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**Ursodeoxycholic acid differentially affects three types of sphingomyelinase in human colon cancer Caco 2 cells**

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## **Abstracts**

We investigated the effects of UDCA on sphingomyelinase (SMase) in Caco 2 cells cultured under monolayer and polarized conditions. Alkaline SMase activity was high in polarized cells whereas acid and neutral SMase activities were high in monolayer cells. In polarized cells UDCA increased alkaline SMase expression and caspase 3 activity but had no effect on acid and neutral SMases. In monolayer cells, UDCA reduced both acid and neutral SMase activities, inhibited cell proliferation, but had little effect on alkaline SMase and caspase 3 activities. In conclusion, UDCA differentially affects SMase activity, cell proliferation, and apoptosis in colonic cells, depending on the cell conditions.

**Key words: Sphingomyelinase; Colon cancer; UDCA; Caco 2 cells**

## 1. Introduction

Ursodeoxycholic acid (UDCA) is a type of bile salt naturally occurring in human bile in a relatively small amount. A novel chemopreventive effect of UDCA against colonic carcinogenesis was first identified by Earnest et al in an animal study [1] and confirmed by others in both animal and human studies [2,3]. The mechanism underlying the anticancer effects of UDCA is not fully understood, and multiple molecules that are effected by UDCA have been reported, including Cox 2, phospholipase A , cycline D, E-cadherin, and Ras [2,4,5].

Sphingomyelin (SM) metabolism generates multiple lipid messengers such as ceramide, sphingosine and sphingosine-1-phosphate which regulate cell proliferation and apoptosis [6,7]. A particular link between SM and colon cancer has been indicated. SM was found to be accumulated and ceramide reduced in precancer lesions and cancer tissues in either animal models or human beings [8,9]. Supplement of SM and other sphingolipids in the diet could inhibit the formations of aberrant crypt foci and carcinomas in animals treated with a chemical carcinogen [10]. Since SM and glycosphingolipids can not be absorbed intact [11], the hydrolysis of sphingolipids in the gut may be a prerequisite for their anticancer effects. In the intestinal tract, there are at least three types of SMase called acid, neutral and alkaline SMase. The acid and neutral SMases are common enzymes whereas alkaline SMase is specifically present in the intestinal mucosa as an ecto enzyme with the highest hydrolytic capacity among these SMases [12-14]. The alkaline SMase has been shown to be significantly decreased in both precancer lesions such as ulcerative colitis [15] and in colonic adenocarcinoma [16]. A mutation of the enzyme caused by an alternative splicing which inactivates the enzyme has been identified in HT 29 cells but not in Caco-2 cells [17]. Caco-2 cells cultured in monolayer conditions express little alkaline SMase and the expression increases significantly in polarized conditions undergoing differentiation [17].

We reported previously that feeding rats UDCA significantly increased alkaline SMase activity in the colonic mucosa in positive correlation with the activity of caspase 3, a key enzyme for apoptosis [18]. In the present study, we examined the direct effect of UDCA on three types of SMase in Caco-2 cells cultured in both monolayer and polarized conditions.

## **2. Materials and methods**

*2.1 Materials* UDCA (purity >98%) was provided by Dr. Falk Pharma GmbH (Freiburg, German). SM was purified from bovine milk and labelled with [<sup>14</sup>C-CH<sub>3</sub>] choline ([<sup>14</sup>C-SM]) at Astra Zeneca (Stockholm, Sweden). Anti-human alkaline SMase antibody was developed in AgriSera AB (Vännäs, Sweden) using purified human alkaline SMase as an antigen [13]. All cell culture mediums and other chemicals used were purchased from Sigma Co. (Stockholm, Sweden).

*2.2 Cell cultures* The Caco 2 cells were cultured in both monolayer and polarized conditions as described [17]. The monolayer cells at about 70% confluence were stimulated with UDCA for 3 days. The polarized cells were cultured in filter inserts (1.0 μm , BD Falcon, Bedford, USA) in a 6-well plate. At day 14 when the cells were confluent and started polarization, UDCA was added in the cultured medium and the cells were further cultured for 3 days. Both monolayer and polarized cells were scraped, centrifuged, washed, and lysed in lysis buffer as described previously [17]. The lysate was centrifuged at 20,000 g for 10 min and the supernatant was used for biochemical analysis and Western blotting.

*2.3 SMase assays* The activities of three types of SMases were determined with different buffers as described previously [19]. Alkaline SMase assay was assayed in 50mM Tris-HCl buffer, pH 9.0, containing 0.15M NaCl, 2 mM EDTA, and 6 mM taurocholate. Neutral SMase was assayed in 50 mM Tris buffer containing 2 mM MgCl<sub>2</sub>, 0.15 M NaCl, and 0.12% Triton X 100, pH 7.5. Acid SMase was assayed in 50 mM Tris-maleate buffer containing 0.15 NaCl and 0.12% Triton X 100, pH 5.0. For each determination, 5μl sample

was mixed with 95  $\mu$ l of buffer and 0.80  $\mu$ M [ $^{14}$ C]-SM and incubated at 37 °C for 30 min. The reaction was terminated by adding 0.4 ml of chloroform/methanol (2:1) and the cleaved phosphocholine in the upper phase was counted by liquid scintillation.

*2.4 Western blotting* Western blotting of alkaline SMase was performed as described [13]. Fifty microgram of proteins were subjected to 10% SDS PAGE and transferred to a nitrocellulose membrane by electrophoresis. After washing and blocking, the membrane was probed with anti-human alkaline SMase antibody (1:500) and then with a second antibody conjugated with alkaline phosphatase (1:2000). Colour development was carried out by a kit from Bio-Rad. For a loading control, parallel Western blotting was performed and the membrane was blotted with anti-actin antibody.

*2.5 Assay caspase 3 activity and cell proliferation* Caspase 3 activity was determined using N-acetyl-Asp-Glu-Val-Asp-pNa as substrate in 50 mM Hepes buffer containing 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol, pH 7.4 [20]. The cell proliferation was assayed by the cleavage of tetrazolium salt (WST-1 reagent) to formazan by mitochondria dehydrogenase as described previously [20]. The protein was assayed by a kit from Bio-Rad using serum albumin as a standard.

*2.6 Statistical analysis* The results are present as Mean  $\pm$  standard error obtained from duplicate determinations in at least 3 separate experiments. The statistical significance was determined by Student *t* test and  $p < 0.05$  was considered to be statistically significant.

### **3. Results**

*3.1 Effects of UDCA on alkaline SMase* As shown in the top panel of Fig. 1, alkaline SMase activity was much lower in monolayer cells than in polarized cells. UDCA increased the activity of alkaline SMase in polarized cells dose-dependently, with 75% increase obtained by 0.5 mM UDCA. UDCA at concentrations less than 0.5 mM had no effect on monolayer cells, and 1 mM UDCA only induced a slight increase of alkaline SMase activity. The

increased activity in polarised cells may be due to an enhanced expression, as Western blotting showed that UDCA at 0.5 mM increased the alkaline SMase protein in the polarized cells (lower panel). For monolayer cells, Western blot showed very faint bands of alkaline SMase and the changes were not detectable after UDCA stimulation (data not shown).

*3.2 Effects of UDCA on acid and neutral SMases* As shown in Fig. 2, acid SMase activity in monolayer cells was 3.6 fold higher than in polarized cells. Similar phenomenon was found for neutral SMase. UDCA decreased both acid and neutral SMase activities in monolayer cells dose-dependently. The effects were much smaller for polarized cells, and 1 mM UDCA only caused 20% decrease in acid SMase activity but not neutral SMase activity.

*3.3 Effects of UDCA on caspase 3 activity and cell proliferation* The effects of UDCA on caspase 3 activity are shown in the top panel of Fig. 3. UDCA increased caspase-3 activity markedly in polarized cells but not in monolayer cells. At high concentration (1 mM), UDCA even reduced caspase 3 activity in monolayer cells. The low panel shows that UDCA inhibited cell proliferation in monolayer cells. The effects of UDCA on cell proliferation under polarized conditions were not studied since the cells were confluent on the filter insert already before UDCA stimulation.

#### **4. Discussion**

The present work demonstrates that the activities of SMases in the colon varied significantly with the conditions of the cells. Alkaline SMase activity was high in polarized cells whereas acid and neutral SMase activities were high in monolayer cells. UDCA affected SMase activities, cell proliferation, and apoptosis, depending on the cell conditions, stimulating alkaline SMase expression and apoptosis in polarized cells, and inhibiting acid and neutral SMase activities and cell proliferation in monolayer cells.

Human colon cancer Caco-2 cells can be cultured conventionally in monolayer conditions or in an insert filter which allows the cells to undergo differentiation and

polarization [21]. The polarized cells resemble the matured absorptive enterocytes on the microvilli, which under normal conditions are shortly alive and are destined to apoptosis. UDCA increased the activity of caspase 3, the executor of apoptosis [22], in polarized cells, indicating that UDCA is able to stimulate the apoptotic process of the matured epithelial cells. Alkaline SMase is the major SMase in the intestinal tract that hydrolyses both endogenous and exogenous SM in the intestinal lumen and generates ceramide, the important lipid messenger with anticancer properties [6,7]. The increased alkaline SMase expression by UDCA may contribute to the changes of caspase 3, as ceramide has been shown to be able to activate caspases, and a positive correlation between alkaline SMase activity and caspase 3 activity has been demonstrated [18,23]. In contrast to the polarized cells, UDCA did not show a decent effect on either alkaline SMase or caspase 3 in monolayer cells. We previously reported that little alkaline SMase was expressed in Caco-2 cells cultured in monolayer conditions and the expression increased significantly in polarized cells associated with differentiation [17], indicating that some unknown factors that are present in differentiated Caco-2 cells are required for triggering the expression of alkaline SMase. UDCA can not markedly enhance the expression of alkaline SMase in monolayer cells where the prerequisite factors are absent. Of interest is the finding that UDCA, although did not activate caspase 3 in monolayer cells, inhibited the cell proliferation. The monolayer Caco 2 cells resemble the non-differentiated enterocytes migrating up on the crypts with rapid division. The finding indicates that UDCA can inhibit the migration and division of the enterocytes along the crypts. In association with such an inhibition, UDCA dose-dependently reduced acid and neutral SMase activities. Whether the reduced activities are contributable to the changed cell proliferation is unknown. Although ceramide can be generated by any types of SMase, the biological functions of ceramide vary with how and where it is generated and which pathway it activates [24]. High concentration of acid SMase is probably in favour of cell survival as the basal activity is much



higher in monolayer cells under growing than in polarized cells awaiting apoptosis as shown in this paper. In agreement to this hypothesis, we have shown that the expression of acid SMase was enhanced by high fat diet, the noxious factor for colon cancer in vivo [23] and inhibited by anticancer compounds such as ursolic acid in vitro [25].

Finally, the concentrations used in this study is compatible with others, but the relevance of the doses to in vivo situations is hard to estimate. The normal dose of UDCA for patients with liver diseases is 12-15 mg/kg, i.e. about 3 mmole for a person with 75 kg body weight. The exact concentration of UDCA in the colon in these patients is unknown. UDCA is highly hydrophilic and has protective effect on intestinal mucosa. Animal study showed that concentration of UDCA in the colon can be significantly increased by 7-sulfate conjugation without induction of toxic effect [22].

### **Acknowledgement**

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**Fig 1.**

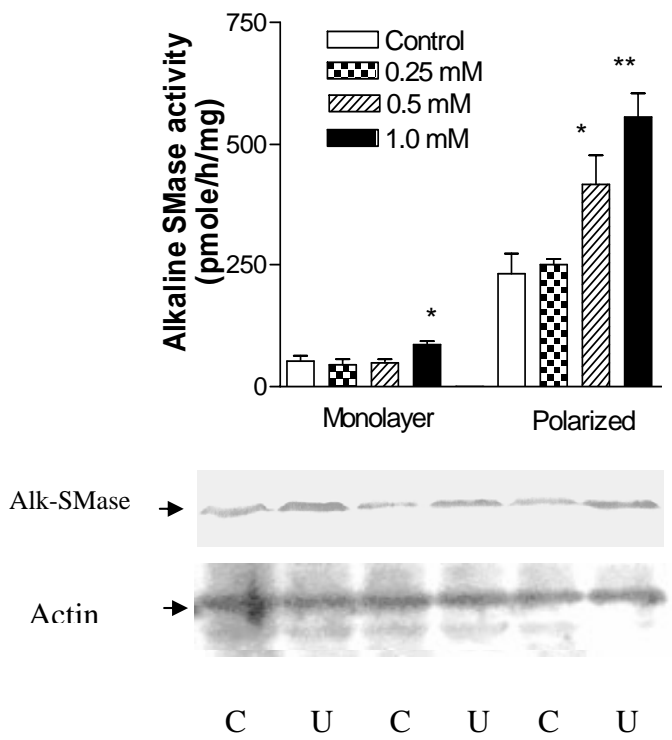
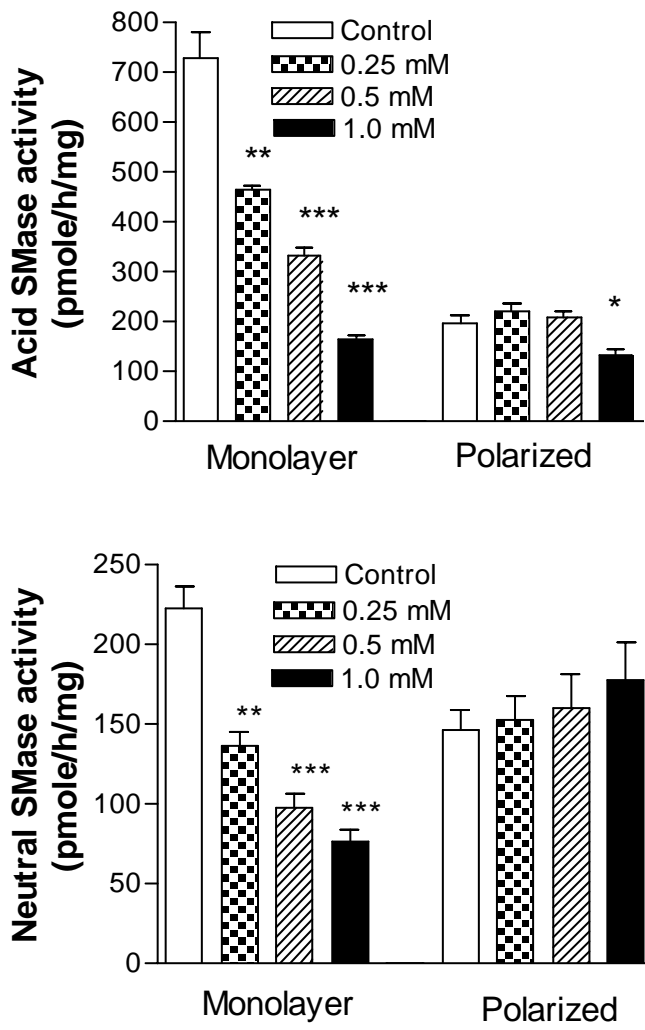
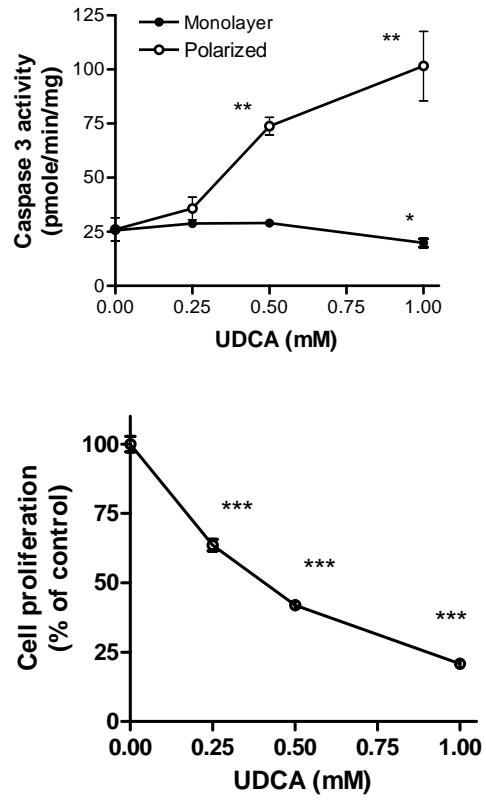


Fig. 2



**Fig. 3.**





## Figure legend

Fig. 1. Effects of UDCA on alkaline SMase. In the upper panel, both monolayer and polarized cells were treated with UDCA for 3 days. The cell-free extracts were prepared and alkaline SMase activity determined. \*  $P < 0.05$ , \*\* $P < 0.01$  compared with control. In the lower panel are Western blottings of alkaline SMase in polarized cells treated with and without UDCA. Results from 3 pair samples are demonstrated. C: control, U: UDCA treated. Western blotting of actin was used as a loading control.

Fig. 2. Effects of UDCA on acid and neutral SMase activities. Both monolayer and polarized cells were incubated with UDCA for 3 days. The activities of acid (upper panel) and neutral SMase (lower panel) were determined. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  compared with control.

Fig. 3. Effects of UDCA on caspase 3 activity and cell proliferation. In the upper panel, caspase 3 activity was determined in both monolayer and polarized cells treated with UDCA. In the lower panel, monolayer cells were incubated with UDCA for 3 days and the cell proliferation was determined. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  compared with control.