Ursodeoxycholic Acid and F$_6$-D$_3$ Inhibit Aberrant Crypt Proliferation in the Rat Azoxymethane Model of Colon Cancer: Roles of Cyclin D1 and E-Cadherin

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Abstract

We have previously demonstrated that ursodeoxycholic acid (UDCA) and a fluorinated analogue of vitamin D$_3$, F$_6$-D$_3$, inhibited colonic carcinogenesis in the azoxymethane (AOM) model. Generalized colon mucosal hyperproliferation and aberrant crypt foci (ACF) are intermediate biomarkers of colon cancer. Using these biomarkers, in this study we examined the anticarcinogenic mechanisms of these chemopreventive agents. Rats were maintained on AIN-76A chow or supplemented with 0.4% UDCA or F$_6$-D$_3$ (2.5 nmol/kg chow) and treated weekly with AOM 20 mg i.p./kg wt or saline × 2 weeks. F$_6$-D$_3$ was continued for an additional 2 weeks and UDCA for the duration of the study. At 40 weeks, animals received bromoexodouridin (Brdu) i.p. 2 h before sacrifice. A portion of each tumor was fixed in formalin and the remainder flash frozen. Colons were divided longitudinally and half-fixed in formalin and half in ethanol. The size and location of methylene blue-stained ACF were recorded. Cell proliferation (Brdu labeling) and apoptosis (terminal deoxynucleotidyl transferase-mediated nick end labeling assay) were measured in colonic crypts and tumors. Protein expression levels of several regulators of cell proliferation were analyzed by immunostaining and Western blotting. Colonic crypt cyclin D1 and E-cadherin mRNA levels were measured by real-time PCR. In saline injected controls, neither UDCA nor F$_6$-D$_3$ alone had any effect on cytokinetic parameters or on the expression of mitogenic regulators. AOM significantly increased the proliferation (percentage of BrdUrd-positive cells) of both ACF (23.1 ± 1.7%) and non-ACF crypts (17.6 ± 1.6%), compared with normal colonic crypts (4.5 ± 0.8%; P < 0.05). This hyperproliferation was accompanied by a 5-fold increase in cyclin D1 and >50% decrease in E-cadherin protein (P < 0.05) in ACF, both of which are predicted to be growth-enhancing alterations. UDCA and F$_6$-D$_3$ significantly (P < 0.05) inhibited AOM-induced crypt cell hyperproliferation, ACF development, and tumor burden. These chemopreventive agents also significantly blocked AOM-induced alterations in cyclin D1 and E-cadherin protein in ACF and tumors. In ACF, changes in mRNA levels of cyclin D1, but not E-cadherin, paralleled alterations in protein expression. Cyclooxygenase-2 and inducible nitric oxide synthase were increased in AOM tumors but not in ACF, and these changes were blocked by UDCA and F$_6$-D$_3$. UDCA and F$_6$-D$_3$ significantly inhibited ACF development and hyperproliferation, in part, by preventing carcinogen-induced alterations in cyclin D1 and E-cadherin. In established tumors, UDCA and F$_6$-D$_3$ also limited inductions of cyclooxygenase-2 and inducible nitric oxide synthase, which together with their effects on cyclin D1 and E-cadherin, contribute to their chemopreventive actions.

Introduction

Colon cancer is one of the most prevalent causes of cancer-related deaths in both males and females in the Western world. Both endogenous factors such as tumor-promoting secondary bile acids, as well as exogenous factors, including xenobiologic dietary procarcinogens, have been implicated. This disease is characterized by the progressive accumulation of genetic abnormalities, including inactivating mutations in tumor suppressor genes, and activating mutations in proto-oncogenes (1, 2). These genetic changes are accompanied by epigenetic alterations that include increases in cyclin D1 and COX-2 and loss of E-cadherin (3–5). Both the genetic and epigenetic changes lead to increases in cell proliferation and loss of normal responsiveness to growth inhibiting signals (6). Because there is no effective therapy for advanced disease, there are continuing efforts to identify intermediate biomarkers and early premalignant markers.
Ursodeoxycholic Acid and F6-D3 Inhibit AOM-induced ACF

The rodent AOM model of colonic carcinogenesis recapitulates many of the clinical, pathologic, and molecular features of human colon cancer, including hyperproliferation in the premalignant phase. Studies of colon cancer in both humans and the AOM model have identified generalized colonic mucosal hyperproliferation and ACF as intermediate biomarkers (7–12). ACF are collections of abnormal crypts that are characterized by hyperproliferation, increased size, expanded pericryptal zones, and elongated or serrated crypt lumens. In experimental models, agents that enhance or inhibit colon cancer development have frequently been found to cause parallel changes in colonic proliferation and ACF formation (13–16). Expansion of the crypt proliferative zone and the presence of larger and more dysplastic ACF are thought to be associated with an increased risk for the development of colon cancer (17, 18). ACF have served, moreover, as surrogate biomarkers to screen numerous potential chemopreventive agents (19). The recent identification of the monoclonal nature of ACF has strengthened the assertion that they have a malignant potential (20). Very little is known, however, of the mechanisms that drive hyperproliferation and ACF development and growth.

We have previously shown that UDCA and F6-D3, an analogue of 1,25-dihydroxyvitamin D3, are potent chemopreventive agents in this model (21, 22). It was, therefore, of interest to examine the effects of UDCA and F6-D3 on the AOM-induced hyperproliferative state in ACF and non-ACF mice. A number of vitamin D3 analogues have also been implicated as chemopreventive agents in human and experimental models, agents that enhance or inhibit colon cancer development (22). Both UDCA and F6-D3, moreover, inhibited proliferation and induced a G1 arrest in vivo in some colon cancer-derived cell lines (27, 28). In this study, we have elucidated the roles of proliferation and cell death in the chemopreventive actions of UDCA and F6-D3 in the AOM model. We have also investigated potential mechanisms involved in their anticarcinogenic actions. Specifically, we have characterized alterations in several regulators of cell proliferation implicated in colonic carcinogenesis. In this regard, we previously observed increases in cyclin D1, COX-2, and iNOS in AOM tumors (29, 30). Cyclin D1 is an important positive regulator of the G1 to S cell cycle transition that is up-regulated in colon cancer (3, 29). COX-2, an inducible isoform of COX, regulates the rate-limiting step in prostanoid biosynthesis and is intimately linked to colonic carcinogenesis (5). iNOS is also increased in colon cancer, including AOM tumors, and might be important in activating COX-2 and increasing angiogenesis (31). E-cadherin, a Ca2+ regulated component of the zona adherens junctions, is an important negative regulator of cellular proliferation involved in cell contact-mediated growth inhibition and maintenance of a polarized epithelium. E-cadherin is down-regulated in many colon cancers (32) but has not been examined in the AOM model. Our findings regarding the effects of UDCA and F6-D3 on AOM-induced hyperproliferation and on these regulators of proliferation serve as the basis for this report.

Materials and Methods

Materials. Male Fisher 344 rats were procured from Harlan Sprague Dawley, Inc. (Indianapolis, IN). AOM and BrdUrd were obtained from Sigma Chemical Co. (St. Louis, MO). UDCA was generously provided by Dr. Horst-Dietmar Tauschel of Falk Pharma GmbH (Freiburg, Germany), and the fluorinated derivative of 1,25-dihydroxyvitamin D3, 1α,25-dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (F6-D3), was kindly provided by Dr. Milan Uskokovic (Hoffman LaRoche Pharmaceuticals, Nutley, NJ). AIN-76A chow and AIN-76A chow supplemented with 0.4% (w/w) UDCA or F6-D3 (2.5 nmol/kg chow) were prepared by ICN (Aurora, OH). Diets were prepared fresh each month, protected from fluorescent light, and stored at 4°C. The ApopTag assay kit for nicked DNA detection was purchased from Intergen Co. (Purchase, NY). Mouse monoclonal anti-BrdUrd, anti-E-cadherin, and anti-β-actin antibodies were obtained from Zymed (San Francisco, CA). Rabbit polyclonal anticytokerin 12 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 polyclonal antibodies were from Cayman Chemical (Ann Arbor, MI). Mouse monoclonal iNOS antibodies were purchased from Transduction Laboratories (Lexington, KY). The ABC peroxidase detection kit and Vectabond coating reagent were purchased from Vector Laboratories (Burlingame, CA). Primers for real-time PCR were obtained from Invitrogen and Sybr Green from Qiagen. Enhanced chemiluminescence reagents were obtained from Amersham Pharmacia (Piscataway, NJ). Unless otherwise noted, all other reagents were obtained from Sigma Chemical Co. and were of the highest quality available.

Experimental Animal Protocol. Male Fisher 344 rats, initially weighing 80–100 g, were divided into six groups and fed the following experimental diets: groups 1 and 2, AIN-76A alone; groups 3 and 4, AIN-76A + 0.4% (w/w) UDCA; and groups 5 and 6, AIN-76A + F6-D3 (2.5 nmol/kg chow). After 2 weeks, animals in groups 2, 4, and 6 were treated with AOM (20 mg i.p./kg body wt) 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 the time of sacrifice (40 weeks). For groups 5 and 6, the F6-D3 supplementation was continued for 2 weeks after the second AOM/saline injection (5 weeks total), and these rats were then switched to the AIN-76A diet alone. We have previously demonstrated that this protocol for F6-D3, given only during the initiation phase, significantly inhibited AOM-induced tumor development (22). In contrast, the chemopreventive effects of UDCA were previously demonstrated in a protocol that supplemented UDCA throughout the study (21). We, therefore, used these two different protocols of dietary supplementation, which differed in their duration of administration because UDCA and F6-D3 had each been established to be chemopreventive when supplemented on these schedules. 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%). All animal procedures followed the guidelines approved by the University of Chicago Animal Care Committee. All rats were sacrificed in the nonfasted state 40 weeks after the beginning of the study. Two h before sacrifice, rats were given i.p. injections of BrdUrd (50 mg/kg body wt) to label S-phase cells. Animals were sacrificed between 10–12 a.m. to control for diurnal
variation in cell cycling. Colons were opened longitudinally and examined for the presence of tumors. Tumors were excised and a portion fixed in formalin. The remainder of each tumor was flash frozen for later Western blotting analyses. Tumor specimens were classified as either benign (adenoma) or malignant (carcinoma in situ or adenocarcinoma) as described previously (21). Colons were divided longitudinally, and one hemicolonic segment fixed flat in formalin and the other segment in 70% ethanol and both stored at 4°C. Colonic segments were stained for 5 min in 0.02% methylene blue and destained in PBS. Methylene blue-stained ACF, identified with a dissecting microscope, appeared as collections of elevated crypts with increased staining and expanded pericryptal spaces. The ACF location (distance from the anus) and size (number of component crypts/ACF) were recorded.

**Immunohistochemical Staining.** Formalin-fixed AOM tumors and hemicolons from six animals in each AOM or saline-treated groups were excised from the distal colon of each rat. In the saline-treated groups, which included unsupplemented rats and rats supplemented with UDCA or F6-D3, four biopsies were obtained from the distal colon of each rat. In the saline-treated groups, which included unsupplemented rats and rats supplemented with UDCA or F6-D3, four biopsies were obtained from colonic sites comparable with the biopsies from AOM-treated rats. Five-μm paraffin-embedded sections, mounted on Vectabond-coated Superfrost™ slides, were heated to 60°C for 1 h, deparaffinized, hydrated by a graded series of ethanol rinses, and washed in PBS. Antigen retrievals for BrdUrd, cyclin D1, COX-2, and iNOS were accomplished by microwave heating in 10 mM citrate buffer (pH 6) for 5 min × 3 cycles. Epitope retrieval for E-cadherin was achieved by treating with 1% Triton-X-100 for 15 min. Endogenous peroxidase activity was blocked by incubation for 5 min in 3% H2O2, and nonspecific binding was inhibited with blocking reagent (Zymed). Sections were incubated at 4°C in appropriate primary antibodies [anti-BrdUrd (Zymed kit), anticyclin D1 (1:40), anti-E-cadherin (1:50), anti-COX-2 (1:50), anti-iNOS (1:50) antibodies] followed by incubation with 1:200 dilution of appropriate biotinylated secondary antibodies. After washing, sections were incubated with avidin-biotin peroxidase complex using the Vectastain Elite ABC kit (Vector Laboratories) following the recommendations of the manufacturer. As negative controls, sections were incubated with nonimmune serum. Inter-gen’s Apoptag kit was used for TUNEL assays following the manufacturer’s recommendations. Only complete crypts, extending from the muscularis mucosa to the colonic lumen, were counted for BrdUrd and TUNEL labeling. Biopsies contained on average ≥4 complete crypts. BrdUrd labeling was expressed as percentage of crypt cells positive for BrdUrd immunostaining. The proliferative zone was defined as the ratio of the crypt height of the most proximally labeled cell to the total crypt height, measured in cells, and expressed as a percentage. For AOM tumors proliferation was measured by counting the number of BrdUrd positive nuclei in 1000 cells and expressing the results as percentage of BrdUrd positive.

**Western Blotting.** We quantified the protein expression levels of cyclin D1, E-cadherin, iNOS, and COX-2 by Western blotting in colonic crypts and tumors. For crypt studies, we examined six animals in each group. For these studies, we used colonic segments fixed flat in 70% ethanol that allowed methylene blue-stained ACF to be identified while preserving proteins for later extraction. Randomly chosen large ACF (≥4 aberrant crypts/aberrant crypt focus) were excised with a 2-mm biopsy punch. ACF biopsies were trimmed under a dissecting microscope to remove surrounding normal appearing crypts. We obtained separate biopsies of non-ACF crypts. An identical procedure was followed to obtain colonic biopsies of normal mucosa from saline-treated rats in each group: unsupplemented rats or rats supplemented with UDCA or F6-D3. Because the protein recovered from biopsies was limited, four to six biopsies were pooled from the distal left colon of each animal. AOM tumors were homogenized in Laemmli buffer. Protein measurements and Western blotting were performed as previously described (29), using rabbit polyclonal anti-cyclin D1 (2 μg/ml), mouse monoclonal anti-E-cadherin (2.5 μg/ml), rabbit polyclonal anti-COX-2 (2 μg/ml), or mouse monoclonal anti-iNOS antibodies (2 μg/ml). Immunoreactive bands were visualized by chemiluminescence captured by xerography. Xerograms were scanned to digitize images, which were analyzed by IP Lab Gel (Scanalytics, Fairfax VA). Blots were stripped and reprobed with anti-β-actin antibodies to confirm equal protein loading.

**Real-Time PCR.** Colonic crypt biopsies were homogenized in 1 ml of TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD), and total RNA was extracted according to the manufacturer’s protocol. RNA was dissolved in diethylpyrocarbonate-treated water. Residual genomic DNA was removed by DNase-I using the DNA-free kit (Ambion, Austin, TX). Total RNA was quantified by absorbance measured at 260 nm. RNA was reverse transcribed in a 20-μl reaction containing 1 μg of RNA. 5 mM MgCl2, 1× PCR Buffer II (Applied Biosystems), 4 mM deoxynucleotide triphosphates, 20 units of RNase inhibitor, 2.5 μM random hexamers, and 50 units of Moloney murine leukemia virus reverse transcriptase. Samples were reverse transcribed in a DNA Thermal Cycler 480 (Perkin-Elmer Corp., Boston, MA) at 42°C for 60 min and the temperature then raised to 95°C for 5 min to denature the reverse transcriptase. For real time PCR, a Cepheid Smart Cycler real-time PCR machine was used with a Sybr Green I PCR kit as recommended by the manufacturer. Each reaction contained 2.5 μl of the 10× Sybr Green buffer, 200 μM of forward and reverse primers, 200 μM deoxynucleotide triphosphate, 5 mM MgCl2, 0.75 units of HotStarTag DNA polymerase, 5 μl of a 1:5 dilution of the cDNA, and water added to a final volume of 25 μl. The cyclin D1 forward primer was 5′-GCACAACG-CACCCTTTCTCTTTCTACA-3′ and the reverse primer was 5′-GCAGGCTTGACTCCAGAAGAC-3′, with predicted product size of 96 bp. The E-cadherin forward primer was 5′-ATCGTGGGCAGAAAGCCCTTT-3′ and the reverse primer 5′-TGGGCTGGATGCCAGCTTGTT-3′, with predicted product size of 100 bp. The rat cytoplastic β-actin forward primer was 5′-AAGCCAACCGTGATGGAAGATG-3′ and the reverse primer 5′-GGACGAAACAGGCCTGGATGG-3′, with predicted product size of 86 bp. The reactions were incubated at 95°C for 15 min to activate the DNA polymerase followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. Separate parallel reactions were run for rat β-actin to normalize for total RNA. The PCR reactions were subjected to a melt curve analysis to verify the presence of a single amplicon using the Cepheid Smart Cycler (software version 1.2). In preliminary experiments, PCR products of the expected size were visualized on an agarose gel and their predicted sequences confirmed by automated sequencing. All cDNA samples were synthesized in parallel reactions and PCR reactions run in triplicate. The mRNA levels were expressed as relative changes after normalization to β-actin mRNA abundance.
Statistical Methods. ANOVA for a nested design was used to examine the effects of carcinogen treatment and chemoprevention supplementation on the BrdUrd labeling and proliferative zone height. Tukey’s procedure was used to adjust for multiple comparisons. A nonparametric Kruskal-Wallis test was performed to compare ACF numbers and ACF size and tumor multiplicity among the groups because these variables were not normally distributed. Quantitative densitometry values were compared by unpaired Student’s t test. Differences with \( P < 0.05 \) were considered statistically significant. Values were expressed as means ± SD or means ± SE, as indicated.

Results
UDCA and F6-D3 Inhibit the Development and Growth of AOM-induced ACF. In agreement with our earlier studies (21, 22), there were no significant differences in weight gain among any of the dietary and carcinogen-treated groups (data not shown). As expected, in animals without carcinogen treatment, there were no tumors or ACF present, regardless of dietary supplementation. As assessed by tumor multiplicity (Table 1) and in agreement with our earlier studies (21, 22), both UDCA and F6-D3 significantly decreased tumor development compared with AOM alone, with 1.9 tumors/TBR, and 1.8 tumors/TBR, compared with 3.1 tumors/TBR (\( P < 0.05 \)), respectively.

The mean number of ACF in the proximal, middle, and distal colonic segments are shown in Table 2. Both UDCA and F6-D3 significantly decreased the total number of ACF in the distal colon, the segment previously shown to have the greatest number of carcinogen-induced ACF and tumors (16). Compared with the AOM-alone group with a mean total number of ACF/rat = 65 ± 8, UDCA and F6-D3 significantly reduced the number of ACF to 34 ± 4 and 23 ± 2, respectively (\( P < 0.05 \)). UDCA and F6-D3 also inhibited the growth of larger ACF (≥4 crypts), decreasing the mean number of larger ACF from 26 ± 3 for AOM alone to 13 ± 2 (UDCA) and 7 ± 1 (F6-D3) (\( P < 0.05 \)). The numbers of dysplastic ACF in the groups were too small to make meaningful comparisons (<5% total). The decreases in the number and size of ACF that we found are in keeping with the lower tumor burdens observed in the groups receiving these chemopreventive agents (Table 1).

UDCA and F6-D3 Inhibit AOM-induced Colonic Epithelial Hyperproliferation. Because the development of ACF may reflect an increase in the proliferative response of the epithelium and/or a decrease in the rate of apoptosis, we next examined these cytokerin parameters by BrdUrd incorporation and TUNEL assay, respectively. In the absence of carcinogen treatment, UDCA or F6-D3 did not alter the BrdUrd labeling or the height of the proliferative zone (data not shown). Control animals (saline-treated groups 1, 3, and 5) were, therefore, combined for the subsequent proliferation analyses and labeled as “Control.” The rate of BrdUrd incorporation in control colonic crypts was 4.5 ± 0.8%, and the proliferative zone extended to ~15% of the crypt height in normal crypts from vehicle-treated rats (Figs. 1 and 2). Compared with the colonic crypt proliferative rate (BrdUrd incorporation) in saline-treated control animals (4.5 ± 0.8%), AOM significantly increased the cell proliferation within both ACF (23.1 ± 1.7%) and non-ACF crypts (17.6 ± 1.6%). In the AOM-treated group, supplemented with UDCA, the BrdUrd incorporation was reduced to 15.4 ± 1.3% in ACF and 9.8 ± 1.5% in non-ACF crypts. The effects of AOM on BrdUrd labeling were reflected by parallel changes in the proliferative zones of the ACF and non-ACF crypts, with increases from 13.7 ± 4.0% (normal crypts from saline-treated animals) to 29.0 ± 2.8% in ACF crypts and 25.2 ± 2.0% in non-ACF crypts in AOM-treated animals (Fig. 2B). UDCA significantly limited this AOM-induced increase in the proliferative zone to 20.0 ± 2.0% in ACF and 18.1 ± 1.6% in non-ACF crypts. As shown in Fig. 2, supplementation with F6-D3 caused inhibition of similar magnitudes of both the AOM-induced increase in crypt cell proliferation and expansion of the proliferative zone of both ACF and non-ACF crypts. Thus, UDCA induced a significant increase in the BrdUrd labeling in both ACF and non-ACF crypts, and UDCA or F6-D3 significantly inhibited these hyperproliferative responses.

Unlike the differences in proliferation observed between control and carcinogen-treated animals, the measured rates of apoptosis did not differ among any of the control or carcinogen-treated dietary groups. These rates were low, ranging from 1–2 TUNEL-positive cells/5 crypts. These low rates of detectable apoptosis were confirmed by the rare occurrence of apoptotic bodies in H&E-stained sections of ACF and non-ACF crypts (data not shown).

We also examined the effects of these agents on proliferation and apoptosis in tumors. As shown in Table 3, in AOM tumors, UDCA supplementation significantly inhibited BrdUrd incorporation compared with unsupplemented animals. There was also a trend toward lower proliferation in the F6-D3 group. There were no significant differences in rates of apoptosis among the tumor groups, although occasional tumors from the F6-D3 supplemented animals showed striking numbers of TUNEL-positive cells (data not shown).

AOM Increases Cyclin D1 and Decreases E-Cadherin in ACF Crypts and Tumors. Cyclin D1 is an important positive cell cycle regulator of the G1-S transition (33). This cyclin is up-regulated by growth factors that induce cellular proliferation and is overexpressed in some malignancies, including colon cancer (3). Because AOM induced hyperproliferation in both ACF and non-ACF crypts, we asked whether this carcinogen altered cyclin D1. There were no differences in cyclin D1 expression in the saline-treated animals on the unsupplemented diet, compared with those on UDCA or F6-D3 supplementation, consistent with comparable rates of crypt cell proliferation among these groups. In contrast, in carcinogen-treated rats, we detected increased immunostaining for cyclin D1 expression in both ACF and non-ACF crypts in colonic mucosa (Fig. 3 and data not shown). Compared with AOM

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colonic segment</th>
<th>Total ACF</th>
<th>Large ACF</th>
</tr>
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<tr>
<td>AOM (18)</td>
<td>4 ± 1</td>
<td>13 ± 2</td>
<td>48 ± 10</td>
</tr>
<tr>
<td>AOM + UDCA</td>
<td>4 ± 2</td>
<td>11 ± 2</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>AOM + F6-D3 (9)</td>
<td>4 ± 1</td>
<td>8 ± 3</td>
<td>13 ± 3</td>
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</tbody>
</table>

* Mean ± SE.
\( a P < 0.05 \), compared with AOM alone (Kruskal-Wallis test).

Table 1  Tumor multiplicity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Tumor no.</th>
<th>Tumors/TBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOM alone</td>
<td>18</td>
<td>55</td>
<td>3.1 (2.5/0.6)*</td>
</tr>
<tr>
<td>AOM + UDCA</td>
<td>11</td>
<td>19</td>
<td>1.9 (1.7/0.7)**</td>
</tr>
<tr>
<td>AOM + F6-D3</td>
<td>9</td>
<td>14</td>
<td>1.8 (1.0/0.8)**</td>
</tr>
</tbody>
</table>

* Adenomas/TBR/carcinomas/TBR.
** \( P < 0.05 \), compared with AOM alone (Kruskal-Wallis test).
alone, supplementation with UDCA or F₆-D₃ limited this carcinogen-enhanced expression of cyclin D1 (Fig. 3). As shown in Fig. 4 and quantified in Table 4, we found by Western blotting that AOM significantly up-regulated cyclin D1 protein expression ~5-fold in ACF and >2-fold in non-ACF crypts, compared with normal mucosa (P < 0.05). Consistent with the partial reversal of AOM-induced hyperproliferation by UDCA and F₆-D₃, these chemopreventive agents also significantly
BrdUrd positivity.

tumor, six representative fields with 3000 total cells were counted and scored for BrdUrd positivity.

In contrast, in carcinogen-treated animals, cadherin expression in the saline-treated animals on the un-
molecule in this model. There were no differences in E-
we examined alterations in the expression of this cell adhesion

dscribed in a number of tumors, including colon cancer (4, 32),

imentation (Fig. 5).

increases were inhibited by UDCA or F6-D3 supplementation as
(data not shown). In contrast, in AOM-treated rats, iNOS and

creased in AOM-induced tumors from unsupplemented rats,

cases of ACF, cyclin D1 protein levels were significantly in-

TC in aberrant crypts. Furthermore, UDCA and F6-D3 significantly inhibited cyclin D1 mRNAs in ACF and non-ACF crypts (Table 5). As in the case of ACF, cyclin D1 protein levels were significantly increased in AOM-induced tumors from unsupplemented rats, and these increases were inhibited by UDCA or F6-D3 supplementation (Fig. 5).

E-cadherin is a negative regulator of colonic epithelial cell proliferation (34). Because loss of E-cadherin has been de-
scribed in a number of tumors, including colon cancer (4, 32), we examined alterations in the expression of this cell adhesion molecule in this model. There were no differences in E-cadherin expression in the saline-treated animals on the unsupplemented diet compared with those supplemented with UDCA or F6-D3. In contrast, in carcinogen-treated animals, E-cadherin expression was decreased in ACF as assessed by immunostaining (Fig. 3). Furthermore, this loss of E-cadherin expression was reversed by UDCA and F6-D3 supplementation (Fig. 3). We also measured this loss in E-cadherin expression by quantitative Western blotting. As shown in Fig. 4 and quantified in Table 6, AOM significantly inhibited E-cadherin expression by 70% in ACF but not in non-ACF crypts, and this down-regulation was reversed by UDCA and F6-D3. As shown in Table 5 and in contrast to cyclin D1, E-cadherin mRNA levels in ACF were not significantly influenced by AOM or by the chemopreventive agents UDCA or F6-D3. In AOM tumors, as in the case of ACF, E-cadherin protein levels were significantly down-regulated, and these losses were reversed by UDCA or F6-D3 supplementation (Fig. 5).

AOM Increases iNOS and COX-2 Expression in AOM-

duced Tumors but not in ACF. Increased iNOS and

X expression have been implicated in the development of sporadic human colon cancer (35, 36), as well as in experimental models of colon carcinogenesis (31, 37). In agreement with others (31, 37), we previously found that iNOS and COX-2 were increased in AOM-induced tumors (29, 30). In this study, the expression levels of iNOS and COX-2 were very low in saline-treated animals that were given an unsupplemented diet or a diet supplemented with UDCA or F6-D3. Moreover, there were no detectable changes in the epithelial expression of these proteins in ACF or non-ACF crypts in the AOM-alone group or in the groups supplemented with UDCA or F6-D3 as assessed by immunostaining or Western blotting (data not shown). In contrast, in AOM-treated rats, iNOS and COX-2 were both significantly increased in tumors, and these increases were inhibited by UDCA or F6-D3 supplementation as shown in Fig. 6.

Discussion

We have previously shown that UDCA and F6-D3 inhibited AOM-induced colon cancer (21, 22). This is the first report of an effect in vivo of these chemopreventive agents on the proliferative state of aberrant and nonaberrant crypts in the AOM model. Cellular hyperproliferation is a premalignant intermediate biomarker of colon cancer (7–9, 12). ACF are also biomarkers and putative premalignant precursors. Furthermore, ACF demonstrate even greater cellular proliferation than that of the generalized hyperproliferative mucosa (12). In the present studies, we have demonstrated that AOM increased the proliferation and expanded the proliferative zone of both ACF and non-ACF colonic crypts. UDCA and F6-D3 effectively decreased both the number and size of ACF and inhibited their cellular proliferation. Our study demonstrates for the first time that UDCA and F6-D3 are antiproliferative in this model and decreased the incidence of ACF (38, 39). Furthermore, reductions in the number of larger ACF are consistent with the ability of these two agents to inhibit the development of colon cancer because larger ACF, with higher component crypt multiplicity, are thought to be better intermediate biomarkers of tumor occurrence than the number of ACF alone (40). Our observations, moreover, that these chemopreventive agents significantly decreased both the number of large ACF and tumor multiplicity, additionally support the role of ACF as precursors to colon cancer.

The development of colon tumors requires alterations in the balance of cell renewal and cell death that regulate normal cellular homeostasis in the colon (6). UDCA and F6-D3 did not cause an increase in apoptosis in this model in contrast to other chemopreventive agents such as retinoids (41). These results are in agreement with others who have noted increased AOM-induced colonic proliferation with no detectable change in apoptosis weeks after carcinogen administration (42). It should be noted, however, that the number of apoptotic cells in the mucosa of AOM-treated rats is small and that their rapid elimination (1–2 h) may have precluded detection of meaningful differences (43). In preliminary studies from our laboratory, these chemopreventive agents have been shown to induce apoptosis in colon cancer cells (44, 45).

In AOM-treated rats, both the increased proliferation induced by this carcinogen and the antiproliferative actions of UDCA and F6-D3 are field effects involving not only the ACF, but also to a lesser extent, the epithelium diffusely. It is likely that some of the alterations that drive proliferation in both ACF and non-ACF crypts involve similar mechanisms. In this regard, we have demonstrated for the first time increases in cyclin D1 in both ACF and non-ACF crypts, compared with crypts from control animals. By quantitative Western blotting, we have shown, moreover, that cyclin D1 expression in ACF crypts was significantly greater than that of non-ACF crypts, consistent with the differences in proliferation observed between aberrant and nonaberrant crypts. Additionally supporting this role of cyclin D1 in AOM-induced hyperproliferation, we have found that UDCA and F6-D3 inhibited the carcinogen-induced increase in cyclin D1 in both ACF and non-ACF crypts, concomitant with their inhibition of crypt cell hyperplasia. In the premalignant mucosa, changes in the levels of cyclin D1 protein expression were accompanied by parallel changes in mRNA expression, indicating that, in part, these changes are mediated by alterations in cyclin D1 transcription or mRNA stabilization. These agents also inhibited the increase in cyclin D1 in AOM-induced tumors. Taken together, these results indicate that the mechanisms responsible for AOM-induced
hyperproliferation and progression to ACF and their inhibition by UDCA and F6-D3, although likely complex, involve alterations in cyclin D1.

In addition to cyclin D1, E-cadherin, a Ca2+-activated homotypic component of the zonula adherens junctions, is another important regulator of intestinal cell proliferation and cell death (34). In intestinal crypts, dominant negative inactivation of E-cadherin led to adenoma formation, whereas overexpression of E-cadherin suppressed crypt proliferation (46, 47). E-cadherin, moreover, is often down-regulated in colon cancer (4, 32). In this study, AOM treatment inhibited E-cadherin expression in ACF. E-cadherin down-regulation, moreover, was significantly blocked by UDCA and F6-D3 supplementation.

Table 4  Cyclin D1 protein expression in ACF and non-ACF Crypts*

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>ACF crypts</th>
<th>Non-ACF crypts</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOM</td>
<td>486 ± 210(^{\circ}) (^{b,c})</td>
<td>252 ± 110(^{\circ})</td>
</tr>
<tr>
<td>AOM + UDCA</td>
<td>137 ± 17(^{\circ})</td>
<td>87 ± 43(^{\circ})</td>
</tr>
<tr>
<td>AOM + F6-D3</td>
<td>67 ± 24(^{d})</td>
<td>104 ± 53(^{e})</td>
</tr>
</tbody>
</table>

* Cyclin D1 was assessed by quantitative Western blotting as described in the “Materials and Methods” (n = 6 animals in each group, with 4 ACF and 4 non-ACF biopsies from each carcinogen-treated rat and 4 normal biopsies from each of the control groups). Data are expressed as % cyclin D1 in normal crypts from vehicle-treated rats (mean ± SD). Cyclin D1 expression did not differ among the saline-treated unsupplemented rats and UDCA and F6-D3-supplemented rats.

\(^{b}\) P < 0.05 compared with control.

\(^{c}\) P < 0.05, compared with non-ACF crypts in AOM-alone group.

\(^{d}\) P < 0.05, compared with ACF in AOM-alone group.

\(^{e}\) P < 0.05, compared with non-ACF crypts in AOM-alone group.
Ursodeoxycholic Acid and F6-D3 Inhibit AOM-induced ACF formation.

**Table 5** Cyclin D1 and E-cadherin mRNA in ACF crypts

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cyclin D1</th>
<th>E-cadherin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOM</td>
<td>817 ± 167</td>
<td>91 ± 58</td>
</tr>
<tr>
<td>AOM + UDCA</td>
<td>75 ± 20</td>
<td>170 ± 75</td>
</tr>
<tr>
<td>AOM + F6-D3</td>
<td>78 ± 34</td>
<td>165 ± 72</td>
</tr>
</tbody>
</table>

a Cyclin D1 and E-cadherin mRNA levels in the indicated groups assessed by real-time PCR as described in the “Materials and Methods” (n = 6 animals in each group, with 4 ACF biopsies from each carcinogen-treated rat and 4 normal biopsies from controls). Data are expressed as % cyclin D1 or E-cadherin mRNA in normal crypts from saline-treated rats (mean ± SE). Cyclin D1 and E-cadherin mRNA were comparable among the saline-treated unsupplemented group and UDCA and F6-D3-supplemented groups.

b P < 0.05, compared with control crypts.

c P < 0.05, compared with ACF crypts in the AOM-alone group. Samples were normalized to β-actin mRNA expression.

Table 6 E-cadherin protein expression in ACF and non-ACF crypts

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>ACF crypts</th>
<th>Non-ACF crypts</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOM</td>
<td>31 ± 22</td>
<td>89 ± 26</td>
</tr>
<tr>
<td>AOM + UDCA</td>
<td>65 ± 35</td>
<td>103 ± 43</td>
</tr>
<tr>
<td>AOM + F6-D3</td>
<td>64 ± 38</td>
<td>109 ± 41</td>
</tr>
</tbody>
</table>

a E-cadherin expression in the indicated groups assessed by quantitative Western blotting as described in the “Materials and Methods” (n = 6 animals in each group, with 4 ACF and 4 non-ACF biopsies from each carcinogen-treated rat and 4 normal biopsies from each of the saline-treated control groups). E-cadherin expression did not differ among saline-treated unsupplemented rats and UDCA and F6-D3-supplemented rats. Data are expressed as % E-cadherin in normal crypts from saline-treated rats (mean ± SD).

b P < 0.05, compared with control.

c P < 0.05, compared with ACF crypts in AOM-alone group.

involved in the marked morphological features that distinguish aberrant from nonaberrant crypts in situ. In contrast to the parallel changes in cyclin D1 protein and mRNA, the alterations in E-cadherin protein in ACF did not involve significant changes in mRNA expression, suggesting a posttranscriptional mechanism, as proposed for other noncolonial epithelial cells (48). These changes in E-cadherin induced by AOM and reversed by UDCA and F6-D3 also occurred in tumors, paralleling their effects on proliferation.

There are several potential mechanisms by which loss of E-cadherin may alter cyclin D1 and stimulate cell proliferation. For example, because E-cadherin sequesters β-catenin, the loss of E-cadherin may contribute to an increase in activating β-catenin/Tcf-4 transcription factor complexes (49). These complexes, in turn, may drive the increase in cyclin D1 in colonic carcinogenesis (3, 50). In this regard, others have reported increased nuclear β-catenin in non-ACF crypts in the AOM model (51). The nature of these crypts remains controversial, however, with others suggesting these crypts are dysplastic ACF (52). E-cadherin also negatively regulates receptor tyrosine kinase signaling (53–56). Loss of E-cadherin could, therefore, increase growth factor activated Ras signaling to enhance cyclin D1 expression (34). Additional studies will be required to examine these and other possibilities. Regardless of a potential underlying cross-talk between E-cadherin and cyclin D1 regulation, AOM-induced hyperproliferation in ACF appears to involve both an increase in cyclin D1 and a loss of E-cadherin. In comparison to nonaberrant crypts, the more accentuated AOM-induced changes in cyclin D1 and E-cadherin in aberrant crypts would favor their greater proliferation. The enhanced changes in proliferation and greater deregulations in mitogenic regulators in aberrant compared with nonaberrant crypts emphasize the use of ACF as biomarkers of colonic carcinogenesis. The ability of UDCA and F6-D3 to inhibit AOM-induced hyperproliferation and reverse growth-promoting alterations in these mitogenic regulators, moreover, support the validity of ACF as biomarkers to assess the efficacy of chemopreventive agents.

The increases in iNOS and COX-2, which were present in AOM tumors but not ACF, indicate that up-regulation of these inducible enzymes occurs at a later stage of colonic carcinogenesis. Thus, these findings suggest that chemopreventive UDCA and F6-D3 act by inhibiting cyclin D1 up-regulation and E-cadherin down-regulation and thereby limit hyperproliferation at the ACF stage. Furthermore, at more advanced stages of tumorigenesis, these agents also lead to a block in iNOS and COX-2.
COX-2 induction. Interestingly, in noncolonic cells, iNOS and COX-2 expression are positively regulated by nuclear factor κB (57, 58). UDCA and 1,25-dihydroxyvitamin D3, the parent compound of F6-D3, moreover, have both been shown to block nuclear factor κB activation (59, 60).

In summary, we have demonstrated that UDCA and F6-D3, two agents previously established to prevent AOM-induced tumor development, limit carcinogen-induced hyperproliferation and ACF formation in this model. These actions are mediated, in part, by inhibiting the growth-enhancing alterations in cyclin D1 and E-cadherin induced by this carcinogen. At more advanced stages, UDCA and F6-D3 also limit iNOS and COX-2 induction. Recent studies in humans, moreover, suggest that UDCA and 1,25-dihydroxyvitamin D3, a congener of F6-D3, might prevent tumor development in individuals at risk for colon cancer (24, 25, 61). Those investigations have also suggested that UDCA and 1,25-dihydroxyvitamin D3 act by anti-proliferative mechanisms (25, 61). Elucidation of the underlying mechanisms that dysregulate cyclin D1 and E-cadherin expression in premalignant mucosa and iNOS and COX-2 with tumor progression will provide important additional insights into colonic carcinogenesis. Understanding the pathways by which UDCA and F6-D3 inhibit these growth-enhancing alterations, moreover, may identify new targets for chemoprevention.

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References


