 hr ($^{\#}p<0.05$, $^{\#\#}p<0.0001$).

The evidences of apoptosis

Most nuclei with condensed chromatin and shrunk cytoplasm were heavily labelled by a positive TUNEL reaction. GCDC induced apoptotic cell death was time- and dose-dependent. After 1 hr and 5 hr incubations with GCDC, condensed chromatin and shrunk cytoplasm were observed at all doses in a dose-dependent manner while no morphological changes were observed in control (Fig. 4).

DISCUSSION

Cholestasis defined as an accumulation in hepatocytes caused by intrahepatic or extrahepatic diseases. Hepatic concentration of toxic bile acids increase during cholestasis and chenodeoxycholic acid (CDC) is one of the major toxic bile acids. CDC is mostly conjugated to glycine or taurine in bile during cholestasis. Glycine conjugate, GCDC is known for the most toxic bile acid among hydrophbic bile acids (Patel et al, 1994). The hepatic tissue concentration of CDC in cholestasis is 415 nmol/g liver tissue. Assuming 1×10^8 hepatocytes/g wet weight and 5 μl of water/10^6 hepatocytes, the intracellular concentration of CDC and its conjugated species are under 800 μM based on measurements of hepatic tissue concentration of CDC in cholestasis. Although hepatic tissue consists predominantly of hepatocytes, it also contains bile canaliculi and bile ducts leading to an overestimate of the hepatocellular bile salt concentration. Therefore, we were especially interested in the toxicity of bile salt concentrations below this value.

Over the range of 0~400 μM of GCDC, cell viability that measured by staining of PI or FDA was decreased time- and dose-dependently in our study. These results demonstrate that GCDC cytotoxicity in primary cultured rat hepatocytes occurs at bile acid concentrations probably relevant to those found within the hepatocyte in human disease. Botla et al (1995) demonstrated that GCDC decreased cell viability in a time dependent manner and Spivey et al (1993) demonstrated that GCDC cytotoxicity was concen-

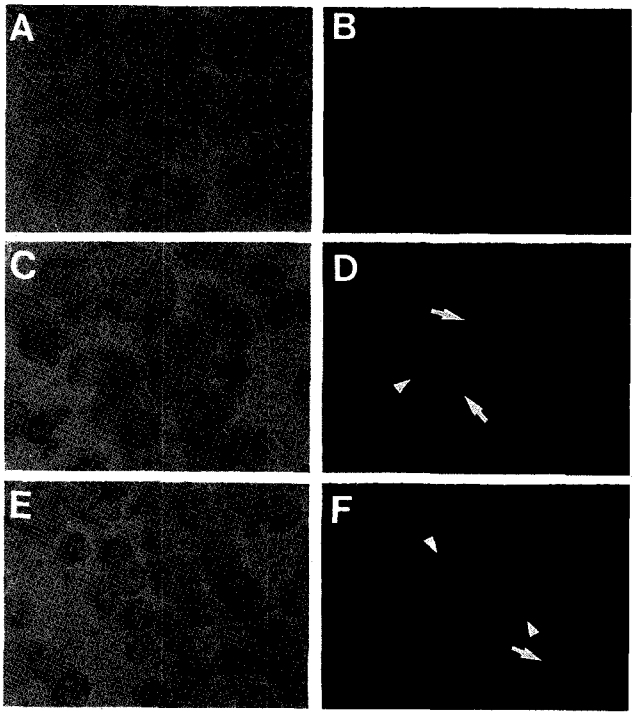

Fig. 2. Fluorescein diacetate (FDA)/propidium iodide (PI) stained cultured rat hepatocytes after 3 hr treatment with GCDC. Viable cells with intact membrane fluoresce bright green, while nonviable cells have red stained nuclei (arrow head). Apoptotic bodies with intact membrane were also observed (arrow). (A) (C) (E): phase contrast microscopy of GCDC treated cells (B) (D) (F): fluorescence microscopy of GCDC treated cells (A) (B) control, (C) (D) 100 μM GCDC, (E) (F) 400 μM GCDC ($\times$200).

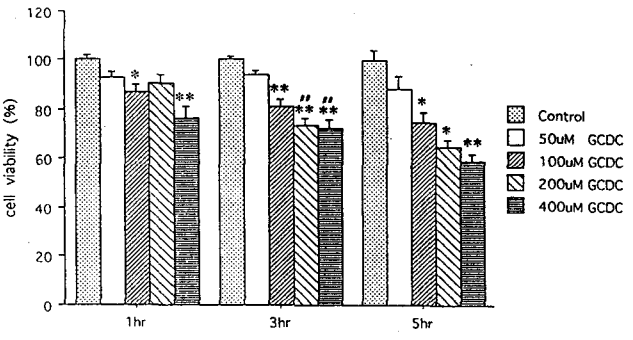

Fig. 3. Cell viability after incubation with 50 μM to 400 μM GCDC for 5 hr. Cell viability is expressed as a percent of control group (mean$\pm$SE). Significant decrease of cell viability was noted (*$p<0.05$, **$p<0.0001$ compared with control, $^{\#\#}p<0.0001$ compared with 1 hr).

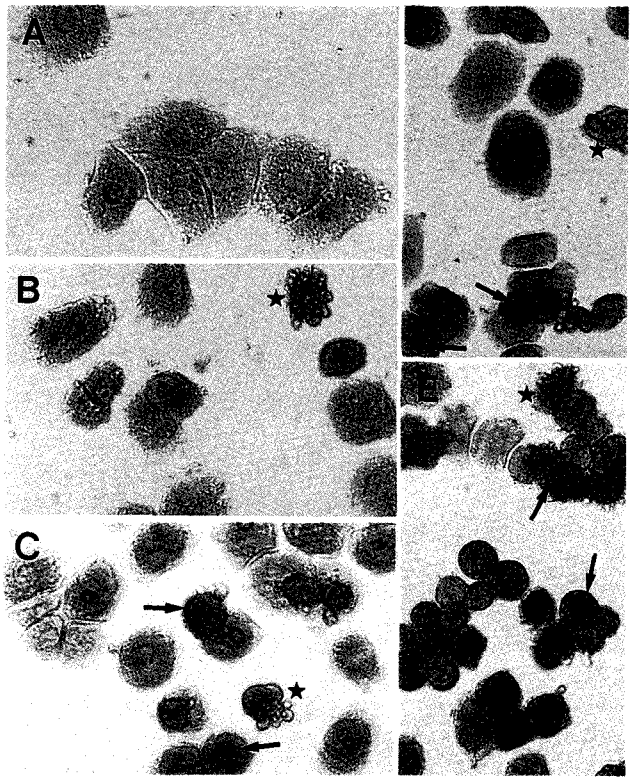

Fig. 4. Apoptosis after incubation with GCDC detected by TUNEL method. Most nuclei with condensed chromatin (arrow) and shrunk cytoplasmn labelled with brown color while no morphological changes were observed in control. Apoptotic bodies were shown (star). (A) control (B) 50 μM GCDC for 1 hr (C) 400 μM GCDC for 1 hr (D) 50 μM GCDC for 5 hr (E) 400 μM GCDC for 5 hr ($\times$400).

tration dependent with near maximal killing at 250 μM during 4 hr incubation. Comparing with other studies, the degree of GCDC toxicity in our study is mild. It is because we used freshly isolated cultured cells on the collagen coated cell culture dishes whereas other groups use freshly isolated cell suspensions. In the condition of suspension, freshly isolated hepatocytes lose cell viability more rapidly than in the condition of attachment. Prolonged survival in the cultured cells may be due to less efficient transport of bile acids by cultured hepatocytes (Frimmer & Ziegler, 1988).

Cytoplasmic enzymes including lactate dehydrogenase (LDH), alkaline phosphatase, acid phosphatase, glutamate oxalacetate transaminase, glutamate pyruvate transaminase, arginosuccinate lyase are released into the extracellular spaces upon damage of

the plasma membrane by necrosis (Li, 1994). Among of them, LDH activity can be commonly used as an indicator of cell necrosis, because of the universal presence of LDH in cells and rapid release. Extracellular LDH release was increased in time and GCDC concentration dependent manner in our study. Benz et al (1998) demonstrated that LDH released in dose dependently when incubated with 100, 250, 500 μM GCDC for 4 hr and time dependently with 100 μM GCDC for 24 hr. Hillaire et al (1995) demonstrated that LDH release from human hepatocytes in primary culture after 24 hr incubation with CDC increased at the concentration of 250 μM and 500 μM.

Recently, apoptosis has been implicated as a mechanism of several different cellular injury in many digestive diseases including toxin induced liver diseases (Que and Gores, 1996). Apoptosis present characteristics morphologically, biochemically. Apoptotic nuclei with condensed chromatin and shrunk cytoplasm are heavily labelled by a positive TUNEL reaction dose- and time- dependently in our study. Many of GCDC-induced apoptosis reported by others were observed under the limited conditions, one concentration at a certain time. Low dose GCDC such as 50 μM or 100 μM was incubated for 2 hr, 3 hr or 4 hr to induce apoptosis with isolated rat hepatocytes (Patel et al, 1994; Kwo et al, 1995; Jones et al, 1997; Patel & Gores, 1997; Roberts et al, 1997; Zeid et al, 1997; Webster & Anwer, 1998).

After exposure of primary cultured rat hepatocytes to GCDC, the resulting pattern of cell death depended on concentration and time. Low concentrations or short exposure to GCDC was sufficient to induce apoptosis whereas high concentraion or long exposure to GCDC induced necrosis accompanied by membrane damage. Because of the time- and dose-dependent transition of apoptosis to necrosis, it seems likely that in mild to moderate cholestasis, apoptosis may be the main cell death mechanism whreas in severe cholestasis, necrosis may play the major role. Therefore identification of potential common mechanisms responsible for bile acid-induced necrosis and apoptosis would be of scientific and clinical importance and may also generate strategies for the treatment of cholestatic liver diseases.

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