Ursodeoxycholic Acid Inhibits the Initiation and Postinitiation Phases of Azoxymethane-induced Colonic Tumor Development[1]

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Abstract

Colonic tumorigenesis involves the processes of initiation and promotion/progression from normal epithelial cells to tumors. Studies in both humans and experimental models of colon cancer indicate that secondary bile acids promote tumor development. In contrast, we have demonstrated previously that another bile acid, ursodeoxycholic acid (UDCA), inhibits the development of azoxymethane (AOM)-induced colon cancer in rats. More recently, we have shown that UDCA inhibits AOM-induced hyperproliferation, and aberrant crypt formation and growth. In our previous studies, we supplemented UDCA throughout the experiment. The efficacy of a chemopreventive agent may depend on the timing of administration, which has important clinical implications. In the present investigation, we examined the ability of UDCA, when administered only in the initiation or the promotion/progression phase, to block tumor development. Male Fisher 344 rats were divided in a 2 × 3 factorial design, with animals receiving AOM or vehicle, and fed an unsupplemented diet or diet supplemented with 0.4% UDCA in the initiation or promotion/progression phase. Thirty-two weeks later, rats were sacrificed and tumor histology determined, and colons were examined for aberrant crypt foci (ACF). In the carcinogen-treated dietary control group, tumor incidence was 72.3%, and tumor multiplicity was 1.9 tumors per tumor-bearing rat. UDCA, in the initiation or promotion/progression phase, significantly decreased tumor incidence to 46.2% and 38.4% ($P < 0.05$), respectively; and tumor multiplicity to 1.4 and 1.3 tumors per tumor-bearing rat ($P < 0.05$), respectively. UDCA did not alter tumor size, histology, or location, although there were trends for smaller tumors and less advanced histological grades in the group given UDCA during the promotion phase. UDCA, in the initiation but not the promotion phase, inhibited ACF formation and growth. In summary, UDCA significantly inhibited AOM-induced colonic carcinogenesis during either tumor initiation or in the promotion/progression phase. In contrast, UDCA inhibited ACF formation only when administered in the initiation phase, suggesting that the mechanisms of chemoprevention by this bile acid differ in these two phases.

Introduction

Colon cancer is a leading cause of cancer-related deaths in the Western world (1). Environmental factors, especially dietary constituents and xenobiotic procarcinogens, are thought to contribute to the development of colonic cancers (2). Diets high in animal fats, which are associated with increased fecal bile acids, have been implicated in the increased occurrence of this malignancy in the industrialized world (2, 3). These lipid rich diets, moreover, promote tumor growth in the AOM[3] model of colon cancer (4). Dietary animal fat increases the fecal concentration of secondary bile acids that are known to enhance cell proliferation and promote tumor development in this model (5–11). Whereas the mechanisms that underlie dietary animal fat-induced tumor promotion remain speculative, this link with secondary bile acids is intriguing.

Despite advances in our understanding of many of the genetic and epigenetic features of this malignancy, survival of individuals with advanced colon cancer remains poor. Therefore, efforts have focused increasingly on possible chemopreventive strategies in high-risk individuals (12). Recent studies in patients with conditions predisposing to colon cancer, moreover, suggest that a low abundance primary bile acid, UDCA, may prevent human colonic neoplasms (13, 14). Our laboratory has shown that UDCA supplemented in the diet of AOM-treated rats significantly inhibited both carcinogen-induced and cholic acid-promoted tumor development (15). In our previous studies UDCA was supplemented throughout the course of the experiment (15). More recently, we have found that UDCA also inhibited the development and growth of ACF (16). ACF are the earliest identifiable putative premalignant precursors of AOM and human colon cancers (17, 18). Whether the protective effects of UDCA occur during tumor initiation and/or at a later stage of tumor growth is uncertain, but of potentially great clinical significance. Pyrazole and disulfiram, for example, inhibit tumorigenesis only in the initiation phase (19–21), and inorganic selenium (Na_2SeO_3) only in the post-initiation phase (22), whereas Cox-2 inhibitors block tumor

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[3] The abbreviations used are: AOM, azoxymethane; ACF, aberrant crypt foci; Cox-2, cyclooxygenase-2; UDCA, ursodeoxycholic acid; TBR, tumor-bearing rat.

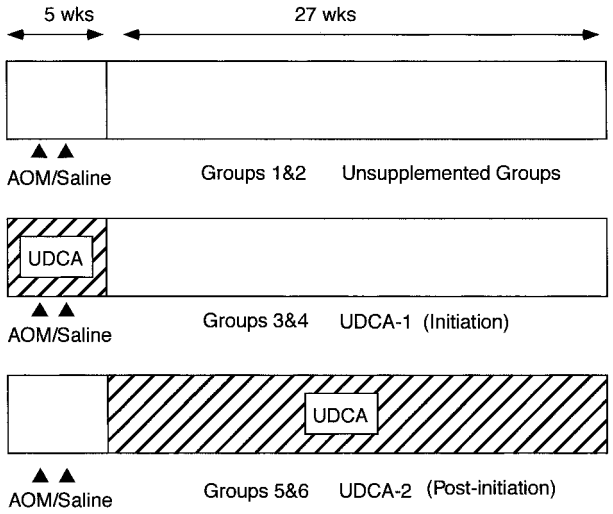

Fig. 1. Animal treatment protocol. Animals in groups 1 and 2, and 5 and 6 were fed AIN-76A alone, and animals in groups 3 and 4 were supplemented with UDCA [*UDCA-1 (initiation)*]. After 2 weeks of diet rats received either AOM or vehicle (▲) as described in "Materials and Methods," and the diets were continued. Two weeks after the second AOM/vehicle injection (▲), animals in groups 3 and 4 were switched to AIN-76A alone, and animals in groups 5 and 6 were started on UDCA supplementation [*UDCA-2 (postinitiation)*]. The study was terminated 32 weeks after diets were started.

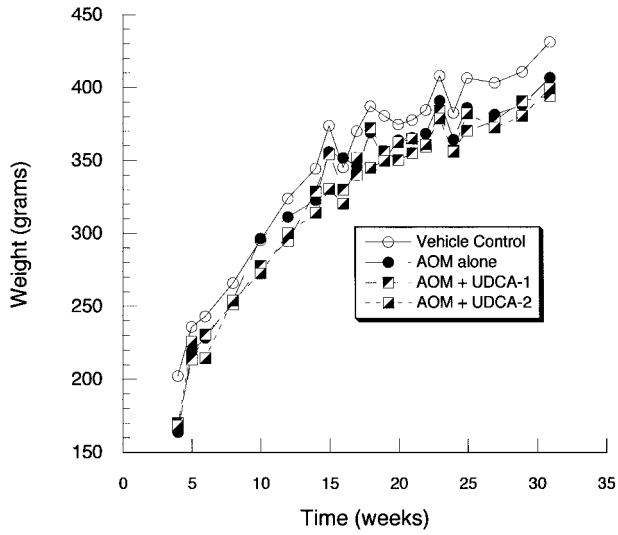

Fig. 2. Effect of AOM and UDCA supplementation on rat growth. Animals were fed AIN-76A alone or supplemented with UDCA. After 2 weeks of diet they were treated with AOM or vehicle, and the dietary protocols continued as described in "Materials and Methods," and summarized in Fig. 1. At weekly intervals animals were weighed. Unsupplemented and UDCA-supplemented control animals that received saline (AOM vehicle) were combined for this analysis (*vehicle control*), because there were no differences in their weights.

development when given in the initiation or the promotion/progression phase in this model (23). Chemopreventive agents effective in the initiation phase are often carcinogen-specific. These agents may act by altering the metabolism or distribution of the carcinogen, for example, by blocking the conversion of the procarcinogen to active carcinogen, or by accelerating detoxification or elimination of the carcinogen. Agents that block tumor development in the promotion/progression phase presumably inhibit downstream effector pathways initiated by mutations and epigenetic signaling that drive progressively dysregulated growth. To additionally understand the chemopreventive mechanisms of UDCA, in the present study we examined the effects of UDCA on tumor incidence and ACF formation, when this bile acid was supplemented during carcinogen administration, or beginning several weeks after AOM treatment. These effects were compared with those in AOM-treated rats maintained on standard chow.

Materials and Methods

Materials. Male Fisher 344 rats were procured from Harlan Sprague Dawley Inc. (Indianapolis, IN). AOM was obtained from Sigma Chemical Co. (St. Louis, MO). UDCA was generously provided by Falk Pharma GmbH (Freiburg, Germany). AIN-76A rat chow and AIN-76A chow supplemented with 0.4% (w/w) UDCA were prepared by ICN (Aurora, OH). Diets were prepared fresh each month and stored at 4°C. Unless otherwise noted, all of the other reagents were obtained from Sigma and were of the highest quality available.

Experimental Animal Protocol. The animal treatment protocols are summarized in Fig. 1. Male Fisher 344 rats, initially weighing 80–100 grams, were divided into the following experimental groups: groups 1 and 2, AIN-76A alone (unsupplemented); groups 3 and 4, AIN-76A +0.4% UDCA (w/w; UDCA-1, initiation); and groups 5 and 6, AIN-76A alone. After 2 weeks, animals in groups 2, 4, and 6 were treated with AOM (20 mg i.p./kg body weight) weekly for 2 weeks, and animals in groups 1, 3, and 5 received injections of saline (AOM-vehicle). For groups 3 and 4, the UDCA diet was continued until 2 weeks after the second AOM/saline injection (week 5 of the study), and then UDCA was discontinued. For groups 5 and 6, 0.4% UDCA (w/w) supplementation was begun 2 weeks after the second AOM/saline injection and continued until the time of sacrifice (UDCA-2, postinitiation). It should be noted that AOM is completely metabolized to CO_2 within 72 h of administration (24). Rats were provided water *ad libitum* and housed in polycarbonate cages in a room with environmental control (12 h light and 12 h dark cycles, 22–24°C and a relative humidity of 30–70%). Rats were weighed weekly. All of the animal procedures followed the guidelines approved by the University of Chicago Animal Care Committee.

All of the rats were sacrificed in the nonfasted state 32 weeks after the beginning of the study. Colons were removed, and after flushing with normal saline, opened longitudinally and examined macroscopically for the presence of visible tumors (>1 mm in size). Tumor locations (distance from anus) were recorded, and tumors were rapidly excised and weighed. Tumors, fixed in 10% buffered formalin, were paraffin-embedded, sectioned, and stained with H&E for histological classification. All of the specimens were evaluated by a pathologist (J. H.), who was unaware of the treatment groups, and classified as adenomas, carcinomas *in situ*, or invasive adenocarcinomas, as described previously (15).

To identify ACF, colons were fixed flat and stained with 0.2% methylene blue for 30 s. After destaining in PBS, ACF were identified using a dissecting microscope equipped with a ×25 objective. ACF appeared as collections of slightly elevated crypts with increased staining, expanded pericryptal spaces, and crypt lumens varying in shape from circular to elongated or serrated. In carcinogen-treated animals, the location (distance from the anus) and size of the ACF (number of component crypts per ACF) were recorded.

Table 1 Tumor incidence

UDCA-1 is the dietary protocol with UDCA administered during the first 5 weeks of the study (initiation phase). UDCA-2 is the dietary protocol with UDCA administered beginning 2 weeks after the second AOM injection and continued until the time of sacrifice (promotion/progression phase).

Diet group	Number of animals	Number of rats with tumors	Number of tumors	Tumor incidence	Tumor multiplicity[a]
AOM alone	47	34	64	72.3	1.9
AOM + UDCA-1	39	18	26	46.2[b]	1.4[c]
AOM + UDCA-2	39	15	20	38.5[b]	1.3[c]

[a] Tumor multiplicity: average number of tumors per TBR.
[b] $P < 0.05$ compared with AOM alone.
[c] $P < 0.05$ compared with AOM alone.

Table 2 Tumor histology

UDCA-1 is the dietary protocol with UDCA administered during the first 5 weeks of the study (initiation phase). UDCA-2 is the dietary protocol with UDCA administered beginning 2 weeks after the second AOM injection and continued until the time of sacrifice (promotion/progression phase).

Diet group	Adenomas	Carcinoma *in situ*	Invasive carcinomas
AOM alone	25 (39%)	28 (44%)	11 (17%)
AOM + UDCA-1	15 (60%)	7 (28%)	3 (12%)
AOM + UDCA-2	7 (35%)	11 (55%)	2 (10%)

Table 3 Tumor weight and location

UDCA-1 is the dietary protocol with UDCA administered during the first 5 weeks of the study (initiation phase). UDCA-2 is the dietary protocol with UDCA administered beginning 2 weeks after the second AOM injection and continued until the time of sacrifice (promotion/progression phase). Values are means ± SE.

Diet group	Average tumor weight (mg)	Tumor size range (mg)	Mean distance from rectum (cm)
AOM alone	91.2 ± 10.4	13–206	3.6 ± 2.3
AOM + UDCA-1	114.8 ± 15.4	27–139	4.0 ± 2.4
AOM + UDCA-2	59.5 ± 10.8	19–98	3.5 ± 2.5

Statistics. Values are expressed as means ± SD or SE as indicated. Statistical tests for tumor incidence were calculated by χ^2 analysis. Nonparametric Kruskal-Wallis tests were performed to compare tumor multiplicities, as well as ACF numbers and ACF size among the groups. Values of $P < 0.05$ were considered significant.

Results

There were no significant differences in body weights among the saline-treated (AOM vehicle) groups given AIN-76A alone or supplemented with UDCA (data not shown). Therefore, for purposes of weight gain the vehicle-treated groups were combined. As shown in Fig. 2, the vehicle and carcinogen-treated groups did not differ significantly in weight gain, in agreement with our previous studies (15, 25).

No tumors were present in the groups given saline (AOM vehicle) on either the AIN-76A diet alone (group 1, Fig. 1), or in the groups supplemented with UDCA alone in the initiation (group 3, Fig. 1) or promotion arms (group 5, Fig. 1). There were 3 animals in the AOM alone group, and 1 each in the AOM+UDCA initiation arm and in the AOM+UDCA promotion/progression arm that died before completion of the study. As summarized in Table 1, the tumor incidence in the AOM group fed AIN-76A alone was 72.3%, and the tumor multiplicity or average number of tumors per TBR was 1.9. UDCA, supplemented during carcinogen administration or beginning 2 weeks after the second AOM dose, significantly inhibited tumor development, decreasing the tumor incidence from 72.3% to 46.2% and 38.5%, respectively ($P < 0.05$). Tumor burden was also significantly reduced by each of these dietary protocols for UDCA supplementation, decreasing tumor multiplicity from 1.9 tumors per TBR to 1.4 tumors per TBR and 1.3 tumors per TBR ($P < 0.05$), respectively (Table 1).

Whereas tumor incidence and tumor multiplicity were significantly decreased, the histological distributions of tumors were not different among the carcinogen-treated groups. As shown in Table 2, however, there were numeric reductions in invasive cancers of 12% and 10% in the UDCA initiation and UDCA postinitiation groups, respectively, compared with 17% in the AOM alone group. In addition, the groups did not differ in tumor location or weight. However, as shown in Table 3, there was a trend toward smaller tumors in the postinitiation UDCA-supplemented group, with a mean tumor weight of 59.5 ± 10.8 mg compared with 91.2 ± 10.4 mg ($P = 0.13$) in the AOM alone group and 114.8 ± 15.4 mg in the group supplemented with UDCA during tumor initiation.

ACF are the earliest identified putative premalignant precursors of both human and experimental colon cancer (17, 18). In previous studies we have shown that UDCA, when supplemented throughout the study, not only decreased AOM tumor formation, but also concomitantly inhibited the development and growth of ACF (16). Therefore, we also examined the effects of UDCA supplementation on ACF when administered in the initiation *versus* postinitiation phase. In animals without carcinogen treatment there were no ACF present, regardless of dietary supplementation. As shown in Fig. 3, UDCA, when supplemented during the initiation phase (UDCA-1), significantly decreased the total number of ACF, as well as the growth of larger ACF. In contrast, UDCA, when supplemented during the promotion/progression phase (UDCA-2), failed to alter the number or size of ACF. Thus, whereas UDCA, given in the promotion/progression phase, was as effective as UDCA in the initiation phase in preventing tumor occurrence, supplementation in the postinitiation phase did not inhibit ACF development.

Discussion

Tumor development has been divided into stages of initiation, promotion, and progression. The initiation phase involves irreversible genetic changes (mutations) in normal cells. Initiated cells are then promoted to preneoplastic lesions by epigenetic and potentially reversible events. The progression phase involves additional DNA mutations (genetic instability phase), as well as epigenetic changes that together drive mitogenesis and apoptotic resistance to eventual neoplasia (26). In experimental

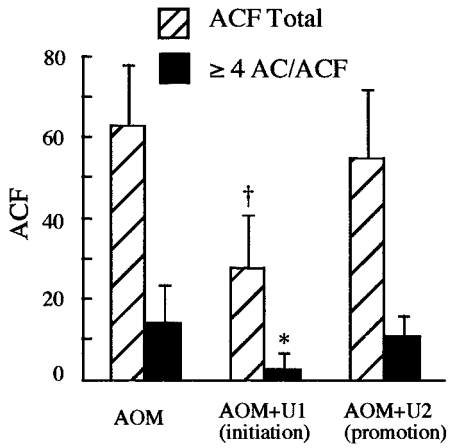

Fig. 3. ACF incidence and size. The total number of ACF and number of ACF containing 4 or more aberrant crypts were enumerated. Shown are means for the indicated carcinogen-treated groups; *bars*, ± SE. $*P < 0.05$, compared with AOM alone.

models of colon cancer, this operational classification has been useful because, in general, agents initiating carcinogenesis such as AOM, are mutagenic, whereas tumor-promoting agents are usually nonmutagenic. In this regard, a number of epidemiological reports in humans and experimental studies in animals have indicated that both primary and secondary bile acids, whereas not mutagenic, promote colonic carcinogenesis (6).

Chemopreventive agents can be classified by their timing of anticarcinogenic actions into those that inhibit tumor initiation, and those that block tumor promotion and progression. We have reported previously that UDCA, the 7β epimer of tumor-promoting chenodeoxycholic acid, is chemopreventive in the AOM model of colon cancer when administered throughout the study (15). In the present study we have extended these investigations to examine the ability of UDCA to block tumor development when supplemented only during the initiation or the promotion/progression phase of AOM-induced carcinogenesis. We report now for the first time that both protocols were effective in significantly reducing tumorigenesis, as assessed by decreases in tumor incidence and tumor multiplicity. Both initiation and postinitiation UDCA supplementation also showed a tendency to decrease invasive tumors, in agreement with our previous report when UDCA was supplemented throughout the experiment (15). Moreover, the smaller tumor size in the UDCA postinitiation group likely reflects the long duration of UDCA treatment. We have demonstrated previously this bile acid is antiproliferative in the AOM model (16), in agreement with the findings of others in human colonic polyps (13).

Whereas previous studies have shown that AOM treatment alone did not alter the colonic bile acid composition, UDCA supplementation caused prominent changes in fecal bile acid profiles in the rat (27). Much of supplemented UDCA is converted by bacterial 7β-dehydroxylase to lithocholic acid, a bile acid with low aqueous solubility (28). In addition, UDCA increased the more hydrophilic bile acids, β- and ω-muricholic acid, and decreased the amount of deoxycholic acid in the aqueous phase (27). These changes in the water-soluble bile acid profile may contribute to the chemopreventive actions of UDCA, because deoxycholic acid possesses detergent-like properties that damage cells. By accelerating cell death, this tumor-promoting bile acid might induce compensatory epithelial hyperproliferation and thereby increase the risk of acquiring growth-promoting or survival-enhancing mutations (29, 30). In this regard, studies have demonstrated increased colonic epithelial proliferation in response to cholic acid feeding in rats (31). Moreover, in humans, serum deoxycholic acid levels correlated with increased colonic proliferation (32).

Cholic acid, the precursor to deoxycholic acid, has been shown previously to promote tumors in the AOM model when given in the initiation phase, but not the postinitiation phase (11). Therefore, this suggests that the postinitiation chemopreventive actions of UDCA are not limited to reductions in deoxycholic concentration. We have also observed *in vitro* that UDCA can directly inhibit deoxycholic acid induction of Cox-2 in colon cancer cells (33). Cox-2 is intimately involved in colonic carcinogenesis, and these *in vitro* effects of UDCA additionally support a direct chemopreventive effect of this agent that is independent of decreases in deoxycholic acid concentration. Taken together, these findings suggest that the chemopreventive actions of UDCA are complex, and not limited to physical-chemical alterations in net hydrophobicity and cytotoxicity of the fecal bile acid constituents.

In addition to reductions in colonic deoxycholic acid, a number of other potential chemopreventive mechanisms of UDCA have been proposed. These include induction of antioxidants (34), inhibition of several AOM-induced enzymes, including phospholipase A2, inducible nitric oxide synthetase, and Cox-2 (16, 35), alterations in nuclear receptor signaling mediated by glucocorticoid or farsenoid X bile acid nuclear receptors (36, 37), preservation of intestinal alkaline sphingomyelinase (38), enhancement of immune surveillance (39), and inhibition of protein kinase C-ζ down-regulation in AOM tumors (40). It will be of interest to assess whether UDCA differentially induces alterations in any of these signaling pathways in the initiation *versus* postinitiation phases that may be chemopreventive.

In this regard, the differential effect of UDCA on ACF formation, when administered in the initiation *versus* postinitiation phase, is especially intriguing. ACF are the earliest identifiable putative precursors of both human and experimental colon cancers (17, 18). ACF have been used to evaluate chemopreventive efficacy of many agents (41). In agreement with others, we have shown previously that UDCA given throughout the study inhibited ACF development (16, 42). In the present study, UDCA inhibited ACF growth, but only when given during carcinogen treatment. In contrast, UDCA, given in the postinitiation phase, even when administered for many weeks, was unable to block ACF development despite its ability to inhibit tumor formation. These results would suggest that UDCA prevents tumor development during the initiation phase by blocking ACF development, whereas UDCA in the postinitiation phase might prevent tumorigenesis by blocking the progression of ACF to more dysplastic lesions.

This differential effect of UDCA on ACF suggests that unique targets mediate the chemopreventive actions of this agent during the initiation and postinitiation phase. As noted earlier, UDCA was antiproliferative when given throughout the study (16). We have more recently begun to examine rates of crypt cell proliferation when UDCA was administered in the initiation or postinitiation phase. In preliminary studies, we found that postinitiation UDCA inhibited crypt cell proliferation,[4] in agreement with our earlier experiments (16). In con-

[4] Unpublished observations.

trast, in rats receiving UDCA in the initiation phase, which suppressed ACF, the crypt cells remained hyperproliferative, with proliferation rates comparable with the group receiving AOM alone when assessed at 32 weeks. This suggests that signaling pathways that mediate focal alterations in crypt morphology are likely targets of UDCA in the initiation phase. Cytoskeletal regulators and other morphogens, for example, are candidate targets, because ACF are focal lesions displaying marked crypt architectural changes. In contrast, UDCA in the postinitiation phase might have prevented tumors by limiting proliferation and, thereby, preventing ACF progression to adenomas, similar to the apparent action of this agent to limit tumor growth when administered in this phase. Hyperproliferation may be required in this phase, for example, to expand mutant clones. Potential targets of UDCA in the postinitiation phase include regulators of the cell cycle. In this regard, we have observed previously that when supplemented throughout the study, UDCA inhibited cyclin D1 expression (16). Additional studies will be required to identify the molecular targets of UDCA in the initiation *versus* postinitiation phases.

Regardless of the chemopreventive mechanisms of action of UDCA, this study is especially noteworthy because we have shown for the first time that this bile acid blocks colonic carcinogenesis during tumor initiation, as well as tumor promotion/progression. It is estimated that individuals at increased risk for colon cancer require 5–10 years between tumor initiation and the development of invasive cancers (43). Agents capable of inhibiting tumor growth during the promotion/progression phase merit special interest in the clinical setting. Moreover, it should be emphasized that agents effective in this phase are clearly not acting only to inhibit the activity of the initiating carcinogen, for example, by blocking metabolic activation or accelerating detoxification of the carcinogen. The application of a chemopreventive agent active only during tumor initiation might be more restrictive, because the specific tumor initiators and the onset of human tumorigenesis are unknown. However, it should be noted that a chemopreventive agent effective in the initiation phase might also have an important role in tumor prevention. Theoretically, for example, individuals who have had a resection of adenomatous colonic polyps might benefit if such an initiation-specific inhibitor were begun shortly after a follow-up colonoscopy was found to be normal, *i.e.*, no remaining polyps were present. Ongoing studies to assess the efficacy of UDCA to prevent recurrent colonic polyps in this setting are in progress and will be of great interest.

In summary, we have established that UDCA, when administered during tumor initiation or in the postinitiation (promotion/progression) phase, inhibits tumor development in the AOM model. Furthermore, our studies suggest that preventing ACF formation may be involved in the ability of UDCA to block tumor development in the initiation phase. UDCA may act by additional or alternative mechanisms to block tumor development in the postinitiation phase, perhaps at a step distal to ACF, because in this phase UDCA prevents tumors but not ACF formation. Taken together with previous studies in humans (13, 14), these results additionally support the potential efficacy of this bile acid as a chemopreventive agent in individuals at risk for colon cancer. Elucidation of the mechanisms by which UDCA inhibits colonic carcinogenesis may provide important insights into the signaling pathways involved in tumor promotion by secondary bile acids. Moreover, these studies may identify new targets for chemopreventive strategies.

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