**Research Article**

**Vitamin D Receptor and Retinoic X Receptor α Status and Vitamin D Insufficiency in Models of Murine Colitis**

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**Abstract**

The anti-inflammatory actions of vitamin D have long been recognized and its importance in modulating colon cancer and colitis development is becoming apparent. The vitamin D receptor (VDR) is down-regulated in human ulcerative colitis and colitis-associated cancer (CAC); however, its status in murine models of colitis has yet to be explored. Snail and Snail2, zinc-finger transcription factors regulated by inflammatory pathways and able to transcriptionally silence VDR, are upregulated in human Ulcerative Colitis and are associated with localized VDR silencing. To signal, VDR must heterodimerize with retinoic X receptor α (RXRα). If either VDR or RXRα are compromised, vitamin D cannot regulate inflammatory pathways. RXRα is downregulated in human colorectal cancer, yet its expression in human and murine colitis has yet to be investigated. To explore the importance of vitamin D and VDR in murine colitis, we used acute and chronic azoxymethane/dextran sulfate sodium models of murine colitis. VDR was downregulated early in the onset of colitis, whereas RXRα downregulation only occurred as colitis became chronic and developed into CAC. Receptor downregulation was associated with an early increase in the expression of the inflammatory markers, Snail and Snail2. The acute colitis model induced in combination with a vitamin D-deficient diet resulted in increased morbidity, receptor downregulation, inflammatory marker expression, and Snail and Snail2 upregulation. These experiments show the importance of vitamin D and VDR in modulating murine colitis development. Cancer Prev Res; 6(6); 585–93. ©2013 AACR.

**Introduction**

Vitamin D and its receptor are intimately involved in the regulation of inflammation (1–5) and have been implicated in the progression of inflammatory diseases, such as diabetes, cardiovascular disease, and cancer (6). This has been supported by *in vitro* data where vitamin D provided anti-tumor activity in numerous cell lines (7–9) and by epidemiologic evidence showing that reduced vitamin D intake or production increases cancer prevalence (10–15).

The role of vitamin D in the development of colitis, a disease state that can develop into colitis-associated cancer (CAC), has yet to be elucidated. Low-circulating 25(OH) vitamin D has been associated with an increased risk for colorectal cancer (CRC) (16–18), and as vitamin D supplementation has been proposed as a potential chemo-preventive tool for CRC (19), it follows that vitamin D may play a role in the development and prevention of colitis. The association between vitamin D and colitis is based on the observation that colitis incidence is proportional to the distance from the equator and thus, sunlight exposure and dermal vitamin D production (20). Vitamin D deficiency is common in patients with both Ulcerative Colitis and Crohn’s disease (21), but it has yet to be determined whether this deficiency is a cause or effect of colitis.

The importance of vitamin D in colitis development is illustrated by the observation that vitamin D receptor (VDR)−− mice, when challenged with a chemically induced colitis, exhibit increased mortality as compared with wild-type mice (22). It has been shown that VDR expression is downregulated in human Ulcerative Colitis (23), yet the mechanism behind its downregulation has yet to be elucidated. VDR can be silenced at the transcription level via the zinc-finger transcription factors Snail and Snail2 (24–26), which are upregulated or stabilized by inflammatory mediators (27–31). Snail and Snail2 expression are increased in ulcerated tissue of patients with Ulcerative Colitis and in CRC with expression corresponding to a localized downregulation in VDR (25, 32–34). It has yet to be investigated whether the upregulation of Snail and Snail2 and subsequent, localized decrease in VDR expression is a factor in human and murine colitis and CAC.

To study colitis and CAC, the dextran sulfate sodium (DSS) model, a well-accepted proxy for human Ulcerative Colitis can be used in combination with azoxymethane.

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(AOM), a carcinogen (35). Our study shows the importance of VDR and vitamin D in modulating colitis development and severity. By examining localization of VDR downregulation and Snail and Snail2 expression, a potential mechanism for VDR downregulation is suggested. Also, the role that dietary vitamin D has on colitis is exemplified by the severe colitis we observed in vitamin D–deficient mice.

Materials and Methods

Mice

Female, 6-week-old C57BL/6j mice weighing approximately 20 g were used in all experiments (Jackson Laboratories). Mice were cared for within Institutional Animal Care and Use Committee (IACUC) guidelines, and all procedures were approved by the Medical University of South Carolina (MUSC) IACUC. Mice were housed in groups of 5 at 22 to 24°C using a 12-hour light–12-hour dark cycle with lights on at 6:00 am. Animals were fed normal chow (Harlan Teklad Diet 2918) except for experiments using a deficient vitamin D diet. For animals receiving DSS, automatic water was removed from cages at 6 weeks of age or 3 weeks of age for the mice on the vitamin D-deficient diet and replaced with bottled water.

Induction of colitis

To induce colitis, DSS (MW 36,000–50,000D, MP Biomedical) and AOM (Sigma-Aldrich) were used. Thirty mice were divided into control and treatment groups for 4 different treatment modalities. For the acute AOM/DSS model, mice were allowed to acclimate for one week and were then injected intraperitoneally with 10 mg/kg AOM or saline (control) on day 8. The mice recovered for one week with water, and on day 15, the mice injected with AOM were given 2% DSS in water for 7 days and the mice injected with saline remained on normal water. The mice were sacrificed on the seventh day of the DSS/water treatment. For the CAC model, the same procedure was conducted with 2 additional DSS/water cycles. For the CAC model, 12 mg/kg of AOM was used with 4% DSS and this protocol is associated with a high degree of colon tumor development (36).

Study design

Fifteen animals per treatment group were used. Colons from 10 mice were used for RNA and protein extraction and the remaining 5 mice had their colons Swiss-rolled as described (37) for colitis scoring and immunohistochemistry (IHC).

Sacrifice and tissue harvesting

Mice were sacrificed via CO2 inhalation followed by cervical dislocation. Blood was removed via cardiac puncture, allowed to clot at room temperature for one hour, centrifuged for 15 minutes at 1,000 x g, and plasma was removed. The colon of each mouse was removed, measured, flushed with ice-cold PBS, flayed, and the mucosa was scraped and separated into 2 fractions. One fraction was flash frozen in liquid nitrogen and the other fraction was placed in RNAlater (Ambion) and then flash frozen in liquid nitrogen.

Vitamin D quantification

Plasma was transported to the laboratory of Dr. Bruce Hollis (Medical University of South Carolina, Charleston) for quantification of systemic 25(OH)-vitamin D via a 25 (OH)-vitamin D ELISA as described (38).

Colitis scoring

The colons from mice reserved for colitis scoring and IHC were removed, flushed with ice-cold PBS, flayed, and Swiss-rolled. Colons were fixed overnight in 70% ethanol and paraffin-embedded. Five-micrometer sections were cut and then stained for hematoxylin and eosin (H&E) or left unstained for IHC. H&E–stained slides were scored blindly by a MUSC pathologist on a scale from 0 to 4 as described (39). Briefly, grade 0 was normal colon tissue, grade 1 was mild focal ulceration, grade 2 was moderate multifocal ulceration, grade 3 was moderate to severe multifocal ulceration, and grade 4 was widespread ulceration.

Immunohistochemistry

Paraffin sections were rehydrated through xylens and graded ethanol. Cells were permeabilized with 0.5% Triton X-100 (USB Products) for 5 minutes at room temperature, rinsed, and then incubated with 3% hydrogen peroxide for 20 minutes. Slides were washed with PBS, boiled for 20 minutes in 10 mmol/L sodium citrate buffer (Vector Labs), rinsed with water to cool, and then placed in iced methanol for 10 minutes, and rinsed with PBS. Slides were serum blocked with the appropriate species Vectorstain ABC system (Vectastain) for one hour at room temperature. Slides were then incubated with the primary antibody (anti-VDR, -RXRα, -PCNA; Santa Cruz Biotechnology) -Snail, -Snail2 (Abcam), -COX-2 (Cayman Chemical), -iNOS (Calbiochem) diluted in water 1:50 to 1:2,000. The appropriate species secondary antibody and ABC reagent (Vectorstain) were added for 30 minutes each at room temperature with a PBS wash between solution incubation. DAB solution (Vectastain) was added for 3 minutes, slides were rinsed with PBS, and then counterstained with hematoxylin (Thermo Scientific) for 30 seconds or until adequate staining had occurred. Slides were dehydrated through graded ethanol and xylene and coverslipped using Cytosol 60 (Thermo Scientific). Specimens were visualized by a Zeiss Axiophot Microscope (Carl Zeiss AG) and pictures were taken with an Insight digital camera (Spot Imaging, Sterling Heights) at ×10 to ×20 magnification.

Protein extraction and immunoblotting

The colonic mucosa fraction not placed in RNAlater was homogenized in 500 μL of T-Per tissue protein extraction (Thermo Scientific) and 0.05% protease inhibitor cocktail via sonification. The homogenate was centrifuged at 10,000 x g for 5’ and supernatant was collected. Protein purity and concentration was quantified with a GE
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RNA extraction and real-time PCR

RNA was isolated with a miRNAeasy Kit (Qiagen) according to manufacturer’s instructions and each sample was resuspended in 40 μL RNAse-free water. RNA purity and concentration was quantified with a GE Nanovue. Reactions were set up in duplicate for each sample. PCR was conducted in a 25 μL reaction containing 12.5 μL of the 2× SYBR PCR reaction mix, 300 nmol/L of each primer, 0.5 μL of iScript reverse transcriptase, and 25 to 150 ng of RNA. Reaction protocol was as follows: initial incubations at 50°C for 10 minutes to allow for cDNA synthesis, reverse transcriptase inactivation at 95°C for 1 minute, and then 40 cycles of PCR cycling and detection with 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute. The melt curve was done at 55°C for one minute and 80 cycles of 0.5°C increments from 55°C to 95°C. Primers sequences were: RXRα (antisense), VDR (antisense), -RXRt (Millipore) and -GAPDH (Santa Cruz Biotechnology) as a loading control. A Student’s t test was carried out to determine statistical significance.

Vitamin D deficiency studies

For the vitamin D intervention experiment, female, C57BL/6J mice were fed a diet deficient in vitamin D (TD.89123; Harlan) beginning at 3 weeks of age. Thirty mice were placed on each diet, half of which would be induced to have colitis and the other half serving as diet controls. The diets were maintained for the duration of the experiment.

Statistical analysis

For the statistical analysis of immunoblotting and real-time PCR data, expression levels from the animals were averaged in the control or treated group. Treatment comparisons are as follows. For the acute and CAC models, the averages of treated and control mice were compared via a Student’s t test. For the vitamin D intervention trials, the averages of the treated and control mice on the same diet were compared via a Student’s t test. To carry out comparisons between the acute and CAC trials and the vitamin D-deficient diet and acute normal diet, a 2-way ANOVA with interactions was used to evaluate differences in treatment effects.

Results

Challenge with AOM/DSS results in acute colitis with receptor downregulation and expression of inflammatory markers

To study murine colitis, the AOM/DSS model was used. Mice were injected intraperitoneally with 10 mg/kg of AOM and after a week of recovery, were challenged with 2% DSS for 7 days. This resulted in a decrease in body weight that became significant at the third week (P = 0.02; Fig. 1A) with gross blood loss, an indicator of disease severity, apparent 2 days before sacrifice. Challenged mice exhibited a shortened colon of 57.6 ± 9.4 mm as compared with 67.8 ± 6.1 mm in control mice (P < 0.05). Challenged mice had a decreased systemic 25(OH)-vitamin D of 20.1 ± 5.1 ng/mL as compared with control mice with 36.5 ± 4.7 ng/mL (P < 0.005).

To analyze receptor expression, PCR results or immunoblots were quantified and the average expression levels were calculated for the treated and control groups. VDR mRNA (Fig. 1B) and protein (Fig. 1C) were downregulated in challenged mice, but RXRt mRNA and protein were not significantly decreased (Fig. 1B and C). COX-2 mRNA was upregulated in challenged mice (Fig. 1B). The expression of amphiregulin (Fig. 1B), a downstream vitamin D target, was not significantly downregulated in challenged mice.

Upon microscopic analysis, challenged mice showed a loss of crypts and ulcerations most prevalent in the distal colon. The average colitis score of challenged mice was 3.5. IHC indicated that the downregulation of VDR was confined to ulcerated tissue with normal adjacent tissue exhibiting nuclear VDR expression. RXRt protein was downregulated in ulcerated tissue. Localization with the receptor downregulation was expression of iNOS and COX-2, which were not evident in normal tissue or in
the proximal colons of treated mice. Proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, was present in ulcerated tissue and normal adjacent crypts, indicating cell turnover due to tissue damage (Fig. 1D).

Colitis-associated cancer results in further receptor downregulation and expression of inflammatory markers

To model CAC, an extended study of 13 weeks was conducted with an initial 12 mg/kg AOM injection and 3 cycles of 4% DSS and water. Challenged mice showed fluctuations in weight with decreases corresponding to DSS challenges (Fig. 2A). This model had a mortality rate between 10% and 40%. Challenged mice had a shortened colon of 52.5 ± 3.5 mm as compared with control mice with a colon length of 74.6 ± 6.8 mm (P < 0.005). Challenged mice had a decreased systemic 25(OH)-vitamin D of 14.7 ± 6.1 ng/mL as compared with control mice with 30.8 ± 9.6 ng/mL (P < 0.005), but this was not significantly decreased as compared with acute treated mice.

Real-time PCR showed that challenged mice had a down-regulation of VDR mRNA (Fig. 2B) and protein (Fig. 2C), but in this model, RXRa mRNA (Fig. 2C) was also downregulated. RXRa protein, although appearing to be downregulated in treated mice, did not demonstrate statistically different expression than control mice, likely due to the sample size. COX-2 mRNA was upregulated in CAC-challenged mice (Fig. 2B), and it was upregulated more significantly than in the acute model (P = 0.02). There was no difference in amphiregulin (Fig. 2B) expression between CAC-challenged and control mice.

In CAC-challenged mice, microscopic analysis showed phases of damage and regeneration with ulcerated areas and loss of crypts along with areas of abnormal appearing, branched crypts consistent with regeneration. Three out of 5 mice developed intramucosal adenocarcinomas. IHC showed a downregulation of VDR and RXRa in ulcerated and dysplastic tissue. Normal adjacent tissue in CAC-challenged mice and control mice exhibited nuclear VDR and RXRa. Dysplastic tissue and nearby normal tissue exhibited a high degree of iNOS and
COX-2 expression with no staining evident in the proximal colon. PCNA was present in ulcerated tissue, dysplastic tissue, normal adjacent crypts, and in control mice (Fig. 2D).

Snail and Snail2 expression in acute colitis and CAC
To investigate a possible mechanism through which VDR was being silenced, the expression of Snail and Snail2 and their upstream regulator, TNFα, were investigated. TNFα and Snail mRNA were significantly upregulated in acute colitis with Snail2 just short of significance. In the CAC model, the upregulation of TNFα was still present, but Snail2 and Snail were no longer upregulated (Fig. 3A). Snail and Snail2 were evident in ulcerated areas or dysplastic tissue in the acute and CAC models, thus, in the same locations where RXRa and VDR were downregulated. No Snail or Snail2 expression was in normal adjacent tissue or in control mice (Fig. 3B).

The effect of vitamin D deficiency on colitis development and nuclear receptor expression
To study the effects of vitamin D deficiency on the development and progression of acute AOM/DSS colitis, mice were started on a vitamin D–deficient diet at 3 weeks of age and the diet was continued for the duration of the experiment. Acute AOM/DSS colitis was introduced after 3 weeks on the diet at 6 weeks of age. Treated mice exhibited a decreased body weight that became significant at the third week \((P < 0.005; \text{ref. Fig. 4A})\) and gross blood loss 4 days before sacrifice. Mice on a vitamin D-deficient diet had mortality rate between 7% and 10%. A shortened colon of 44.1 ± 4.6 mm in challenged mice was found as compared with 63.3 ± 3.8 mm in control mice \((P < 0.005)\). This decrease in colon length was greater than the decrease observed in acute treated mice on normal chow \((P = 0.02)\). AOM/DSS-treated mice had a systemic 25(OH)-vitamin D of 4.9 ± 4.1 ng/mL as compared with saline/water...
control mice on the vitamin D-deficient diet that evidenced an average systemic vitamin D level of 2.6 ± 2.1 ng/mL, a difference that was not statistically significant. This can be contrasted to mice fed normal chow where AOM/DSS-treated mice had a decreased systemic 25(OH)-vitamin D of 20.1 ± 5.1 ng/mL as compared with saline/water control mice with 36.5 ± 4.7 ng/mL (P < 0.005). Thus, the vitamin D-deficient diet reduced systemic vitamin D levels in both AOM/DSS-treated and saline/water control mice; DSS treatment also reduced circulating vitamin D levels, likely due to induction of colitis. VDR mRNA was significantly downregulated (Fig. 4B), but not significantly more so than the downregulation observed in the normal chow colitis. RXRα mRNA was significantly downregulated in this model (Fig. 4B and C). COX-2 mRNA was upregulated in treated mice on a vitamin D-deficient diet but not significantly more so than the upregulation observed in acute treated mice on a normal chow. No difference in the expression of amphiregulin was found between treated and control mice (Fig. 4B). VDR protein was reduced, but not significantly more so than observed downregulation found in acute treated mice on a normal chow (Fig. 4C). In this vitamin D-deficient acute model, TNFα, Snail, and Snail2 mRNA were all significantly upregulated (Fig. 4B).

The average colitis score was 3.8 to 4 out of 4. This was more than 3.5 observed in acute treated mice but not significantly so. Ulceration was observed not only in the distal colon, but throughout the entire colon. Very few normal appearing mucosal areas remained with almost the entire colon being ulcerated. COX-2 and iNOS, as well as Snail and Snail2, had widespread expression in the colon, both distally and proximally, in the ulcerated tissue and stroma (Fig. 4D).

Discussion

Vitamin D has been implicated in the pathogenesis of numerous inflammatory diseases (6) and its anti-inflammatory effect (1–5) is being explored as a potential interventional agent. It has been shown in vitro that vitamin D can prevent the growth of cancer cells (7–9), and epidemiologic data suggests reduced vitamin D intake or production can increase rates of various cancers (10–15). Because of the epidemiologic relationship between vitamin D status and Ulcerative Colitis, we probed the influence of RXRα and VDR in acute and chronic animals models of colitis.

To study an acute murine colitis, the AOM/DSS model was used as this model is accepted as a proxy for human Ulcerative Colitis. We found a loss of crypts and the development of colonic ulcerations in treated mice that, along with the decreased systemic 25-(OH) vitamin D, resembles findings in patients with Ulcerative Colitis (40).

We have shown that the downregulation of VDR occurs early in acute murine colitis at both the protein and the mRNA level, which agrees with data from patients with Ulcerative Colitis (23). This downregulation was confined to ulcerated tissue and corresponded to a localized increase of COX-2 and iNOS. RXRα was not downregulated in acute colitis at the protein or mRNA level, suggesting that VDR and RXRα undergo different mechanisms of silencing. Upon immunohistochemical analysis, it was clear that...
RXRα was downregulated in areas of ulceration in the acute colitis. The apparent disagreement in expression of RXRα between IHC compared with real-time PCR and immunoblotting is likely due to the fact that for IHC, the entire colon can be visualized and it was noted that the RXRα down-regulation was confined to distal tissue. However, for mRNA and protein extraction, the entire colon was scraped, as it was not feasible to separate out ulcerated and normal tissue. The introduction of unaffected, proximal tissue with normal RXRα expression likely masked any receptor down-regulation that was occurring distally.

As colitis develops into CAC, mice exhibited a 10% to 40% mortality rate with 60% of surviving mice developing adenomas. It has been proposed that the development of AOM/DSS CAC is due to TNFα production that encourages inflammatory cell invasion and the subsequent production of COX-2, a major player in CAC development (41). In fact, it has been shown that dietary calcium and active vitamin D in tandem were able to reduce colitis in vitamin D-deficient IL-10−/− mice due to a suppression of the TNFα pathway (42). Our finding of increased COX-2 protein and mRNA in challenged mice in ulcerated and cancerous tissue supports this notion and it is likely that other proinflammatory mediators are involved.

To investigate a potential mechanism for VDR down-regulation Snail and Snail2, two transcription factors that have shown to inhibit VDR transcription (24–26), were accessed. We found an early upregulation of Snail mRNA in the acute model, but Snail2 mRNA upregulation was short of significance. Snail and Snail2 expression correlated with mediators are involved.

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been linked to cancer progression. However, previous studies were mostly conducted in cell lines and, to our knowledge, expression patterns have not been reported in murine colitis or CAC models (43). IHC showed the upregulation of Snail and Snail2 to be confined to ulcerated tissue, similar to findings in patients with Ulcerative Colitis (29). The fact that Snail and Snail2 were found to be upregulated in CAC on IHC, but not via real-time PCR, was once again, likely due to the method of colon scraping and subsequent RNA extraction.

A previous study showed that a vitamin D-deficient diet leads to increased weight loss and colitis scores in mice (44). Other studies have shown that VDR−/− mice challenged with DSS exhibit early mortality (22), that VDR deficiency results in a worsened colitis in the CD45RB transfer model (45), and that VDR−/− mice have a loss of intestinal transepithelial resistance post-DSS challenge (46). We attempted, in this study, to make our model clinically relevant by starting mice on a vitamin D-deficient diet 3 weeks before colitis induction to model a human with low serum vitamin D. Both AOM/DSS-challenged mice and saline/water control mice on the vitamin D-deficient diet showed systemic vitamin D levels that were decreased as compared with AOM/DSS-challenged mice and saline/water control mice on normal chow. However, mice on the vitamin D-deficient diet did not show observable symptoms of vitamin D deficiency, such as osteomalacia. We did not expect systemic signs of vitamin D deficiency due to the short nature of our experiment. It was shown by Lagashetty and colleagues (44) that a one-week course of DSS after time on a vitamin D-deficient diet did not lead to clinical symptoms of vitamin D deficiency, such as hypocalcemia. However, a vitamin D-deficient diet fed to mice, subsequently challenged with AOM/DSS, resulted in an intensity of vitamin D deficiency due to the method of colon scraping and subsequent RNA extraction.

This study shows the importance of VDR and vitamin D status in controlling murine colitis development. Although a definitive mechanism for VDR downregulation could not be approached because of the lack of an accepted in vitro model for colitis, our work suggests the transcription factors Snail and Snail2 may be, in part, responsible. The downregulation of RXRα in the vitamin D-deficient mice suggests that severe colitis allows for an unknown mechanism to downregulate RXRα expression and a plausible explanation is through epigenetic silencing. More work must be done to elucidate the mechanism(s) responsible for the downregulation of these receptors with the hopes that therapy could be targeted at restoring the expression of VDR and RXRα to reduce colitis progression and CAC development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: R.W. Knackstedt, M.J. Wargovich
Development of methodology: R.W. Knackstedt
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.W. Knackstedt
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