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Characteristics of Apoptosis in HCT116 Colon Cancer Cells Induced by Deoxycholic Acid

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Hydrophobic bile acids induce apoptosis in both colon cancer cells and hepatocytes. The mechanism by which colon cancer cells respond to bile acids is thought to be different from that of hepatocytes. Therefore, we investigated the characteristics of apoptosis in colon cancer cell line HCT116. Hydrophobic bile acids, *i.e.*, deoxycholic acid (DCA), and chenodeoxycholic acid, induced apoptosis in HCT116 cells. Apoptotic indications were detectable at as early as 30 min and the extent increased in time- and concentration-dependent manners. SDS and a hydrophilic bile acid, cholic acid, did not induce apoptosis even at cytotoxic concentrations. Pretreatment with cycloheximide failed to inhibit apoptosis, suggesting that protein synthesis is not involved in the apoptotic response. Release of cytochrome *c* from mitochondria and activation of caspase-9 were detectable after 5 and 10 min, respectively, whereas remarkable activation of Bid was not detected. Ursodeoxycholic acid (UDCA) protected HCT116 cells from DCA-induced apoptosis but a preincubation period of ≥5 h was required. Nevertheless, UDCA did not inhibit cytochrome *c* release from mitochondria. Our results indicate that hydrophobic bile acids induce apoptosis in HCT116 cells by releasing cytochrome *c* from mitochondria *via* an undefined but specific mechanism, and that UDCA protects HCT116 cells by acting downstream of cytochrome *c* release.

Key words: bile acids, cycloheximide, cytotoxicity, cytochrome *c*, initiator caspases.

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; PBS, phosphate-buffered saline; UDCA, ursodeoxycholic acid.

The primary bile acids, which are amphiphilic derivatives of cholesterol synthesized in the liver, are excreted into the duodenum, where they facilitate the digestion and absorption of dietary lipids. Most bile acids are reabsorbed in the terminal ileum and then are efficiently transported by the portal vein back to the liver. Small amounts of unabsorbed bile acids pass into the colon, where they are converted to secondary bile acids, such as deoxycholic and lithocholic acids (DCA and LCA, respectively), by enteric bacteria.

Secondary bile acids and a hydrophobic primary bile acid, chenodeoxycholic acid (CDCA), induce apoptosis in particular types of cells such as hepatocytes and colon cancer cells. Caspase-dependent apoptosis is initiated by cell surface death receptors or cytochrome *c* release from mitochondria. Apoptotic signals have been reported to differ among cell types, and have been characterized as "type I" or "type II" cellular responses (*1*, *2*). In type I cells, activated initiator caspases (caspase-8 and caspase-10) cleave, and thereby activate, effector caspases (caspase-3, caspase-6 and caspase-7), which are responsible for execution of the cell death program. In type II cells, such as hepatocytes, a sufficient degree of activation of the effector caspases requires the involvement of a mitochondrial amplification pathway. Activated initiator caspases cleave Bid, a proapoptotic member of the Bcl-2 family, which, in turn, induces mitochondrial dysfunction. Truncated Bid transmigrates to mitochondria, where it induces cytochrome *c* release. Once in the cytosol, cytochrome *c* binds to apoptosis-activating factor–1 (Apaf-1), resulting in the activation of caspase-9, which can activate effector caspases.

Current reports suggest that bile acid–mediated apoptosis in hepatocytes is, at least in part, dependent on death receptor signaling (*2–6*). Glycochenodeoxycholic acid (GCDC) in particular triggers the oligomerization of Fas in a ligand-independent manner by promoting trafficking of intracellular Fas to the plasma membrane (*7*, *8*). The involvement of another death receptor, TRAIL-R2/DR5, is also suggested (*9–11*). In contrast, hydrophilic ursodeoxycholic acid (UDCA) has been demonstrated to prevent bile acid–induced apoptosis through modulation of the mitochondrial membrane perturbation and the signaling pathway in hepatocytes (*12–14*).

While present knowledge of the mechanisms by which bile acids induce cellular response is drawn mostly from studies on hepatocytes, the molecular mechanisms involved in the apoptotic process in colon cancer cell lines have not yet been fully evaluated. In Fas-positive SW480 and HT-29 colon cancer cells, DCA induces apoptosis associated with activation of caspase-8 within 1 h (*3*). It is suggested, however, that DCA-induced apoptosis in colon cancer cells is independent of Fas expression since 1-h incubation with agonistic anti-Fas antibodies failed to induce apoptosis in SW480 cells, and a rapid apoptotic response was also induced by DCA in Fas-negative

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SW620 colon cancer cells (*3*). Bile acids have been reported to cause oxidative stress, DNA damage, and mitochondrial membrane instability in colon cancer cells (*15–17*), but the precise mechanism by which apoptosis is initiated has not been clearly elucidated. Secondary bile acids, particularly deoxycholic acid (DCA), function as tumor promoters, although the mechanism by which they do so remains unclear. The general consensus is that bile acids contribute to colonic carcinogenesis by disturbing the fine balance among proliferation, differentiation, and apoptosis in colonic epithelial cells (*18–20*). Numerous studies have suggested that the induction of apoptosis in colon cancer cells is the mechanism by which the tumor promoting activity of bile acids is expressed.

The human colon cancer cell line HCT116 requires activation of caspase-9 for TRAIL-induced apoptosis, and thus appears to show a type II response to proapoptotic stimuli (*1*, *21*, *22*). In the present study, we examined the characteristics of DCA-induced apoptosis in HCT116 cells to obtain a better understanding of how hydrophobic bile acids induce apoptosis in colon cancer cells. At the concentration of 500 μM DCA induced apoptosis in HCT116 cells at as early as 30 min, through a process that was started by cytochrome *c* release from mitochondria. Following pretreatment for periods of ≥5 h, UDCA suppressed DCA-induced apoptosis. It was demonstrated that pretreatment with UDCA efficiently inhibits the activation of both caspase-8 and caspase-9, but cytochrome *c* release into the cytosol was not apparently inhibited, suggesting a novel mechanism by which UDCA protects cells from apoptosis caused by mitochondrial dysfunction.

MATERIALS AND METHODS

Materials—The primary antibodies used were anti–caspase-3 (Imgenex), anti–caspase-8 (Neo Markers), anti–caspase-9 (Sigma-Aldrich), anti-Bid (MBL), and anti–cytochrome *c* (MBL). The secondary antibodies used were peroxidase-labeled anti–mouse IgG (H+L) and anti–rabbit IgG (H+L) (Vector). Caspase-family inhibitor Z-VAD-FMK was obtained from MBL.

Cell Culture—Human colon cancer cell line HCT116 was grown in McCoy's 5A medium supplemented with 10% fetal bovine serum and 0.1 mg/ml kanamycin at 37°C under 5% CO_2.

Induction of Apoptosis—Cells were seeded into 3.5-cm dishes and allowed to grow to 80% to 90% confluency. For the induction of apoptosis, the medium was replaced with fresh medium containing an appropriate concentration of DCA, CA, CDCA, or UDCA. The concentration of the vehicle (dimethylsulfoxide) was kept at <0.5% and uniformly adjusted among the dishes.

Hoechst Staining—Cells grown on cover slips were incubated in Hoechst 33342 (10 μg/ml) for 5 min at 37°C. The cells were then washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 10 min at 4°C. Cells were examined under a BX51 microscope (Olympus), with excitation and emission at 360 and 510 nm, respectively. Images were captured with a digital camera (DP50; Olympus). Nuclei were identified as being normal, fragmented, or condensed. Fragmented and condensed nuclei were classified as apoptotic. For determination of the proportion of apoptotic cells, pictures were taken from five random fields (×400 or 600) and the cells were counted.

DNA Fragmentation—Low molecular weight DNA was extracted by suspending cells in 100 μl of lysis buffer comprising 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100 for 10 min at 4°C. After centrifugation for 5 min at 13,000 rpm, RNase A was added to the supernatant, to a final concentration of 20 μg/ml, and incubation was continued for 1 h at 37°C. Then, Proteinase K was added to a concentration of 20 μg/ml, followed by incubation for a further 30 min at 50°C. DNA was precipitated with 42 mM NaCl and 50% isopropanol overnight at –20°C. After centrifugation for 15 min at 13,000 rpm, the DNA pellet was resuspended in 20 μl TE buffer (10 mM Tris-HCl and 1 mM EDTA), and 10 μl of the DNA solution was run on a 2% agarose gel containing 0.5 μg/ml ethidium bromide in TBE buffer (89 mM Tris base, 89 mM boric acid, and 2.6 mM EDTA).

Western Blotting—Cells were lysed in a buffer comprising 0.5 mM sodium phosphate (pH 7.0), 0.1 mM EDTA, and 0.1% SDS, supplemented with protease inhibitors (2 mM PMSF, 0.022 TIU/ml aprotinin, 5 μg/ml leupeptin, and 0.1 μg/ml pepstatin) by sonication for 5 s. For cytochrome *c* analysis, a cytoplasmic fraction was prepared as described previously (*23*). Briefly, cell pellets were resuspended in cold mitochondrial buffer (10 mM HEPES, 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 1 mM PMSF, 1 μM pepstatin, and 10 μg/ml leupeptin), lysed by passing six times through a 25-gauge needle, and then centrifuged for 3 min at 4°C at 10,000 × *g*. The supernatant contained the released cytochrome *c*. Total protein (80 μg) was resolved by SDS-PAGE on a 10% to 15% gel under reducing conditions. After electrophoresis, the protein was transferred to a nitrocellulose membrane. Membranes were blocked with 1% skim milk, 1% albumin, and 0.05% Tween 20 in PBS. The blots were reacted with primary antibodies diluted in blocking buffer overnight at 4°C. The blots were washed 3 × 10 min and then incubated with secondary antibodies (anti–mouse horseradish peroxidase or anti–rabbit peroxidase) diluted 1:1,000 in blocking buffer for 1 h at room temperature. The blots were washed 3 × 10 min and then chemiluminescense was detected with an image analyzer (LAS-1000; FujiFilm).

RESULTS

Hydrophobic bile acid DCA had a cytotoxic effect on HCT116 human colon cancer cells at concentrations of ≥200 μM (Fig. 1A). Cells treated with 600 μM DCA were all dead after 24 h. To determine if this cytotoxicity was apoptotic in nature, we examined the cells for DNA fragmentation, a typical sign of apoptosis. DNA fragmentation was detectable after incubation with 300 μM DCA for 1 h, and its extent increased in a concentration-dependent manner (Fig. 1B, left panel). A lesser extent of DNA fragmentation was observed after the cells had been treated with 600 μM DCA, and thus it appeared that higher concentrations of DCA caused necrosis rather than apoptosis. DNA fragmentation was induced by 500 μM DCA at as early as 30 min (Fig. 1B, right panel). Figure 1C shows the morphological changes occurring in

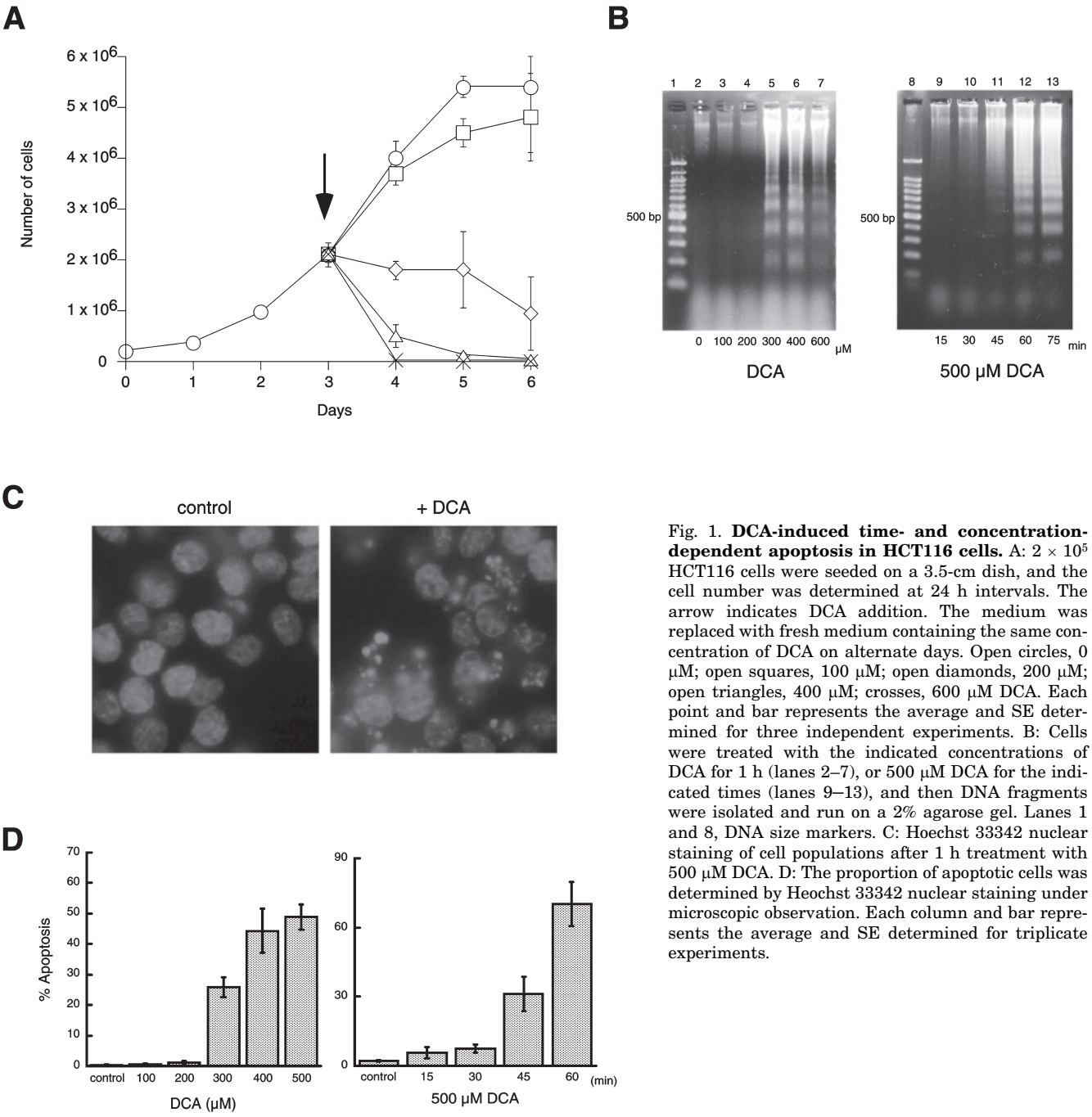

Fig. 1. **DCA-induced time- and concentration-dependent apoptosis in HCT116 cells.** A: 2×10^5 HCT116 cells were seeded on a 3.5-cm dish, and the cell number was determined at 24 h intervals. The arrow indicates DCA addition. The medium was replaced with fresh medium containing the same concentration of DCA on alternate days. Open circles, 0 μM; open squares, 100 μM; open diamonds, 200 μM; open triangles, 400 μM; crosses, 600 μM DCA. Each point and bar represents the average and SE determined for three independent experiments. B: Cells were treated with the indicated concentrations of DCA for 1 h (lanes 2–7), or 500 μM DCA for the indicated times (lanes 9–13), and then DNA fragments were isolated and run on a 2% agarose gel. Lanes 1 and 8, DNA size markers. C: Hoechst 33342 nuclear staining of cell populations after 1 h treatment with 500 μM DCA. D: The proportion of apoptotic cells was determined by Heochst 33342 nuclear staining under microscopic observation. Each column and bar represents the average and SE determined for triplicate experiments.

apoptotic cells after the addition of DCA are nuclear condensation and nuclear fragmentation. The proportion of apoptotic cells after treatment with DCA was determined based on this morphological change. As shown in Fig. 1D, the proportion of the cells with an apoptotic morphology increased in time- and concentration-dependent manners. This is consistent with the results of DNA fragmentation analysis. Cleavage of caspase-3 provided evidence that DCA treatment induces caspase-3 activation in time- and concentration-dependent manners (Fig. 2A). To determine if DCA-induced apoptosis in HCT116 cells is dependent on caspases, we examined the effect of caspase-family inhibitor Z-VAD-FMK (Fig. 2B). Z-VAD-FMK completely inhibited DCA-induced apoptosis.

Hydrophilic bile acid CA was cytotoxic at the concentration of 600 μM (Fig. 2A). Treatment with CA for 48 or 72 h, however, did not induce DNA fragmentation with any examined concentration (Fig. 3B). Thus, these data suggest that DCA-induced apoptosis in HCT116 cells is not caused simply by a detergent effect, but that a specific mechanism is involved.

This is supported by the observation that 500 μM SDS failed to induce DNA fragmentation in spite of causing cell death (Fig. 4A). Like DCA, hydrophobic bile acid CDCA induced DNA fragmentation at concentrations of ≥400 μM (Fig. 4B). The addition of protein synthesis inhibitor cycloheximide (10 or 100 μg/ml) revealed that

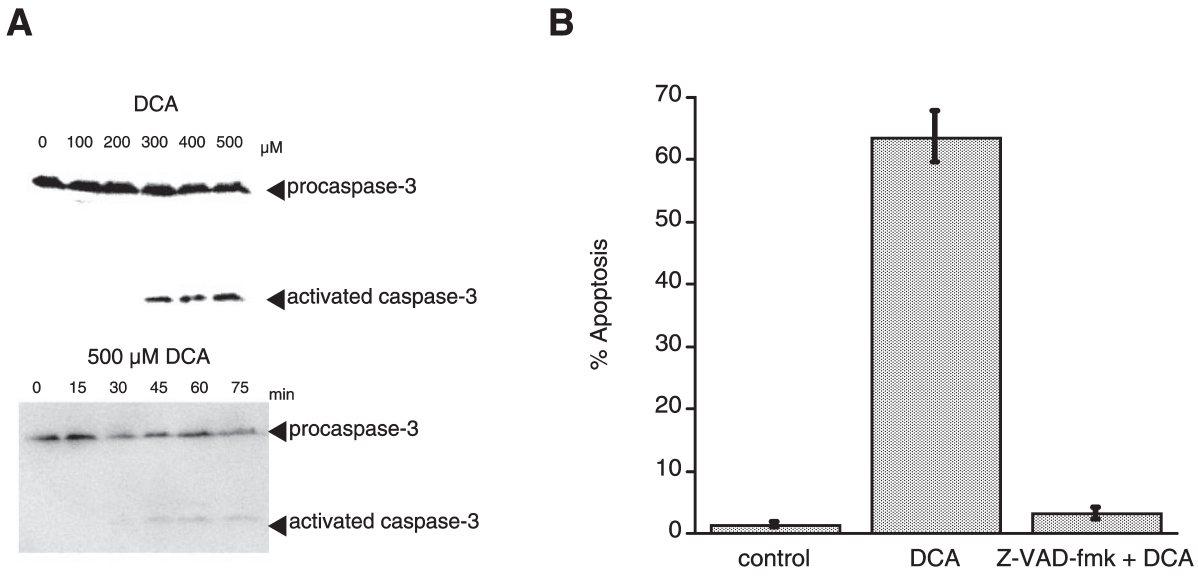

Fig. 2. **DCA-induced apoptosis in HCT116 cells is dependent on caspase activity.** A: HCT116 cells were treated with the indicated concentrations of DCA for 1 h (top), or with 500 μM DCA for the indicated times (bottom). A whole cell lysate was analyzed by Western blotting to detect caspase-3. Upper bands, uncleaved caspase-3. B: HCT116 cells were preincubated for 30 min in the presence of 20 μM caspase-family inhibitor Z-VAD-FMK, and then treated with 500 μM DCA for 1 h. Each column and bar represents the average and SE determined for triplicate experiments.

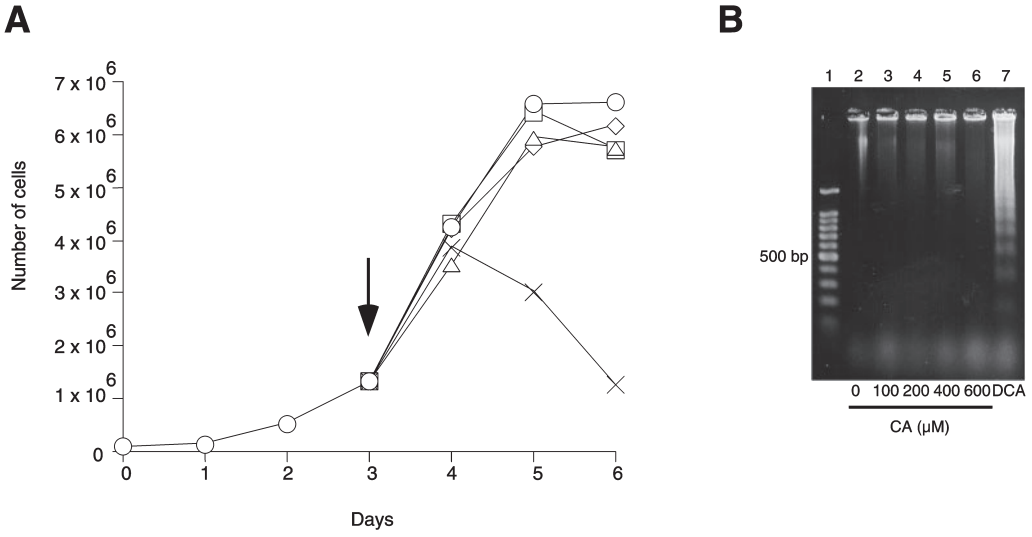

Fig. 3. **CA does not induce apoptosis in HCT116 cells.** A: The cytotoxicity of CA was examined as in Fig. 1A. The graph shows the data for a single experiment, representative of four in which similar results were obtained. B: DNA fragments from cells treated with the indicated concentrations of CA for 48 h were isolated and run on a 2% agarose gel. Lane 7 represents a positive control, *i.e.*, cells treated with 500 μM DCA for 1 h.

DCA-induced apoptosis is not dependent on protein synthesis (Fig. 4C).

After determining the kinetics and concentration dependence of DCA-mediated apoptosis in HCT116 cells, the signal transduction pathways involved in the intracellular transmission of the apoptotic stimulus induced by DCA were investigated (Fig. 5). Effecter caspase caspase-3 and initiation caspase caspase-8 were both activated by 500 μM DCA-treatment atas early as 30 and 45 min, respectively, while another initiation caspase, caspase-9, was activated after 10 min. Cytochrome *c* was detected in the cytosolic extract of DCA-treated cells at as early as 5 min. Activation of Bid, a proapoptotic member of the Bcl-2 family known to induce mitochondrial dysfunction, could not be clearly detected in DCA-treated HCT116 cells.

It has previously been reported that UDCA protects both hepatocytes and nonhepatic cells from DCA-induced apoptosis in rats (*12*, *24*). We therefore examined whether UDCA has any protective effect on human colon cancer cells (Fig. 6). Preincubation with UDCA for 1 h had no proapoptotic effect on HCT116 cells with the concentra-

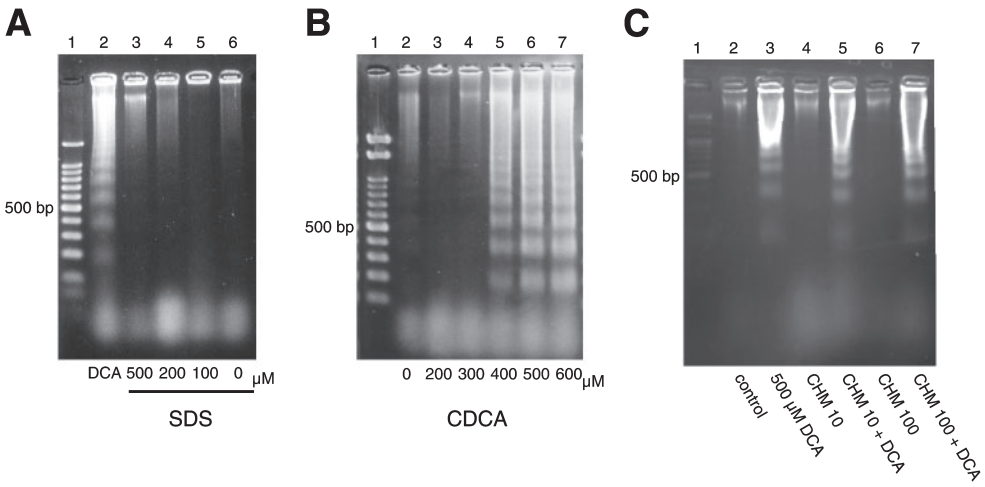

Fig. 4. **Apoptosis in HCT116 cells is not induced simply by a detergent effect, and is independent of protein synthesis.** A and B: Cells were treated with SDS or CDCA, respectively, for 1 h and then DNA fragmentation was examined. Lane 1 in A, a DNA extract of cells treated with 500 μM DCA for 1 h was used as a positive control. C: Cells were incubated with 10 or 100 μg/ml cycloheximide for 10 min prior to incubation with 500 μM DCA for 1 h.

tions examined. Prolonged treatment with UDCA for at least 5 h, however, completely prevented DCA-induced apoptosis (Fig. 6A). In contrast to UDCA, CA failed to protect HCT116 cells from DCA-induced apoptosis (Fig. 6B). We therefore examined the effects of UDCA on the signal transduction pathway involved in DCA-mediated apoptosis. DCA-induced activation of caspase-8 and caspase-9 was blocked by pretreatment with UDCA for 5 h (Fig. 6C). The release of cytochrome *c* into the cytosol, however, was not apparently blocked by pretreatment with UDCA, suggesting that UDCA protects cells by acting upstream of caspase-9 but downstream of cytochrome *c* release from mitochondria.

DISCUSSION

Since DCA does not induce apoptosis in some cell lines even at cytotoxic concentrations (S. Yui, A. Fujiwake, and T. Saeki, unpublished observation), hydrophobic bile acids are thought to react specifically with a proapoptotic switch harbored by restricted types of cells such as hepatocytes and colon cancer cells. This is consistent with the finding that SDS and a hydrophilic bile acid, CA, did not induce apoptosis at concentrations at which they exhibited cytotoxicity via a detergent effect. The hydrophobic bile acids used in the present study induced apoptosis after 30 min treatment. It has been reported that DCA induces GADD153 gene expression and activates AP-1 *via* the EGFR/Raf-1/ERK signal transduction pathway at the concentration of 250 μM after incubation for ≥2 h (*25*, *26*). Our present observations, however, suggest it is unlikely that gene expression or protein synthesis is involved in the rapid apoptotic response of HCT116 cells to 500 μM DCA since pretreatment with cycloheximide failed to suppress DCA-induced apoptosis. The DCA concentration of colonic contents varies depending on the physiological and pathological conditions. It has been reported that a high-fat diet could make the DCA concentration in fecal water reach 700 μM in humans (*27*), with which epithelial cells could be in contact theoretically. Further investigation is required if such a high concentration of hydrophobic bile acids could damage epithelial cells in the colonic mucosa.

It has been shown that some anticancer drugs induce Fas clustering in a Fas ligand-independent manner in HCT116 cells resulting in caspase-8 activation via FADD (*28*). In other cell lines, however, a rapid DCA-induced apoptotic response was found to be Fas-independent (*3*, *17*). In the present study, it was confirmed that DCA rapidly induces cytochrome *c* release from mitochondria in HCT116 cells after 5 min, followed by subsequent activation of caspase-9. These results suggest that caspase-9 is

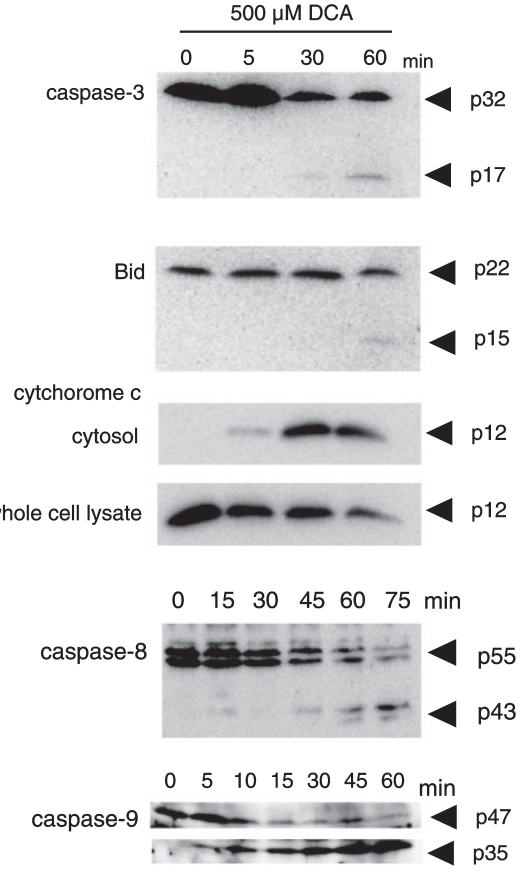

Fig. 5. **DCA-induced activation of caspase-8 and caspase-9, and cytochrome *c* release.** A: Cells were treated with 500 μM DCA for 1 h, and caspase-8, caspase-9, cytochrome *c*, and Bid were detected by Western blotting. A whole cell lysate or cytoplasmic extract was used for the detection of caspases and Bid or cytochrome *c*, respectively.

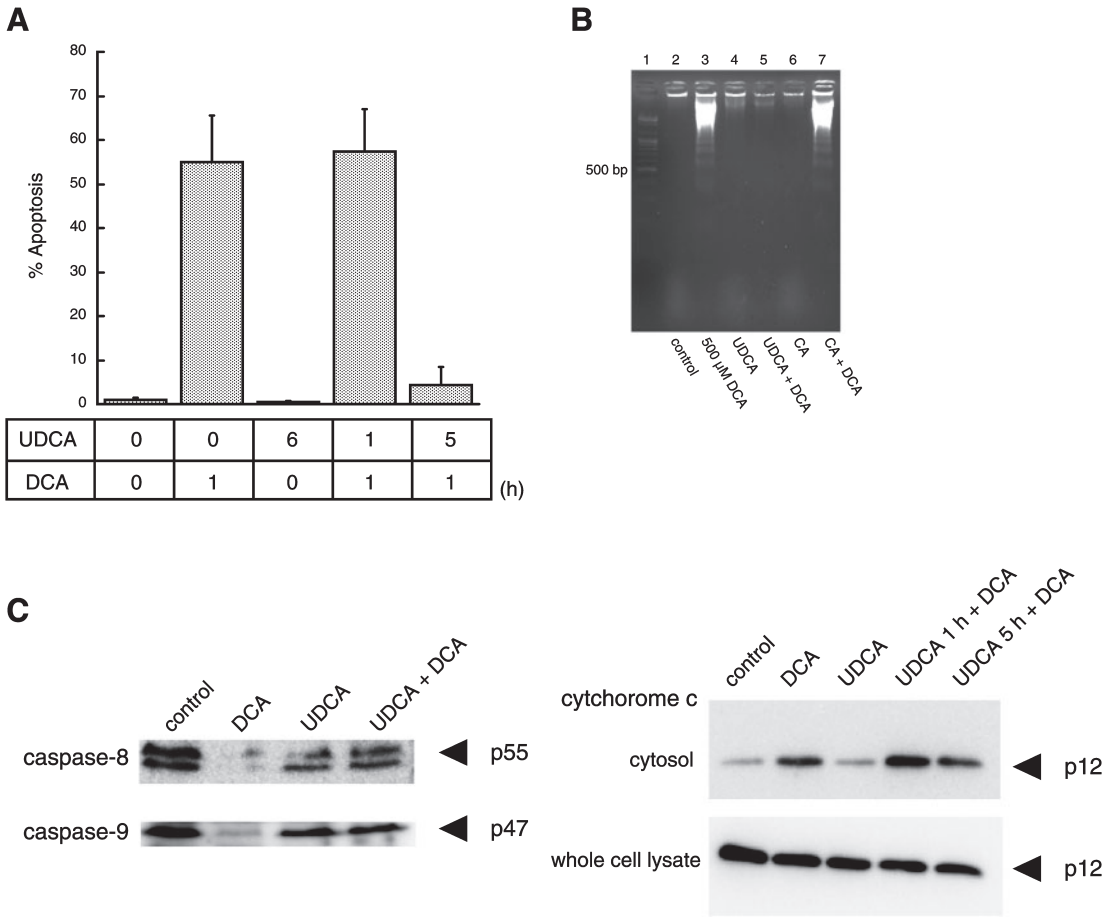

Fig. 6. **UDCA suppresses DCA-induced apoptosis.** A: HCT116 cells were treated with 500 μM UDCA for 1–6 h prior to treatment with 500 μM DCA for 1 h. The proportion of apoptotic cells was determined by Hoechst 33342 staining. Each column and bar represents the average and SE for triplicate experiments. B: Cells were treated with 500 μM UDCA or CA for 1 h prior to treatment with 500 μM DCA for 1 h. DNA fragmentation was examined. C: The effects of pretreatment with UDCA on DCA-mediated activation of caspase-8 and caspase-9, and cytochrome *c* release from mitochondria were examined by Western blotting. For caspase-8 and caspase-9, the cells were pretreated with 500 μM UDCA for 5 h. For cytochrome *c* release, the cells were pretreated with UDCA for 1 or 5 h.

the initiator caspase in the apoptotic process induced by DCA in HCT116 cells. Previously, it was reported that TRAIL-induced apoptosis in HCT116 cells was dependent on caspase-9, suggesting that HCT116 has the characteristics of type II cells (*1*, *21*). In this study, however, Bid, which is a proapoptotic member of the Bcl-2 family activated by caspase-8 and which transmigrates to the mitochondrial membrane to induce cytochrome *c* release, was not significantly truncated, suggesting that DCA-mediated caspase-9 activation is independent of Bid.

Although 1-h pretreatment with UDCA has been demonstrated to protect hepatocytes and hepatic cancer cells from bile acid-induced apoptosis, HT-29 colon cancer cells were not protected by 1-h pretreatment with UDCA (*3*, *12*, *24*). In the present study, pretreatment with 500 μM UDCA for 1 h had no effect on DCA-induced apoptosis in HCT116 cells, while pretreatment for more than 5 h completely suppressed apoptosis. Since HCT116 cells lack an active uptake system for bile acids (S. Yui, M. Iwabuchi, and T. Saeki, unpublished observations), it is conceivable that UDCA requires at least 5 h to reach an intracellular concentration sufficient to exhibit a protective effect. Alternatively, UDCA might modulate the expression of genes related to apoptosis or cell survival during the 5-h pretreatment period. It is interesting that UDCA completely blocked the activation of caspase-8 and caspase-9, but cytochrome *c* release from mitochondria was not inhibited in HCT116 cells, suggesting that UDCA exerts its effect between cytochrome *c* release and caspase-9 activation in HCT116 cells. This is in striking contrast to findings for hepatocytes, in which UDCA exerts a protective effect by blocking the release of cytochrome *c* from mitochondria.

Collectively, our results show that DCA induces apoptosis in HCT116 colon cancer cells by releasing cytochrome *c* from mitochondria, followed by subsequent activation of caspase-9 and caspase-8. The apoptotic response was rapid, and did not involve protein synthesis. The protective effect of UDCA on HCT116 cells was not demonstrated to result from the inhibition of cytochrome *c* release from mitochondria, in contrast to the situation in hepatocytes and hepatic cancer cells. Further investigations are necessary to determine how DCA causes cytochrome *c* release from mitochondria, and how UDCA has a protective effect on DCA-mediated apoptosis.

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