Korean J Physiol Pharmacol
1: 107−115, February, 1997

Induction of Apoptosis by Bile Acids in HepG2 Human Hepatocellular Carcinoma Cells

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We studied the effects of bile acids on the induction of apoptosis in HepG2 human hepatocellular carcinoma cells. Treatment with either ursodeoxycholic acid (UDCA) or lithocholic acid (LCA) resulted in a dose- and time-dependent decrease in cell viability assessed by MTT assay. Both UDCA and LCA also induced genomic DNA fragmentation, a hallmark of apoptosis, indicating that the mechanism by which these bile acids induce cell death was through apoptosis. Cycloheximide, a protein synthesis inhibitor, blocked the apoptosis induced by these bile acids, implying that new protein synthesis may be required for the apoptosis. Intracellular Ca^{2+} release blockers (dantrolene and 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester) inhibited decreased cell viability and DNA fragmentation induced by these bile acids. Treatment of HepG2 cells with calcium ionophore A23187 induced DNA fragmentation. These results suggest that UDCA and LCA induce apoptosis in the HepG2 cells and that the activation of intracellular Ca^{2+} signals may play an important role in the apoptosis induced by these bile acids.

Key Words: Apoptosis, Bile acids, HepG2 cells, Intracellular Ca^{2+}

INTRODUCTION

Apoptosis has been reported to be involved in the carcinogenic process (Isaacs, 1993; Thompson, 1995) and in tumor therapy and prevention (Bursch et al, 1992). Many of the presently used chemotherapeutic agents induce apoptosis (Miyashita & Reed, 1993). Apoptosis has been shown to be induced by increased intracellular Ca^{2+} concentration in a variety of cells (Jiang et al, 1994; Dow, 1995).

Hepatoma is the most life-threatening cancer in Asian population, especially in Koreans. Since targeting of chemotherapeutic agents to the cancer site reduces drug's side effect, it is important to develop chemotherapeutic agents which can be delivered to the site of hepatocellular carcinoma. Bile acids, the major degradation products of cholesterol, are made in the liver and circulate through the intestine via the enterohepatic circulation. Moreover, tumor-differentiating and apoptosis-inducing activities of bile acids in F9 teratocarcinoma cells have been observed (Baek et al, 1996).

In this study we investigated the effect of bile acids on the induction of apoptosis in HepG2 human hepatocellular carcinoma cells. Since intracellular Ca^{2+} is known to mediate apoptosis (Dow, 1995), we determined whether intracellular Ca^{2+} signals are involved in the apoptosis induced by bile acids.

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METHODS

Materials

HepG2, human hepatocellular carcinoma cell line was purchased from American Type Culture Collection (Rockville, MA). The cell culture powder, minimum essential medium (MEM), fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). Bis-(*o*-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester (BAPTA-AM) was from Molecular Probes, Inc. (Eugene, OR). Dantrolene (Dant), 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester

(TMB-8), cycloheximide (CHX) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were obtained from Sigma Chemical Co. (St. Louis, MO). Unless otherwise indicated, all other chemicals were the purest grade available and were obtained from Sigma. Dant was dissolved in dimethylsulfoxide (DMSO), and other drugs were dissolved in distilled water. These stock solutions were sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

HepG2 cells were maintained in MEM medium supplemented with 10% FBS, 200 U/ml penicillin and 200 μg/ml of streptomycin at 37°C in a humidified incubator under 5% CO_2/95% air. The cells were split in a 1:5 ratio every two or three days.

Cell viability test (MTT staining)

Cell viability was assessed as described by Mosmann (1983). Briefly, the cells were incubated in 100 μl of media in 96-well plates with the indicated concentration of the compounds for 24 hr at an initial cell density of 3×10^5 cells/ml. An appropriate volume of drug vehicle was added to untreated cells. After each period of incubation, 10 μl of MTT solution (5 mg MTT/ml in H_2O) were added and the cells were further incubated for 4 hr. After washing out the MTT-containing media, 100 μl of acid-isopropanol (0.04 N HCl in isopropanol) were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. The relative cell viability was obtained by scanning with an ELISA reader (Molecular Devices Corp., USA) with a 570 nm filter.

Cell morphology

For assessments of altered morphology, HepG2 cells were washed twice with cold serum-free MEM, cytocentrifuged onto slide glass and fixed in 95% ethanol for 30 min at room temperature. The fixed cells were rinsed with distilled water, stained with hematoxylin and eosin and examined under the light microscope ($\times$1000). Apoptotic cells were identified by morphological features characteristic of apoptosis (e.g., cell shrinkage, nuclear condensation, and formation of membrane blebs and apoptotic bodies) on stained cell preparations.

DNA isolation and electrophoresis

Following incubation with the drugs, HepG2 cells were collected by centrifugation (200 *g*, 5 min), washed twice in phosphate-buffered saline, pH 7.4 and resuspended at the density of 2×10^6 cells/200 μl in lysis buffer containing 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0. To each 200 μl of cell suspension, 25 μl of proteinase K (10 μg/ml) and 25 μl of 10% sodium dodesyl sulfate (SDS) were added. Samples were incubated at 70°C for 90 min. Ten μl of RNAse A (100 units/200 μl) was added and the incubation continued for a further 60 min.

The total purified DNA from each sample was dissolved in 15 μl of loading buffer (10 mM EDTA, 0.25% (w/v) bromophenol blue, 50% (w/v) glycerol), before application onto 1.5% agarose gels. Electrophoresis was carried out in TAE buffer (0.04 M tris-acetate, 0.01 M EDTA), and the gels were run at 55 V for 4 hr. The pattern of DNA fragmentation was visualized under UV light, after staining the gel with ethidium bromide (0.5 μg/ml in TAE buffer).

Quantitation of DNA fragmentation

For quantitative analysis of fragmented and intact DNA, the cells were harvested, washed with cold PBS and lysed with extraction buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 0.2% Triton X-100) for 30 min on ice (Sentman, 1991). Low and high molecular weight DNAs were separated by centrifugation at 15,000 *g* at 4°C for 30 min. The supernatant was collected and the pellet was resuspended in 0.5 ml of extraction buffer. DNA from both pellet and supernatant was precipitated by the addition of 0.5 ml of 1 N perchloric acid. After centrifugation at 15,000 g at 4°C for 30 min, the supernatant was discarded and pellets were resuspended in 0.5 ml of 0.5 N perchloric acid. The DNA was hydrolyzed by incubation at 70°C for 20 min. The amount of DNA was quantified by the diphenylamine (DPA) method (Burton, 1968). Percent fragmentation refers to the ratio of DNA in the supernatant to the total DNA recovered in the supernatant plus pellet.

Effect of intracellular Ca^{2+} release blockers on the apoptosis induced by UDCA or LCA

To investigate involvement of intracellular Ca^{2+} in UDCA- or LCA-induced apoptosis, intracellular Ca^{2+}

release blockers, Dant and TMB-8 (Rittenhouse-Simmons & Deykin, 1978; Ehrlich et al, 1994), were used. HepG2 cells were seeded in 12 multiwell-plates at a density of 2×10^6 cell/ml. The cells were pretreated with each blocker for 4 hrs, and then incubated with UDCA or LCA. The cells were assayed for cell viability and DNA fragmentation.

Data analysis

All experiments were performed four times. All data were displayed as % of control condition. All the control experiments were carried out in the same media containing drug-free vehicle. Data were expressed as mean±standard error of the mean (S.E.M.) and were analyzed using one way analysis of variance and Student-Newman-Keul's test for individual comparisons. *P* values less than 0.05 are considered to be statistically significant.

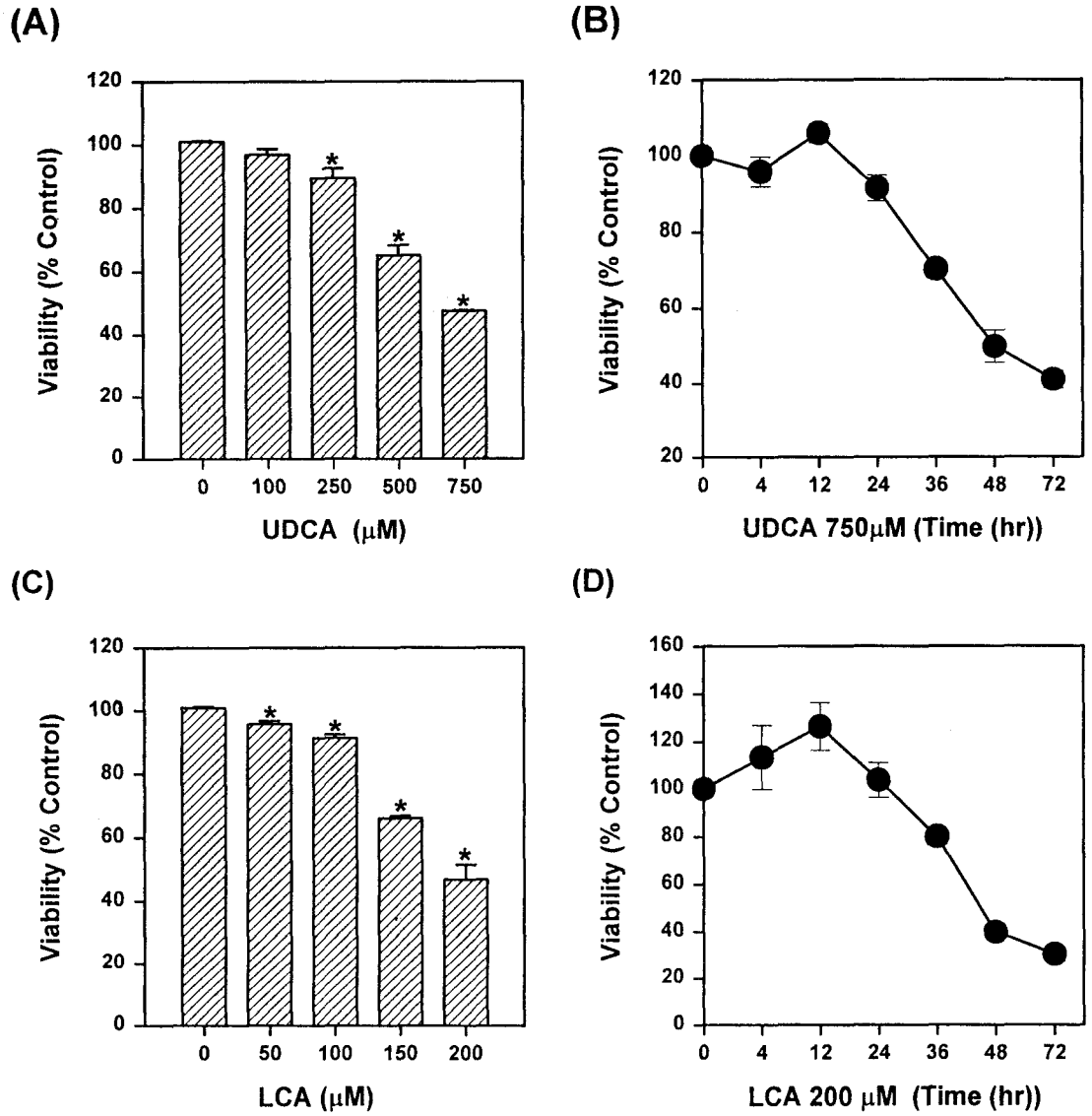


Fig. 1. Effects of UDCA or LCA on the viability of HepG2 cells. The relative viability of HepG2 cells was assessed by MTT assay. HepG2 cells were treated with various concentrations of UDCA for 48 hrs (A) or treated with 750 μM UDCA for 4, 12, 24, 36, 48 and 72 hrs (B). HepG2 cells were treated with various concentrations of LCA for 48 hrs (C) and treated with 200 μM LCA for indicated duration (D). *p < 0.05 compared to control.

RESULTS

Induction of apoptosis by UDCA and LCA in HepG2 cells

Treatment of HepG2 cells with UDCA decreased cell viability in a concentration- and time-dependent fashion (Fig. 1A and B). The viability of HepG2 cells treated with LCA was also decreased in a similar manner as UDCA (Fig. 1C and D). In order to determine whether cell death induced by these bile acids occurs through an apoptotic pathway, the genomic DNA fragmentation, a hallmark of apoptotic cell death (Wyllie et al, 1980), and cell morphology were examined. As shown in Fig. 2, treatment with UDCA or LCA at concentrations associated with a significant reduction of cell viability, produced cell morphology corresponding to the apoptotic features including cell shrinkage, cytoplasmic and nuclear membrane blebbing, and chromatin condensation. DNA fragmentation on a agarose gel and quantitation of the DNA fragments by the DPA method showed that UDCA and LCA caused DNA fragmentation in a dose-dependent manner (Fig. 3), indicating that the bile acids induced apoptosis in HepG2 cells.

Effect of CHX on the DNA fragmentation of HepG2 cells treated with UDCA or LCA

Since new protein synthesis was reported to be required for the induction of apoptosis (Altman, 1992), the effect of CHX, a protein synthesis inhibitor (Okuda & Kimura, 1988), on the cell death induced by these bile acids, was investigated. Pretreatment with CHX for 4 hrs at the concentration which did not affect the viability nor the DNA fragmentation of the HepG2 cells, prevented decreased cell viability and DNA fragmentation induced by UDCA and LCA (Fig. 4).

Effect of intracellular Ca^{2+} release blockers on the apoptosis

Intracellular Ca^{2+} is required for endonuclease activity which is responsible for internucleosomal cleavage in apoptosis. We, therefore, tested whether UDCA or LCA induces apoptotic cell death via increased intracellular Ca^{2+} levels. Treatment with BAPTA-AM, an intracellular Ca^{2+}-chelating agent, blocked cell death induced by UDCA and LCA (Fig.5). Since bile acids have been reported to induce Ca^{2+} release from endoplasmic reticulum (ER) (Laurent et al, 1988) independently of external Ca^{2+} (Combettes et al, 1990), we tested the effects of Dant and TMB-8, inhibitors of intracellular Ca^{2+} release from the ER (Rittenhouse-Simmons & Deykin, 1978; Zhang & Melvin, 1993), on the apoptosis induced by these bile acids. Dant and TMB-8 blocked the bile acid-induced decrease in cell viability (Fig. 6A and B) and DNA fragmentation (Fig. 6C), whereas these inhibitors alone did not significantly affect the

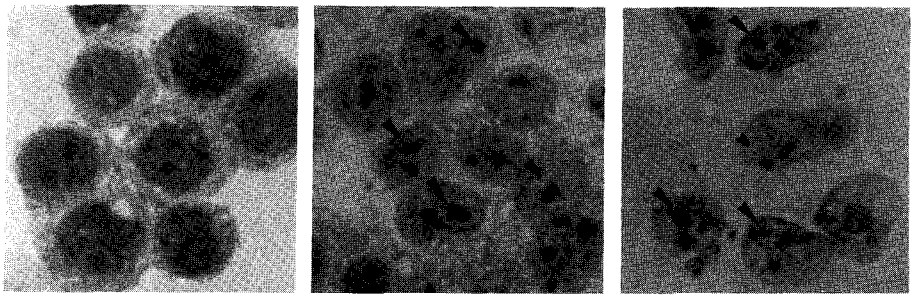


Fig. 2. Morphology of UDCA- or LCA-treated HepG2 cells. The cells treated with 750 μM UDCA or 200 μM LCA for 48 hrs were stained with hematoxylin and eosin. Untreated HepG2 cells showed normal distribution of chromatin (A), but UDCA- (B) or LCA-treated cells (C) showed local distribution of spots of condensed chromatin. The arrow-heads indicate the spots of condensed chromatin in nuclei. The bile acid treated cells also showed decreased cell volume and apoptotic bodies.

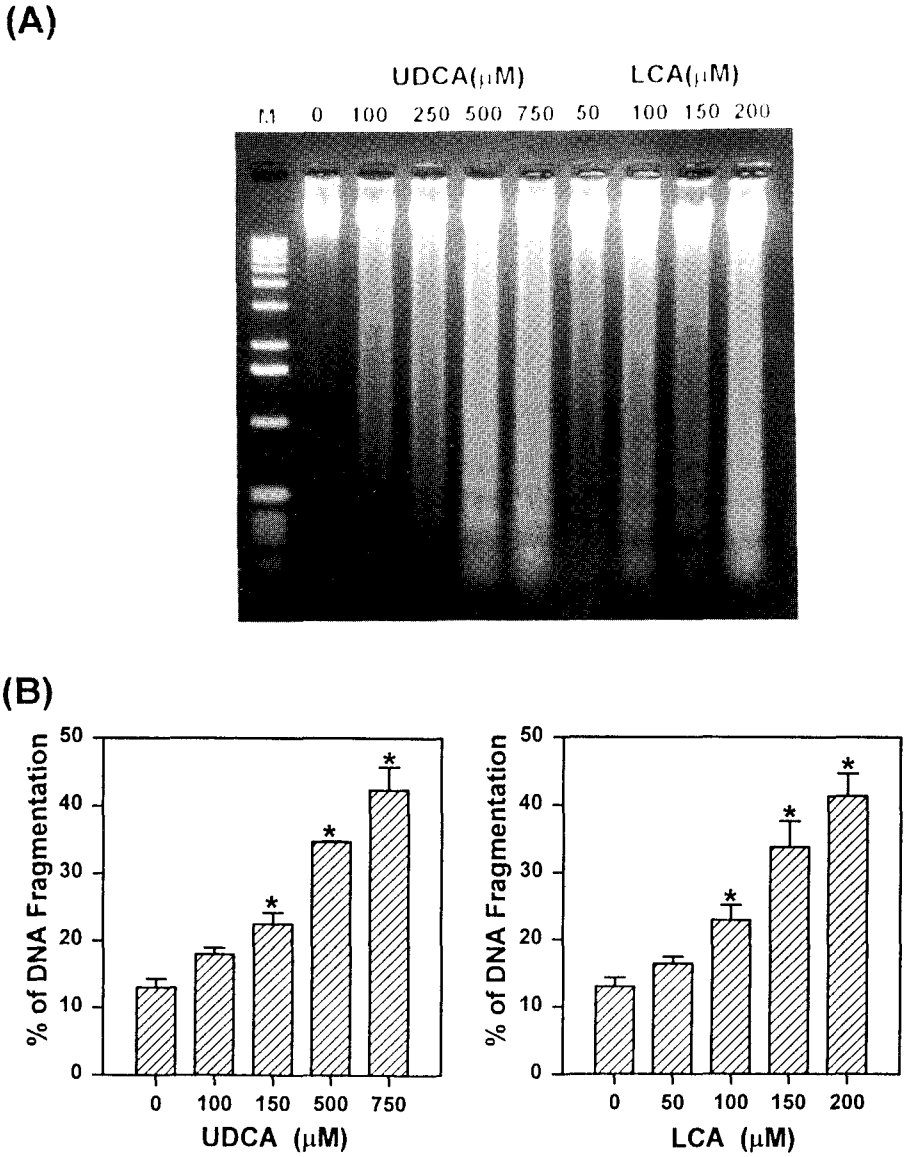


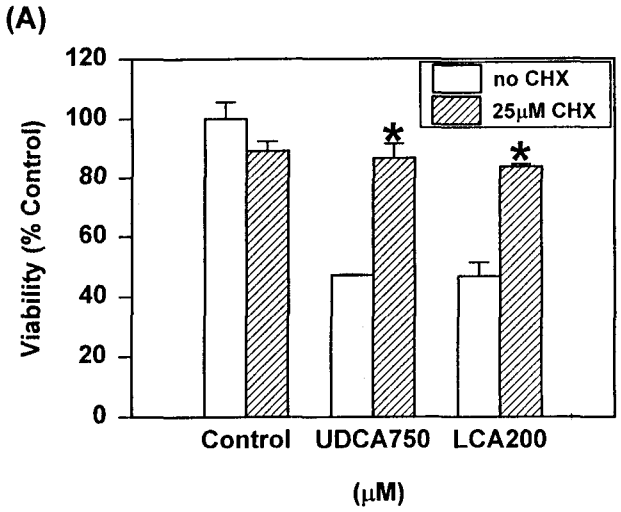
Fig. 3. DNA fragmentation induced by UDCA or LCA. HepG2 cells were treated with UDCA or LCA for 48 hrs. Total DNA was isolated by phenol extraction and electrophored on a 1.5% agarose gel. Gel was photographed under UV light with polaroid film (A). HepG2 cells were treated with UDCA or LCA. The amount of DNA fragments was quantitated by DPA-method compared to total DNA (B). Data were presented as the mean ± SEM. *p < 0.05 compared to control.

viability of HepG2 cells. In addition, Ca^{2+} ionophore A23187 which increases intracellular Ca^{2+} concentration (Bassukevitz et al, 1992), induced DNA fragmentation in HepG2 cells (Fig. 7).

DISCUSSION

The application of anticancer drugs for the treatment of cancer has been mostly based on the empirical grounds. Although the development of effective drugs for the treatment of cancer has progressed, it is unlikely that drugs with relatively low side-effects can be readily found.

Bile acids are secreted by the liver in the form of bile salts, reabsorbed in the intestine, and returned to liver. These bile acids have been observed to induce



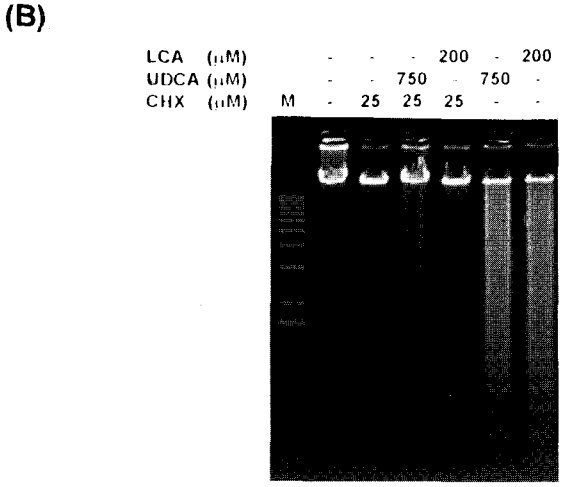


Fig. 4. Effect of CHX on the apoptosis of hepG2 cells induced by UDCA or LCA. The effect of CHX on the cell viability was assessed by MTT assay (A). DNA was isolated and electrophored after treating with 25 μM CHX for 4 hrs prior to the treatment with UDCA or LCA. The gel was photographed under UV light with polaroid film. *p < 0.05 compared to UDCA- or LCA-treated group.

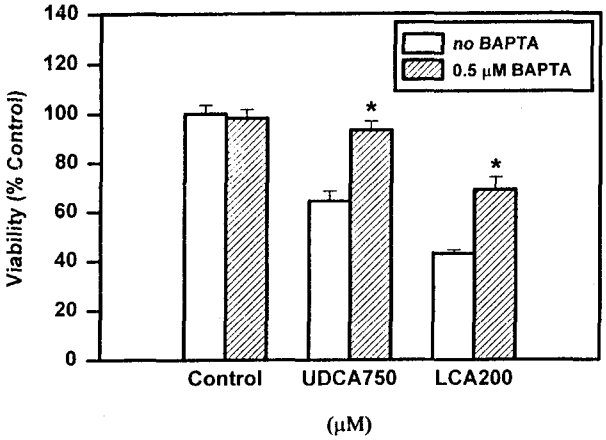


Fig. 5. Blocking Effect of BAPTA-AM on the bile acid-induced cell death of HepG2 cells. The effect of BAPTA-AM on the viability of UDCA- or LCA-treated HepG2 cells were assessed by MTT assay. Data were presented as the mean ± SEM. *p < 0.05 compared to UDCA- or LCA-treated group.

differentiation and apoptosis in F9 teratocarcinoma cells (Baek et al, 1996). Bile acids which cycle through the enterohepatic circulation, may also regulate the growth of Hepatoma cells.

The results of the present study clearly showed that bile acids, UDCA and LCA, induced apoptosis in the HepG2 cells in culture (Figs. 1, 2, and 3). Blockade of UDCA- or LCA-induced cell death by BAPTA-AM, an intracellular Ca^{2+} chelating agent (Fig. 5), indicates that the bile acid-induced apoptosis may be mediated through increased intracellular Ca^{2+} concentration. Apoptosis-blocking effect by intracellular Ca^{2+} release blockers, Dant and TMB-8 (Fig. 6) represents that the source of increased intracellular Ca^{2+} would be mobilisation from internal sources rather than extracellular Ca^{2+} influx since bile acids have been shown to release Ca^{2+} from endoplasmic reticulum in several cell types (Combettes et al, 1989; Combettes et al, 1990; Coquil et al, 1991). In addition, induction of apoptosis by Ca^{2+} ionophore A23187 (Fig. 7) implicates that increased intracellular Ca^{2+} concentration may be important in the mechanism of apoptosis in this cell line. Moreover, since the apoptosis induced by UDCA and LCA was prevented by CHX (Fig. 4), the apoptosis may be an active process which requires synthesis of new proteins. Intracellular Ca^{2+} has been known as one of important transducers in the signal transduction pathways (Hepler, 1994; Whitfield, 1992) through which multiple factors regulate cell survival and cell death (Raff, 1992; Raff et al, 1993). Although the precise mechanism is unknown, the disruption of intracellular Ca^{2+} concentration by bile acids may deregulate these factors, resulting in cell death.

Based on these results, we suggest that bile acids induce apoptosis in HepG2 cells and that the bile

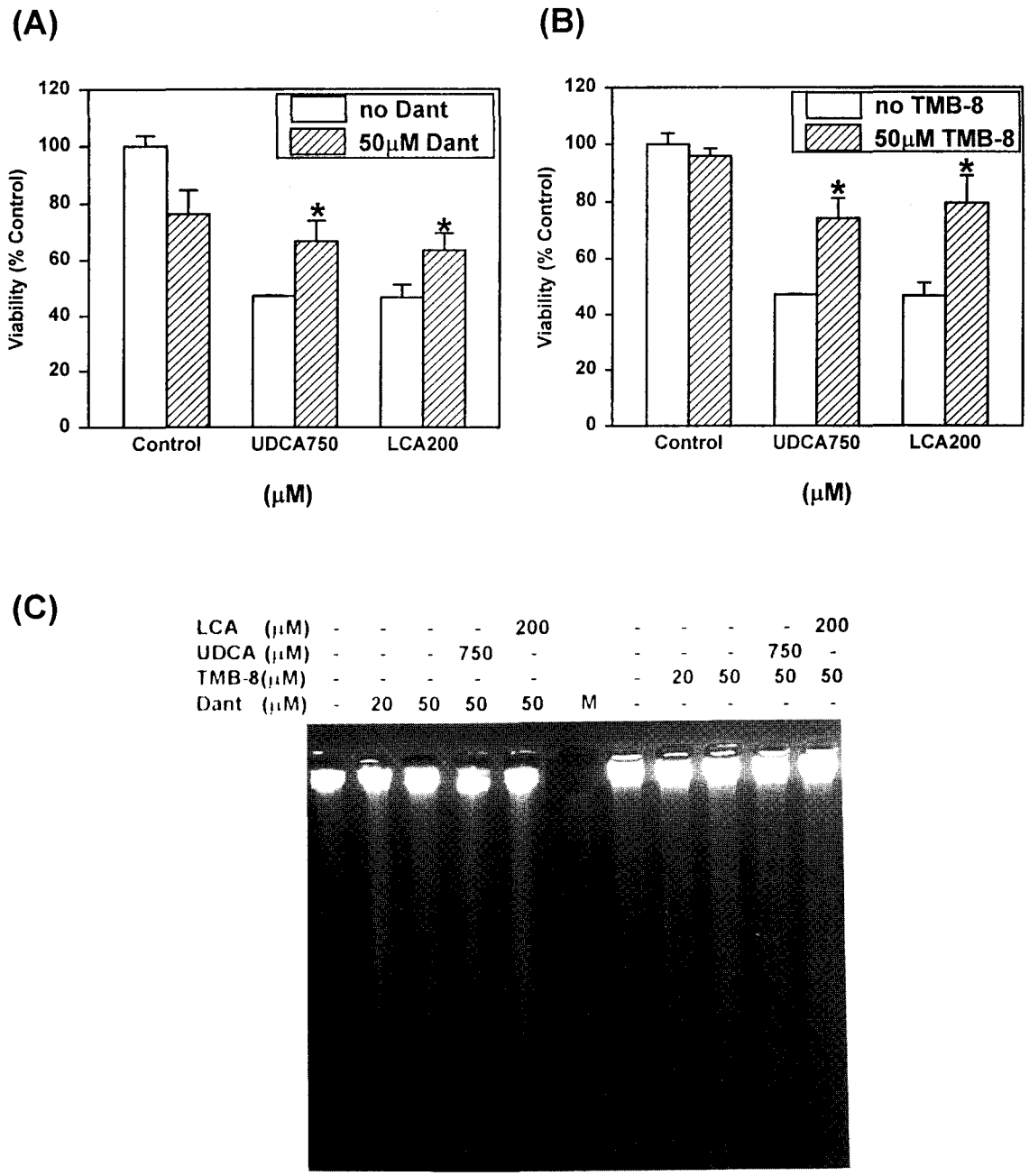


Fig. 6. Effect of intracellular Ca^{2+} release blockers on the apoptosis induced by UDCA or LCA. The effects of Dant (A) and TMB-8 (B) on the viability of UDCA- or LCA-treated HepG2 cells were assessed by MTT assay. The effects of Dant and TMB-8 on the UDCA- or LCA-induced DNA fragmentation are shown in (C). *p < 0.05 compared to UDCA- or LCA-treated group.

acid-induced apoptosis may be mediated through increased intracellular Ca^{2+} levels and new protein synthesis. Furthermore, these results suggest that bile acids may be involved, at least to the some extent, in the mechanism of physiological protection from the genesis of liver cancer. In addition, apoptosis induction by these bile acids may be a potential tool for the therapy-related studies of human hepatoma.

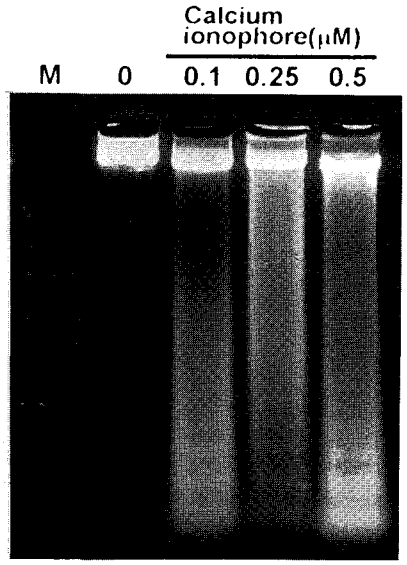


Fig. 7. Effect of Ca^{2+} ionophore A23187 on the apoptosis of HepG2 cells. The DNA isolated from Ca^{2+} ionophore-treated HepG2 cells was electrophoresed on agarose gel. Lane M represents standard DNA marker.

ACKNOWLEDGMENT

This work was supported by the Genetic Engineering Research Grant funded in 1996 from the Ministry of Education and the Research Grant (KOSEF-SRC-56-94K3-0401-03-01-3) from the Korea Science and Engineering Foundation through the Cancer Research Center.

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