SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 28: 1429-1434, 2012

Pro-apoptotic role of the MEK/ERK pathway in ursodeoxycholic acid-induced apoptosis in SNU601 gastric cancer cells

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Received April 24, 2012; Accepted June 15, 2012

DOI: 10.3892/or.2012.1918

Abstract. Ursodeoxycholic acid (UDCA) has been regarded as a suppressor of gastrointestinal cancer, but the mechanisms underlying its antitumor effects are not fully understood. Previously, we reported the antitumor effect of UDCA by demonstrating that UDCA induces apoptosis of gastric cancer cells. Bile acids are known to activate the ERK pathway and ERK is a representative oncogenic kinase in cancer cells. Here, we investigated the role of ERK in UDCA-induced gastric cancer cell apoptosis. We found that UDCA enhanced the phosphorylation of ERK1/2 and MEK1/2. The prevention of MEK by the pharmacologic inhibitors PD98059 and U0126, resulted in decreased UDCA-induced apoptosis as shown by the reduction of apoptotic body formation, caspase-8 activity, and caspase-3, -6 and PARP cleavage, indicating that ERK exerts pro-apoptotic activity upon exposure to UDCA. In addition, U0126 reduced UDCA-triggered TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2/DR5) expression. In gene silencing studies, we observed that RNA interference of ERK2 decreased apoptosis and reduced DR5 overexpression. Lipid raft disrupting agent, methyl-β-cyclodextrin, blunted the phosphorylation of ERK1/2, indicating that ERK activation is regulated in a lipid raft-dependent manner. On the other hand, tumor-promoting bile acid, deoxycholic acid (DCA), also phosphorylated ERK in SNU601 cells. However, the DCA-triggered ERK pathway exerted anti-apoptotic function in the cells. Suppression of the ERK pathway enhanced DCA-induced apoptosis, and ERK activation was observed to be lipid raft-independently controlled. These results indicated that UDCA and DCA may cause differential responses in gastric cancer cells through the ERK signaling molecule. Thus, ERK activation may be a possible mechanism by which UDCA and DCA represent differential activities in gastrointestinal cancer.

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Key words: ursodeoxycholic acid, ERK, gastric cancer, apoptosis

Introduction

Bile acids are amphipathic molecules that are synthesized from cholesterol in the liver. They are essential to the digestion and absorption of lipids, but high concentration of bile acids exert pathological activities in hepatic and colorectal tissues (1). The hydrophobicity of bile acids seems to be closely linked to their pathological activities, and highly hydrophobic bile acids, such as deoxycholic acid (DCA), are potent apoptotic inducers and have been identified as tumor promoters (2). Several studies have demonstrated that bile acid-mediated hepatic injury is mainly due to hepatocellular apoptosis and colonic carcinogenesis caused by alterations in cell signaling and gene expression. In contrast, less hydrophobic (hydrophilic) bile acids such as ursodeoxycholic acid (UDCA) possess an opposite activity against hydrophobic bile acids. UDCA relieves cholestatic liver diseases by exerting cytoprotective and anti-apoptotic activities in hepatocytes, and it is implicated in the prevention of colonic cancer through cell cycle arrest and suppression of oncogenic factors including Ras and COX-2 (3,4).

Bile acids are known to induce both apoptotic and survival mechanisms in parallel (5,6), and the regulatory mechanism components governing cell death and survival include death receptor signaling, epidermal growth factor receptors (EGFR) and mitogen-activated protein kinases (MAPKs) (2,7). Death receptor-mediated apoptosis is controlled by membrane translocation of Fas/CD95 and overexpression of TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2/DR5) (2,7-9). Several lines of research have reported the importance of TRAIL-R2/DR5 induction in bile acid-triggered apoptosis (7,10). MAPKs are well understood enzymes that play critical roles in various cellular responses including cell growth, differentiation and apoptosis.

MAPKs belong to an evolutionarily conserved family of enzymes that includes three subfamilies: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK1/2) and p38 MAPK. In hepatocytes, prolonged activation of JNK1/2 or p38 MAPK promotes bile acid-induced apoptosis, whereas ERK1/2 are mainly involved in the cell survival pathway and their inhibition enhances bile acid-induced apoptosis (5,11,12). Hydrophobic bile acid, DCA, and hydrophilic bile acid, UDCA, have both been shown to activate the ERK pathway, and bile acid-mediated ERK activation seems to be essential in the cytoprotective pathway that prevents liver damage. However, when

it comes to cancer cells, hydrophobic bile acid-induced ERK activation seems to be oncogenic because the ERK pathway has been observed to be involved in COX-2 expression in esophageal cancer cells, it increases the invasiveness of colon cancer cells and chemoresistance in hepatocellular carcinoma cells, and it suppresses apoptosis in colon cancer cells (13-15). Indeed, deregulation of the ERK pathway has often been correlated with the malignant progression of human cancers (16,17).

In our previous study, we observed that UDCA performs a tumor preventing role in gastric carcinoma cells (18). However, the role of bile acids in the ERK pathway of gastric cancer cells remains unclear. In this study, we explored the effect of UDCA on the ERK pathway and found that the pro-apoptotic ERK pathway is activated in SNU601 gastric cancer cells. However, DCA-mediated ERK activation exerted an anti-apoptotic activity in this cell line, and this finding may point to one of the possible mechanisms of the anti-tumor effect of UDCA in gastric cancer cells.

Materials and methods

Cell culture and dosing. The SNU601 human gastric cancer cell line was obtained from the Korea Cell Line Bank and grown in RPMI-1640 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and 1% antibiotics at 37˚C in a 5% CO_2 atmosphere. Dosing of the cells was performed by adding 600-1000 μM UDCA (ICN Biomedicals) or 300 μM DCA (Calbiochem) to the culture medium and incubation for 48 h, unless otherwise specified. Cells were pretreated for 1 h with 30 μM of a MEK1 inhibitor (PD98059), 10 μM MEK1/2 inhibitor (U0126), 10 μM EGFR inhibitor (AG1478), 5 mM N-acetyl cysteine, 100 μM BHA (butylated hydroxyanisole) or 1 mM methyl-β-cyclodextrin (MBCD).

Apoptosis measurement. Treated cells were stained with 1 μg/ml Hoechst 33342 (HO) for 15 min at room temperature in the dark. Then, both the floating and attached cells were collected and centrifuged. The pooled cell pellets were washed with ice-cold phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde on ice, washed again with PBS, resuspended, and then a fraction of the suspension was centrifuged in a cytospinner (Thermo Shandon). Slides were prepared, air-dried, mounted in anti-fade solution and observed under a fluorescence microscope (DM5000, Leica) as described elsewhere (19). Any condensed/fragmented nuclei were assessed as apoptotic cells. A total of 500 cells from randomly chosen microscope viewing fields were counted and the number of apoptotic cells was expressed as a percentage of the total number of cells counted.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assays. For performance of the MTT assay, cells were plated in the wells of a 96-well plate at a density of $1x10^4$ cells/well, incubated for 24 h and then treated with drugs for 48 h. The MTT solution (0.5 mg/ml) was added to the wells and incubated for 4 h. The plates were centrifuged at 600 g for 10 min, and then the culture medium was removed. The cells were solubilized using dimethyl sulfoxide (DMSO) and the solubilized formazan product was quantified using an enzyme-linked immunosorbent assay (ELISA) plate reader at 595 nm. The absorbance of the untreated cells was designated as 100% and the cell survival was expressed as a percentage of this value.

Immunoblotting. Using a standard technique, equal amounts of protein were electrophoretically separated using SDS-PAGE and then transferred to a nitrocellulose membrane. Antibodies were used to probe for active caspase-6, -3, phospho-p38, p38, phosphor-MEK1/2, MEK1/2 (Cell Signaling Technology), PARP, phospho-ERK1/2, ERK2, phosphor-JNK1/2, JNK1/2, α-tubulin (Santa Cruz) and DR5 (Pro Sci). An image analyzer (Image Station 4000MM, Kodak) was used for acquisition of the probe signals.

Caspase-8 activity assay. According to the manufacturer's protocol, a FADD-like IL-1β-converting enzyme (FLICE) colorimetric assay kit (BioVision) was used to perform the caspase-8 activity assay. Briefly, 200 μg of protein lysates in a 50-μl volume was mixed with reaction buffer, mixed with IETD-pNA substrate, and then incubated for 90 min. The resulting absorbance was measured at a wavelength of 405 nm. Fold increase in FLICE activity was determined by comparison of the results of the treated samples with the level of the untreated control.

RNA interference (RNAi). For the RNAi experiment, siRNA of ERK1, 5'-CUC UCU AAC CGG CCC AUC U(dTdT)-3' (S) and 5'-AGA UGG GCC GGU UAG AGA G(dTdT)-3' (AS), ERK2, 5'-CAC CAU UCA AGU UCG ACA U(dTdT)-3' (S) and 5'-AUG UCG AAC UUG AAU GGU G(dTdT)-3' (AS), EGFR, 5'-GAU CCA CAG GAA CUG GAU A(dTdT)-3' (S) and 5'-UAU CCA GUU CCU GUG GAU C(dTdT)-3' (AS), and control siRNA, 5'-CCUACGCCACCAAUUUCGU(dTdT)-3' (S) and 5'-ACGAAAUUGGUGGCGUAGG(dTdT)-3' (AS) were purchased from Bioneer (Daejeon, Korea). Using an Amaxa transfection kit, cells (10^6) were transfected with 5~8 μg siRNA, and the transfected cells were then stabilized for 24 h prior to dosing.

Results and Discussion

UDCA induces pro-apoptotic ERK1/2 activation in SNU601 cells. Generally, the bile acid-induced ERK pathway in gastrointestinal cancer stimulates cell proliferation, inhibits apoptosis and causes chemoresistance, as observed in other human carcinomas (5,14,15). However, the precise role of hydrophilic bile acid in the ERK pathway in gastric carcinoma cells remains unclear. In this study, we examined the role of the ERK pathway in UDCA-induced apoptosis of the SNU601 gastric carcinoma cell line. First, we examined the effect of UDCA on the MAPK family members. SNU601 cells were exposed to 600 μM UDCA for various time intervals, and as active MAPKs can be estimated by measuring the appearance of phosphorylated forms of MAPKs, the phosphorylation patterns of ERK1/2, p38, JNK1/2 and MEK1/2 were determined. As shown in Fig. 1, treatment by UDCA increased the phosphorylation levels of ERK1/2 and MEK1/2, but had no effect on p38 and JNK1/2 in SNU601 cells. In order to elucidate the effect of activation of the ERK pathway upon exposure to UDCA, the SNU601 cells were pre-incubated with 30 μM PD98059 (MEK1 inhibitor) or 10 μM U0126 (MEK1/2 inhibitor) for 1 h, and then further exposed to UDCA for 48 h. As reported

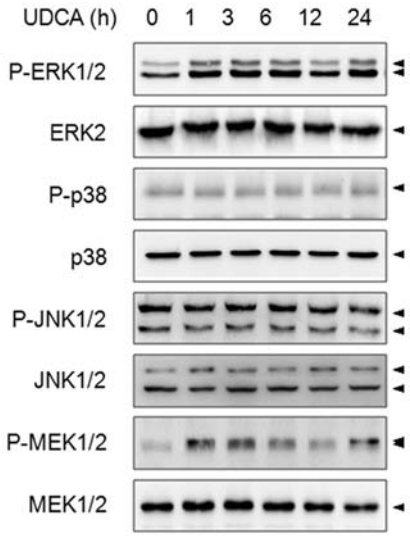

Figure 1. UDCA activates the MEK/ERK pathway. SNU601 cells were exposed to 0.6 mM UDCA for various durations and the results were analyzed by immunoblotting using antibodies to phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK, JNK, phospho-MEK1/2 and MEK1/2.

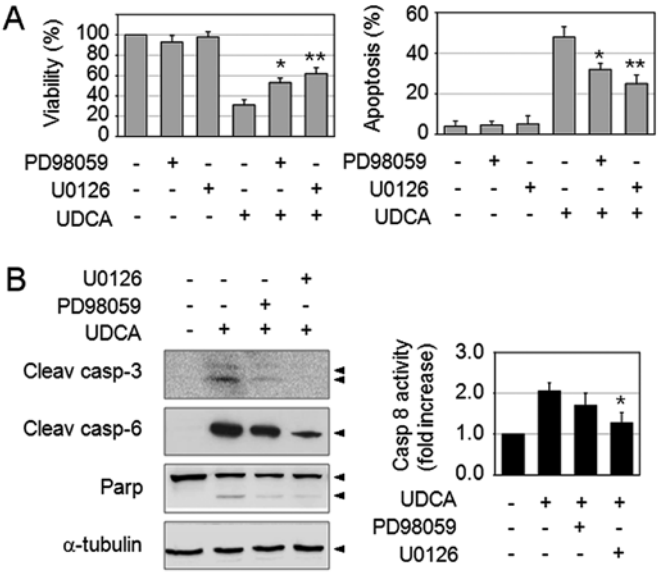

Figure 2. MEK inhibitors reduce UDCA-induced apoptosis. (A) Cells were exposed to 0.6 mM of UDCA in the absence or presence of 30 μM PD98059 and 10 μM U0126 for 48 h. Viability and apoptosis were measured by MTT assay and apoptotic body formation, respectively. (B) Cells were treated with 0.6 mM UDCA either alone or in combination with 30 μM PD98059 or 10 μM U0126 for 48 h, and the same amount of extracted protein was analyzed using immunoblotting (left) or caspase-8 activity (right). *p<0.01; **p<0.005 versus UDCA-treated sample.

above, UDCA significantly reduced cell viability and increased apoptosis in SNU601 cells. Interestingly, combined treatment of UDCA with MEK inhibitors partially enabled the recovery of cell viability and reduced the amount of apoptosis (Fig. 2A). In addition, as detected by immunoblotting and FLICE-like enzyme activity assay, MEK inhibitors also reduced the quantity of the active form of caspase-3, -6 and resulting PARP cleavage, as well as caspase-8 activity (Fig. 2B). These results indicated that UDCA-induced ERK activation plays a pro-apoptotic role in SNU601 cells. Although it plays an anti-apoptotic role in general, recent studies have shown several exceptional roles for ERK. α-Tocopheryl succinate-induced apoptosis has been reported to be modulated by ERK1/2 in gastric cancer cells (20). In addition, ERK has been shown to play a pro-apoptotic function upon exposure to cisplatin in multiple cancer cells including cervical carcinoma, osteosarcoma, neuroblastoma and myeloid leukemia (21-25). Hence, various cancer cells may be able to use the ERK pathway to mediate a pro-apoptotic signal under certain conditions.

The ERK pathway is involved in UDCA-induced DR5 overexpression. In our previous study, we found that DR5 overexpression was largely responsible for the UDCA-induced apoptosis in gastric cancer cells (18). Although DR5 induction was shown to be regulated by PKCδ activation (18), we questioned whether or not the ERK pathway is also connected to UDCA-induced DR5 expression signaling. To this end, we assessed the DR5 expression level under suppression of ERK activation. SNU601 cells were treated with UDCA in the absence or presence of 10 μM U0126 (MEK1/2 inhibitor) for 24 h, and then analyzed by immunoblotting using anti-DR5 antibody. As shown in Fig. 3A, treatment by UDCA highly increased the expression of DR5, and treatment in combination with U0126 partially decreased the DR5 expression level as compared to UDCA-only treated samples. This result suggested the partial involvement of the ERK pathway in the UDCA-induced DR5 expression pathway.

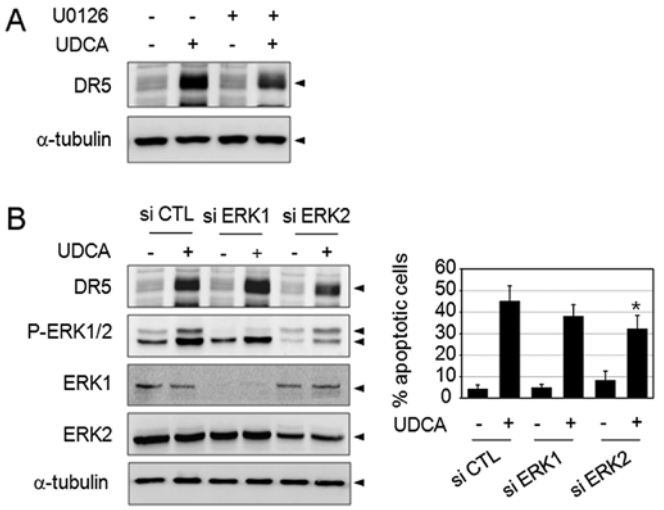

Figure 3. The ERK pathway is associated with DR5 induction in UDCA-triggered apoptosis. (A) Cells were exposed to 1 mM of UDCA for 24 h, either with or without 10 μM U0126, and the same amount of extracted protein was analyzed using immunoblotting with anti-DR5 antibody. (B) Cells were transfected with siRNA for ERK1 and ERK2, and scrambled RNA (siCTL) for 24 h, and then treated with 1 mM UDCA for 24 h. Cells were analyzed by immunoblotting (left) and the resulting apoptosis was assessed after nuclear HO staining as described in Materials and methods (right). *p<0.01 versus control RNA transfected UDCA-treated sample.

Then, in order to confirm the role of ERK in UDCA-induced apoptosis, we silenced ERK expression using siRNA specific to ERK1 and ERK2, and examined its effects on UDCA-induced DR5 expression and apoptosis. The result of the silencing effect in the reduction of ERK1 and ERK2 protein levels was confirmed by immunoblotting. Transfection with siRNA targeting ERK1

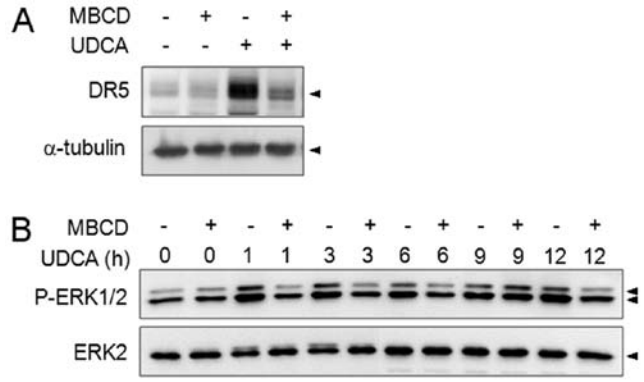

Figure 4. UDCA-induced ERK phosphorylation is lipid raft-dependently controlled. (A) Cells were exposed to 1 mM UDCA, either with or without 1 mM MBCD, for 24 h and analyzed by immunoblotting to measure the resulting DR5 expression level. (B) Cells were pre-exposed with 1 mM MBCD for 1 h and further incubated with 1 mM UDCA for the indicated durations. Then, the protein extracts were collected and analyzed by immunoblotting to detect phosphorylated ERK1/2 and total ERK1/2.

appeared to slightly reduce the number of apoptotic cells, but the results were statistically insignificant and did not affect the DR5 expression level as compared with the control siRNA. However, siRNA targeting ERK2 reduced the UDCA-induced DR5 expression level and significantly decreased the apoptotic cell rate. These results suggested that ERK2 activity may be linked to the pro-apoptotic signaling that contributes to DR5 upregulation in response to UDCA exposure.

ERK phosphorylation is lipid raft-dependently controlled. In order to examine the upstream regulation of the MEK/ERK pathway, we assessed the role of lipid rafts in ERK phosphorylation. Previously, PKCδ-mediated DR5 expression was shown to be controlled by lipid rafts upon exposure to UDCA (18). The role of lipid rafts in DR5 induction was reconfirmed using lipid raft disrupting agent, MBCD (Fig. 4A) and MBCD clearly reduced UDCA-induced ERK phosphorylation at all measured time-points. However, the suppression of PKCδ did not affect

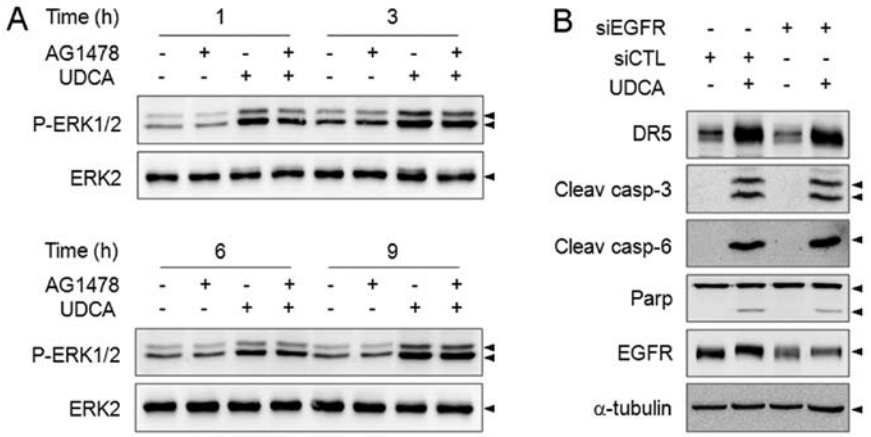

Figure 5. UDCA-induced pro-apoptotic ERK activation is not signaled from EGFR. (A) Cells were exposed to 1 mM UDCA, either with or without 10 μM AG1478, for the indicated times and analyzed by immunoblotting in order to detect phosphorylated ERK1/2 and total ERK1/2. (B) Cells were transfected with siRNA targeting EGFR and scrambled RNA (siCTL) for 24 h, and then treated with 1 mM UDCA for 24 h. The cells were then subjected to immunoblotting in order to detect cleaved caspase-3 and -6, PARP, EGFR and α-tubulin.

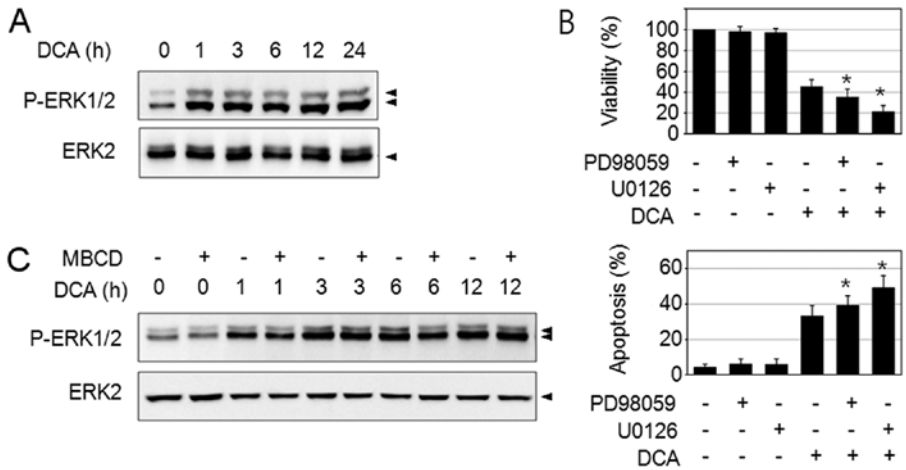

Figure 6. The DCA-induced ERK pathway exerts anti-apoptotic activity. (A) Cells were exposed to 0.3 mM DCA for the indicated durations and then analyzed by immunoblotting. (B) Cells were exposed to 0.3 mM of DCA either in the absence or presence of 30 μM of PD98059 and 10 μM of U0126 for 48 h. Viability and apoptosis were measured by MTT assay and apoptotic body formation, respectively. (C) Cells were pre-exposed with 1 mM MBCD for 1 h and further incubated with 0.3 mM DCA for the indicated durations. Then, the protein extracts were analyzed by immunoblotting. $^{*}p<0.01$ versus DCA-treated sample.

ERK activation and vice versa (data not shown). These results indicated that the ERK pathway is also lipid raft-regulated but that it is a PKCδ-independently activated pathway. We further explored whether or not EGFR is involved in UDCA-induced ERK activation. The role of EGFR signaling has been implicated in bile acid-induced ERK activation in hepatocytes, but UDCA did not induce EGFR activation in colon cancer cells (26). A specific inhibitor of EGFR, AG1478 scarcely affected UDCA-induced ERK phosphorylation (Fig. 5A). Furthermore, although the interference of EGFR expression was confirmed, silencing of the expression of EGFR by specific siRNA did not alter the DR5 protein level, or cleavage of caspase-3, -6 and PARP in response to UDCA (Fig. 5B). Therefore, UDCA-triggered ERK activation may not be regulated by the EGFR pathway in SNU601 cells.

DCA-induced ERK activation plays an anti-apoptotic role. Tumor promoting hydrophobic bile acids such as DCA have also been reported to activate the ERK pathway in hepatocytes and colon cancer cells. The hydrophobic bile acid-induced ERK pathway has been suggested to be associated with various tumor promoting properties in cancer cells. Therefore, we questioned whether or not hydrophobic bile acid DCA can also trigger ERK activation in SNU601 cells. DCA treatment strongly increased ERK1/2 phosphorylation as shown in Fig. 6A. Then, we examined the role of DCA-induced ERK activation in SNU601 cells. When cells were treated with DCA their viability was reduced and apoptosis was observed. The combination of DCA with MEK inhibitors, 30 μM of PD98059 or 10 μM of U0126, further reduced cell viability and increased apoptosis (Fig. 6B). These results agreed with previous reports in which ERK was observed to play an anti-apoptotic role in DCA-induced apoptosis (5). Therefore, the results obtained above indicated that the DCA-induced ERK pathway exerts an opposite activity from which it was induced by UDCA in SNU601 gastric cancer cells. Next, we examined if DCA-induced ERK activation requires lipid rafts or not. We found that combined treatment of MBCD with DCA did not alter the ERK phosphorylation level as compared to the DCA treated samples (Fig. 6C), indicating that DCA-induced ERK activation is not mediated by lipid rafts. Therefore, UDCA and DCA seem to regulate ERK activation via a differential signaling mechanism and lead to opposite responses through the ERK signaling molecule.

Previously, we hypothesized UDCA anti-tumor activity by demonstrating that UDCA induces apoptosis of gastric cancer cells. We found that UDCA triggered DR5 overexpression through lipid rafts, ROS, and in a PKCδ-dependent manner, and the overexpressed DR5 was translocated to the lipid raft region and recruited by DISC proteins to initiate caspase-8 activation (18). However, tumor-promoting hydrophobic bile acids are also strong apoptotic inducers and it was observed that DCA induced apoptosis as well as necrosis in gastric cancer cells (18). In hepatocytes, the cytoprotective and tumor preventing features of UDCA that distinguishes it from other hydrophobic bile acids is often assumed to be the result of its mild hydrophobicity, because strong hydrophobicity will destroy membrane structures by detergent effects. Nevertheless, we found that the ERK pathway triggered by UDCA and DCA played an opposite role in the viability of SNU601 cells. This result indicated that, more than simply having a milder effect as compared to DCA, UDCA may possess tumor-preventing activities by inducing differential responses. Indeed, strong hydrophobic bile acid-induced ERK activity has been reported to be linked with various tumor-promoting functions. Lithocholic acid (LCA) has been shown to induce expression of urokinase-type plasminogen activator receptor (uPAR) and enhances cell invasiveness in colon cancer cells (14). Furthermore, the ERK pathway is involved in DCA-upregulated mucin gene transcription, which is often implicated in colon neoplasia (27). Our finding suggested that UDCA and DCA trigger differential responses in cancer cells using ERK. Thus, UDCA exposure can produce an anti-tumor effect.

Acknowledgements

This study was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (2009-0075493) and through the Research Center for Resistant Cells (R13-2003-009).

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