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[1]

Ramesh K. Wali,[2] Sharad Khare, Maria Tretiakova, Greg Cohen, Lan Nguyen, John Hart, Julia Wang, Ming Wen, Akila Ramaswamy, Loren Joseph, Michael Sitrin, Thomas Brasitus, and Marc Bissonnette[3]

Departments of Medicine [R. K. W., S. K., G. C., L. N., J. W., A. R., M. S., T. B., M. B.], Pathology [M. T., J. H., L. J.], and Health Studies [M. W.], University of Chicago Hospitals and Clinics, Chicago, Illinois 60637

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 colonic 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 bromodeoxyuridine (BrdUrd) 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 (BrdUrd 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 xenobiotic 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[4] 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 premalig-

Received 5/2/02; revised 9/18/02; accepted 9/27/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] These studies were funded, in part, by the following grants: USPHS Grants CA36745 (to T. B. and M. B.), DK39573 (to T. B., M. S., M. B.), P30DK42086 (to T. B.) [Digestive Diseases Research Core Center], and CA69532 (to M. B.), as well as by the Samuel Freedman Research Laboratories for Gastrointestinal Cancer Research. Additional funding was provided by the Department of Pathology Research Core Facilities of the University of Chicago. T. B. is the recipient of a Merit Award from the National Cancer Institute, NIH.
[2] Present address: Evanston Northwestern Health Care, Evanston, IL 60201.
[3] To whom requests for reprints should be addressed, at Department of Medicine, MC 4076 University of Chicago Hospitals and Clinics 5841 South Maryland Avenue, Chicago, IL 60637. Phone: (773) 702-8597; Fax: (773) 702-2182; E-mail: mbissonn@medicine.bsd.uchicago.edu.

[4] The abbreviations used are: COX, cyclooxygenase; ACF, aberrant crypt foci; AOM, azoxymethane; BrdUrd, 5-bromo-2′-deoxyuridine; F_6-D_3, 1α,25 dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol; iNOS, inducible nitric oxide synthase; TBR, tumor-bearing rat; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; UDCA, ursodeoxycholic acid.

nant lesions to risk stratify patients for colon cancer surveillance. In addition, increasing efforts are being made to develop efficacious chemopreventive agents.

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 F_6-D_3, an analogue of 1,25-dihydroxyvitamin D_3, are potent chemopreventive agents in this model (21, 22). It was, therefore, of interest to examine the effects of UDCA and F_6-D_3 on the AOM-induced hyperproliferative state in ACF and non-ACF crypts, as well as in AOM tumors to elucidate their potential chemopreventive mechanisms of action. UDCA is a low abundance primary bile acid. In cholestatic disorders, UDCA is cytoprotective against toxic hydrophobic bile acids such as chenodeoxycholic acid or deoxycholic acid (23). UDCA has recently been suggested to protect against the development of human colon cancer in several premalignant conditions (24, 25). A number of vitamin D_3 analogues have also been implicated as chemopreventive agents in human and experimental colon cancer (26). F_6-D_3, a fluorinated synthetic derivative of 1,25-dihydroxyvitamin D_3, unlike the parent secosteroid, does not cause hypercalcemia at chemopreventive doses in the AOM model (22). Both UDCA and F_6-D_3, moreover, inhibited proliferation and induced a G_1 arrest *in vitro* 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 F_6-D_3 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 G_1 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 Ca^{2+} regulated component of the zonula 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 F_6-D_3 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 D_3, 1α,25-dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (F_6-D_3), 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 F_6-D_3 (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 anticyclin D1 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 + F_6-D_3 (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 F_6-D_3 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 F_6-D_3, 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 F_6-D_3 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 were further examined to determine rates of crypt cell proliferation and apoptosis and to assess changes in cyclin D1, E-cadherin, iNOS, and COX-2 by immunostaining. In the AOM-treated groups, four random large ACF (≥4 aberrant crypts/focus) and four additional biopsies containing no ACF were excised from the distal colon of each rat. In the saline-treated groups, which included unsupplemented rats and rats supplemented with UDCA or F_6-D_3, 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% H_2O_2, 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 sera. Intergen'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 colon 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 F_6-D_3. 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 $MgCl_2$, 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-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 nM of forward and reverse primers, 200 μM deoxynucleotide triphosphate, 5 mM $MgCl_2$, 0.75 units of HotStarTaq 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-CACTTTCTTTCTTTCCA-3′ and reverse primer was 5′-CG-CAGGCTTGACTCCAGAAG-3′, with predicted product size of 96 bp. The E-cadherin forward primer was 5′-ATCTGTT-GCAGAAGGCGCTCTT-3′ and the reverse primer 5′-TG-TAGGCGATGGCAGCATTGT-3′, with predicted product size of 100 bp. The rat cytoplasmic β-actin forward primer was 5′-AAGGCCAACCGTGAAAAGATG-3′ and the reverse primer was 5′-GGACAACACAGCCTGGATGG-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 F_6-D_3 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 F_6-D_3 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 F_6-D_3 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 F_6-D_3 significantly reduced the number of ACF to 34 ± 4 and 23 ± 2, respectively ($P < 0.05$). UDCA and F_6-D_3 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 (F_6-D_3) [$P < 0.05$]. 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).

Table 1 Tumor multiplicity

Treatment	No. of animals	Tumor no.	Tumors/TBR
AOM alone	18	55	3.1 (2.5/0.6)[a]
AOM + UDCA	11	19	1.9[b] (1.7/0.2)[a]
AOM + F_6-D_3	9	14	1.8[b] (1.0/0.8)[a]

[a] Adenomas/TBR/carcinomas/TBR.
[b] $P < 0.05$, compared with AOM alone (Kruskal-Wallis test).

Table 2 ACF distribution[a]

Treatment (no. of animals)	Colonic segment			Total ACF	Large ACF ≥4 crypts/ACF
	Proximal	Middle	Distal		
AOM (18)	4 ± 1	13 ± 2	48 ± 10	65 ± 8	26 ± 3
AOM + UDCA (11)	4 ± 2	11 ± 2	19 ± 5[b]	34 ± 4[b]	13 ± 2[b]
AOM + F_6-D_3 (9)	4 ± 1	8 ± 3	11 ± 3[b]	23 ± 2[b]	7 ± 1[b]

[a] Mean ± SE.
[b] $P < 0.05$, compared with AOM alone (Kruskal-Wallis test).

UDCA and F_6-D_3 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 cytokinetic parameters by BrdUrd incorporation and TUNEL assay, respectively. In the absence of carcinogen treatment, UDCA or F_6-D_3 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. 2*B*). 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 F_6-D_3 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, AOM induced a significant increase in the BrdUrd labeling in both ACF and non-ACF crypts, and UDCA or F_6-D_3 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/5–8 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 F_6-D_3 group. There were no significant differences in rates of apoptosis among the tumor groups, although occasional tumors from the F_6-D_3 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 G_1-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 the expression of 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 F_6-D_3 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

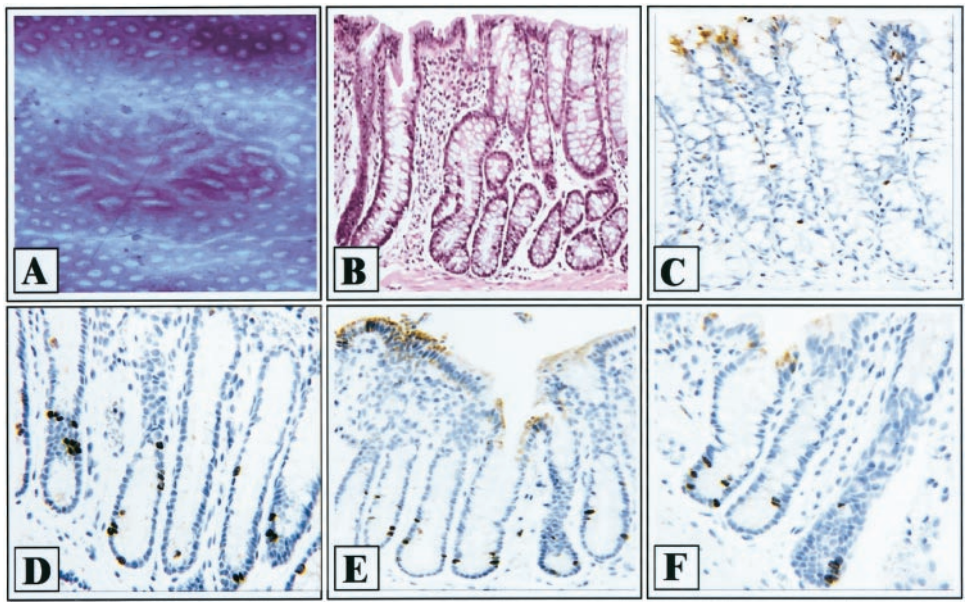

Fig. 1. BrdUrd incorporation in normal and ACF crypts. Unsupplemented rats or rats supplemented with UDCA or F_6-D_3 were treated with AOM or saline (vehicle) and the protocol followed as described in "Materials and Methods." Forty weeks after diet initiation, rats were injected with BrdUrd and sacrificed 2 h later. Colons were fixed in formalin for 4 h, and cell proliferation measured by BrdUrd incorporation. Shown in the figure are representative micrographs from normal crypts and ACF from the indicated groups. *A*, AOM-induced ACF *in situ*, ×25. (Note expanded pericryptal space for crypts comprising the ACF compared with adjacent crypts). *B*, ACF stained with H&E (×200); *C*, BrdUrd-labeled crypt from normal rat (×200); *D*, BrdUrd-labeled ACF from AOM-treated animal fed AIN-76A alone (×200). *E*, BrdUrd-labeled ACF from AOM-treated animal supplemented with UDCA (×200). *F*, BrdUrd-labeled ACF from AOM-treated animal supplemented with F_6-D_3 (×200). Note ACF crypts are larger with increased BrdUrd labeling, expanded proliferative zones, and mucin depletion, compared with normal crypts (compare *D* to *C*). Treatment with UDCA or F_6-D_3 partially reversed carcinogen-induced hyperproliferation (compare *E* and *F* to *D*).

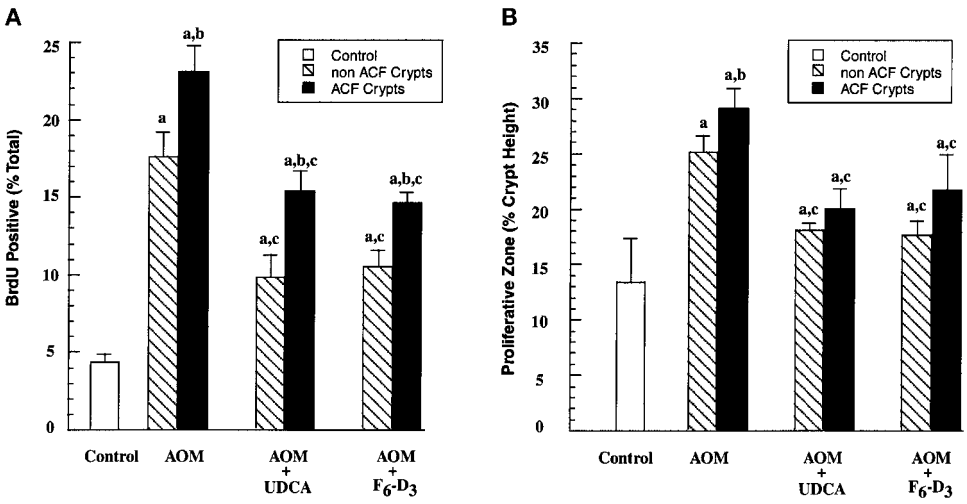

Fig. 2. Effects of AOM treatment and UDCA and F_6-D_3 supplementation on proliferation in ACF and non-ACF crypts. Six animals, prepared as described in Fig. 1, were examined in each group. From each carcinogen-treated rat, four ACF and four non-ACF biopsies were obtained, and from each saline-treated rat, four normal biopsies were obtained. BrdUrd labeling and the proliferative zone height were assessed as described in "Materials and Methods" and expressed as means ± SD. (The saline-treated groups were combined as "Control" in the figure because neither UDCA nor F_6-D_3 alone altered the BrdUrd labeling index or height of the proliferation zone, compared with the unsupplemented group). *A*, BrdUrd labeling. $^aP < 0.005$, compared with normal (control) crypts; $^bP < 0.005$, compared with nonaberrant crypts; $^cP < 0.005$, compared with their respective nonaberrant or aberrant crypts in the carcinogen-treated unsupplemented group. *B*, proliferative zone. $^aP < 0.05$, compared with normal (control) crypts; $^bP < 0.05$, compared with nonaberrant crypts; $^cP < 0.005$, compared with their respective nonaberrant or aberrant crypts in the carcinogen-treated unsupplemented group.

alone, supplementation with UDCA or F_6-D_3 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 F_6-D_3, these chemopreventive agents also significantly

Table 3 BrdUrd labeling in AOM tumors[a]

Group	% BrdUrd positive
AOM alone	13.7 ± 3.1
AOM + UDCA	5.1 ± 1.2[b]
AOM + F_6-D_3	7.8 ± 1.5

[a] BrdUrd labeling expressed as mean ± SE.
[b] $P < 0.05$ compared to AOM alone group. $n = 6$ tumors in each group. For each tumor, six representative fields with 3000 total cells were counted and scored for BrdUrd positivity.

limited ($P < 0.05$) the increased expression of cyclin D1 in ACF induced by this carcinogen. These agents caused >70% reduction in cyclin D1 levels in ACF and >50% reduction in cyclin D1 in non-ACF crypts (Fig. 4, Table 4). To assess the level of cyclin D1 gene regulation, we measured cyclin D1 mRNA by real-time PCR. As shown in Table 5, AOM significantly up-regulated cyclin D1 mRNA in aberrant crypts. Furthermore, UDCA and F_6-D_3 significantly inhibited this carcinogen-induced increase in cyclin D1 mRNA (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 F_6-D_3 supplementation (Fig. 5).

E-cadherin is a negative regulator of colonic epithelial cell proliferation (34). Because loss of E-cadherin has been described 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 F_6-D_3. 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 F_6-D_3 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 F_6-D_3. 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 F_6-D_3. 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 F_6-D_3 supplementation (Fig. 5).

AOM Increases iNOS and COX-2 Expression in AOM-induced Tumors but not in ACF. Increased iNOS and COX-2 expression have been implicated in the development of sporadic human colon cancer (35, 36), as well as in experimental models of colonic 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 F_6-D_3. 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 F_6-D_3 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 F_6-D_3 supplementation as shown in Fig. 6.

Discussion

We have previously shown that UDCA and F_6-D_3 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 F_6-D_3 effectively decreased both the number and size of ACF and inhibited their cellular proliferation. Our study demonstrates for the first time that UDCA and F_6-D_3 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 colonic tumors requires alterations in the balance of cell renewal and cell death that regulate normal cellular homeostasis in the colon (6). UDCA and F_6-D_3 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 F_6-D_3 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 F_6-D_3 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

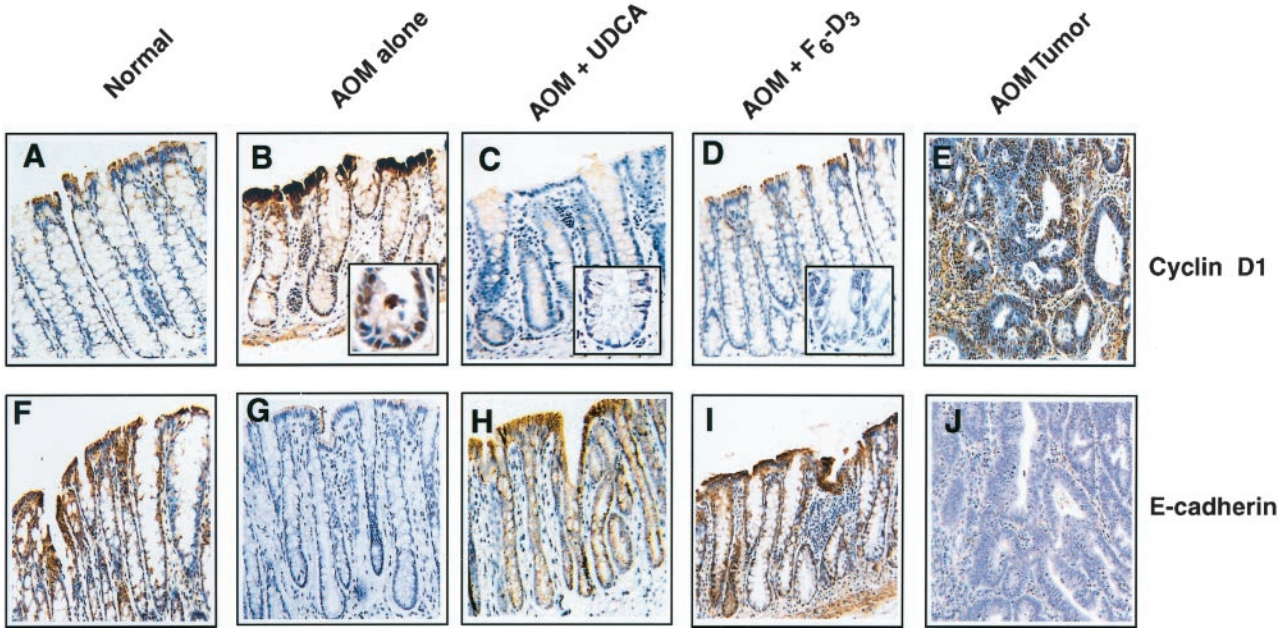

Fig. 3. Cyclin D1 and E-cadherin expression in normal and ACF crypts by immunostaining. Colonic biopsies of control and ACF crypts were immunostained for cyclin D1 and E-cadherin. There were no differences in cyclin D1 or E-cadherin staining among the saline-treated unsupplemented and UDCA and F_6-D_3 supplemented groups (normal). *Upper panel:* cyclin D1 expression in representative normal crypt (*A*) and ACF crypts (*B–D*) and in AOM-induced tumor from an unsupplemented animal (*E*). *Insets* in *B–D* show higher magnification (×400) to demonstrate nuclear staining of cyclin D1. *Lower panel:* E-cadherin expression in representative normal crypt (*F*) and ACF crypts (*G–I*) and in AOM-induced tumor from an unsupplemented animal (*J*). (n = 6 animals in each group). E-cadherin is membrane associated, with increasing expression from crypt base to colonic surface. Compared with normal mucosa, there was an increase in cyclin D1 and a decrease in E-cadherin expression in ACF crypts from the carcinogen-treated unsupplemented animals. These changes were partially inhibited by UDCA or F_6-D_3 supplementation.

Table 4 Cyclin D1 protein expression in ACF and non-ACF Crypts[a]

Treatment group	ACF crypts	Non-ACF crypts
AOM	486 ± 210[b,c]	252 ± 110[b]
AOM + UDCA	137 ± 87[d]	87 ± 43[e]
AOM + F_6-D_3	67 ± 24[d]	104 ± 53[e]

[a] 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 (means ± SD). Cyclin D1 expression did not differ among the saline-treated unsupplemented rats and UDCA and F_6-D_3-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.

hyperproliferation and progression to ACF and their inhibition by UDCA and F_6-D_3, although likely complex, involve alterations in cyclin D1.

In addition to cyclin D1, E-cadherin, a Ca^{2+}-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 F_6-D_3. Because E-cadherin plays a critical role in cell-cell adhesion, this loss in ACF crypts, but not in non ACF crypts, may be

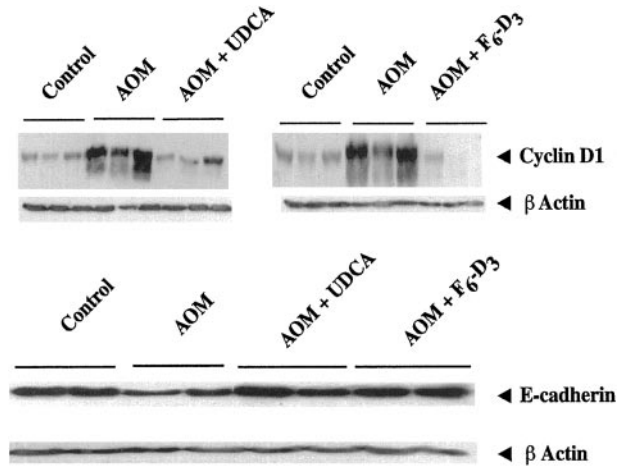

Fig. 4. Cyclin D1 and E-cadherin expression in normal and ACF crypts by Western blotting. Ethanol-fixed biopsies of crypts from saline injected rats (control) and of ACF and non-ACF crypts from AOM treated animals were examined for cyclin D1 and E-cadherin expression by Western blotting as described in the "Materials and Methods" (n = 6 animals in each group). There were no differences in cyclin D1 or E-cadherin expression among the saline-treated unsupplemented and UDCA and F_6-D_3 supplemented groups (control). *Top panel:* representative Western blot of cyclin D1 in normal (control) and ACF crypts from three animals in each group. Blots were reprobed for β-actin expression to confirm comparable protein loading. *Bottom panel:* representative Western blots of E-cadherin in normal (control) and ACF crypts from two animals in each group. Blots were reprobed for β-actin expression to confirm comparable protein loading.

Table 5 Cyclin D1 and E-cadherin mRNA in ACF crypts[a]

Treatment group	Cyclin D1	E-cadherin
AOM	817 ± 167[b]	91 ± 55
AOM + UDCA	75 ± 20[c]	170 ± 75
AOM + F_6-D_3	78 ± 34[c]	165 ± 72

[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 (means ± SE). Cyclin D1 and E-cadherin mRNA were comparable among the saline-treated unsupplemented group and UDCA and F_6-D_3-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.

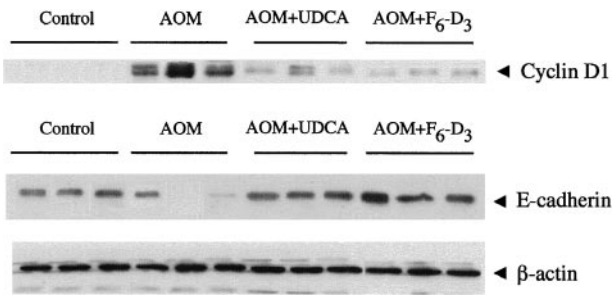

Fig. 5. Cyclin D1 and E-cadherin expression in AOM tumors by Western blotting. Normal colonic mucosa and AOM tumors from the indicated dietary groups were homogenized and lysates (30 μg of protein) subjected to Western blotting as described in "Materials and Methods." There were no differences in cyclin D1 or in E-cadherin in colonic mucosa among the saline-treated animals in the unsupplemented and the UDCA and F_6-D_3 supplemented animals (controls). *Top panel:* representative Western blot of cyclin D1 in normal (control) and AOM tumors from three animals in each group. The relative cyclin D1 densitometric values (mean ± SD) were 0, (normal mucosa), 100 ± 37% (AOM alone), 17 ± 9% (AOM + UDCA), and 5 ± 2% (AOM + F_6-D_3). *Middle panel:* representative Western blots of E-cadherin in normal (control) and AOM tumors from three animals in each group. The relative E-cadherin densitometric values (mean ± SD) were 100 ± 24% (normal mucosa), 31 ± 17% (AOM alone), 141 ± 14% (AOM + UDCA), and 193 ± 39% (AOM + F_6-D_3). *Bottom panel:* β-actin expression in control and tumor samples.

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

Treatment group	ACF crypts	Non-ACF crypts
AOM	31 ± 22[b]	89 ± 26
AOM + UDCA	65 ± 35[c]	103 ± 43
AOM + F_6-D_3	64 ± 38[c]	109 ± 41

[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 F_6-D_3-supplemented rats. Data are expressed as % E-cadherin in normal crypts from saline-treated rats (means ± 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 noncolonic epithelial cells

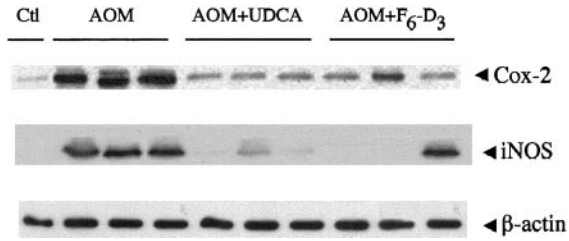

Fig. 6. COX-2 and iNOS expression in AOM-induced tumors by Western blotting. Normal colonic mucosa and AOM tumors from the indicated dietary groups were homogenized and lysates (30 μg of protein) subjected to Western blotting as described in "Materials and Methods." There were no differences in colonic mucosal COX-2 or iNOS in the saline-treated animals among the unsupplemented rats and the UDCA or F_6-D_3 supplemented animals (controls). *Top panel:* representative Western blot of COX-2 in normal (control, Ctl) and tumors from three animals in each group. The relative COX-2 densitometric values (mean ± SD) were 10% (normal mucosa), 100 ± 7% (AOM alone), 24 ± 4% (AOM + UDCA), and 34 ± 16% (AOM + F_6-D_3). *Middle panel*: representative Western blot of iNOS in normal (control, Ctl) and tumors from three animals in each group. The relative iNOS densitometric values (mean ± SD) were 0% (normal mucosa), 100 ± 14% (AOM alone), 11 ± 6% (AOM + UDCA), and 55 ± 30% (AOM + F_6-D_3). *Bottom panel:* loading control of β-actin expression in control and tumor samples.

(48). These changes in E-cadherin induced by AOM and reversed by UDCA and F_6-D_3 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 derangements in mitogenic regulators in aberrant compared with nonaberrant crypts emphasize the use of ACF as biomarkers of colonic carcinogenesis. The ability of UDCA and F_6-D_3 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 F_6-D_3 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 induction. Interestingly, in noncolonic cells, iNOS and COX-2 expression are positively regulated by nuclear factor κB (57, 58). UDCA and 1,25-dihydroxyvitamin D_3, the parent compound of F_6-D_3, moreover, have both been shown to block nuclear factor κB activation (59, 60).

In summary, we have demonstrated that UDCA and F_6-D_3, 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 F_6-D_3 also limit iNOS and COX-2 induction. Recent studies in humans, moreover, suggest that UDCA and 1,25-dihydroxyvitamin D_3, a congener of F_6-D_3, 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 D_3 act by antiproliferative 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 F_6-D_3 inhibit these growth-enhancing alterations, moreover, may identify new targets for chemoprevention.

Acknowledgments

We thank Dr. Theodore Karrison for additional statistical advice during the analysis of data and Gordon Bowie for excellent assistance with photomicroscopy. We also thank Can Gong for excellent technical assistance with immunohistochemical staining.

References

1. Vogelstein, B., and Kinzler, K. W. The multistep nature of cancer. Trends Genet., *9:* 138–141, 1993.

2. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science (Wash. DC), *275:* 1787–1790, 1997.

3. Arber, N., Hibshoosh, H., Moss, S. F., Sutter, T., Zhang, Y., Begg, M., Wang, S., Weinstein, I. B., and Holt, P. R. Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. Gastroenterology, *110:* 669–674, 1996.

4. Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T., and Hirohashi, S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc. Natl. Acad. Sci. USA, *92:* 7416–7419, 1995.

5. Fosslien, E. Biochemistry of cyclooxygenase (COX)-2 inhibitors and molecular pathology of COX-2 in neoplasia. Crit. Rev. Clin. Lab. Sci., *37:* 431–502, 2000.

6. Eastwood, G. L. A review of gastrointestinal epithelial renewal and its relevance to the development of adenocarcinomas of the gastrointestinal tract. J. Clin. Gastroenterol., *21:* S1–S11, 1995.

7. Risio, M., Lipkin, M., Candelaresi, G., Bertone, A., Coverlizza, S., and Rossini, F. P. Correlations between rectal mucosa cell proliferation and the clinical and pathological features of nonfamilial neoplasia of the large intestine. Cancer Res., *51:* 1917–1921, 1991.

8. Terpstra, O. T., van Blankenstein, M., Dees, J., and Eilers, G. A. Abnormal pattern of cell proliferation in the entire colonic mucosa of patients with colon adenoma or cancer. Gastroenterology, *92:* 704–708, 1987.

9. Ponz de Leon, M., Roncucci, L., Di Donato, P., Tassi, L., Smerieri, O., Amorico, M. G., Malagoli, G., De Maria, D., Antonioli, A., Chahin, N. J., *et al.* Pattern of epithelial cell proliferation in colorectal mucosa of normal subjects and of patients with adenomatous polyps or cancer of the large bowel. Cancer Res., *48*: 4121–4126, 1988.

10. Takayama, T., Katsuki, S., Takahashi, Y., Ohi, M., Nojiri, S., Sakamaki, S., Kato, J., Kogawa, K., Miyake, H., and Niitsu, Y. Aberrant crypt foci of the colon as precursors of adenoma and cancer. N. Engl. J. Med., *339:* 1277–1284, 1998.

11. Bird, R. P., and Good, C. K. The significance of aberrant crypt foci in understanding the pathogenesis of colon cancer. Toxicol. Lett. (Amst.), *112–113*: 395–402, 2000.

12. McLellan, E. A., Medline, A., and Bird, R. P. Dose response and proliferative characteristics of aberrant crypt foci: putative preneoplastic lesions in rat colon. Carcinogenesis (Lond.), *12:* 2093–2098, 1991.

13. Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res., *58:* 409–412, 1998.

14. Mori, H., Sugie, S., Yoshimi, N., Hara, A., and Tanaka, T. Control of cell proliferation in cancer prevention. Mutat. Res., *428:* 291–298, 1999.

15. Reddy, B. S., Rao, C. V., and Seibert, K. Evaluation of cyclooxygenase-2 inhibitor for potential chemopreventive properties in colon carcinogenesis. Cancer Res., *56:* 4566–4569, 1996.

16. Rao, C. V., Hirose, Y., Indranie, C., and Reddy, B. S. Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids. Cancer Res., *61:* 1927–1933, 2001.

17. McLellan, E. A., Medline, A., and Bird, R. P. Sequential analyses of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. Cancer Res., *51:* 5270–5274, 1991.

18. Maskens, A. P., and Deschner, E. E. Tritiated thymidine incorporation into epithelial cells of normal-appearing colorectal mucosa of cancer patients. J. Natl. Cancer Inst. (Bethesda), *58:* 1221–1224, 1977.

19. Pereira, M. A., Barnes, L. H., Rassman, V. L., Kelloff, G. V., and Steele, V. E. Use of azoxymethane-induced foci of aberrant crypts in rat colon to identify potential cancer chemopreventive agents. Carcinogenesis (Lond.), *15:* 1049–1054, 1994.

20. Siu, I. M., Robinson, D. R., Schwartz, S., Kung, H. J., Pretlow, T. G., Petersen, R. B., and Pretlow, T. P. The identification of monoclonality in human aberrant crypt foci. Cancer Res., *59:* 63–66, 1999.

21. Earnest, D. L., Holubec, H., Wali, R. K., Jolley, C. S., Bissonnette, M., Bhattacharyya, A. K., Roy, H., Khare, S., and Brasitus, T. A. Chemoprevention of azoxymethane-induced colonic carcinogenesis by supplemental dietary ursodeoxycholic acid. Cancer Res., *54:* 5071–5074, 1994.

22. Wali, R. K., Bissonnette, M., Khare, S., Hart, J., Sitrin, M. D., and Brasitus, T. A. 1α,25-dihydroxy-16-ene-23-yne-26, 27-hexafluorocholecalciferol, a noncalcemic analogue of 1α, 25-dihydroxyvitamin D_3, inhibits azoxymethane-induced colonic carcinogenesis. Cancer Res., *55:* 3050–3054, 1995.

23. Beuers, U., Boyer, J. L., and Paumgartner, G. Ursodeoxycholic acid in cholestasis: potential mechanisms of action and therapeutic applications. Hepatology, *28:* 1449–1453, 1998.

24. Tung, B. Y., Emond, M. J., Haggitt, R. C., Bronner, M. P., Kimmey, M. B., Kowdley, K. V., and Brentnall, T. A. Ursodiol use is associated with lower prevalence of colonic neoplasia in patients with ulcerative colitis and primary sclerosing cholangitis. Ann. Intern. Med., *134:* 89–95, 2001.

25. Serfaty, L., Deleusse, A., Rosmorduc, O., Flejoux, J-F, Chazouilleres, O., Poupon, R., and Poupon, R. Ursodeoxycholic acid decreases recurrence of colorectal adenomas in patients with primary biliary cirrhosis. Gastroenterology, *122:* A70, 2002.

26. Brasitus, T. A., and Sitrin, M. D. Chemoprevention of colon cancer by vitamin D_3 and its metabolites/analogues. *In:* D. D. Feldman, F. Glorieux, and W. Pike (eds.). Vitamin, pp. 1141–1154. New York: Academic Press, Inc., 1997.

27. Martinez, J. D., Stratagoules, E. D., LaRue, J. M., Powell, A. A., Gause, P. R., Craven, M. T., Payne, C. M., Powell, M. B., Gerner, E. W., and Earnest, D. L. Different bile acids exhibit distinct biological effects: the tumor promoter deoxycholic acid induces apoptosis and the chemopreventive agent ursodeoxycholic acid inhibits cell proliferation. Nutr. Cancer, *31:* 111–118, 1998.

28. Scaglione-Sewell, B. A., Bissonnette, M., Skarosi, S., Abraham, C., and Brasitus, T. A. A vitamin D_3 analog induces a G_1-phase arrest in CaCo-2 cells by inhibiting cdk2 and cdk6: roles of cyclin E, $p21^{Waf1}$, and $p27^{Kip1}$. Endocrinology, *141:* 3931–3939, 2000.

29. Bissonnette, M., Khare, S., von Lintig, F. C., Wali, R. K., Nguyen, L., Zhang, Y., Hart, J., Skarosi, S., Varki, N., Boss, G. R., and Brasitus, T. A. Mutational and nonmutational activation of $p21^{ras}$ in rat colonic azoxymethane-induced tumors: effects on mitogen-activated protein kinase, cyclooxygenase-2, and cyclin D1. Cancer Res., *60:* 4602–4609, 2000.

30. Wali, R., Bissonnette, M., Nguyen, L., Hart, J., Skarosi, S., Suh, W., Sitrin, M., and Brasitus, T. Chemopreventive agents, ursodeoxycholate and F_6-D_3, inhibit iNOS and COX-2 expression in AOM-induced colonic tumors. Gastroenterology, *116:* A526, 1999.

31. Takahashi, M., Fukuda, K., Ohata, T., Sugimura, T., and Wakabayashi, K. Increased expression of inducible and endothelial constitutive nitric oxide synthases in rat colon tumors induced by azoxymethane. Cancer Res., *57:* 1233–1237, 1997.

32. Dorudi, S., Sheffield, J. P., Poulsom, R., Northover, J. M., and Hart, I. R. E-cadherin expression in colorectal cancer. An immunocytochemical and *in situ* hybridization study. Am. J. Pathol., *142:* 981–986, 1993.

33. Sherr, C. J., Kato, J., Quelle, D. E., Matsuoka, M., and Roussel, M. F. D-type cyclins and their cyclin-dependent kinases: G_1 phase integrators of the mitogenic response. Cold Spring Harb. Symp. Quant. Biol., *59:* 11–19, 1994.

34. Conacci-Sorrell, M., Zhurinsky, J., and Ben-Ze'ev, A. The cadherin-catenin adhesion system in signaling and cancer. J. Clin. Investig., *109:* 987–991, 2002.

35. Ambs, S., Merriam, W. G., Bennett, W. P., Felley-Bosco, E., Ogunfusika, M. O., Oser, S. M., Klein, S., Shields, P. G., Billiar, T. R., and Harris, C. C. Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. Cancer Res., *58:* 334–341, 1998.

36. Sano, H., Kawahito, Y., Wilder, R. L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M., and Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res., *55:* 3785–3789, 1995.

37. DuBois, R. N., Radhika, A., Reddy, B. S., and Entingh, A. J. Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. Gastroenterology, *110:* 1259–1262, 1996.

38. Seraj, M. J., Umemoto, A., Kajikawa, A., Mimura, S., Kinouchi, T., Ohnishi, Y., and Monden, Y. Effects of dietary bile acids on formation of azoxymethane-induced aberrant crypt foci in F344 rats. Cancer Lett., *115:* 97–103, 1997.

39. Steele, V. E., Moon, R. C., Lubet, R. A., Grubbs, C. J., Reddy, B. S., Wargovich, M., McCormick, D. L., Pereira, M. A., Crowell, J. A., Bagheri, D., Sigman, C. C., Boone, C. W., and Kelloff, G. J. Preclinical efficacy evaluation of potential chemopreventive agents in animal carcinogenesis models: methods and results from the NCI Chemoprevention Drug Development Program. J. Cell. Biochem. Suppl., *20:* 32–54, 1994.

40. Magnuson, B. A., Carr, I., and Bird, R. P. Ability of aberrant crypt foci characteristics to predict colonic tumor incidence in rats fed cholic acid. Cancer Res., *53:* 4499–4504, 1993.

41. Zheng, Y., Kramer, P. M., Lubet, R. A., Steele, V. E., Kelloff, G. J., and Pereira, M. A. Effect of retinoids on AOM-induced colon cancer in rats: modulation of cell proliferation, apoptosis and aberrant crypt foci. Carcinogenesis (Lond.), *20:* 255–260, 1999.

42. Latham, P., Lund, E. K., and Johnson, I. T. Dietary n-3 PUFA increases the apoptotic response to 1,2-Dimethylhydrazine, reduces mitosis and suppresses the induction of carcinogenesis in the rat colon. Carcinogenesis (Lond.), *20:* 645–650, 1999.

43. Grossmann, J., Mohr, S., Lapetina, E. G., Fiocchi, C., and Levine, A. D. Sequential and rapid activation of select caspases during apoptosis of normal intestinal epithelial cells. Am. J. Physiol., *274:* G1117–G1124, 1998.

44. Bissonnette, M., Johnson, J., Wexler, S., Skarosi, S., and Wali, R. Ursodeoxycholate, a chemopreventive bile salt in colonic carcinogenesis, stimulates apoptosis and inhibits Bcl-2 expression in Caco-2 cells. Gastroenterology, *112:* A539, 1997.

45. Skarosi, S., Wali, R. K., Scaglione-Sewell, B., Bissonnette, M., Sitrin, M. D., and Brasitus, T. A. A fluorinated synthetic analogue of 1,25-dihydroxyvitamin D_3, F_6-D_3, stimulates apoptosis in Caco-2 cells and alters the expression of Bcl-X_L and the pro-form of caspase-3. Gastroenterology, *116:* A506, 1999.

46. Hermiston, M. L., Wong, M. H., and Gordon, J. I. Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautonomous regulation of cell fate in a self-renewing system. Genes Dev., *10:* 985–996, 1996.

47. Hermiston, M. L., and Gordon, J. I. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. Science (Wash. DC), *270:* 1203–1207, 1995.

48. Davies, G., Jiang, W. G., and Mason, M. D. Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell-cell dissociation and *in vitro* invasion. Clin. Cancer Res., *7:* 3289–3297, 2001.

49. Stockinger, A., Eger, A., Wolf, J., Beug, H., and Foisner, R. E-cadherin regulates cell growth by modulating proliferation-dependent β-catenin transcriptional activity. J. Cell Biol., *154:* 1185–1196, 2001.

50. Tetsu, O., and McCormick, F. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature (Lond.), *398:* 422–426, 1999.

51. Yamada, Y., Yoshimi, N., Hirose, Y., Kawabata, K., Matsunaga, K., Shimizu, M., Hara, A., and Mori, H. Frequent β-catenin gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. Cancer Res., *60:* 3323–3327, 2000.

52. Pretlow, T. P., and Bird, R. P. Correspondence re: Y. Yamada *et al.*, Frequent β-catenin gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. Cancer Res., *61:* 7699–7701, 2001.

53. Wilding, J., Vousden, K. H., Soutter, W. P., McCrea, P. D., Del Buono, R., and Pignatelli, M. E-cadherin transfection down-regulates the epidermal growth factor receptor and reverses the invasive phenotype of human papilloma virus-transfected keratinocytes. Cancer Res., *56:* 5285–5292, 1996.

54. Jawhari, A. U., Farthing, M. J., and Pignatelli, M. The E-cadherin/epidermal growth factor receptor interaction: a hypothesis of reciprocal and reversible control of intercellular adhesion and cell proliferation. J. Pathol., *187:* 155–157, 1999.

55. Potempa, S., and Ridley, A. J. Activation of both MAP kinase and phosphatidylinositide 3-kinase by Ras is required for hepatocyte growth factor/scatter factor-induced adherens junction disassembly. Mol. Biol. Cell, *9:* 2185–2200, 1998.

56. Takahashi, K., and Suzuki, K. Density-dependent inhibition of growth involves prevention of EGF receptor activation by E-cadherin-mediated cell-cell adhesion. Exp. Cell Res., *226:* 214–222, 1996.

57. Schmedtje, J. F., Jr., Ji, Y. S., Liu, W. L., DuBois, R. N., and Runge, M. S. Hypoxia induces cyclooxygenase-2 via the NF-κB p65 transcription factor in human vascular endothelial cells. J. Biol. Chem., *272:* 601–608, 1997.

58. Hatano, E., Bennett, B. L., Manning, A. M., Qian, T., Lemasters, J. J., and Brenner, D. A. NF-κB stimulates inducible nitric oxide synthase to protect mouse hepatocytes from TNF-α- and Fas-mediated apoptosis. Gastroenterology, *120:* 1251–1262, 2001.

59. Miura, T., Ouchida, R., Yoshikawa, N., Okamoto, K., Makino, Y., Nakamura, T., Morimoto, C., Makino, I., and Tanaka, H. Functional modulation of the glucocorticoid receptor and suppression of NF-κB-dependent transcription by ursodeoxycholic acid. J. Biol. Chem., *276:* 47371–47378, 2001.

60. Yu, X. P., Bellido, T., and Manolagas, S. C. Down-regulation of NF-κB protein levels in activated human lymphocytes by 1,25-dihydroxyvitamin D_3. Proc. Natl. Acad. Sci. USA, *92:* 10990–10994, 1995.

61. Thomas, M. G., Nugent, K. P., Forbes, A., and Williamson, R. C. Calcipotriol inhibits rectal epithelial cell proliferation in ulcerative proctocolitis. Gut, *35:* 1718–1720, 1994.