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# Increased Expression of c-*jun* in the Bile Acid-Induced Apoptosis in Mouse F9 Teratocarcinoma Stem Cells

**Jin-Hyen Baek, Chang-Mo Kang[1], Hae-Young Chung[2], Myung-Hwan Park[3] and Kyu-Won Kim***

*Department of Molecular Biology, [1]Department of Biology, and [2]Department of Pharmacy, Pusan National University, Pusan 609-735, [3]Research and Development Center, DaeWoong Pharmaceutical Co. Sungnam 462-120, Korea*

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**Abstract:** Ursodeoxycholic acid (UDCA) and lithocholic acid (LCA), secondary bile acids, have been shown to have a cell differentiation activity in mouse F9 teratocarcinoma stem cells. Treatment with bile acids induced morphological changes, including cytoplasmic and nuclear membrane blebbing, aggregation of organelles, and chromatin condensation, corresponding to apoptosis. Moreover, the bile acids induced internucleosomal DNA fragmentation, a hallmark of apoptosis. In addition, the expression of c-*jun* was increased, but that of c-*myc* and laminin was decreased during apoptosis induced by the bile acids in F9 cells. These results suggest that the bile acids can induce apoptosis in F9 cells. Furthermore, the c-*jun* expression may be related to the apoptosis induced by UDCA or LCA in F9 cells.

**Key words:** apoptosis, c-*jun*, lithocholic acid, mouse F9 teratocarcinoma stem cells, ursodeoxycholic acid.

Apoptosis (programmed cell death, PCD) is a major form of cell death involved in normal cell turnover, embryonic and T-cell development, and immune and toxic cell killing (Wyllie *et al.*, 1980). It has several well known features distinct from necrosis which is a mode of pathogenic death. In apoptosis the morphological changes are different from those of necrosis (John *et al.*, 1994). The earliest recognized morphologic changes are condensation of the nuclear chromatin and shrinkage of cytoplasm. Progression of the condensation is accompanied by blebbing of the nuclear and cytoplasmic envelope, followed by breaking up into apoptotic bodies. Since the apoptotic bodies are quickly ingested by nearby phagocytic cells and degraded within their lysosomes, there is no associated inflammation which occurs in necrosis (Searle *et al.*, 1982). Although the mechanism of apoptosis is not known, this process appears to require RNA and protein synthesis (Williams, 1991).

The proto-oncogene, c-*myc* is expressed when quiescent cells are stimulated to proliferate (Kelly *et al.*, 1983) or are subjected to external damage (Colotta *et al.*, 1990), which codes for immediate-early response transcription factors like AP-1. The expression of c-*fos* and c-*jun* has been found to be induced during apoptosis of lymphoid cells (Colotta *et al.*, 1992). Fos and Jun are constituents of AP-1 whose activity is known to affect many cellular functions (MacGregor *et al.*, 1990). In addition, members of the extracellular matrix (ECM), except fibronectin and collagen, have been shown to suppress apoptosis of mammary epithelial cells in tissue culture and *in vivo* (Strange *et al.*, 1992). Expression of interleukin-1β converting enzyme (ICE) whose activation is necessary in apoptosis in mammary epithelial cells (Boudreau *et al.*, 1995) has been reported to be related to the disruption of ECM, and an inhibitor of ICE prevented apoptosis (Donald *et al.*, 1995).

Mouse F9 teratocarcinoma cells are undifferentiated stem cells derived from mouse teratocarcinoma (Martin, 1980). The cells provide a good model to study the mechanism of both differentiation and apoptosis. F9 cells show very low spontaneous differentiation under normal culture conditions (Strickland *et al.*, 1978). However, the cells differentiate into primitive endoderm in response to retinoic acid (RA), and into parietal endoderm in response to RA and dibutyryl cyclic AMP (dbcAMP). The F9 cells undergo apoptosis in response to radiation or RA (Atencia *et al.*, 1994). Moreover, other differentiation-inducing agents are reported to induce apoptosis, such as glucocorticoids in immature thymocytes or human acute leukemic cell line (Elisabeth *et al.*, 1995) and dbcAMP in thymocytes (David

*To whom correspondence should be addressed.
Tel: 82-51-510-2277, Fax: 82-51-513-9258.

**Table 1.** Effects of UDCA and LCA on DNA and protein syntheses of F9 cells

| Treatment | Concentration (μM) | $^3$H-Thymidine incorporation (cpm/75 μl) | Protein (μg/10 μl) | cpm/μg of protein |
|---|---|---|---|---|
| Control | | 67,444± 8 | 138.1± 0.3 | 65.1 |
| UDCA | 100 | 59,508± 8,723 | 140.1± 17.2 | 56.6 |
| | 300 | 50,209± 5,375 | 105.0± 4.9 | 63.8 |
| | 500 | 4,553± 117 | 41.5± 1.6 | 14.6 |
| LCA | 50 | 50,328± 5,346 | 104.1± 2.9 | 64.5 |
| | 100 | 9,643± 2,176 | 49.7± 1.0 | 25.9 |
| | 150 | 3,106± 1,114 | 28.3± 4.2 | 14.6 |

DNA synthesis was measured in F9 cells after treatment with UDCA or LCA. F9 cells ($2\times10^5$ cells/ml) were cultured in 24-multiwell plates, and the cells were pulsed with 0.2 μCi of [methyl-$^3$H]-thymidine (5 Ci/mmol) per well. The cells were then fixed with methanol, washed twice with cold PBS, treated with 10% (v/v) trichloroacetic acid (TCA), and solubilized. The radioactivity was measured by a liquid scintillation counter, and results were normalized for protein concentration. The data represent the mean value of three independent experiments with standard deviation.

*et al.*, 1993). In addition, some apoptosis-inducing agents have been suggested to act as anti-cancer agents (John *et al.*, 1994; Barry *et al.*, 1990).

Ursodeoxycholic acid (UDCA) and lithocholic acid (LCA) are secondary bile acids. They are similar in structure to glucocorticoids which are known to induce apoptosis in human thymus-derived cell lines (Lynne *et al.*, 1991; Tanaka *et al.*, 1992; Diane *et al.*, 1992) (Fig. 1). UDCA and LCA have been shown to exhibit cell differentiation activity in F9 cells (unpublished data). Thus, in this study we investigated whether UDCA and LCA can induce apoptosis in F9 cells by employing several techniques, such as gel electrophoresis and transmission electron microscopy. In addition, the gene expression of *c-myc*, *c-jun*, and laminin was examined to determine the possible interrelationship between expression of these genes and the induced apoptotic cell death of F9 cells.

## Materials and Methods

### Materials

Fetal calf serum (FCS), Dulbecco's Modified Eagle's Medium (DMEM), and other chemicals for cell culture were obtained from Gibco BRL (Gaithersburg, USA). Cell culture flasks and plates were purchased from Corning (Cambridge, USA). [Methyl-$^3$H]-thymidine was obtained from Amersham (Arlington Heights, USA). UDCA and LCA were provided by DaeWoong Pharmaceutical Co (Sungnam, Korea). Agarose and all other chemicals were obtained from Sigma Chemical Co (St. Louis, USA).

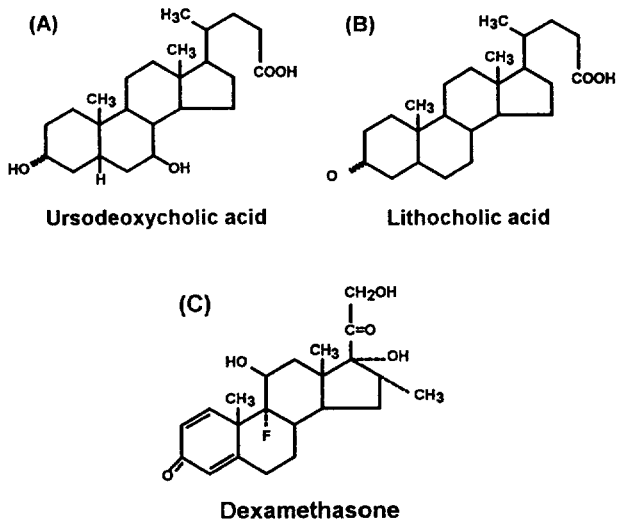



**Fig. 1.** The structures of UDCA (A), LCA (B), and dexamethasone (C).

### Cell culture

The mouse F9 teratocarcinoma cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, USA). F9 cells were maintained as monolayer cultures in DMEM, supplemented with heat-inactivated 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37℃ in a humidified atmosphere containing 5% $CO_2$. The cells used for experiments were between passages 5 and 15.

### Agarose gel analysis of DNA fragmentation

For qualitative analysis, F9 cells were harvested and washed with cold phosphate buffered saline (PBS), and lysed with lysis buffer (500 mM Tris-Cl, pH 9, 2 mM EDTA, 10 mM NaCl, 1% (w/v) sodium dodecyl sulfate (SDS), and 500 μg proteinase K/ml) at 50℃ for 36 h (Goelz *et al.*, 1985). The cells were extracted with phenol and 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 solution was performed on 1.5 % agarose 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. Size of DNA was compared with a standard ladder.

### Transmission electron microscopy

F9 cells were harvested and washed with cold PBS. Cell pellets were fixed in a mixture of 2% paraformaldehyde and 2% glutaraldehyde, and then mixed with 2% agarose gel. The cell block was cut to 1 mm$^3$, and

fixed at 4℃ overnight in mixture of 2% paraformaldehyde and 2% glutaraldehyde. Cell blocks were rinsed in 0.1 M phosphate buffer and post-fixed in 1% osmium tetraoxide solution. The samples were finally dehydrated with Epon 812 and embedded in polar Epon mixture. For electron microscopy, sections were cut 60~90 nm in thickness and stained with uranyl acetate and lead citrate, then examined using a transmission electron microscope (JOEL 1200EXII, Japan) at 80 KV.

### Analysis of DNA synthesis

F9 cells ($2\times10^5$ cells/ml) were cultured with 1 ml of media per well in 24-multiwell plates for 1 day. For the final 4 h of incubation, the cells were pulsed with 0.2 μCi of [methyl-$^3$H]-thymidine (5 Ci/mmol) per well. The cells were then fixed with methanol at 4℃ for 30 min, washed twice with cold PBS, treated with 10% (v/v) trichloroacetic acid (TCA) overnight, and dissolved in 150 μl of 0.2 M NaOH and 0.1% (v/v) SDS. 75 μl aliquots were transferred into 1 ml of scintillation fluid and radioactivity was measured with a liquid scintillation counter, and results were normalized for protein concentrations determined by the bicinchoninic acid (BCA) protein assay method (Chung *et al.*, 1992).

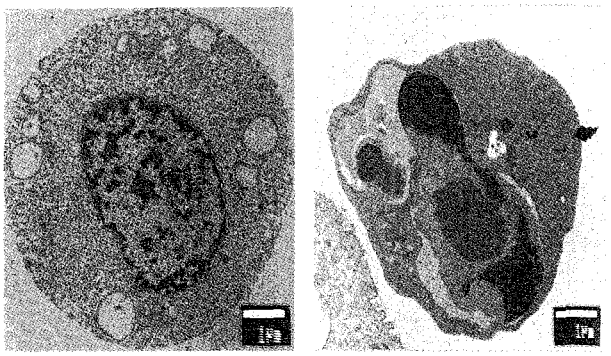
### Northern analysis

Total cellular RNAs were isolated by the acid-guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski, 1987). The Northern blotted nylon membrane was hybridized with $^{32}$P-labeled probes. The filter was dried and exposed to X-ray film for 2 to 5 days.

## Results and Discussion

### Induction of apoptosis by UDCA or LCA

We observed morphological changes corresponding to apoptosis, including cell shrinkage, cytoplasmic and nuclear membrane blebbing, aggregation of organelles, and chromatin condensation in UDCA- or LCA-treated F9 cells. As typically illustrated in Fig. 2, chromatin condensation was observed in the nuclei of UDCA-treated F9 cells. LCA treatment also showed a similar chromatin condensation in the F9 cells. Then we performed a DNA fragmentation assay with gel electrophoresis to confirm whether the mode of UDCA- or LCA-induced cell death is related to apoptosis. The F9 cells were treated with UDCA or LCA for 24 h. As shown in Fig. 3, few or no DNA fragments were observed in the untreated control cells and the cells treated with 100 μM of UDCA or 50 μM of LCA. However, DNA fragmentation was observed in the cells treated with



(A) (B)

**Fig. 2.** Morphological change of UDCA-treated F9 cells. The nucleus of untreated F9 cells shows normal distribution of chromatin (A), but that of UDCA-treated cells shows local distribution of condensed chromatin (B).

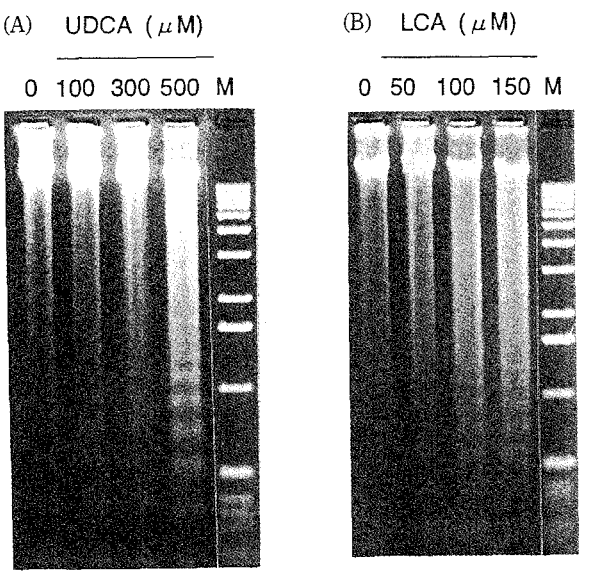



**Fig. 3.** DNA fragmentation in F9 cells treated with UDCA (A) and with LCA (B). The bile acids were treated for 24 h and then total DNAs were isolated, and internucleosomal DNA fragmentation was analyzed by 1.5% agarose gel electrophoresis.

300 and 500 μM of UDCA, or 100 and 150 μM of LCA (Fig. 3). The intensities of the observed DNA fragment bands showed a dose-dependency. In addition, DNA synthesis was inhibited in UDCA- or LCA-treated F9 cells in a dose-dependent manner (Table 1), indicating that UDCA or LCA can inhibit proliferation of F9 cells.

It was previously reported that bile acids can induce $Ca^{2+}$ release from endoplasmic reticulum (ER) (Laurent *et al.*, 1988). Although in this study we did not investigate the mechanism of the apoptosis-inducing action of these bile acids, the possibility may exist that bile acids may induce $Ca^{2+}$ release from ER in F9 cells and activate $Ca^{2+}$ dependent endonuclease, which results in the fragmentation of chromosomal DNA (Cohen

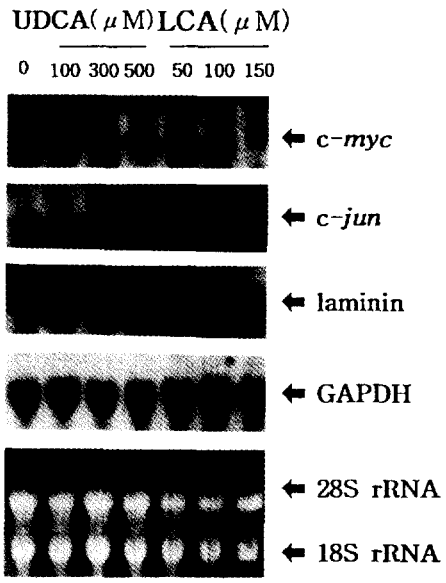



**Fig. 4.** Alteration of gene expression was detected with Northern analysis in UDCA- or LCA-treated F9 cells. Cellular mRNAs were isolated from untreated, UDCA- or LCA-treated F9 cells. Human c-*myc*, human c-*jun*, mouse laminin B1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were labelled with $^{32}$P-dCTP, and then used for northern blot analysis. For RNA loading control, GAPDH and rRNA were used.

*et al.*, 1984).

**Alteration of gene expression in apoptotic process induced by bile acids**

Northern blot analysis was used to determine the expression of genes in the F9 cells. Total RNAs from the untreated F9 cells, and UDCA- or LCA-treated F9 cells for 24 h were isolated. The expression of c-*myc* proto-oncogene was decreased during apoptosis induced by the bile acids in a dose dependent manner as shown in Fig. 4. Recent reports have shown that a high level of c-*myc* expression is correlated with DNA damage in the presence of etoposide, a cytotoxic drug that inhibits topoisomerase activity (Bertrand *et al.*, 1991). In addition, when transfected with a constitutively expressed c-*myc* gene, Rat-1 fibroblasts enter apoptosis (Evan *et al.*, 1992; Heiko *et al.*, 1994). However, in this study we found the down-regulation of c-*myc* gene by the bile acids, but the exact role of the c-*myc* down-regulation in the bile acid-induced apoptosis is not known.

It was found that c-*jun* expression was increased by treatment with UDCA or LCA (Fig. 4). In addition, the expression of laminin was slightly decreased, depending on the concentration of UDCA, but was not altered in LCA-treated F9 cells (Fig. 4), suggesting that the expression of laminin gene may not be related to apoptosis induced by UDCA or LCA. In contrast, the expression of c-*jun* may be related to apoptosis induced by UDCA or LCA. Previously, it was reported that AP-1 was induced in mouse mammary epithelial cells undergoing apoptosis (Buttyan *et al.*, 1988) and that consists of dimeric complexes of different Fos and Jun family members (Hai *et al.*, 1991). Induced AP-1 transcription factor can express several genes which are related to apoptosis (Andreas *et al.*, 1994).

Apoptosis was reported to be induced by disruption of epithelial cell-matrix interactions (Frisch and Francis, 1994). Hence inhibitor of ICE whose activation gives rise to the disruption of ECM prevented apoptosis (Boudreau *et al.*, 1995). In this study the expression of laminin that is a major constituent of ECM, was slightly decreased by UDCA (Fig. 4), indicating that the structure of ECM in the cells may be somewhat disorganized during the apoptotic process. Taken together, these results suggest that apoptosis induced by UDCA or LCA may be related to the activation of AP-1 transcription factor and disorganization of ECM.

In this study we found that bile acids could induce apoptosis in F9 cells. The structures of UDCA and LCA are similar to that of glucocorticoids such as dexamethasone and methylprednisolone (Fig. 1). Since glucocorticoid induces apoptosis through the activation of glucocorticoid receptor (GR) in immature thymocyte (Wyllie, 1980; Cohen *et al.*, 1984), it could be hypothesized that GR also mediates the UDCA or LCA-induced apoptosis. GR is a member of ligand-dependent transcription factors capable of both positive and negative regulation of gene expression (Akerblom *et al.*, 1988; Touray *et al.*, 1991). Therefore, further study will be necessary to confirm whether these activities of UDCA and LCA are mediated by GR.

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