Vitamin D Deficiency Enhances the Growth of MC-26 Colon Cancer Xenografts in Balb/c Mice$^{1,2}$

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ABSTRACT Vitamin D deficiency has been associated with increased risk of colon cancer in epidemiologic and prospective clinical studies. In vitro and in vivo studies demonstrated that 1,25-dihydroxycholecalciferol [1,25(OH)$_2$D$_3$] and its analogs inhibit colon cancer cell proliferation. Few studies have evaluated the effect of vitamin D deficiency on the development and growth of colon cancer. To assess the antiproliferative effects of 25-hydroxyvitamin D [25(OH)D] and 1,25(OH)$_2$D$_3$ in vitro, we cultured MC-26 (a colon cancer cell line) in the presence of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ and performed $[^3]$H]thymidine incorporation. The proliferation of MC-26 was significantly inhibited by both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$. To determine the effect of vitamin D deficiency on colon cancer proliferation, Balb/c mice were rendered vitamin D deficient by feeding them a vitamin D–deficient diet for 3 mo. A group of vitamin D–sufficient mice was given the same diet with supplemental vitamin D. The mice were injected with MC-26 colon cancer cells and the tumors were measured daily for 20 d. Vitamin D–sufficient mice had 40% smaller tumors than vitamin D–deficient mice. The tumors were evaluated for mRNA expression of the vitamin D receptor (VDR) and 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase) by quantitative RT-PCR. The expression of the mRNA for the VDR and the 1α-OHase was 37- and 6-fold higher, respectively, in the vitamin D–sufficient mice compared with the vitamin D–deficient mice. We conclude that vitamin D deficiency enhances the growth of colon cancer in mice. The tumor expression of VDR and 1α-OHase indicates possible autocrine/paracrine cell growth regulation by vitamin D.


KEY WORDS: • vitamin D • colon cancer • 1α-hydroxylase

Epidemiologic studies have suggested that maintaining adequate vitamin D status may prevent colon cancer. Apperly et al. (1) that there was an inverse relation between solar irradiation and cancer mortality, thus establishing a possible connection between sunshine, vitamin D, and cancer. Garland and Garland (2) reported that mortality rates due to colorectal cancer in the United States were highest in areas with the least amount of solar radiation. Several cohort studies were conducted and confirmed these initial observations that vitamin D may be protective against colon cancer (3–6). These studies demonstrated that subjects with low (<12.5 nmol/L) serum 25-hydroxyvitamin D [25(OH)D] levels, the major circulating form of vitamin D, were at a 2- to 3-fold increased risk for developing colon cancer.

Cell culture studies further supported that vitamin D was protective against colon cancer. Halline et al. (7) demonstrated that cultured Caco-2 cells, a human colon cancer cell line, expressed the vitamin D receptor (VDR) and was inhibited in a dose-dependent fashion by 1,25-dihydroxycholecalciferol [1,25(OH)$_2$D$_3$] (8). Further, in vitro studies with several human colon cancer cell lines demonstrated that growth was inhibited in the presence of 1,25(OH)$_2$D$_3$ and less calcemic vitamin D analogs (9–11). Several in vivo rodent models demonstrated that cholecalciferol and its analogs were protective against colon carcinogenesis. These models included carcinogen-induced colon cancer in rats (12–14), and human colon cancer xenografts in nude mice (15,16) and APC mutant mice (17).

A few studies evaluated the effect of vitamin D deficiency on the induction and growth of colon cancer in vivo. Sitrin et al. (12) studied rats fed a vitamin D–deficient diet for 6 wk and found a detrimental effect of vitamin D deficiency in a rat model of carcinogen-induced colon cancer. Mokady et al. (18)
evaluated a low vitamin D diet in a carcinogen-induced colon cancer model in rats and found that vitamin D deficiency increased colon cancer proliferation. Studies by Newmark et al. (19) evaluated a Western style diet in rats low in vitamin D and found that vitamin D deficiency increased colon cancer proliferation. Studies by Newmark et al. (19) evaluated a Western style diet in rats low in vitamin D and found that vitamin D deficiency increased colon cancer proliferation.

We sought to evaluate the effect of vitamin D deficiency (undetectable 25-hydroxyvitamin D) on the development and growth of colon cancer. We used the MC-26 mouse colon cancer model, which closely mimics human colon cancer biology (20). This mouse model was used to test new chemotherapeutic agents (21–25) against colon cancer and to evaluate gene therapy as a treatment in colon cancer (26–31). We also evaluated whether the mouse colon tumors contained the VDR and the 25-hydroxyvitamin D-1α-hydroxylase (CYP27B, 1α-OHase), the enzyme that converts 25(OH)D3 to 1,25(OH)2D3.

**MATERIALS AND METHODS**

**Cell proliferation assay.** MC-26 mouse colon cancer cells were grown in sterile plastic flasks in DMEM supplemented with 10% fetal bovine serum. The MC-26 cells were replated from the flasks into 24-well Petri dishes and grown to ~50% confluence. Treatment conditions [25(OH)D or 1,25(OH)2D] were compared with controls in 12 replicates on each dish. The cells were incubated in the presence of either 1.25(OH)2D3 or 25(OH)D2 [10−8 to 10−6 mol/L] in the presence of 25 μg/L epidermal growth factor for 18 h. Cellular proliferation was assessed by incubation with [3H]thymidine for 2 h as previously described (32). After the 2-h incubation, the cells were rinsed 3 times with ice-cold PBS and incubated in 5% perchloric acid on ice for 30 min. The perchloric acid was aspirated then replaced again with 500 μL of perchloric acid and incubated at 70°C in a hot water bath to precipitate the DNA. The cellular precipitates were combined with scintillation fluid, placed into plastic vials, and counted with a β-liquid scintillation counter. Radioactivity data of test wells were expressed relative to those of control wells.

**In vivo colon cancer assay.** The IACUC at Boston University School of Medicine granted approval to conduct this study. Male Balb/c mice (6 wk old) were obtained from Taconic Farms. They were housed at the Boston University Laboratory Animal Sciences Facility and maintained under standard light:dark cycles. At the end of the experiment, animals were killed with 100% CO2.

The rodent diets were obtained from Harlan-Teklad. We used 2 different diets for our studies: a vitamin D–sufficient diet (cholecalciferol) (0.3%) and a vitamin D–deficient diet (the exact same diet without cholecalciferol) (Table 1). The absence of cholecalciferol was confirmed by HPLC analysis of the diet. The mice were randomized into 2 groups of 9 or 10. The mice were fed their respective diets until the group consuming the vitamin D–deficient diet developed vitamin D deficiency. Every 4–6 wk, 3 mice from each group were bled from the dorsal tail vein. Blood was collected in plastic microcentrifuge tubes containing a serum separating additive (Microtainer SST, Benton-Dickerson). The blood was centrifuged at 1000–1500 rpm for 10 min and the serum collected for 25(OH)D analysis. After 12 wk, the vitamin D–deficient mice had a serum 25(OH)D concentration < 12.5 nmol/L.

The MC-26 cells were grown to near confluence in 100-mm Petri dishes. The cells were then trypsinized and gently mixed in a sterile beaker with a stir-bar. With the use of a hemocytometer, the cells were diluted with DMEM to a final concentration of 10,000 cells/0.1 mL. The mice were shaved on the lower posterior trunk and 0.1 mL containing 10,000 MC-26 cells was injected subcutaneously through a 27-gauge needle.

The size of the tumors was recorded on a daily basis starting on d 7 after the MC-26 cells were injected. Using a digital caliper, we recorded the length and width of the tumors. The volume of each tumor was calculated using the following formula: volume = length × width × thickness / 2.

**TABLE 1**

**Composition of the vitamin D–deficient and vitamin D–sufficient diets**

<table>
<thead>
<tr>
<th>Vitamin D–deficient diet</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, “vitamin-free” test</td>
<td>180.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>2.0</td>
</tr>
<tr>
<td>Dextrose, monohydrate</td>
<td>644.18</td>
</tr>
<tr>
<td>Corn oil</td>
<td>100.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>30.0</td>
</tr>
<tr>
<td>Mineral mix (#TD 79055)</td>
<td>13.37</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>11.63</td>
</tr>
<tr>
<td>Potassium phosphate, dibasic</td>
<td>4.9</td>
</tr>
<tr>
<td>Potassium phosphate, monobasic</td>
<td>3.9</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>4.672</td>
</tr>
<tr>
<td>Choline dihydrogen citrate</td>
<td>3.497</td>
</tr>
<tr>
<td>Dry vitamin E acetate</td>
<td>0.242</td>
</tr>
<tr>
<td>Dry vitamin A palmitate</td>
<td>0.0396</td>
</tr>
<tr>
<td>p-Aminobenzoic acid</td>
<td>0.1101</td>
</tr>
<tr>
<td>Ascorbic acid, coated (97.5%)</td>
<td>1.0166</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.0004</td>
</tr>
<tr>
<td>Vitamin B-12 (0.1% in mannitol)</td>
<td>0.0297</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>0.0661</td>
</tr>
<tr>
<td>Folic acid</td>
<td>0.002</td>
</tr>
<tr>
<td>Inositol</td>
<td>0.1101</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.0496</td>
</tr>
<tr>
<td>Niacin</td>
<td>0.0991</td>
</tr>
<tr>
<td>Pyridoxine · HCl</td>
<td>0.022</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.022</td>
</tr>
<tr>
<td>Thiamin · HCl</td>
<td>0.022</td>
</tr>
<tr>
<td>Ethoxyquin</td>
<td>0.02</td>
</tr>
</tbody>
</table>

1 Dry cholecalciferol (500,000 IU/kg) was included in the vitamin D–sufficient diet.
2 Harlan Teklad, Madison, WI.

![FIGURE 1](http://example.com/figure1.png)

**FIGURE 1** Antiproliferative effect of 1,25(OH)2D3 (A) and 25(OH)D3 (B) on [3H]thymidine incorporation in a mouse cancer cell line (MC-26). Values are means ± SEM, n = 12, *different from the control (no treatment), P < 0.001.
The mice were observed for 20 d before killing. At the time of killing, the mice were bled via the dorsal tail vein for calcium and 25(OH)D determinations (Hitachi 747 Colorimetric Assay). Serum was sent to Antech Diagnostics for calcium determinations. The 25(OH)D concentration was measured by the method of Chen et al. (33). The tumor was dissected from the back for mRNA analysis.

**Isolation of total RNA.** The tumors were flash frozen in liquid nitrogen and stored at −80°C until use. The tissues were washed once with PBS. Total RNA was harvested using the SV Total RNA Isolation System (Promega). The quality of the RNA was confirmed by a 260abs:280abs ratio > 1.8.

**RT-PCR.** 1α-OHase and VDR mRNA levels were determined by quantitative RT-PCR assay using the CyberGreen technology and an ABI Prism™ 7700 Sequence Detector (Perkin Elmer). Total RNA (200 ng) was analyzed for each reaction. The primers for the mouse VDR and 1α-OHase are the same as those previously published (34).

**Statistical methods.** The computer software programs Microsoft Excel™ with the statistical add-in program Analyze-It and SAS 9.1 were used to analyze the results. Student’s t test was used to compare the means of the 2 groups. Repeated-measures ANOVA and post-hoc tests were used to compare tumor sizes in vitamin D–sufficient and–deficient groups each day. Differences were considered significant at $P < 0.05$. Values in the text are means ± SEM.

## RESULTS

**1,25(OH)$_2$D$_3$ effect on MC-26 cell proliferation.** The proliferation of MC-26, a mouse colon cancer cell line, was inhibited 34 and 54% by 10$^{-7}$ and 10$^{-6}$ mol/L 1,25(OH)$_2$D$_3$, respectively ($P < 0.05$; Fig. 1A). Treatment of MC-26 with 25(OH)D$_3$ inhibited proliferation by 47 and 40% at concentrations of 0.5 × 10$^{-7}$ and 10$^{-6}$ mol/L, respectively ($P < 0.05$; Fig. 1B).

**Serum vitamin D and calcium concentrations.** Mice fed the vitamin D–deficient diet had serum 25(OH)D concentrations below the limit of detection in our assay (<12.5 nmol/L). The concentration in mice fed the vitamin D–sufficient diet was 65.6 ± 15 nmol/L. Serum calcium concentrations did not differ between the vitamin D–sufficient and–deficient groups and were 2.3 ± 0.1 and 2.3 ± 0.03 mmol/L (9.1 ± 0.3 and 9.0 ± 0.1 mg/dL), respectively.

**Growth of colon cancer xenografts in vitamin D–deficient mice.** The tumors in the vitamin D–sufficient mice were significantly smaller than those in the vitamin D–deficient mice starting at d 9. This difference was maintained until the end of the study (Fig. 2). The final tumor sizes in the vitamin D–sufficient mice were 40% smaller (340 ± 70 mm$^3$) compared with those in the vitamin D–deficient mice (560 ± 110 mm$^3$) ($P < 0.05$, t test; Fig. 2 and 3).

### FIGURE 2
MC-26 tumor size in vitamin D–deficient (n = 9; solid line, black diamonds) and vitamin D–sufficient (n = 10; dashed line, black triangles) mice. Values are means ± SEM. Repeated-measures ANOVA: $P = 0.002$.
*Different from vitamin D–sufficient mice at that time, $P < 0.05$.

A

**Expression of 1α-OHase and VDR in mouse tumors.** Quantitative RT-PCR of the tumors revealed that the colon cancer xenograft tumors contained the mRNA for the 1α-OHase and the VDR. The expression of the VDR and 1α-OHase mRNA was 37- and 6-fold higher in the vitamin D–sufficient mice than in the vitamin D–deficient mice.
D–sufficient mice compared with the vitamin D–deficient mice, respectively (Fig. 4).

**DISCUSSION**

Higher 25(OH)D levels in humans is protective against the development of colon cancer (3–6). Increased sunlight exposure and vitamin D sufficiency in humans were reported to be protective against the development of colon, prostate, and breast cancers (35). However, there has never been a study demonstrating that documented undetectable 25(OH)D accelerated colon cancer growth. This study evaluated whether vitamin D deficiency [25(OH)D < 12.5 nmol/L] in mice would result in more aggressive growth of colon cancer. We demonstrated that vitamin D–deficient mice had significantly larger tumors than mice who had a 25(OH)D concentration of 65 nmol/L. These data support the 8-yr prospective study that reported an 80% reduced risk of developing colon cancer in individuals who had a serum 25(OH)D concentration > 67.5 nmol/L (4).

Vitamin D must undergo 2 sequential hydroxylations to form the steroid hormone 1,25(OH)2D (35). The major antiproliferative effects occur when 1,25(OH)2D binds to the VDR to activate transcription of vitamin D–responsive genes (36,37). Previously it was thought that the 1α-OHase was expressed mainly in the kidney. However, our laboratory and others demonstrated its presence in normal and malignant colon tissue (9,38). The presence of the 1α-OHase in the mouse colon cancer tumors further supports the role of this enzyme in regulating colon cancer proliferation. The presence of the 1α-OHase in colon cancer tumors suggests that circulating 25(OH)D could be converted to 1,25(OH)2D within the cancer cell. This is supported by the observation that 25(OH)D3 was effective in inhibiting the proliferation of MC-26 cells in vitro (Fig. 1B). This is similar to the observations in prostate cancer cells (38) and in the prostate cancer cell line LNCAP transfected with the 1α-OHase gene (39). The 1,25(OH)2D could then activate vitamin D–responsive genes that regulate cellular proliferation and differentiation. The expression of the 1α-OHase and VDR was higher in the tumors of the vitamin D–sufficient mice, which was not unexpected because 1,25(OH)2D upregulates the expression of its receptor (40).

Vitamin D deficiency is now recognized as a major health problem. Nearly one third of healthy adults are vitamin D deficient at the end of winter in Boston (41). Nearly half of all patients diagnosed with cancer have vitamin D deficiency in the summer months, a time in which vitamin D deficiency is less common (42). Combined with the epidemiologic data demonstrating a protective effect of vitamin D on colon cancer proliferation, this study supports the notion that vitamin D nutrition could play an important role in either preventing or limiting the progression of colon cancer (43).

In summary, vitamin D deficiency results in the aggressive growth of colon cancer. The presence of the VDR and the 1α-OHase mRNA in these tumors raises the possibility that 25(OH)D may be locally converted to 1,25(OH)2D to bind the VDR to active transcription of vitamin D–dependent genes. Our in vivo mouse model of colon cancer supports the deleterious effect of vitamin D deficiency on colon cancer progression, which further supports the epidemiologic data.

**LITERATURE CITED**


