A Vitamin D Analogue Inhibits Colonic Carcinogenesis in the AOM/DSS Model

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Background. The azoxymethane (AOM) model recapitulates many features of human colon cancer, lacking an inflammatory component. Dextran sulfate sodium (DSS) induces colitis and promotes AOM-induced colon cancer in mice. Vitamin D analogues are anti-inflammatory and chemopreventive in models of colon cancer. Our aim was to evaluate the anti-inflammatory and chemopreventive efficacy of the vitamin D analogue Ro26-2198 in the AOM/DSS model and in vitro in HCA-7 colon cancer cells.

Materials and methods. A/J mice received Ro26-2198 (0.01 µg/kg body wt/day × 28 days) or vehicle by mini-osmotic pump. Animals were treated with a single dose of AOM (5 mg/kg body wt) or vehicle 1 week after pump insertion. Mice received 3% DSS or water × 7 days beginning week 3. Animals were sacrificed after 8 weeks and colon segments were fixed in formalin or flash-frozen. Hematoxylin and eosin colonic sections were examined for dysplasia and colonic lysates were assessed for c-Myc, cyclooxygenase 2, and phospho-active extracellular signal regulated kinase (ERK) by Western blotting. For in vitro studies, HCA-7 cells were treated with Ro26-2198 followed by interleukin-1beta (IL-1β). Proliferation was measured by WST-1 assay.

Results. Ro26-2198 delayed the onset of clinical colitis. Several dysplastic foci were present in the AOM/DSS group; none were found in the Ro26-2198 group. Compared with control, AOM/DSS significantly increased c-Myc (15-fold), cyclooxygenase 2 (COX-2) (2.5-fold), and pERK (10-fold), and Ro26-2198 abolished these increases. In vitro, Ro26-2198 inhibited IL-1β-induced ERK activation and COX-2 induction and decreased HCA-7 cell proliferation.

Conclusions. Ro26-2198 inhibited proliferative (ERK, c-Myc) and pro-inflammatory (COX-2) signals and progression to dysplasia, suggesting chemopreventive efficacy in this model of colitis-associated carcinogenesis. © 2007 Elsevier Inc. All rights reserved.

Key Words: inflammatory bowel diseases; colorectal cancer; vitamin D; chemoprevention; COX-2; c-Myc; ERK; HCA-7.

INTRODUCTION

Ulcerative colitis and Crohn’s disease are inflammatory diseases of the bowel (IBD) that are associated with an increased risk of colon cancer [1–3]. Colitis-associated crypt destruction and secondary regeneration predispose to tumorigenesis. This increased risk depends on several factors, including duration, activity, and extent of the disease [2]. Compared to sporadic tumors, colitis-associated tumors are often flat and deeply invasive. While stage for stage these tumors have comparable prognosis, colitis-associated colon cancers are often multifocal and diagnosed at an advanced stage [3]. The molecular signatures also differ from sporadic colon cancers, with early mutations in p53 and greater increases in pro-inflammatory mediators cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) in IBD-associated cancers [4, 5]. In patients with IBD, total colectomy is advised if preneoplastic dysplasia is identified. Since dysplasia is often patchy and potentially missed upon surveillance colonoscopy, and cancers are frequently invasive at the time of diagnosis, effective chemopreventive strategies are needed to prevent the neoplastic complications of these diseases.

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Despite intensive research, the etiology and pathogenesis of IBD remain elusive. Several animal models have been proposed to further elucidate the pathogenesis of these disorders [6]. The results obtained from these models provide strong support for an immunological hypothesis in that a deregulated mucosal immune response to enteric bacterial antigens in a genetically susceptible host initiates or sustains chronic inflammation [6]. Dextran sulfate sodium (DSS) induces acute ulcerations in mice with clinical colitis manifested by bloody diarrhea and weight loss. Pathologically, there is a shortening and edema of the colon, mucosal ulcerations, possibly containing neutrophilic infiltration, Th1-like immune responses, and increases in pro-inflammatory mediators COX-2 and iNOS. The earliest change of acute DSS-induced colitis is a progressive noninflammatory shortening and loss of colonic crypts [7], suggesting that epithelial cells are the primary target. In fact, DSS has been demonstrated to inhibit proliferation of mouse epithelial cells in vivo [8]. Early lesions occur mainly in the left colon and often involve lymphoid aggregates. There are several advantages to the DSS model of inflammation and colon cancer. This model provides a simple method to induce colonic epithelial damage in most mouse strains. The lesions are reproducible and the clinical and histological severity can be reliably and reproducibly quantified [7]. For these reasons, DSS colitis has been used to screen potential chemopreventive or anti-inflammatory drug efficacy. Several interventions have shown beneficial effects, supporting the use of this model to test therapeutic agents. Several models have been proposed to further elucidate the pathogenesis of IBD remain elusive. Several animal models were also shown to inhibit hyperproliferation [15]. Vitamin D analogues have been extensively studied as possible treatments for cancer [9, 13, 16, 17]. Several vitamin D analogues are chemopreventive in experimental models of colon cancer, including the AOM model. These vitamin D analogues activate the vitamin D receptor (VDR). In sporadic colon cancer, the growth promoting transcription complex β-catenin/Tcf-4 is activated [18, 19]. Furthermore transformed colonic epithelial cells become resistant to growth inhibitory transforming growth factor-beta (TGF-β) [20]. Recent studies suggest that activated VDR competes with Tcf-4 to bind β-catenin and inhibit β-catenin/Tcf signals [21]. In human colon cancer, VDR can also bind SMAD3, a TGF-β effector, to enhance TGF-β signaling [22]. Vitamin D analogues through the VDR are also known to suppress signaling by nuclear factor kappa beta (NFκB), a key pro-inflammatory activator of the innate and adaptive immune systems. VDR suppresses NFκB signaling by increasing IκBα, the major inhibitor of NFκB, and by directly binding NFκB and thereby inhibiting its function [23, 24].

In the current study, we used the AOM/DSS model to evaluate the potential chemopreventive efficacy and signaling changes induced by a novel noncalcemic vitamin D analogue, Ro26-2198, that is structurally related to Ro24-5531. Compared to Ro24-5531, Ro26-2198 possesses higher potency for VDR activation in vitro and lower hypercalcemic potential in vivo in dose-escalating murine toxicity studies (personal communication, Dr. Milan Uskokovic, Bioxell, Nutley, NJ). We also investigated the in vitro effects of Ro26-2198 on interleukin-1beta (IL-1β)-induced extracellular signal regulated kinase (ERK) activation and COX-2 up-regulation, as well as its effects on human HCA-7 colon cancer cell growth. The results of these studies and a discussion of their implications form the basis of this report.

MATERIALS AND METHODS

Materials

Male A/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME).AIN-76A rodent chow diet was purchased from Harlan Teklad Laboratories (Madison, WI). AOM was obtained from the NCI Chemical Carcinogen Reference Standard Repository, Midwest Research (Kansas City, MO). DSS was purchased from MP Biomedical (Solon, OH). Ro26-2198 was generously provided by Milan Uskokovic (Bioxell, Nutley, NJ). Mouse monoclonal antibodies to phospho-(active) ERK-1 and ERK-2 (SC-7383) and c-Myc antibodies (9E10) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). COX-2 rabbit monoclonal antibodies (#160106) were purchased from Cayman Chemicals (Ann Arbor, MI). Mouse monoclonal anti-jun antibody was obtained from Zymed Laboratories Inc. (South San Francisco, CA). Rabbit monoclonal antibody against Ki-67 (clone SP1) was from Neomarkers (Fremont, CA).Electrophoretic grade acrylamide, bisacrylamide, Tris, SDS, and precasted molecular weight markers were from Bio-Rad Labs (Richmond, CA). The X-OMAT AR film was supplied by Kodak (Rochester, NY). PVDF membranes (Immobilon-P) were purchased from Millipore Inc. (Bed-
fixed flat in 10% buffered formalin for 4 h and paraffin embedded as
mittee and followed NIH-approved guidelines. The University of Chicago Institutional Animal Care and Use Com-
sion (Fig. 1). All animal procedures were reviewed and approved by
sacrificed by euthanasia at 8 weeks under ketamine-xylazine anes-
or given 3% DSS in their drinking water for 7 days. Animals were
injection, animals were continued on regular water or given 3% DSS in their
drinking water for 7 days. Mice were sacrificed (Sacrified) at week 8.

Methods

Animal Treatment and Tissue Harvest

Male A/J mice (25 g) were prepped in a sterile fashion and anes-
thetized by subcutaneous injection with 70 mg ketamine/kg body weight and 7 mg xylazine/kg body weight. A flank incision was performed to expose the subcutaneous space. Mini-osmotic pumps (ALZET, Cupertino, CA) were implanted subcutaneously to deliver Ro26-2198 (0.01 µg/kg body wt/day × 28 days) or vehicle (1:1 PEG: DMSO). This dose of Ro26-2198 was 30% of the maximal tolerated daily dose. One week after pump insertion, animals received one injection of AOM (5 mg/kg body wt) or saline (vehicle). One week after AOM/saline injection, animals were continued on regular water or given 3% DSS in their drinking water for 7 days. Animals were sacrificed by euthanasia at 8 weeks under ketamine-xylazine anesthesia (Fig. 1). All animal procedures were reviewed and approved by the University of Chicago Institutional Animal Care and Use Committee and followed NIH-approved guidelines.

For tissue harvest, left hemi-colons from five animals in each group were flash frozen for Western blotting and another five were fixed flat in 10% buffered formalin for 4 h and paraffin embedded as Swiss rolls [25]. Multiple formalin-fixed colonic sections were stained with hematoxylin and eosin (H&E) and examined for dysplasia and adjacent sections used for immunostaining. Proteins from frozen colons were extracted to measure changes in c-Myc, COX-2, and phospho-ERK expression by Western blotting.

Assessment of Inflammation and Colitis

Clinical severity of colitis was assessed using the Disease Activity Index, a semiquantitative scoring system that incorporates (a) the presence of occult or gross bleeding, (b) diarrhea, and (c) weight loss as previously described [7]. This clinical index has been shown to correlate closely with histological changes [7]. Using H&E sections from Swiss rolls [25], we evaluated several histological features of inflammation, including extent of crypt loss, inflammatory cell infiltration, and mucosal ulceration following previously validated methods and scoring systems [7, 25].

Cell Culture

Dr. Susan Kirkland (ICRF, London, United Kingdom) generously provided HCA-7 cells that were derived from a mucinous colon carcinoma [26]. Cells were grown in McCoy’s media containing 10% fetal calf serum and penicillin/streptomycin mixture. Preconfluent cells (70–80% confluent) were used for experiments. Cells were treated with Ro26-2198 (100 nM) overnight or ethanol (vehicle), followed by IL-1β (10 ng/mL) or vehicle (water) for 1 h. Proliferation was mea-
sured using the WST-1 assay (Roche Applied Sciences, Indianapolis, IN). This assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. For proliferation assay cells were incubated with Ro26-2198 (50 and 100 nM) or ethanol (vehicle) overnight. Cells were then assayed at 48 h. WST-1 reagent (20 µL) was added and the plate was incubated at 37°C for 1 to 2 h. Sample absorbance was measured at 450 nm using a microtiter plate (ELISA) reader and cell proliferation rates were calculated from absorbance changes [27].

Western Blotting

Frozen left colons were mechanically pulverized in liquid nitrogen with a mortar and pestle chilled in a liquid nitrogen bath. Colons were sonicated and then homogenized with a Polytron (power setting 4 × 30 s) in 1.5 mL non-denaturing lysis buffer containing 25 mM HEPES pH 7.5, 125 mM NaCl, 1% IGEPAL, 10 mM MgCl2, 1 mM EDTA, and 2% glycerol. Colonic or HCA-7 cell proteins were ex-
tracted in Laemmli buffer and measured by Bio-Rad RC-DC protein assay. Protein extracts were subjected to quantitative Western blotting. Briefly, proteins were separated by SDS-PAGE using a 10% resolving polyacrylamide gel and electroblotted. Blots were incubated overnight at room temperature with specific primary antibodies (c-Myc 1:100, COX-2 1:1000, and pERK 1:1000) followed by 2 h incubation with appropriate peroxidase-coupled secondary antibod-
ies and subsequent detection on XOMAT film by enhanced chemilu-
minescence. Xerograms were digitized with an Epson scanner and band intensity quantified using IP Lab Gel.

Statistical Methods

Data were expressed as means ± SD or SE as indicated. Western blotting band intensities were expressed in arbitrary units and nor-
malized to fold increases compared to control samples. Differences between treated and control samples were compared by Student’s t-test. Differences in average clinical and pathologic scores were compared by ANOVA. Values of P < 0.05 were considered statisti-
cally significant.

RESULTS

Ro26-2198 Delayed the Onset of Colitis

We initially assessed the clinical effects of Ro26-2198 on inflammation in the AOM/DSS model in male A/J mice during the acute phase. Clinically, DSS-treated mice developed signs of colitis with occult bleeding, followed by gross bleeding and weight loss. Ro26-2198 delayed the onset of colitis, with the appearance of bleeding 4 days later than symptom onset in the AOM/DSS group. Ro26-2198 also inhibited the severity of colonic bleeding (Fig. 2). Interestingly, while this vita-
mnin D analogue delayed the onset of diarrhea and bleeding, it was associated with significantly more weight loss at the peak of inflammation, compared to the AOM/DSS alone group (Fig. 3). In agreement with these observations, the mortality in the group receiving Ro26-2198 was higher at 52%, compared to 40% in the group given AOM/DSS alone (data not shown).

Ro26-2198 Inhibited Proliferation in the AOM/DSS Model

We evaluated colonic crypt cell proliferation by Ki-67 immunostaining in the postcolitis recovery phase. The
colonic crypt cell proliferative rates in animals receiving Ro26-2198 were lower than those in animals in the AOM/DSS alone group or control mice (Fig. 4), suggesting that Ro26-2198 inhibited proliferation. In contrast, there were no differences in inflammation at this stage in the AOM/DSS group, compared to the group also receiving Ro26-2198 (data not shown).

Ro 26-2198 Inhibited the Development of Dysplasia in the AOM/DSS Model

Mice were sacrificed at 8 weeks during the pre-malignant phase. By this time the histological features of acute colitis had largely resolved. Colonic sections stained with H&E were examined for dysplasia (Fig. 5). There were several dysplastic foci in AOM/DSS-treated mice, but none were identified in matching number of multiple sections from AOM/DSS mice treated with Ro26-2198. Except for focal dysplastic foci in the AOM/DSS group, colonic histology among groups and controls was similar (Fig. 5, and data not shown).

Ro26-2198 Inhibited AOM/DSS-Induced c-Myc and COX-2 Up-Regulation and ERK Activation

ERK and c-Myc are important mitogenic signals that regulate proliferation [28, 29]. COX-2 is the rate-limiting enzyme for prostanooid biosynthesis and is a downstream effector of ERK [30]. COX-2 in vitro has been shown to inhibit apoptosis and increase cell invasiveness, while in vivo COX-2 is up-regulated in ulcerative colitis and colon cancer. Furthermore the biosynthetic product of COX-2, PGE2 stimulates proliferation and angiogenesis in colonic carcinogenesis [5]. Shown in Fig. 6 are Western blots of colonic lysates from control, AOM/DSS alone, and AOM/DSS + Ro26-2198-treated mice at 8 weeks. Compared to control, AOM/DSS treatment up-regulated c-Myc and COX-2 expression and increased phospho-(active) ERK. In contrast to AOM/DSS treatment alone, Ro26-2198 inhibited these increases, suggesting that this analogue possesses anti-inflammatory and antiproliferative effects in the AOM/DSS model (Fig. 6).

Ro26-2198 Inhibits HCA-7 Cell Proliferation and IL-1β-Induced ERK Activation and COX-2 Up-Regulation

We next assessed the effects of Ro26-2198 on inflammatory signals in HCA-7 colon cancer cells. HCA-7 cells were stimulated with pro-inflammatory IL-1β. HCA-7 cells were pretreated with 50 and 100 nM Ro26-2198.

FIG. 3. Ro26-2198 increased weight loss during DSS treatment. Ro26-2198 caused greater weight loss at the peak of inflammation, compared to the group receiving AOM/DSS alone. Weight loss score: 0: None; 1: 1–5%; 2: 5–10%; 3: 10–20%; 4: >20% [7].

FIG. 4. Ro26-2198 inhibited proliferation in the AOM/DSS model in the regenerative phase. Ki-67 immunohistochemistry. Left panel: Control. Middle panel: AOM/DSS alone. Note the short crypts in the regenerating mucosa. Right panel: AOM/DSS plus Ro26-2198. Note the lower Ki-67 staining in the colonic crypts of animals treated with AOM/DSS plus Ro26-2198 compared with AOM/DSS alone. (n = two animals in each group.)

FIG. 5. Ro26-2198 inhibited the development of dysplasia in the premalignant phase of the AOM/DSS model. H&E sections. Left panel: Control. Middle panel: AOM/DSS alone. Right panel: AOM/DSS plus Ro26-2198. Note the dysplastic foci of crypt epithelial cells (white arrowheads) in AOM/DSS alone compared with AOM/DSS plus Ro26-2198. (n = two animals in each group.)
2198 or vehicle followed by IL-1β (10 ng/mL) or vehicle. As expected, IL-1β activated ERK and up-regulated COX-2 in these cells (Fig. 7). ERK activation occurred within 15 min of IL-1β treatment, while COX-2 levels were increased by 1 h. Similar to our in vivo observations, Ro26-2198 inhibited IL-1β-induced ERK activation and COX-2 up-regulation in vitro. Ro26-2198 also inhibited HCA-7 cell proliferation (Fig. 7).

**DISCUSSION**

In this study we have investigated the anti-inflammatory, antiproliferative, and chemopreventive effects of vitamin D analogue, Ro26-2198, in a mouse model of colitis-associated colon cancer. We found that Ro26-2198 decreased up-regulation of growth-promoting c-Myc, pro-inflammatory COX-2, and inhibited ERK activation in the premalignant phase. This vitamin D analogue also decreased the incidence of dysplasia in the premalignant phase of the AOM/DSS model. It should be noted that the major limiting clinical toxicity of 1,25(OH)2D3 is hypercalcemia and that serum calcium levels were normal in these animals at the time of sacrifice. In separate in vitro studies using HCA-7 colon cancer cells, we showed this analogue blocked ERK activation and c-Myc and COX-2 up-regulation induced by pro-inflammatory IL-1β. IL-1β treatment results in the activation of nuclear factor-kappa B, by signaling the dissociation and degradation of IκBα [31]. Active nuclear factor-kappa B is then translocated to the nucleus where it binds to target genes including COX-2 [32] and other proliferative and inflammatory mediators [33]. Vitamin D analogues through the VDR are known to suppress NFκB signaling by increasing IκBα, the major inhibitor of NFκB, and by directly binding NFκB and thereby inhibiting its function [23, 24]. Ro26-2198 also inhibited HCA-7 cell proliferation. Our in vitro and in vivo results are in agreement with recent studies showing that active vitamin D3 and VDR inhibited inflammation in immune models of colitis [34, 35].

Although several studies have suggested that vitamin D, or one or more of its metabolites, may inhibit inflammation and prevent colon cancer, there is considerable concern about their potential toxicity, particularly with respect to hypercalcemia and its consequences, such as deposition of this mineral in soft tissues. The practical use of these seco steroids for prevention of various cancers will depend on the development of synthetic analogues that do not elevate serum calcium levels. In this regard, a number of analogues have recently been synthesized that are markedly less calcemic, yet have sufficient affinity for the VDR. One of these compounds, Ro26-2198, has significantly lower tendency (efficacy) to raise calcium than 1,25(OH)2D3, which may account, at least in part, for these observed differences.

Patients with IBD have a higher risk for the development of intestinal neoplasms compared to the general population. Cancer risk correlates with the duration, extent, and severity of disease and is similar in Crohn’s disease and ulcerative colitis [36]. Recurrent inflammation, increased mutagenic reactive oxygen, and nitrogen intermediates and accompanying secondary regeneration are believed to predispose to malignant transformation in ulcerative colitis [1, 2]. At a molecular level, up-regulation of COX-2 appears to play a key role linking inflammation to cellular transformation [37]. Up-regulation of COX-2 increases β-catenin and EGFR signals that in turn induce c-Myc and ERK to promote cell-cycle progression and inhibit apoptosis [28, 29]. In our study we demonstrated that Ro26-2198 delayed the initial clinical manifestations of colitis and in-
hibited inflammatory and proliferative signals and progression to dysplasia in the premalignant phase. In the DSS model there is a well-characterized sequence of changes beginning with loss of mucosal permeability, arrest of epithelial crypt cell proliferation, and eventual ulceration followed by inflammation. After DSS withdrawal, these events are followed by epithelial restitution accompanied by cell migration and proliferation. With the addition of AOM to this model, there is accelerated malignant progression. In accordance with previous studies demonstrating antiproliferative effects of vitamin D and its analogues in vitro and in vivo [12, 13, 38], we have shown that Ro26-2198 inhibited proliferation in this model as assessed by Ki-67 immunostaining during the regenerative phase in vivo. This effect of the analogue was also mimicked in vitro in HCA-7 colon cancer cells. The antiproliferative effects of this vitamin D analogue might have contributed to the increased weight loss and mortality observed in the group receiving Ro26-2198 during the acute phase by slowing mucosal repair. Although the onset of colitis is delayed by this vitamin D analogue, once ulceration has occurred, the antiproliferative effects of this vitamin D analogue might paradoxically inhibit mucosal restitution and thereby increase disease severity. Future studies will be necessary to identify an optimal dose of Ro26-2198 that inhibits inflammation and prevents neoplastic progression, without compromising mucosal repair.

In the original description by Tanaka et al., after a single intraperitoneal injection of AOM and a 1-week exposure to 2% DSS in the water, 100% of animals harbored colonic neoplasms by 20 weeks [11]. In our study, animals were sacrificed after 8 weeks to characterize premalignant events. Multiple dysplastic lesions were noted in the AOM/DSS group, but none were identified in the AOM/DSS group receiving Ro26-2198. Ulcerative colitis associated dysplasia is a prognostic marker for progression to cancer [39]. Hence, the ability of Ro26-2198 to inhibit dysplasia in the AOM/DSS model suggests this secosteroid might be efficacious in preventing colitis-associated colon cancer in chronic IBD. Future studies will explore dose optimization of Ro26-2198 to reduce acute morbidity in the active colitis phase and to assess the chemopreventive efficacy of this analogue in the malignant phase of this model.

FIG. 7. Ro26-2198 inhibited IL-1β-induced ERK activation and COX-2 induction and HCA-7 colon cancer cell proliferation. Upper panel: HCA-7 human colon cancer cells were treated with Ro26-2198 (100 nM) or vehicle followed by IL-1β (10 ng/mL) or vehicle. Cells were lysed and proteins were separated by SDS-PAGE. Lysate proteins were probed by Western blotting for phospho-(active) ERK and COX-2. Ro26-2198 reduced ERK activation and COX-2 up-regulation by IL-1β. Lower panel: HCA-7 cells were treated with Ro26-2198 (50–100 nM) or vehicle and proliferation was measured by WST-1 assay. Ro26-2198 inhibited HCA-7 cell proliferation. *P < 0.05 compared to control.

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