Increased Dietary Vitamin D Suppresses MAPK Signaling, Colitis, and Colon Cancer

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Abstract

Epidemiologic studies associate low serum vitamin D levels with an increased risk of colon cancer and inflammatory diseases such as inflammatory bowel disease (IBD). 129-Smad³⁺/−Par¹ (Smad³⁻⁻) mice are a model of bacteria-driven colitis and colon cancer when infected with Helicobacter bilis (H. bilis). Thus, we used this mouse model to determine whether increased dietary vitamin D would reduce inflammation and colon cancer. Smad³⁻⁻ mice were fed purified diet with either maintenance (1 IU vitamin D/g diet; maintenance) or increased concentrations of vitamin D (5 IU vitamin D/g diet; high vitamin D). One week after diet initiation, mice were inoculated with broth or H. bilis and were necropsied at several time points postinoculation to assess inflammation, dysplasia, and neoplasia incidence. At 16 weeks postinfection, 11% of mice fed high vitamin D diet had cancer compared with 41% of mice fed maintenance diet (P = 0.0121). Evaluation at an early time point (1 week postinfection) showed that animals fed high vitamin D had decreased MAPK (p-P38 and p-JNK) activation in lamina propria leukocytes as well as decreased NFκB activation in colonic epithelial cells. Reduction in MAPK and NFκB activation correlated with decreased IBD scores (2.7 vs. 15.5; P < 0.0001) as well as decreased inflammatory cell infiltrates and reduced expression of proinflammatory cytokines in cecal tissue. These findings suggest that increased dietary vitamin D is beneficial in preventing inflammation-associated colon cancer through suppression of inflammatory responses during initiation of neoplasia or early-stage carcinogenesis. Cancer Res; 74(16): 4398–408. ©2014 AACR.

Introduction

Colorectal cancer is the third most commonly diagnosed cancer in both men and women (1). Studies indicate that diet and lifestyle choices play a significant role in the development of and prognosis of colon cancer (2). Vitamin D status of an individual is also influenced by both diet and lifestyle (3). The link between vitamin D and colon cancer was first suggested by Garland and colleagues who observed that populations residing in the northeastern United States had an increased incidence of colon cancer–related mortality compared with those living in the southern United States (4). Since then, adequate serum vitamin D levels have been associated with decreased incidence of colon cancer and decreased mortality in patients diagnosed with colon cancer (5, 6). Adequate serum vitamin D levels also correlate with reduced risk for developing inflammatory diseases, such as Crohn’s disease, a risk factor for colon cancer (3, 7).

The risk of colon cancer is increased in patients diagnosed with IBD (Crohn’s disease or ulcerative colitis) compared with the general population, supporting the notion that colonic inflammation affects cancer development (8). The chronic inflammation seen in IBD, likely due in part to dysregulated mucosal immune responses to enteric antigens (8), is believed to progress to cancer through the promotion of angiogenesis, tumor-promoting cytokine production, tumor cell–invasive behavior and cellular proliferation (9).

Vitamin D has a protective effect against colon cancer in various mouse models (10, 11). However, these cancer models, such as ApcMin+/- mice or mice given a chemical mutagen such as azoxymethane (AOM), are not driven by inflammation (12–15). Currently, the protective effect of vitamin D on inflammation-associated colon cancer is not known.

To investigate the potential chemopreventive effects of elevated dietary vitamin D on inflammation-associated colon cancer, we utilized Smad³⁻⁻ (Smad³⁺/−Par¹) mice, which have defective TGFβ signaling due to the absence of the transcription factor Smad3 (16). In humans, the TGFβ signaling pathway is commonly mutated in colon cancer, including colitis-associated colorectal cancer (17). After being infected with an enteric microorganism, Helicobacter bilis (16), Smad³⁻⁻ mice develop transient colitis followed months later by colon cancer making them a useful model for studying inflammation-associated colon cancer. Using this model, we demonstrate that elevated...
dietary vitamin D increases serum vitamin D and protects _H. bilis_ (HB)-infected _Smad3^−/−_ mice from developing colon cancer. These protective effects are mediated through the interactions between vitamin D and proinflammatory signaling pathways during the early stages of disease development.

**Materials and Methods**

**Mice and diets**

Study mice were colony-bred 129-Smad3<sup>−/−</sup>Par/C0 (Smad3<sup>−/−</sup>) mice (age 6–14 weeks) housed in a specific pathogen-free facility. Mice were screened for rodent pathogens as previously described (18) except that sentinel were collected three times yearly rather than quarterly. In addition to referenced pathogens, annual screens were performed for minute virus of mice, lymphocytic choriomeningitis virus, and ectromelia virus. Mice were maintained in a _Helicobacter_ and Mouse Norovirus-free colony as previously described (19). The mice were group housed in ventilated cages and fed a purified, irradiated diet with 1 IU vitamin D (5SRH, maintenance), 5 IU vitamin D (5AV4, AIN93Null). All diets were manufactured by PMI Nutrition International (LabDiet/TestDiet) based on AIN93M diet that is AIN93Null diet, high vitamin D), or 0 IU vitamin D per gram (5AV4, AIN93Null). All diets were manufactured by PMI Nutrition International (LabDiet/TestDiet) based on AIN93M diet that is formulated for the maintenance of rodents’ health. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

**Experimental design**

While no age-associated alterations in endpoint disease have been noted in our experience with _Smad3^−/−_ mice, care was taken to evenly distribute mice across treatment groups with regard to age and sex. Independent studies were performed to evaluate the effects of high and low dietary vitamin D levels on cancer. For high vitamin D studies, mice were started on either maintenance or high vitamin D diets one week before infection. For vitamin D–deficient studies, maintenance or AIN93Null diets were initiated two weeks before infection (Supplementary Fig. S1). For each diet, mice were infected with either approximately 2 × 10<sup>6</sup> CFU HB in Brucella broth or Brucella broth alone (controls) by oral gavage as previously described (Supplementary Fig. S1; ref. 16). _Helicobacter_ infection status was monitored by fecal PCR using previously published primer sequences (18). Fecal samples were collected at 3, 6, and 14 days postinfection for subjective fecal scoring and at 7 days postinfection for fecal cytokine analysis. Mice were weighed weekly and monitored at least three times weekly for dehydration, diarrhea, lethargy, or weight loss. Animals were euthanized by CO<sub>2</sub> asphyxiation at the designated end points.

**Serum vitamin D and calcium determination and tissue collection**

Following euthanasia, blood was obtained via cardiac puncture. Serum samples were submitted to Heartland Assays for quantification of 25-hydroxyvitamin D (radioimmunoassay) and calcium levels. Mesenteric lymph nodes, cecum, colon, and rectum were fixed in 10% phosphate-buffered formalin and processed for routine histologic examination. For study of the early inflammatory phase, cross-sections of proximal colon (5 mm) were prepared for immunohistochemistry (IHC) and histology, and cross-sections of proximal colon (5 mm) and cecum (3 mm) were stored in RNA later (Qiagen) for cytokine analysis by quantitative real-time PCR (qRT)-PCR. For evaluation of HB colonization, 3 cm sections of mid-jejunum, proximal colon, distal colon, and whole cecum were harvested, rinsed gently in sterile PBS to remove fecal material, and stored at −20°C until DNA extraction. For fecal cytokine analysis, individual fecal samples were collected, homogenized in 250 µL RNAlater stabilization solution (Qiagen), and stored at −80°C until RNA extraction. At the time of collection, a subjective fecal score was assigned to each animal, ranking presence of diarrhea and blood in the stool as a clinical measure of IBD (20).

**Histopathology and immunohistochemistry**

Whole colon and cecum were evaluated by a board-certified veterinary pathologist (P.M. Treuting) blinded to experimental groups to assess the severity of colitis and incidence of neoplasia. An overall IBD score was determined as described (21) with the exceptions that scores were summed from cecum, proximal, mid, and distal colons and none were weighed. Analysis of the rectum was included with the distal colon. IBD scores incorporate the severity of mucosal epithelial changes, degree of inflammation, and extent of lesions. A dysplasia score was also generated by determining the degree of dysplasia present in each of four segments as described (22): cecum, proximal colon, mid colon, and distal colon. For each segment, a score ranging from 0–4 was assigned: 0, none; 1, indefinite; 2, low grade; 3, high grade; and 4, high grade with frank invasion beyond tunica muscularis and distinguished from mucosal herniation. Cancers were classified as adenocarcinomas and mucinous adenocarcinomas (16, 22). The four individual segment scores were summed to generate the overall dysplasia score for each animal.

For study of the early inflammatory phase, colonic expression of CD3, F4/80, cleaved caspase-3, and MHCIId were evaluated on transverse cross-sections of proximal colon in animals euthanized 1 week postinfection. IHC staining within the mucosa, excluding any gastrointestinal lymphoid tissue, was scored by a pathologist blinded to groups (P.M. Treuting), using a range of 0–4: 0, no positive cells; 1, few single positive cells; 2, few (3 or less) scattered small clusters of positive cells; 3, many (>3) small clusters of positive cells or larger clusters of positive cells; and 4, large or coalescing clusters of positive cells. For early time points, in addition to IHC staining, cecum and serial transverse cross-sections of proximal colon were evaluated histologically for evidence of IBD.

All IHC staining was performed by Experimental Histopathology Services at Fred Hutchinson Cancer Research Center (Seattle, WA). Rat monoclonal antibodies were used to detect MHC class II (1-A/1-E, BD Pharmaningen), CD3 (MCA1477, Serotec), and F4/80 (MCA497, Serotec). Signals were detected using biotinylated goat anti-rat (Jackson ImmunoResearch) followed by streptavidin HRP (Jackson ImmunoResearch). Cleaved caspase-3 antibody (Biocare Medical CP229B) was followed by Mach 2 anti-rabbit HRP-labeled polymer (Biocare...
Medical RHPR520L). Staining was visualized with 3,3′-diaminobenzidine (Dako) and counterstained with hematoxylin (Dako). Concentration-matched isotype control slides were run for each tissue sample (Jackson ImmunoResearch).

Cytokine analysis and Helicobacter quantification by quantitative real-time PCR analysis

For cytokine analysis, RNA was extracted from cecum, colon, and feces using the RNeasy Kit (Qiagen). Fecal RNA samples were further concentrated using the Qiagen mini-elution/clean-up kit. RNA was converted to cDNA using SuperScript First Strand Synthesis System (Invitrogen) followed by qRT-PCR using Power Sybr Green Master Mix (Applied Biosystems) and a Stratagene Mx3005P analyzer (Agilent Technologies). Samples were run in duplicate. Cytokine levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and were run in duplicate. Cytokine levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and were run in duplicate. Cytokine levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and were run in duplicate.

Western blotting

Protein was extracted by homogenizing cell pellets in RIPA buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Western blot analysis was performed according to a previously published protocol (19) with the following modifications. Proteins (40 μg/lane) were run on gradient (4%–15%) Tris-HCl polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes using 100 mmol/L N-cyclohexyl-3-amino-propanesulfonic acid buffer with 10% methanol. Primary antibodies used were vitamin D receptor (VDR, SC-1008; Santa Cruz Biotechnology), NFκB signaling (Phospho-NF-κB p65 (Ser536), and 1kBα, L33A5; Cell Signaling Technology), MAPK signaling (Phospho-MAPK Family, 9910, and MAPK Family, 9926, Antibody sampler kits; Cell Signaling Technology), Bcl-xl (5H16; Cell Signaling Technology), and proliferating cell nuclear antigen (PCNA, PC10; Cell Signaling Technology). β-Actin (A5441; Sigma-Aldrich) was used as a loading control.

Statistical analysis

Before statistical analysis, distribution of data was assessed for normality. If data were not normally distributed, transformation was attempted; if transformation did not normalize the distribution, nonparametric tests were performed. Serum vitamin D, serum calcium, histologic scoring, fecal scoring, and densitometries were analyzed using either an unpaired or Mann–Whitney t test, qRT-PCR data were analyzed using the Krukal-Wallis nonparametric test followed by the Dunn post hoc test to adjust for multiple comparisons. Cancer and dysplasia incidence significance was determined by the Fisher exact test. All data are presented as mean ± SEM. Differences with a P value of 0.05 or less were considered significant. All statistical analyses were performed using GraphPad Prism software (Version 5.04, GraphPad Software Inc).

Results

Increased dietary vitamin D significantly increases serum 25-hydroxyvitamin D without altering serum calcium levels

To determine whether high dietary vitamin D increases serum vitamin D status without causing toxicity in Smad3−/− mice, we measured serum 25-hydroxyvitamin D and serum calcium in mice fed high vitamin D diet or maintenance diet (control) for one week. The high vitamin D diet significantly increased serum 25-hydroxyvitamin D levels without altering serum calcium (25-hydroxyvitamin D mean: 37.6 vs. 17.6 ng/mL, P = 0.016; calcium mean: 9.6 vs. 10.6 mg/dL, P = 0.1), demonstrating that the dietary regimen rapidly elevated serum vitamin D levels without causing hypercalcemia. Similarly, after 16 weeks on diet, Smad3−/− mice fed high vitamin D diet had serum vitamin D levels that were roughly double that of mice fed maintenance diet (mean: 38.4 vs. 14.4 ng/mL, P < 0.0001; Fig. 1A) while serum calcium levels remained unchanged (Fig. 1B).

Treatment with increased dietary vitamin D significantly reduces cancer incidence in Hb-infected Smad3−/− mice

Hb-infected mice fed increased dietary vitamin D had a significantly reduced incidence of invasive colon cancer compared with Hb-infected mice fed maintenance diet (11% vs. 41%, P = 0.0121; Fig. 1C). No neoplastic lesions developed in uninfected mice on either diet (Fig. 1C). Mucinous adenocarcinomas located in the proximal colon were the primary neoplasm diagnosed, as previously noted in this model (Fig. 1D; ref. 16). Well-differentiated mucinous adenocarcinomas were characterized by expansile mucin-filled, epithelial-lined cysts that disrupt the muscular tunics, serosa and expand into mesentery (Fig. 1D). Consistent with the decreased incidence of invasive adenocarcinoma, Hb-infected mice fed high vitamin D diet had an average 4-fold decrease in dysplasia scores compared with mice fed maintenance diet (mean: 0.71 vs. 2.85, P < 0.001; score range 0–16; Fig. 1E) with a significantly higher percentage of animals with no evidence of dysplasia (83% vs. 40%, P = 0.0005). Dysplasia was primarily observed in the cecum and proximal colon. Minimal dysplasia was observed in the mid colon of mice fed maintenance diet but not in high vitamin D fed mice. No dysplasia was observed in the distal colon regardless of diet.

Clinical disease and colonic inflammatory cell infiltrates are reduced during the inflammatory phase in Hb-infected Smad3−/− mice fed increased dietary vitamin D

Smad3−/− mice develop acute inflammation approximately 3 to 7 days after Hb infection, which is characterized by diarrhea, frank blood in the stool, dehydration, lethargy, and loss of body...
condition. Clinical signs typically resolve within 7 to 14 days until the time that cancers develop (unpublished observations; ref. 16). To determine the effects of elevated dietary vitamin D on early disease stages, we assessed clinical disease parameters and alterations in inflammatory infiltrates in the colons of H. bilis-infected mice fed high vitamin D diet compared with H. bilis-infected mice fed maintenance diet. During the initial inflammatory period, mice were assigned a subjective fecal score to assess clinical evidence of IBD. Animals fed high vitamin D diet had significantly decreased fecal scores at both 3 and 6 days postinfection (mean 0.1 vs. 0.8, P = 0.0015 and 0.2 vs. 1.0 P = 0.0003, respectively; Supplementary Fig. S2A and S2B) compared with animals fed maintenance diet. Broth-treated animals showed no evidence of diarrhea, as expected, and by 14 days postinfection all animals had minimal evidence of diarrhea regardless of diet (Supplementary Fig. S2C). There was no significant difference in body weight change associated with diet following H. bilis infection (data not shown).

To determine whether improved clinical signs associated with increased dietary vitamin D correlate with decreased colonic inflammation during early disease, proximal colon, and cecum of mice fed high vitamin D or maintenance diet were analyzed for inflammation one week after H. bilis infection. H. bilis-infected animals fed high vitamin D diet had significantly reduced colitis compared with infected animals fed maintenance diet (mean 2.7 vs. 15.5, P < 0.0001; Fig. 2 and Supplementary Fig. S3A). Immunohistochemical studies further demonstrated that infected mice fed high vitamin D diet had decreased inflammatory infiltrates compared with those fed maintenance diet [F4/80+ cells (mean ± SEM: 2.7 ± 0.19 vs. 3.7 ± 0.11, P = 0.0003), CD3+ T cells (mean ± SEM: 2.1 ± 0.15 vs. 2.6 ± 0.17, P = 0.04), and MHC II+ cells (mean ± SEM: 2.05 ± 0.20 vs. 2.85 ± 0.13, P = 0.001); Fig. 2 and Supplementary Fig. S3B–S3D]. Broth-treated controls had minimal inflammation regardless of diet.

Because inflammation is often associated with increased cell proliferation, we evaluated if increased dietary vitamin D was associated with changes in cell proliferation in either lamina propria or epithelial cell populations within the colon one week after H. bilis treatment. PCNA was used as a marker of cellular proliferation by Western blot analysis. Analysis of PCNA

Figure 1. Increased dietary vitamin D increases serum vitamin D and decreases dysplasia and cancer in H. bilis-infected Smad3−/− mice. Serum 25-OH Vitamin D (A) and serum calcium (B) were measured 16 weeks after H. bilis infection in H. bilis-infected/maintenance (n = 13) vs. high vitamin D (n = 13) diet. ***, P < 0.0001; Mann-Whitney U test. C–E, colon and cecum were analyzed at 16 weeks after H. bilis infection for histopathologic evidence of invasive adenocarcinoma and dysplasia. C, cancer incidence is reduced in mice fed high vitamin D diet (*, P = 0.0121; Fisher exact test). D, H. bilis-infected mice typically develop grossly visible tumors in the cecum or proximal colon as represented by the pale, multilobulated mass in the proximal colon (*). Note the mucin lakes and neoplastic epithelial cells penetrating the colonic wall and proliferating within the muscularis and serosa. H&E staining; original magnification, ×20. Inset, subgross of the whole colon section; blue box, magnified region. E, H. bilis-infected mice fed high vitamin D diet had decreased mean dysplasia scores and an increased number of animals with no evidence of dysplasia. *, P < 0.01; **, P < 0.001.
showed no significant differences associated with diet in either cell population (LPL mean 0.73 vs. 0.67, epithelial cell mean 0.99 vs. 0.85, high vitamin D diet vs. maintenance diet respectively, data not shown).

**High dietary vitamin D decreases cecal, proximal colon, and fecal proinflammatory cytokines 1 week after H. bilis infection**

Because we previously observed elevated inflammatory cytokines in this model associated with H. bilis infection (16), proinflammatory cytokine expression in cecal and proximal colon tissues one week after H. bilis infection were evaluated to determine whether increased vitamin D would dampen the inflammatory response induced by infection. Increased expression of IL1β, macrophage chemotaxis factor 1α (Mip1α), IL6, Tnfα, and Ifnγ were noted in cecal tissue from H. bilis-infected animals fed maintenance diet compared with broth-treated controls (Fig. 3) as seen previously with this model (16). However, there was a significant reduction in expression of those same cytokines in H. bilis-infected mice fed increased dietary vitamin D compared with those fed maintenance diet (Fig. 3A–E). Interestingly, there were no changes in expression of the anti-inflammatory cytokine, IL10 in response to diet or H. bilis infection (Fig. 3F). Expression patterns of proinflammatory cytokines in the proximal colon were similar to those observed in cecal tissue (Supplementary Fig. S4A–S4F).

Fecal cytokines have been used in the H. bilis-infected Smad3−/− mouse model to characterize the inflammatory response and predict development of cancers (24). To determine whether fecal cytokine expression correlated with tissue cytokine expression, expression of IL1β and Mip1α was evaluated in fecal pellets collected from animals 1, 2, and 3 weeks after H. bilis infection. Similar to the expression pattern of IL1β and Mip1α observed in cecal and proximal colon tissues at 1 week after infection (Fig. 3A and B and Supplementary Fig. S4A and S4B), expression of IL1β and Mip1α in feces were significantly increased in H. bilis-infected animals fed maintenance diet compared with broth-treated animals (Supplementary Fig. S4G and S4H). Accordingly, H. bilis-infected mice fed high vitamin D diet had an average 3-fold decrease in fecal IL1β and 1.5-fold decrease in fecal Mip1α expression compared with mice fed the maintenance diet. However, these fecal cytokine changes were transient as there were no significant differences in IL1β and Mip1α expression between any treatment group at 2 or 3 weeks after infection (data not shown).

**Increased dietary vitamin D decreases p-P38 MAPK in the colon**

*Helicobacter* species have been shown to elicit proinflammatory cytokine production through TLR4-dependent activation of the MAPK and NFκB pathways (25). Thus, we determined whether decreased colonic inflammation in Smad3−/− mice in response to increased dietary vitamin D was associated with altered MAPK and NFκB signaling pathways during early disease (1 week after H. bilis infection).
For changes in the MAPK pathway, relative levels of protein expression of activated forms of P38 (p-P38), JNK (p-JNK), and ERK1/2 (p-ERK) in LPL and colonic epithelial cell populations were determined. LPLs of Hb-infected animals fed increased dietary vitamin D had a 7-fold decrease in p-P38 compared with maintenance diet-fed animals ($P = 0.012$, Fig. 4A and Supplementary Fig. S5). Interestingly, similar changes were not detected in colonic epithelial cells (Fig. 4A and Supplementary Fig. S5). There was a trend toward decreased p-JNK expression in colonic tissue from animals fed increased dietary vitamin D; however, these differences were not statistically significant (LPL: 4.5-fold decrease, $P = 0.14$ and epithelial cell: 2-fold decrease, $P = 0.052$; Fig. 4B and Supplementary Fig. S5). There were no notable differences in p-ERK1/2, total P38, or total JNK in either cell population (data not shown).

For alterations in the NFκB pathway, phosphorylated P65 (p-P65) was evaluated by Western blot analysis in LPL and epithelial cell populations. Mice fed high vitamin D diet had a 4.5-fold decrease in p-P65 in colonic epithelial cells compared with mice fed maintenance diet ($P = 0.028$, Fig. 4C and Supplementary Fig. S5). We did not detect p-P65 in LPL regardless of diet while 1kBz, an inhibitor of NFκB activation,
was present in both LPL and colonic epithelial cells. (Supplementary Fig. S5).

To determine whether the changes in p-P38 or p-P65 were associated with decreased Tlr4 expression, qRT-PCR was performed on cecal tissues collected from mice 1 week after Hb infection. Average Tlr4 expression in Hb-infected animals was modestly increased (1.3-fold) compared with broth-treated controls (P < 0.01; Fig. 4D). Although Tlr4 expression in Hb-infected mice was lower when fed high vitamin D diet, the difference was not statistically significant.

High dietary vitamin D does not alter Hb colonization in Smad3/−/− mice
Changes in the gut microbiome or changes in bacterial load can influence disease severity in both human patients (26) and animal models of IBD (26, 27). As Tlr4 expression levels tend to be lower in high vitamin D-fed Smad3/−/− mice following Hb infection, we determined whether vitamin D alters Hb colonization in cecum and colon where they preferentially reside (16). qRT-PCR was used to compare the relative amount of Helicobacter organisms in cecal, proximal, and distal colonic tissues collected 8 weeks after Hb infection from mice fed either increased or maintenance levels of vitamin D. While cecal tissues had the highest concentration of Helicobacter as previously reported (16), no significant differences in Helicobacter colonization were associated with diet (Supplementary Fig. S6).

High dietary vitamin D does not alter cecal expression of vitamin D receptor or enzymes involved in vitamin D metabolism
To determine whether the protective effect of increased dietary vitamin D was associated with changes in proteins involved in vitamin D signaling and/or metabolism, qRT-PCR was used to evaluate RNA expression of vitamin D receptor (Vdr) as well as two enzymes involved in conversion of vitamin D into its active and inactive forms, 25(OH)D3-1α-hydroxylase (Cyp27b1) and 1, 25(OH)2D3 24 hydroxylase (Cyp24a1) in cecal tissue at one week after Hb infection. A small yet significant increase in VDR expression was detected in mice fed high vitamin D diet following Hb infection (Fig. 5A). However, there were no changes in mRNA levels of Cyp27b1 and Cyp24a1 associated with either diet or infection status (Fig. 5B and C). Although Vdr mRNA was altered with diet, differences in VDR protein expression were not detected using Western blot analysis of proximal colon tissue (data not shown).
A vitamin D–deficient diet did not exacerbate colitis or colitis-associated colon cancer in Hb-injected Smad3−/− mice

Because decreased serum vitamin D is associated with an increased risk for developing IBD as well as colon cancer in humans (4–7), we hypothesized that a diet deficient in vitamin D would exacerbate inflammation and potentially increase the incidence of colon tumors in Hb-injected Smad3−/− mice. We tested this hypothesis by feeding mice maintenance diet or diet devoid of vitamin D (AIN93Null) and induced infection by Hb infection. Serum 25-hydroxyvitamin D levels were significantly decreased in AIN93Null-fed mice compared with mice fed maintenance diet (mean: 5.7 vs. 12.2 ng/mL, P = 0.01) after 2 weeks on the diet and were below the limit of detection at the end of the 16-week study (Fig. 6A and B). Despite decreased serum vitamin D levels, mice maintained on AIN93Null diet showed no differences in serum calcium compared with maintenance diet–fed controls (Fig. 6C). Clinical disease during the early inflammatory phase was assessed by monitoring subjective fecal scores and body weight change following Hb infection. Mice maintained on AIN93Null diet showed no differences in fecal scores or body weight change compared with maintenance diet–fed controls (data not shown). To determine whether decreased dietary vitamin D exacerbated IBD at early disease stages, proximal colon and cecal tissue of mice fed maintenance diet or AIN93Null were analyzed for inflammation one-week after Hb infection. There were no differences in IBD associated with diet (Fig. 6D). To further evaluate any effect of AIN93Null diet, proinflammatory cytokine expression in proximal colon tissues was measured to determine whether decreased dietary vitamin D would exacerbate the inflammatory response induced by Hb. In correlation with the lack of effect on IBD scores, no differences were noted in cytokine gene expression associated with diet (Supplementary Fig. S7). Consistent with the lack of altered inflammation early in disease, there were no differences in cancer incidence or dysplasia noted between AIN93Null-fed mice compared with maintenance diet–fed animals (Fig. 6E and F) when necropsied after 16 weeks of Hb infection.

Discussion

Using Smad3−/− mice, we have shown that increased dietary vitamin D affords protection against the development of colon cancer. In this model, we have demonstrated that increased dietary vitamin D (i) induces elevated serum 25-hydroxyvitamin D without causing hypercalcemia, (ii) significantly decreases inflammation, dysplasia, and tumor incidence following infection with Hb, and (iii) is associated with decreased p-P38 (MAPK) and p-P65 (NFκB) expression during the acute inflammatory stage of disease. These studies provide evidence that the protective effect(s) of elevated dietary vitamin D supplementation in a model of inflammation-associated colon cancer are mediated through suppression of the inflammatory responses triggered following infection with colitogenic bacteria.

Animals fed increased concentrations of dietary vitamin D demonstrated significant protection against inflammation and tumor formation. It should be noted that though serum vitamin D levels are increased in mice fed high vitamin D diet compared with those fed maintenance diet, levels remain within the comparable recommended range for humans without reaching superphysiologic levels (28). Many animal and human studies that have shown antitumor effects of vitamin D administer metabolically active 1,25(OH)2D3, which results in hypercalcemia and vitamin D toxicities (10, 12, 15). Our studies show that dietary vitamin D supplementation offers a way to improve vitamin D status and provide protection from inflammation-associated colon cancer while avoiding vitamin D toxicity.

Vitamin D supplementation decreases cancer incidence in several rodent models of colon cancer (12–15). These models rely on either genetic predisposition for the development of gastrointestinal neoplasias as is the case with APCmin/+ mice or

![Figure 5. Increased dietary vitamin D increases Vdr mRNA expression but does not alter enzymes involved in vitamin D metabolism (Cyp27b1 and Cyp24a1). Expression levels of Vdr (A), Cyp27b1 (B), and Cyp24a1 (C) were determined with qRT-PCR using RNA from cecum (3 mm piece) isolated from mice 1 week after Hb infection. Hb-infected/maintenance diet (n = 20), Hb-infected/high vitamin D diet (n = 20), broth-treated/maintenance diet (n = 10), and broth-treated/high vitamin D diet (n = 10). Kruskal–Wallis nonparametric test and Dunn post hoc test; **, P < 0.001.](image)
Increased dietary vitamin D is effective not only preventing inflammation and dysplasia, but subsequent invasive tumor formation as well.

Chronic inflammation is associated with increased production of proinflammatory cytokines, including TNFα, IL1β, and IL6, which contribute to carcinogenesis through influences on cell proliferation, apoptosis, differentiation, and angiogenesis (34). Dietary vitamin D supplementation significantly lowered inflammatory cytokines induced in response to Hb in Smad3−/− mice. In human patients with colon cancer, these proinflammatory cytokines are positively associated with increased cancer growth, higher neoplastic grade, and increased risk of mortality (34). Proinflammatory cytokines are also upregulated in patients with IBD, even before the onset and progression to dysplasia or neoplasia (35), and vitamin D supplementation has been linked to decreased circulating proinflammatory cytokines in patients with colorectal adenomas (36). Epidemiologic evidence suggests that treatments that limit inflammation may be beneficial in reducing the incidence of inflammation-associated colon cancer in high risk populations (37). Together, these data suggest that dietary vitamin D may be effective at decreasing the proinflammatory milieu in patients with...
IBD and serve as a useful adjunct treatment in certain populations. The mechanism by which vitamin D suppresses colon cancer in Smad3−/− mice is not completely clear, although our data suggest the anti-inflammatory effects of vitamin D are important. Helicobacter species are Gram negative, microaerophilic bacteria that can induce local production of proinflammatory cytokines and chemokines through TLR4 signaling and subsequent activation of the MAPK and NFκB pathways (25). Both of these pathways have been shown to be upregulated in human patients with IBD (38) and are thought to be important links between inflammation and cancer (38–40). In vitro evidence suggests that vitamin D is able to suppress MAPK activity and subsequent proinflammatory cytokine production through the upregulation of MAPK phosphatase-1 (41) and NFκB signaling through the upregulation of IκBα, an inhibitor of NFκB activation (42), or through decreased expression of the NFκB component RelB, which can lead to inhibition of dendritic cell differentiation and maturation (43). During the early inflammatory disease phase in Hb-infected Smad3−/− mice, vitamin D–high diet was associated with dramatic decreases in p-P38 in the lamina propria cells, decreased NFκB activation in epithelial cell populations, and suppressed proinflammatory cytokine expression compared with that observed in infected mice on maintenance diet. On the basis of these data, we propose a model where vitamin D suppresses inflammation by decreasing P38 MAPK activation in lamina propria cells, resulting in decreased proinflammatory cytokine production by those cells, which in turn decreases NFκB activation in colonic epithelial cells.

We have shown that while increased dietary vitamin D affords protection against the development of colon cancer, decreased dietary vitamin D was not sufficient to exacerbate disease in Smad3−/− mice. Because epidemiologic studies (5, 7, 30) as well as studies utilizing mouse models of colitis suggest that vitamin D deficiency or lack of vitamin D signaling can exacerbate IBD (44–46), we hypothesized that decreased dietary vitamin D would exacerbate disease in the Smad3−/− mouse model. Interestingly, although the AIN93Null diet significantly depleted circulating serum 25-hydroxyvitamin D levels, we did not see exacerbation of Hb-induced inflammation or subsequent inflammation-associated colon cancer. These findings are consistent with the idea that modulation of inflammation is likely responsible for the protection afforded by increased dietary vitamin D.

In conclusion, increased dietary vitamin D suppresses acute inflammation and consequently neoplastic development in a mouse model of bacterial-driven colon cancer. While additional studies are needed to elucidate the molecular mechanisms through which vitamin D and TGFβ interact to afford protection in this model, these findings suggest that vitamin D supplementation may prove useful in the treatment of IBD or potentially the prevention of inflammation-associated cancer by limiting inflammation early in disease development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S. Meeker, A. Seamons, J. Paik, P.M. Treuting, T. Brabb, W.M. Grady, L. Maggio-Price
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