# Bile-Acid Induced Apoptosis in HL-60 Leukemia Cells

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We investigated that lithocholic acid (LCA) or ursodeoxycholilc acid (UDCA), components of bile salt, induced apoptosis in HL-60 cells. The growth of HL-60 cells was reduced by these compounds in a time- and a concentration-dependent manners. Moreover, the degree of DNA fragmentation was increased depending on the treatment time and molar concentration of LCA or UDCA on agarose gel electrophoresis. Morphologically, we examined the formation of membrane-enclosed apoptotic bodies in LCA or UDCA-treated HL-60 cells by haematoxylin/eosin staining. The expression of *c-myc* was down-regulated after 24 h treatment of LCA or UDCA. In case of *bcl-2*, there was no change of the expression after 24 h treatment of LCA or UDCA. The DNA fragmentation was reduced when HL-60 cells were treated with 0.5 $\mu$M cycloheximide (CHX) and LCA or UDCA. Therefore, bile acids, LCA and UDCA, induced apoptosis in HL-60 cells, which required *de novo* protein synthesis.

**Key Words:** HL-60 leukemia cells, Apoptosis, LCA, UDCA, c-myc

## INTRODUCTION

Apotosis is a distinct mode of cell death that is responsible for deletion of cells in normal tissues: it also occurs in specific pathologic conditions. A characteristic biochemical feature of apoptosis is double-strand cleavage of nuclear DNA at the linker regions between nucleosomes leading to the production of oligonucleosomal fragments. In many, although not all of the circumstances where apoptosis occurs, it is suppressed by inhibitors of messenger RNA and protein synthesis.[1]

Separation and quantitation of bile acids [cheno-

deoxycholic acid (CDCA), dehydrocholic acid (DHCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA)] from bile are accomplished using high-performance liquid chromatography.[2] Bile acids have several functions; 1) bile acids are the principal endogenous ligands for male-specific and female-specific sterole-binding protein in rat liver cells,[3] 2) UDCA and LCA enhance receptor-dependent low-density lipoprotein uptake and degradation in isolated hamster hepatocytes.[4] 3) UDCA induces cellular chloramphenicol acetyltransferase (CAT) activities in a glucocorticoid receptor-dependent fashion but weak rather than synthetic dexamethasone,[5] 4) LCA affects on the releasing of intracellular $Ca^{2+}$ in liver cells. The effect is neither mediated by Ins (1,4,5) $P_3$ nor via activation of the Ins (1,4,5) $P_3$ receptor, but it is specific for the membrane of the internal pool.[6]

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The structure of bile acids is very similar with that of glucocorticoid. Glucocorticoid hormones also stimulate thymocyte apoptosis[7] by a mechanism that is dependent on a sustained increase in cytosolic $Ca^{2+}$ level. Glucocorticoid treatment of these cells causes a G1 arrest and eventual cell death.[8] A few genes (*bcl-2, myc, ras, jun and fos etc.*) are known to engage apoptosis.[9~12] Constitutive *c-myc* expression prevents growth arrest in serum-deprived Rat-1 fibroblast and induces cell death. This results insisted that deregulated *c-myc* expression is a potent inducer of programmed cell death (apoptosis) when combined with a block to cell proliferation.[13] Bile acids induce apoptosis in Epstein-Barr virus-transformed lymphoid cells. Since these cells contain high levels of bcl-2 protein, the product of a proto-oncogene that maintains cellular viability,[14] the apoptosis-inducing action of bile salts appears to circumvent the protective role of bcl-2.

In this study, the bile acid components, LCA and UDCA were used to examine whether these components induce apoptosis in HL-60 cells biochemically and morphologically. Especially, the effects of UDCA or LCA on the apoptosis-related gene regulation were investigated. Futhermore, the effect of cycloheximide (CHX), protein inhibitor, on LCA or UDCA- induced apoptosis was examined in HL-60 cells.

## MATERIALS AND METHODS

### 1) Cell culture

The HL-60 cells were cultured in a similar manner as described by Collins *et al.*[15] Stock cultures were maintained by growth as suspension cultures and passed by 2 days in RPMI-1640 medium, supplemented with heat inactivated 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin at 37°C in humidified atmosphere containing 5% $CO_2$. Cells were suspended a $5 \times 10^5$ cells/ml in RPMI-1640 and reagents were added so that final concentration of ethanol did not exceed 0.015%. At the end of incubation, the cells were harvested by centrifugation at $200 \times g$ for 10 min.

### 2) Morphological characterization of cell death

The cells were harvested and suspended $2 \times 10^6$ cells/ml in PBS. The cells were cytocentrifuged onto slide glass at 700 rpm for 10 min (Cytospin, SHANDON) and then fixed in 95% ethanol for 30 min at room temperature. Sample was rinsed with distilled water. It was stained with haematoxylin and eosin, and examined under the light microscope ($\times$ 1000).

### 3) DNA fragmentation assay

DNA isolation was done as described by Goels *et al.*[16] Samples was treated for 3 h at 37°C with 300 $\mu$g/ml boiled bovine pancreatic RNase, and loaded onto 1.5% (w/v) agarose horizontal slab gels containing 10 $\mu$g/ml ethidium bromide. DNA from $2 \times 10^6$ cells was loaded into each lane. Electrophoresis was performed in TAE buffer for 4 h at 3 V/cm. Gels were photographed under UV light with instant films.[17]

### 4) Quantification of DNA fragmentation

The extent of DNA fragmentation was determined as follows.[17,18] Briefly, $3 \times 10^6$ cells in complete medium were centrifuged ($200 \times g$, 5 min, 4°C) and the pellet was lysed in 400 $\mu$l hypotonic lysing buffer (10 mM Tris [pH 8.0], 20 mM EDTA, 0.5% Triton X-100) for 15 min. The lysate was centrifuged at $13000 \times g$ for 20 min at 4°C and the chromatin was separated from fragmented DNA. The supernatant was removed, and the pellet resuspended in 400 $\mu$l of lysing buffer. DNA from both pellet and supernatant was precipitated by the addition of 100 $\mu$l of 2.5 N perchloric acid at 4°C during overnight. After centrifugation at $13000 \times g$ for 20 min, the supernatant was discarded, and 0.4 ml of 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 diphenylamin method.[19] 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 3 samples per data point.

## 5) Northern analysis

Total cellular RNAs were isolated by the acid-guanidinium thiocyanate-phenol-chloroform extraction method.[20] The Northern blotted nylon membrane was hybridized with ^{32}P-labeled probe. The filter was dried and exposed to X-ray film for 2 to 5 days.

## RESULTS

Treatment of LCA resulted in dose-dependent decrease of cell growth rate (Fig. 1A). When HL-60 cells were treated with 100 $\mu$M LCA for various periods, the cell growth rate decreased according to the time (Fig. 1C). Similarly, treatment of UDCA caused the growth arrest of HL-60 cells (Fig. 1B
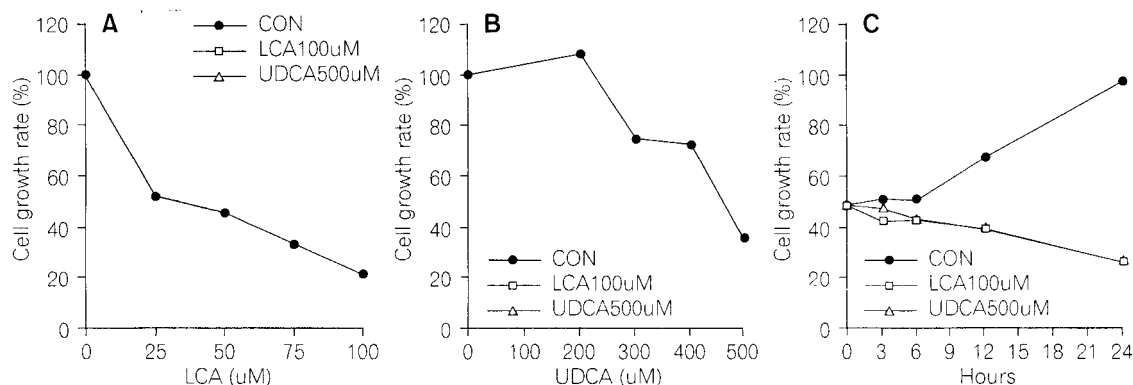


Fig. 1. Growth rate of LCA or UDCA-treated HL-60 cells. The cells were cultured with LCA at the various concentrations for 24 hr (A). The cells were cultured with UDCA at the various concentrations for 24 hr (B). The cells were cultured with 100 $\mu$M LCA and 500 $\mu$M UDCA for the various period (C). The cell number was counted by trypan blue staining. Data are represented as the mean of 3 samples per data point.
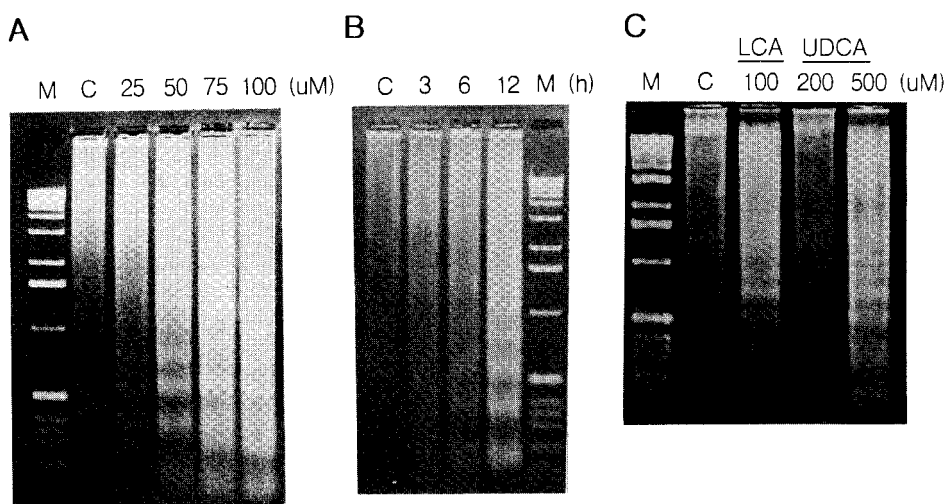


Fig. 2. LCA or UDCA-induced DNA fragmentation in HL-60 cells. A and B show DNA fragmentation on 1.5 % agarose gel in LCA-treated HL-60 cells at the various concentrations for 24 hr and for the various periods, respectively. The LCA-treated cells was compared with the UDCA-treated cells (C). Lane 1 is control, lane 2 is 100 $\mu$M LCA-treated cells, lane 3 is 200 $\mu$M UDCA-treated cells and lane 4 is 500 $\mu$M UDCA-treated cells.

and C). All experiments were started with the same cell number and living cell number was counted by trypan blue staining method. All experiments were done with 0.015% ethanol-treated cell as a control.

**Table 1.** DNA fragmentation rate in bile-acid treated HL-60 cells

| Compounds | Conc. ($\mu$M) | Time (h) | % |
|---|---|---|---|
| Control | — | — | 13.9 ± 1.0 |
| LCA | 25 | 24 | 16.1 ± 0.6 |
| | 50 | 24 | 18.2 ± 1.1 |
| | 75 | 24 | 23.7 ± 2.4 |
| | 100 | 24 | 34.7 ± 1.9 |
| UDCA | 100 | 24 | 16.2 ± 0.2 |
| | 200 | 24 | 16.2 ± 0.4 |
| | 300 | 24 | 19.7 ± 1.3 |
| | 400 | 24 | 28.3 ± 2.5 |
| | 500 | 24 | 39.6 ± 5.0 |
| LCA | 100 | 3 | 18.4 ± 0.7 |
| | | 6 | 22.0 ± 1.0 |
| | | 12 | 28.7 ± 2.1 |
| | | 24 | 33.4 ± 0.9 |
| *CHX + LCA | — | — | 24.7 ± 1.4 |
| UDCA | 500 | 3 | 18.0 ± 1.4 |
| | | 6 | 20.1 ± 1.0 |
| | | 12 | 26.2 ± 5.5 |
| | | 24 | 39.8 ± 3.4 |
| *CHX + UDCA | — | — | 33.3 ± 1.5 |

*100 $\mu$M LCA or 500 $\mu$M UDCA-treated cells with 0.5 $\mu$M CHX for 24 hr after 4 hr treatment of 0.5 $\mu$M CHX. Data are represented as the mean of 3 samples per data point.

LCA or UDCA reduced the growth rate of HL-60 cells in a dose and a time-dependent manners.

DNA fragmentation is the biochemical property of apoptosis. HL-60 cells were treated with bile acids and then DNA was isolated and analyzed by gel electrophoresis. All components of bile acids induced DNA fragmentation and especially UDCA and LCA highly induced the DNA fragmentation (data not shown). However, further experiments were done with LCA and UDCA, because of the fact that LCA induced a differentiation in F9 teratocarcinoma stem cells in our laboratory and UDCA induced cellular CAT activities in a glucocorticoid receptor-dependent fashion.[5]

When the cells cultured with various concentration of LCA for 24 h, DNA fragmentation was increased in proportion to molar concentration of LCA (Fig. 2A, Table 1). In case of treatments with 100 $\mu$M LCA for various time, DNA fragmentation was augmented time dependently (Fig. 2B, Table 1). Similarly, treatment of UDCA increased DNA fragmentation in HL-60 cells (Table 1). But LCA have a similar effect on DNA fragmentation at a lower concentration than that of UDCA (Fig. 2C). This result explained LCA or UDCA induced dose and time-dependent apoptosis in a biochemical point of view.

After treated with 100 $\mu$M LCA or 500 $\mu$M UDCA for 24 h, HL-60 cells were attached to the
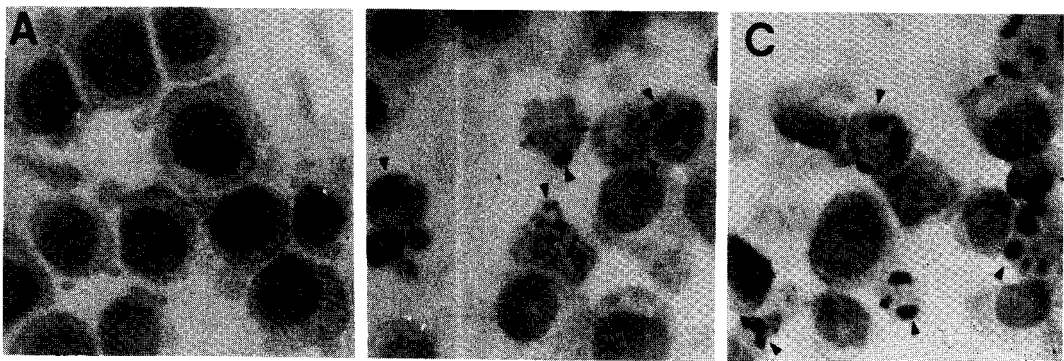


**Fig. 3.** Morphological change of HL-60 cells by LCA or UDCA treatment. Control cells (A), 100 $\mu$M LCA-treated cells for 24 hr (B) and 500 $\mu$M UDCA-treated cells for 24 hr (C) were cytocentrifuged onto slide glass at 700 rev/min for 10 min and then fixed in 95% ethanol for 30 min at room temperature. Sample was rinsed with distilled water. It was stained with hae matoxylin and eosin, and examined under the light microscope (×1000). Arrowheads indicate apoptotic cells.
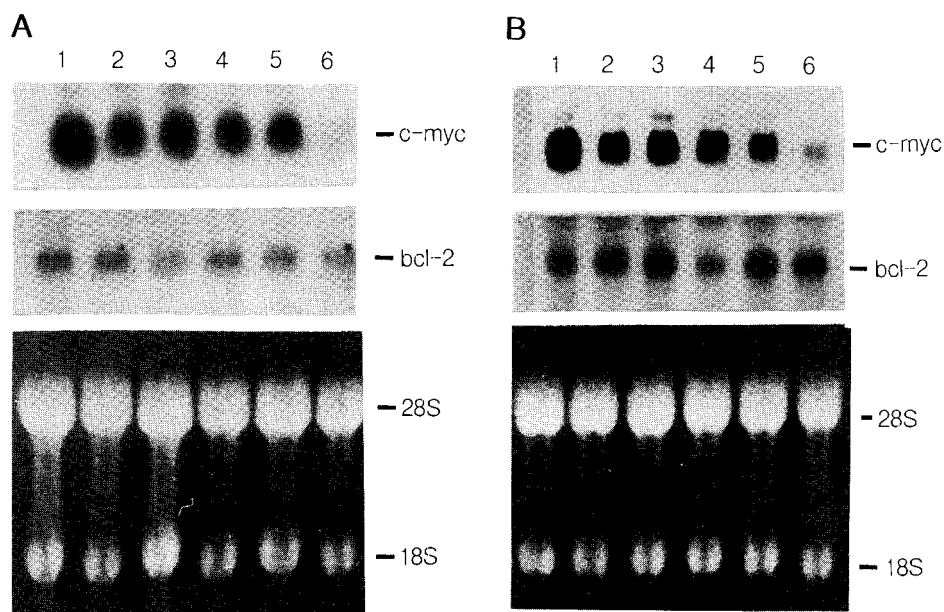
A

B



**Fig. 4.** Expression of *c-myc* and *bcl-2* in the LCA or UDCA-treated cells. A and B showed the expression of *c-myc* and *bcl-2* in the 100 μM LCA-treated cells and 500 μM UDCA-treated cells for various period, respectively. Lane 1 is control. Lane 2, 3, 4, 5 and 6 are LCA or UDCA-treated cells for 1, 3, 6, 12 and 24 hr, respectively. An ethidium bromide stain of the gel prior to transfer is shown. 18S rRNA and 28S rRNA were used as control.

slide glass by cytospin and stained with haematoxylin and eosin, and then examined by light microscopy (×1000). LCA or UDCA induced a chromatin condensation, apoptotic body, and cytosol shrinkage (Fig. 3). Control cells did not show any change in morphology.

The expression of the gene related to apoptosis was investigated by Northern analysis. When the cells cultured with 100 μM LCA for various periods, *c-myc* expression reduced after 1 h (Fig. 4A). Especially, expression of *c-myc* abruptly reduced at 24 h treatment with 100 μM LCA . But the expression of *bcl-2* appeared no change (Fig. 4A). UDCA had the same results as those of LCA (Fig. 4B). These results explained that LCA or UDCA-induced apoptosis may have closely relation to *c-myc* expression but not have any direct concern with *bcl-2* expression.

When the cells cultured with 100 μM LCA or 500 μM UDCA in condition of continuous exposure of 0.5 μM cycloheximide for 24 h after 4 h treatment of cycloheximide, cycloheximide reduced

LCA or UDCA-induced DNA fragmentation (Fig. 5, Table 1). CHX alone did not change the expression of *c-myc*. CHX increased *c-myc* expression of the cells treated with 100 μM LCA or 500 μM UDCA for 24 h (Fig. 6). From this result, *c-myc* expression may have a very close relation to apoptosis and the apoptosis induced by LCA or UDCA needs *de novo* protein synthesis.

## DISCUSSION

From Fig. 1, LCA and UDCA reduced the growth rate of HL-60 cells. But, as shown in Fig. 2 and 3, LCA and UDCA can induce apoptosis in HL-60 cells. It is suggested that these compounds could be used as agents for cancer therapy because most of the cytotoxic anticancer drugs in current use have been shown to induce apoptosis in susceptible cells. The fact that disparate agents, which interact with different targets, induce cell death with some common features (endonucleolytic cleavage of DNA and chromatin condensation) suggests that cytotoxicity is
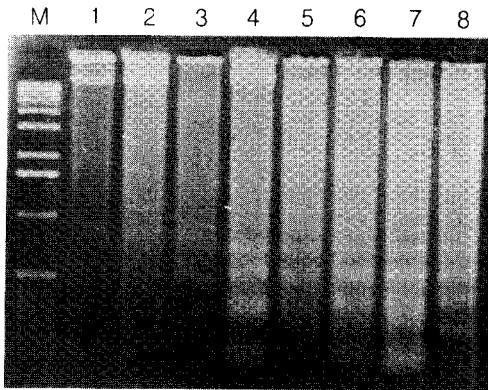
Fig. 5. Suppression of DNA fragmentation in LCA or UDCA-treated HL-60 cells by CHX. This figure shows the DNA fragmentation on the agarose gel (lane 1: control, lane 2: 0.5 $\mu$M CHX-treated cells for 28 hr, lane 3: 100 $\mu$M LCA-treated cells with 0.5 $\mu$M CHX for 24 hr after 4 hr treatment of 0.5 $\mu$M CHX, lane 4: 100 $\mu$M LCA-treated cells without 0.5 $\mu$M CHX for 24 hr after 4hr treatment of 0.5 $\mu$M CHX, lane 5: 100 $\mu$M LCA-treated cells for 24 hr, lane 6: 500 $\mu$M UDCA-treated cells with 0.5 $\mu$M CHX for 24 hr after 4 hr treatment of 0.5 $\mu$M CHX, lane 7: 500 $\mu$M UDCA-treated cells without 0.5 $\mu$M CHX for 24 hr after 4 hr treatment of 0.5 $\mu$M CHX, lane 8: 500 $\mu$M UDCA-treated cells for 24 hr).
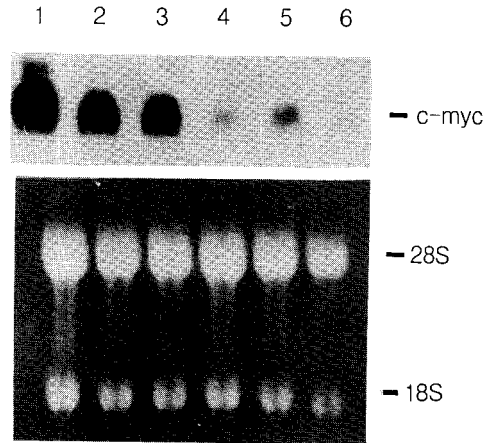


Fig. 6. Change of c-myc expression by CHX. This panel shows change of c-myc expression by CHX (lane 1: 0.015% ethanol treated cell for 24 hr, lane 2: 0.5 $\mu$M CHX-treated cells for 28 hr, lane 3: 100 $\mu$M LCA-treated cells with 0.5 $\mu$M CHX for 24 hr after 4 hr treatment of 0.5 $\mu$M CHX, lane 4: 100 $\mu$M LCA-treated cells for 24 hr, lane 5: 500 $\mu$M UDCA-treated cells with 0.5 $\mu$M CHX for 24 hr after 4 hr treatment of 0.5 $\mu$M CHX, lane 6: 500 $\mu$M UDCA-treated cells for 24 hr). An ethidium bromide stain of the gel prior to transfer is shown. 18S rRNA and 28S rRNA were used as control.

determined by the ability of the cell to engage this so-called 'programmed' cell death. The mechanism of the coupling of a stimulus is not known, but modulation of this coupling may affect the outcome of drug treatment.

As shown in Fig. 4, LCA or UDCA reduced c-myc expression. In recent report, the transient transfection of human leukemic cells with constitutively expressed c-myc-containing constructs inhibited dexamethasone- induced cell death.[21] Similarly, c-myc suppression seems to be involved in the apoptosis of HL-60 cells. The reduction of this gene probably triggers the pathway of apoptosis.

However, LCA or UDCA did not induce any change of bcl-2 expression in HL-60 cells (Fig. 4). LCA or UDCA induced apoptosis may not have direct relation with bcl-2 gene expression. Overexpression of bcl-2 can prevent apoptosis depending on nerve growth factor but not depending upon ciliary neurotrophic factor in neurons.[22] These results suggest the existence of multiple independent

intracellular mechanisms of apoptosis, some of which can be prevented by bcl-2 and others of which are unaffected by this gene. Bcl-x, a bcl-2-related gene can function as a bcl-2-independent regulator of programed cell death.[23] LCA or UDCA-induced apoptosis possibly have a mechanism unrelated to bcl-2.

HL-60 cells originally have an abnormal p53.[24] So, these cells have no relations to the mechanism of p53-dependent apoptosis. Recently, it was reported that p53 is not an activator of apoptosis-mediator genes, but rather represses genes necessary for cell survival or a component of the enzymatic machinery for apoptotic cleavage.[25] Agents that initiate DNA-strand breakage kill thymocytes by the p53-dependent pathway. The p53-independent stimuli include several physiological cell-deletion signals, namely glucocorticoid, calcium associated activation and aging in vitro.[26] So, apoptosis induced by LCA and UDCA may have a novel p53-independent pathway.

As indicated in Fig. 5 and 6, LCA or UDCA-induced apoptosis may need *de novo* protein synthesis. In these cases, new protein synthesis might be required in order to generate an appropriate triggers to activate an endogenous mechanism of cell death. It is supposed that there is a killer gene. Recently, from *C. elegans* to plants, various apoptosis-related genes have been found. During the development of the nematode *Caenorhabditis elegans*, 131 cells undergo programmed cell death. Fourteen genes have been identified that function in different steps of the genetic pathway of programmed cell death in *C. elegans*. Two of these genes, *ced-3* and *ced-4*, play essential roles in either the initiation or execution of the cell death program. CED-3 protein acts as a cystein protease in controlling the onset of programmed cell death in *C. elegans* and its non-serine-rich portions are similar to human interleukin-1 $\beta$-converting enzyme (ICE).[27] Overexpression of ICE causes Rat-1 cells (rat fibroblasts) to undergo programmed cell death.[28] In plants, the hypersensitive response to pathogens involves rapid cell death, which is hypothesized to arise from the activation of a cell death program. *Arabidopsis thaliana* have an accelerated cell death (ACD) gene called ACD2, involved in a pathway that negatively regulates a genetically programmed hypersensitive response.[29] The killer gene may take major role for apoptotic signal.

When differentiation in LCA or UDCA-induced HL-60 was investigated by NBT reduction and morphological observation, LCA or UDCA did not induce the differentiation to granulocyte or macrophages within 24 hr (data not shown). From this result, LCA or UDCA-induced apoptosis probably have the different mechanism from the differentiation of HL-60 cells.

As a result, LCA and UDCA can induce apoptosis in HL-60 cells and will supply the chance to take a step forward to solving the mechanism related with apoptosis.

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