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Induction of Apoptosis in HepG2 Human Hepatocellular Carcinoma Cells by a Novel Derivative of Ursodeoxycholic Acid (UDCA)

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The effects of ursodeoxycholic acid (UDCA) and its novel derivative, named as HS-1030, on the proliferation of HepG2, human hepatocellular carcinoma cells were investigated. Whereas UDCA had no significant effect in a concentration range we have tested, HS-1030 inhibited the proliferation of HepG2 cells in a concentration dependent manner. Surprisingly, HS-1030 had no effect on the proliferation of Human Chang liver cell which is a normal liver cell line. We also found that proliferation-inhibitory effect of HS-1030 was due to the induction of apoptosis of HepG2 cells, which was confirmed by observing the internucleosomal DNA fragmentation and morphological changes (*i.e.*, cell shrinkage, nuclear condensation and the formation of apoptotic bodies). These results suggest that HS-1030 may be a good candidate as a drug for the treatment of liver cancer.

Key words : UDCA, HS-1030, Apoptosis, HepG2 cell

INTRODUCTION

Cell death in cancer cells by inducing apoptosis is of importance in cancer therapy. A variety of cancer chemotherapeutic compounds can induce apoptotic cell death in cancer cells (Gunji *et al.*, 1991). Apoptosis or programmed cell death (PCD) is an active process in which distinct series of biochemical and molecular events lead to the death of cells (Duvall *et al.*, 1986). It has been known that apoptosis is involved in both physiological processes such as normal cell turnover, embryonic and T-cell development, and pathological processes such as immune and toxic cell killing (Wyllie *et al.*, 1980).

Ursodeoxycholic acid (UDCA) is a secondary bile acid metabolized in the liver (Gerald *et al.*, 1990; Gill *et al.*, 1985). Several functions of UDCA are reportedly described as below: 1) UDCA increases low-density lipoprotein binding, uptake and degradation in isolated hamster hepatocyte (Bouscarel *et al.*, 1991). 2) UDCA stimulates proliferation of hepatocytes (Barone *et al.*, 1993). 3) UDCA potentiates the effect of interferone treatment of chronic viral hepatitis (Boucher *et al.*, 1995). 4) UDCA has a similar structure to glucocorticoid (Roda *et al.*, 1990) (Fig. 1A) and induces activation of glucocorticoid receptor (Tanaka *et al.*, 1992). UDCA prompts the nuclear translocation of glucocorticoid receptor in a ligand-independent fashion (Tanaka *et al.*, 1996). Certain glucocorticoids have been shown to inhibit tumor initiation and tumor promotion (Slaga *et al.*, 1973). Dexamethasone, a synthetic glucocorticoid, has been known to induce apoptosis of human thymus-derived cells (Wyllie *et al.*, 1980). The above findings led us to investigate whether UDCA induces apoptosis of HepG2 cell which were originated from human hepatocellular carcinoma and used as a model cell for studies associated with liver cancer. Previously, we have observed apoptosis-inducing activity of UDCA in HepG2 cells (Baek *et al.*, in press) at a high concentration. To find more effective agent than UDCA in inducing apoptosis of HepG2 cells, we synthesized several derivatives of UDCA.

Here we report that HS-1030, glycine methylester conjugate of UDCA (Fig. 1B) was far more powerful than UDCA in eliciting cell death in HepG2 cells. We also treated Chang liver cell, human liver cell line, with HS-1030 to see whether HS-1030 affects the normal cell viability. To assess the cell death pattern induced by HS-1030, the following parameters

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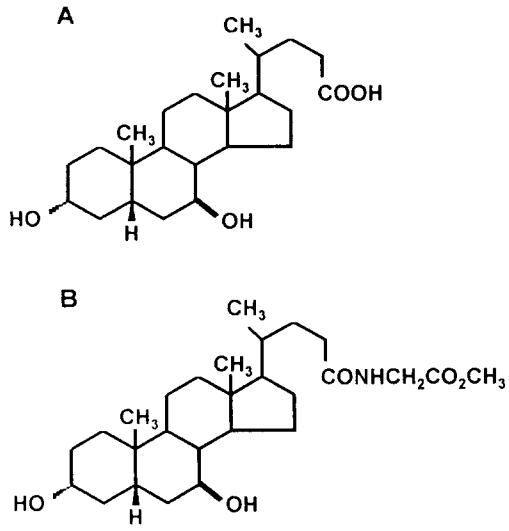

Fig. 1. The structures of UDCA (A) and HS-1030 (B).

were examined, 1) internucleosomal DNA fragmentation by agarose gel electrophoresis, 2) morphological changes of apoptotic cell by staining with propidium iodide, and 3) quantitation of amounts of DNA fragments by diphenylamine (DPA) method.

MATERIALS AND METHODS

Cell culture

A human hepatocellular carcinoma cell line, HepG 2 and normal human liver cell line, Chang liver cell were obtained from American Type Culture Collection (Rockville, U.S.A.). The HepG2 cells were cultured on RPMI supplemented with heat-inactivated 10% FBS, 100 units/ml penicillin, and 100 g/ml streptomycin at 37°C incubator in a humidified atmosphere containing 5% CO_2. Chang liver cells were grown on DMEM supplemented with heat-inactivated 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C incubator in a humidified atmosphere containing 5% CO_2. The number of cells was determined with a hemacytometer.

Assessment of cell proliferation by MTT assay

The proliferation of HepG2 and Chang liver cells was assessed by using MTT assay which is based on the conversion of MTT[3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] to MTT-formazan (Tada *et al.*, 1986) by mitochondrial enzyme. In brief, the cells were cultured with 2 ml medium per well in 12-multiwell plates at 37°C for 24 hrs under 5% CO_2 in air. The cells were then treated with desired concentrations of UDCA and HS-1030. Then, MTT solution (2.5 mg/ml H_2O) was added to the well in a concentration of 0.25 mg/ml. After incubation for 4 hrs, mixture of medium and MTT solution was carefully discarded, then 1 ml acid-isopropanol (0.04 N HCl in isopropanol) was added to the well to extract the crystallized dye. The amount of blue dye formed was determined by measuring the absorbance at 570 nm. Measurements were performed in triplicates.

Morphological characterization of cell death

The cells were harvested and suspended to 2×10^6 cells/ml in phosphate buffered saline (PBS). The cells were fixed in 70% ethanol at 4°C for 60 min, centrifuged, and resuspended in PBS. Then RNase (250 μg/ml) was added, and stained with propidium iodide (25 μg/ml). After 15 min-incubation in the dark at 25°C, the cells were photographed with a fluorescence microscope.

Agarose gel analysis of DNA fragmentation

For the qualitative analysis, the cells were harvested, washed with cold PBS, and lysed with lysis buffer (5 mM Tris-Cl pH 7.4, 20 mM EDTA, and 0.5% Triton X-100) at 4°C for 30 min. After centrifugation at 27,000 g for 15 min supernatant was collected and extracted with phenol-chloroform. The DNAs were precipitated with 0.1 volume of 5 M NaCl and 1 volume of isopropanol. The dried DNA pellet was resuspended in distilled water and treated with 300 μg/ml DNase-free RNase A. Electrophoresis of the DNA was performed on a 1.5% agarose gel in TAE buffer (0.04 M tris-acetate, 0.01 M EDTA). The agarose gel was stained with ethidium bromide and the DNA was visualized on a UV transilluminator. The size of DNA was estimated by comparing with a standard 1kb DNA ladder.

Analysis of DNA fragmentation rate

For the quantitative analysis of fragmented 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 extraction buffer. DNA from both pellet and supernatant was precipitated by the addition of 0.5 ml 1 N perchloric acid. After centrifugation at 15,000 g at 4°C for 30 min, the supernatant was removed, and 0.5 ml 0.5 N perchloric acid was added. 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. Data are represented as the mean of three independently prepared cultures.

RESULTS AND DISCUSSION

Effects of UDCA and HS-1030 treatment on the proliferation of HepG2 and Chang liver cells

MTT assay was performed to test the effects of UDCA and HS-1030 on the proliferation of HepG2 and human Chang liver cells. The cells were treated with UDCA and HS-1030 at the concentration of 25, 50, 75, 100, and 150 μM for 48 hours. As shown in Fig. 2A, HS-1030 reduced the proliferation of HepG2 cells in a concentration-dependent manner whereas UDCA did not have any significant effect on the proliferation of HepG2 cells. In the treatment with 150 μM of HS-1030 in HepG2 cells, the cell viability was decreased to 45% compared to the untreated control.

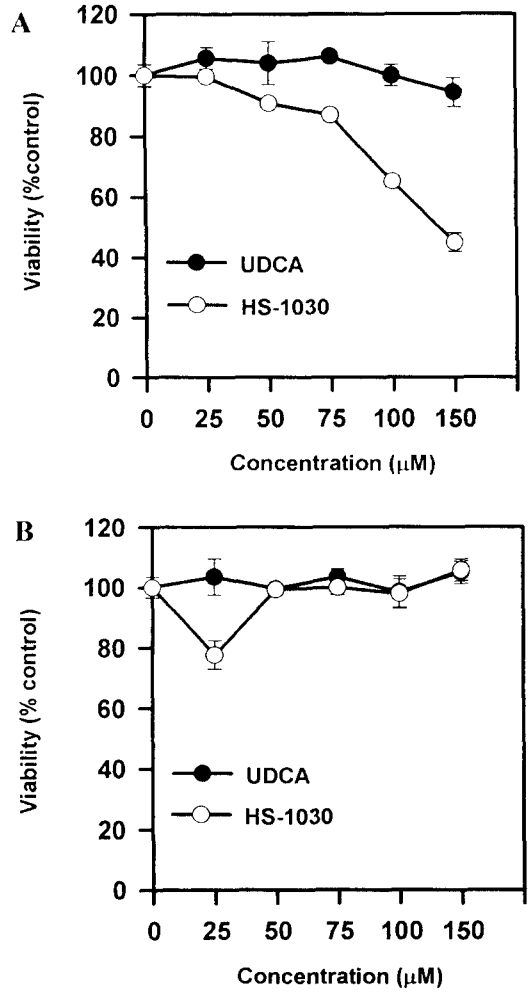

Fig. 2. Effects of UDCA and HS-1030 on the viability of HepG2 and Chang liver cells. The viability was assessed by MTT assay. HepG2 (A) and Chang liver (B) cells were treated with UDCA and HS-1030 for 48 hours.

Previously, we found that IC_{50} of UDCA in the proliferation of HepG2 cell was the concentration of 750 μM (data not shown). Therefore, HS-1030 was more effective than UDCA in reducing proliferation of HepG2 cells. However, treatment of Chang liver cells, the human normal liver cell line, with either UDCA or HS-1030 did not affect the viability of the cells (Fig 2B). These results indicate that HS-1030 inhibits the proliferation of HepG2 cells having possibly no effect on the viability of normal liver cells.

Morphological changes induced by HS-1030

To determine whether decreased proliferation of HepG2 by HS-1030 is due to the cell death, morphological changes were investigated by using propidium-iodide staining. After HepG2 cells were treated with 150 μM of HS-1030 for 48 hours, nuclei were stained with propidium iodide.

Stained nuclei showed nuclear degradation into small, spherical nuclear particles of condensed chromatin which is a characteristic of apoptosis. In contrast, control cells did not show any significant changes (Fig. 3). These results indicated that apoptosis occurred in HepG2 cells by treating with HS-1030.

Analysis of DNA fragmentation

In order to confirm apoptotic cell death in HS-1030 treated HepG2 cells, DNA fragmentation assay was performed. HepG2 cells were treated with 50, 100, and 150 μM of HS-1030 for 48 hours, and then total DNA was isolated and electrophoresed on a 1.5% agarose gel. DNA fragments appeared from the cells treated with 100 and 150 μM of HS-1030 (Fig. 4). Previously, we found that UDCA elicited DNA fragmentation in HepG2 cells at the concentration of 500 and 750 μM (data not shown). We, therefore, conclude that HS-1030 was much more effective than

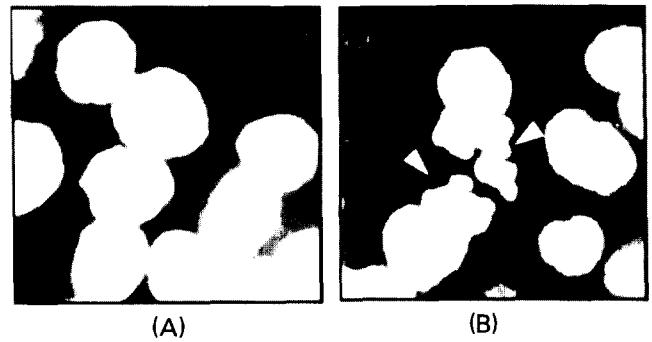

Fig. 3. Morphological change of HS-1030-treated HepG2 cells. The nucleus of untreated HepG2 cells shows normal staining pattern (A), but that of HS-1030-treated cells shows small and spherical nuclear particles of condensed chromatin indicated as arrow heads (B).

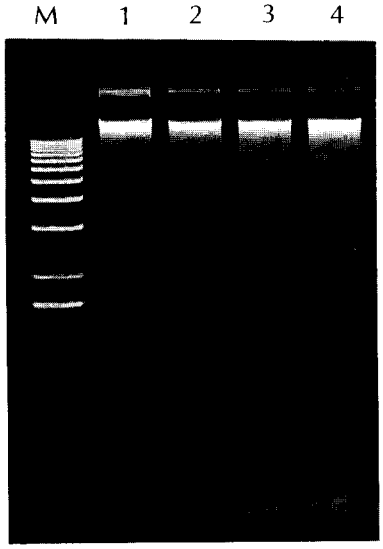

Fig. 4. DNA fragmentation induced by HS-1030. HepG2 cells were treated with HS-1030 for 48 hours. DNA was isolated and electrophoresed on 1.5% agarose gel (M, 1 kb ladder marker; lane 1, control; 2, 50 μM; 3, 100 μM; 4, 150 μM).

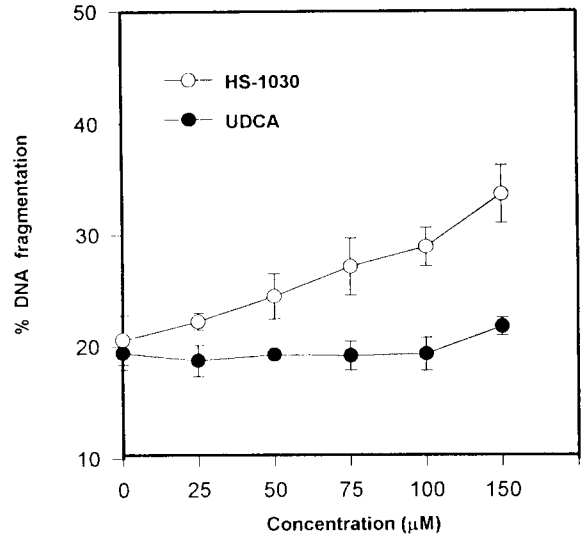

Fig. 5. Quantitation of DNA fragments induced by UDCA and HS-1030. HepG2 cells were treated with UDCA and HS-1030 for 48 hours. The amount of DNA fragments was measured by DPA-method and compared to that of total DNA. Data were expressed as the mean of three different samples.

UDCA in inducing apoptosis of HepG2 cells.

We also quantitated the amount of DNA fragments using DPA-method. Total DNAs isolated from HepG2 cells which were treated with 25, 50, 75, 100, and 150 μM of UDCA and HS-1030 for 48 hours were fractionated into large chromosomal DNA and small DNA fragments. Each DNA was stained with DPA and quantitated by reading absorbance at 560 nm. In Fig. 5, the relative quantity of small DNA fragments was increased in a concentration-dependent manner in HepG2 cells treated with HS-1030. But UDCA did not affect significantly the relative quantity of DNA fragments in HepG2 cells.

The susceptibility of cancer cells to apoptosis-inducing agents is very important in the current chemotherapy. It is, therefore, important to understand the mechanisms involved in induction of apoptotic cell death in order to establish a strategy for cancer treatments. It was previously reported that a bile acid such as UDCA can induce Ca^{2+} release from endoplasmic reticulum (Laurent *et al.*, 1988). Although in this study we have not investigated the mechanism of the apoptosis-inducing action of HS-1030, the possibility may exist that HS-1030 may induce Ca^{2+} release from endoplasmic reticulum in HepG2 cells, and thus, activate Ca^{2+}-dependent endonuclease leading to the fragmentation of chromosomal DNA (Cohen *et al.*, 1984). In addition, there could be other possibilities of apoptosis-inducing mechanism. It has been known that deregulated expression of bcl-2 can inhibit apoptosis (Hockenbery *et al.*, 1990; Sentman *et al.*, 1991) whereas deregulated expression of c-myc can induce apoptosis (Evan *et al.*, 1992). Therefore, further study will be necessary to find relationship between apoptosis-inducing activity of HS-1030 and Ca^{2+} level in the cytosol. Also, we will study whether HS-1030 affects the expression of genes involved in apoptosis such as *bcl-2* and *c*-myc.

In summary, we found that a novel derivative of UDCA, HS-1030, could induce apoptosis much more effectively than UDCA in HepG2 cells.

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