Losartan and Vitamin D Inhibit Colonic Tumor Development in a Conditional Apc-Deleted Mouse Model of Sporadic Colon Cancer

Urszula Dougherty1, Reba Mustafi1, Haider I. Haider1, Abdurahman Khalil1, Jeffrey S. Souris2, Loren Joseph3, John Hart4, Vani J. Konda1, Wei Zhang5, Joel Pekow1, Yan Chun Li1, and Marc Bissonnette1

Abstract

Colorectal cancer is a leading cause of cancer deaths. The renin-angiotensin system (RAS) is upregulated in colorectal cancer, and epidemiologic studies suggest RAS inhibitors reduce cancer risk. Because vitamin D (VD) receptor negatively regulates renin, we examined anticancer efficacy of VD and losartan (L), an angiotensin receptor blocker. Control Apc+/LoxP mice and tumor-forming Apc+/LoxP Cdx2P-Cre mice were randomized to unsupplemented Western diet (UN), or diets supplemented with VD, L, or VD+L, the latter to assess additive or synergistic effects. At 6 months, mice were killed. Plasma Ca2+, 25(OH)D3, 1α, 25(OH)2D3, renin, and angiotensin II (Ang II) were quantified. Colonic transcripts were assessed by qPCR and proteins by immunostaining and blotting. Cancer incidence and tumor burden were significantly lower in Cre+ VD and Cre+ L, but not in the Cre+ VD+L group. In Apc+/LoxP mice, VD increased plasma 1,25(OH)2D3 and colonic VDR. In Apc+/LoxP-Cdx2P-Cre mice, plasma renin and Ang II, and colonic tumor AT1, AT2, and Cyp27B1 were increased and VDR downregulated. L increased, whereas VD decreased plasma renin and Ang II in Cre+ mice. VD or L inhibited tumor development, while exerting differential effects on plasma VD metabolites and RAS components. We speculate that AT1 is critical for tumor development, whereas RAS suppression plays a key role in VD chemoprevention. When combined with L, VD no longer increases active VD and colonic VDR in Cre mice nor suppresses renin and Ang II in Cre+ mice, likely contributing to lack of chemopreventive efficacy of the combination.

Introduction

Colorectal cancer remains a major health problem in the United States, with more than 50,000 deaths expected in 2018 (1). For patients with distant metastatic disease, the prognosis is poor, with less than 15% surviving at 5 years. For these reasons, efforts to prevent colon cancer are a high priority. Aspirin is the only agent shown clinically to inhibit colon cancer development, but serious Gap 1 phase (GI) toxicities have limited its widespread use (2). Vitamin D (VD) is another promising agent for chemoprevention. VD is metabolically activated in vivo to 1α,25-dihydroxyvitamin D3, which binds to VD receptor (VDR), a transcription factor that regulates diverse cellular processes. VD has epidemiologic support and strong preclinical evidence for colon cancer chemoprevention (3–7). However, results from a recent randomized clinical trial to assess VD efficacy to prevent colon adenoma recurrences were negative (8). Several problems were noted in the study design, especially the low dose of VD intervention (9). Subsequent genotype analysis of trial participants suggested that specific SNPs in the VDR gene were associated with VD efficacy to prevent advanced adenoma progression (10). This finding suggests that VD might exert a chemopreventive effect in some populations.

Western diets (WD) are associated with increased colorectal cancer incidence (11). These diets, rich in saturated fats and red meat and low in fiber, are relatively deficient in VD and calcium. Numerous animal studies, using both carcinogen-induced and spontaneous/genetic models of colon cancer, have demonstrated that WDs...
promote colon cancer (12–18). Factors implicated in WD-related tumor promotion include changes in the colonic microbiome and colonic milieu (19), and altered host factors including upregulated growth factors, increased proinflammatory enzymes, and suppressed immune responses (15, 20, 21). The renin-angiotensin system (RAS), which includes circulating and colonic components, is implicated in tumor development (22, 23). The RAS includes angiotensinogen, which is converted to angiotensin I (Ang I) by renin. Angiotensin-converting enzyme (ACE) in turn converts Ang I to Ang II, which binds to Ang II receptors, AT1 and AT2, to modulate numerous cellular functions. We showed that VDR, when bound to 1α,25-dihydroxyvitamin D3, inhibits renin transcription and thus suppresses RAS signals (24). Renin is upregulated in many colon tumors (25–27). Furthermore, we showed that VD suppressed colonic renin levels in mice with wild-type Vdr fed a WD and treated with azoxymethane (AOM)/dextran sulfate sodium (DSS) (6). Intriguingly, renin is required for diet-induced obesity and the metabolic syndrome (28).

Most experimental animal studies showing protective effects of VD against colon cancer have employed 1α, 25-dihydroxyvitamin D3, or an active VD analog (5, 29). Supplemented dietary VD (precursor of active VD) has not been widely studied for chemoprevention in sporadic models of colon cancer, and results have been inconsistent (25, 30, 31). Some of these differences may reflect differences in species (rat vs. mouse), different amounts of dietary fat (standard vs. high fat), and different VD doses. Our laboratory showed that global deletion of the Vdr gene increased inflammation-associated colon cancer, and other groups showed that dietary VD could suppress such tumors (6, 32, 33). Colitis-associated tumors, however, constitute only a small fraction of colon cancers found in humans. Because VD efficacy in a sporadic colon cancer model has only been shown in rats, our goal was to directly test the efficacy of VD in the setting of a WD-fed genetically engineered mouse model that targets tumors to the colon. To also directly test the role of RAS, we examined the chemopreventive effects of losartan (L), an angiotensin receptor (AgtR1) blocker. Because VD not only suppresses RAS signals via renin inhibition, but also exerts RAS-independent chemopreventive effects (34), we also investigated whether the combination of VD and L could exert additive or synergistic effects.

For a colon cancer model, we employed a genetically engineered mouse with a conditional allele for the Apc gene (LoxP-exon14-LoxP). The conditional Apc gene, when deleted in colonic epithelial cells using a constitutively active Cre-recombinase transgene under the control of colonocyte-specific Cdx2 promoter, yields colonocytes with Apc+/Δ genotype. The remaining wild-type Apc allele is subsequently mutated or deleted through LOH. This model phenocopies sporadic human colon cancer in that 85% of human tumors possess APC mutations (35). The model allowed us to assess effects of VD and L on sporadic colonic tumorigenesis. We also explored their effects on ADAM17 and Notch signals that are implicated in tumorigenesis. We demonstrate that as single agents, VD or L suppresses tumor development, but surprisingly the combination is not effective.

Materials and Methods

Materials

Defined diets, enriched in Western fat (WD, 20% fat) and relatively low in VD (100 IU/kg chow) or supplemented with VD (20,000 IU/kg chow), were obtained from Harlan Teklad (14). Rabbit polyclonal anti-ADAM17 antibodies (catalog ab2051) were purchased from Abcam. Mouse monoclonal antibodies to pERK (SC-7383) and rabbit polyclonal antibodies to Jagged 1 (sc-390177) were obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-pAKT antibodies (#9271) and Notch intracellular domain (NICD) antibodies (41477) were obtained from Cell Signaling Technology. Monoclonal β-actin antibodies (#A-5441) were purchased from Sigma-Aldrich. Mouse monoclonal β-catenin antibodies (#13-8400) were obtained from Invitrogen. Polyclonal anti-Hes1 antibodies (AB5702) were obtained from Millipore. Anti-AT1 (PA5-18587) and -AT2 (PA3-210) antibodies were obtained from Thermo-Fisher Scientific. Plasma renin was measured using RayBiotech Mouse Renin1 ELISA (#ELM-Renin1, RayBiotech). Plasma Ang II was measured using Ang II ELISA (ADI-900-204, Enzo Life Sciences). Plasma 25-hydroxyvitamin D3 was measured using Mouse 25-OH Vitamin D ELISA (SKU:VID21-K01, Eagle Biosciences, Inc.). 1α,25-dihydroxyvitamin D3 was measured using EIA (AC-62F1, Immunodiagnostics Systems). See Supplementary Materials and Methods for additional details.

Animals

Mice were used under an approved animal protocol (ACUP 71350). Transgenic mice, expressing constitutively active Cre recombinase controlled by a modified Cdx2 promoter (Cdx2-P-Cre), with expression restricted to distal small intestine and colon, were obtained from The Jackson Laboratory (stock #00935). Genetically engineered mice with a conditional Apc gene (ApcLoxP-exon14-LoxP) were obtained from NCI (strain 01XAA). Both genetically engineered mouse strains were on a C57Bl6/J background. Cdx2-P-Cre and ApcLoxP/LoxP mice were intercrossed. The experimental tumor-forming groups were Apc+/LoxP; Cdx2P-Cre (Cre+) (to generate colonocyte Apc+/Δ), and the control groups were Apc+/LoxP (Cre-). Cre+ and Cre- mouse colonies were expanded.
and control and tumor-forming mice randomized at 6 weeks of age to receive unsupplemented Western diet alone (UN) as described (15), or VD supplemented diet (VD, 500 μg/kg chow), L supplemented diet (L, 160 mg/L drinking water) or VD+L supplemented diet. The experimental protocol, including diets and supplemented agents, is summarized in Fig. 1A. The WD included 100 IU VD/kg chow that reflects the murine equivalent of VD in the American diet (see Supplementary Table S1 in ref. 12). The VD supplemented diet contained 20,000 IU VD/kg chow (500 μg/kg chow). We previously showed that this dose of VD was well tolerated and did not induce hypercalcemia, but suppressed AOM/DSS-induced colonic renin in Vdrtg wild-type mice fed a WD (6). This VD dose was well tolerated in other rodent studies and shown to increase circulating 1α,25-dihydroxyvitamin D3 (32, 36).

We estimated that the reduction in tumor multiplicity (TM) would be approximately 50% based on prior studies in DMH-treated rats fed high-fat diet alone or with VD (30) and that such a reduction would be biologically meaningful. Based on this estimate, we determined that 10 animals per group would be sufficient to provide 80% power to discriminate differences in TM. We included additional animals to compensate for unexpected losses. There were 5 mice in each Cre-control group (Cre- UN, Cre- VD, Cre- L, and Cre- VD+L group). In the Cre+ tumor-bearing groups, there were 15 mice in the unsupplemented group (Cre+ UN), 18 mice in the Cre+ VD group, 17 mice in the Cre+ L group, and 16 mice in the Cre+ VD+L group.

To assess for emergence of overt tumors, colonoscopy was performed in a subset of mice beginning at 5 months of age using Karl Storz-Endoskope under an approved animal protocol (ACUP71350) as described (37). After sedation, the rectal area was cleaned with saline-saturated gauze and mice secured by holding the tail with the left hand and inserting and advancing the scope with the right hand to allow for flexibility and gauge resistance better and allow for active and responsive positioning of the mouse to avoid scope trauma. The scope, lubricated with KY jelly, was inserted into the rectum, and 500 μL air slowly introduced to insufflate the colon and images of tumors captured. After the procedure, the air was evacuated. Mice were killed at 6 months of age, tumors were harvested, and blood was collected for plasma. Tissue aliquots were preserved in RNA later, or flash-frozen or fixed in 10% buffered formalin. Tumor stage was confirmed on hematoxylin and eosin–stained sections by a GI pathologist (J. Hart). Subsequent tissue

Figure 1.
Experimental protocol and effects of vitamin D and L on mouse weight gain and on plasma 25-hydroxyvitamin D3, renin, and Ang II. A, Experimental protocol. B, left, Body weight gain in control (Cre-) mice on the indicated diets (†, P < 0.005 compared with Cre- UN mice). Right, body weight gain in Cre+ tumor-bearing mice. Weights for Cre+ L or Cre+ VD+L were significantly lower than Cre- UN mice for time points > 3 months of age and for Cre- UN or Cre+ VD tumor-bearing mice for time points > 4.5 months of age (†, P < 0.01; ‡, P < 0.005; and ¶, P < 0.001, compared with Cre- UN mice). C, Plasma 25-hydroxyvitamin D3 (ng/mL; †, P < 0.001 and ¶, P < 0.005, compared with Cre- UN mice). D, Plasma renin (pg/mL; †, P < 0.001 compared with Cre- UN mice, ¶, P < 0.01 compared with Cre- UN mice). E, Plasma Ang II levels (pg/mL; †, P < 0.001, compared with treatment-matched Cre- UN mice; ‡, P < 0.01 and ¶, P < 0.001 compared with Cre- UN mice).
analyses included Western blotting, immunostaining, and real-time PCR.

Human tissue
Fresh human colon cancers and adjacent normal-appearing colonic mucosa were obtained under IRB 10-209-A approved by the University of Chicago. Stromal cells and colonocytes were prepared by differential centrifugation in EDTA-EGTA buffer (13). See Supplementary Materials and Methods for further details.

Cell proliferation
HCT116, and HT29 human colon cancer cells and CCD-18Co colonic fibroblasts were obtained from the ATCC and authenticated using short tandem repeat DNA fingerprinting by IDEXX. Cells were cultured in 96-well plates at 37°C in a humidified atmosphere of 5% CO₂ and 95% air as described (13). Preconfluent cells were treated with Ang II (100 ng/mL) or vehicle (PBS). Where indicated, cells were pretreated with AT1 inhibitor L (10 μmol/L) or vehicle (PBS). Where indicated, cells were incubated with isotype-matched nonimmune antibodies. Control sections showed no specific staining. The Image J plug in immunoRatio was used to quantify the number of tumor nuclei with positive Ki67 or β-catenin staining as described (13). Sections from five tumors randomly selected from each Cre⁺ group and coded to blind investigators to treatment conditions were used for quantification. After stained images were scanned and quantified, identifiers were unmasked to compile mean ± SD for each group. See Supplementary Materials and Methods for additional details.

Real-time PCR
Real-time PCR was performed as described (14). Primer sequences are provided below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp27B1</td>
<td>F: 5'-AGA ATG CAC TCC ACT CTG AGA TCA CA-3'</td>
<td>R: 5'-GAT TCC TAC ACC GAG CAT GTC TCT GTC TGG-3'</td>
</tr>
<tr>
<td>Jag1</td>
<td>F: 5'-GAC AAC TGG TAT CGG TGC GA-3'</td>
<td>R: 5'-TGG AGG GCA GAT ACA CTG GT-3'</td>
</tr>
<tr>
<td>Jag2</td>
<td>F: 5'-TGT AAT TGG TCT CAC GGG G-3'</td>
<td>R: 5'-CAC CCC ATG TGG TCT CAC AG-3'</td>
</tr>
<tr>
<td>Notch1</td>
<td>F: 5'-TCT GCA GTG CCA CTG ATT GC-3'</td>
<td>R: 5'-TGC ATA CCC CGC GTG TTT TG-3'</td>
</tr>
<tr>
<td>Notch2</td>
<td>F: 5'-AGA CTA CGT GAT GAA CCG TG-3'</td>
<td>R: 5'-AAG TCA CGA TGG GAG GCA AG-3'</td>
</tr>
<tr>
<td>Hes1</td>
<td>F: 5'-ATA GCT GCC GGC ATT CCA AG-3'</td>
<td>R: 5'-TAT TTCC ACC AAG ACG CTC GG-3'</td>
</tr>
<tr>
<td>Renl</td>
<td>F: 5'-GAC GCC TTG TCC CAT GAC CAA TC-3'</td>
<td>R: 5'-TGT GAA TCC CAC AAG CAA GG-3'</td>
</tr>
<tr>
<td>Agtr1a</td>
<td>F: 5'-CTG CCT TCC CGG ACT TAA CA-3'</td>
<td>R: 5'-CTG GGT TGA TGT GGT CTC AGA-3'</td>
</tr>
<tr>
<td>Agtr2</td>
<td>F: 5'-GGT CCT GAG CAT TCC TA-3'</td>
<td>R: 5'-TCA GGA CTT GGT CAC GGG TA-3'</td>
</tr>
<tr>
<td>Vdr</td>
<td>F: GAT GCC CAC CAC AAG ACC TA-3'</td>
<td>R: 5'-CGG TTC CAT GTC CAG TG-3'</td>
</tr>
<tr>
<td>Actb</td>
<td>5'-TGG CTT GGA GGC ACC GGA GGA TGC GGC A-3'</td>
<td>5'-GGA GGA AGA GGA AGA GGG GCAG-3'</td>
</tr>
</tbody>
</table>

For real-time PCR, we analyzed colonic mRNA from 3 to 4 mice per group. Reactions were run in triplicate, and C_T values were averaged. Relative abundance, expressed as 2exp (−ΔΔC_T), was calculated by exponentiating differences in C_T between target gene and β-actin and normalized to fold of Cre⁺ control (6). There were no detectable amplifications in negative control reactions (reactions omitting reverse transcriptase or reactions lacking template). Fold of control was compared among groups using one-way ANOVA with effects of genotype, treatment conditions, and tissue type (tumor or normal mucosa; ref. 14). For gene expression changes that were significant among the groups by ANOVA, two-group comparisons were made by two-sided unpaired Student t test, and the Bonferroni or Tukey’s honest significance test correction was applied as indicated for multiple comparisons.

Immunohistochemistry
Formalin-fixed paraffin-embedded blocks were stained as described (6, 13). Primary antibody concentrations included 1:500 dilution for anti Ki67 antibodies, 1:150 dilution for anti-β-catenin, 1:200 dilution for anti-AT1 antibodies, and 1:250 dilution for anti-Jagged1 antibodies. Tumors of comparable stage from each group were used for immunostaining comparisons. For negative controls, primary antibodies were omitted, or sections were incubated with isotype-matched nonimmune antibodies. Control sections showed no specific staining. The Image J plug in immunoRatio was used to quantify the number of tumor nuclei with positive Ki67 or β-catenin staining as described (13). Sections from five tumors randomly selected from each Cre⁺ group and coded to blind investigators to treatment conditions were used for quantification. After stained images were scanned and quantified, identifiers were unmasked to compile mean ± SD for each group. See Supplementary Materials and Methods for additional details.

Western blotting
Western blotting was carried out as previously described with primary antibodies (dilutions) to ADAM17 (1:250), AT1 (1:500), AT2 (1:500), NICD (1:200), pERK (1:1000), pAKT (1:400), Hes1 (1:500), Jagged1 (1:500), renin (1:500), VDR (1:200), β-catenin (1:3000), CK20 (1:100), and VIM (1:500) as noted in Materials (13). Proteins were measured using RC-DC assay (Bio-Rad) and lysates adjusted to the same concentration. Separate aliquots were run to blot for β-catenin to confirm equal loading.

Statistical methods
Continuous data (plasma levels of calcium, 25-hydroxyvitamin D3, 1α,25-hydroxyvitamin D3, renin and Ang II, animal weights, tumor sizes, and Western blotting densitometry units) were shown to be normally distributed using the Shapiro–Wilk normality test and summarized as mean ± SD (38). All analyses of normally distributed variables involving more than two groups were calculated by one-way ANOVA and subsequent two group comparisons made using two-sided unpaired Student t test. The Bonferroni correction, or where indicated, Tukey HSD correction, was applied to control
for multiple comparisons for independent univariable tests (e.g., VD alone). For those significant univariables, we evaluated “multivariable models” (e.g., VD+L), and the nominal $P$ value was used to determine statistical significance.

Cancer incidence was defined as the percentage of mice with at least one cancer, and significance was calculated by the $\chi^2$ test adjusted with the Bonferroni correction. TM was defined as the average number of tumors and includes both cancers and adenomas per tumor-bearing mouse. Tumor multiplicities were compared among groups using the nonparametric Kruskal–Wallis (KW) test and significance determined by $P$ values adjusted with the Bonferroni correction.

**Results**

**Effects of VD and L on mouse weights, and on plasma calcium, 25-hydroxyvitamin D3, and RAS components**

VD did not alter mouse weight gain in the Cre- group (Fig. 1B). In preliminary studies, we determined that L, added to the drinking water at a dose of 160 mg/L, was well tolerated. This was equivalent to a dose we had used in prior short-term mouse studies (39). Cre- mice (Fig. 1B, left) showed similar weight gains except for the Cre-L group that showed delayed weight gain ($\ddagger$, $P < 0.005$, compared with unsupplemented Cre- mice). By 6 months, however, weights of Cre- L mice were comparable with other Cre- groups (Fig. 1B, left). Weight gain in the Cre+ groups (all Cre+ mice developed tumors, see below) lagged significantly behind treatment-matched Cre- mice (Fig. 1B, right; $^\ddagger$, $P < 0.01$; $\ddagger$, $P < 0.005$; $\dagger$, $P < 0.001$, compared with Cre- UN mice). The Cre+ L group showed the slowest weight gain. For the Cre+ L group, the average weight at 6 months was 21.5 ± 1.6 g, which was significantly less than other Cre+ groups: 25.0 ± 1.8 g (Cre+ UN, $P < 0.01$); 25.3 ± 1.1 g (Cre+ VD, $P < 0.005$); and 24.6 ± 2.1 g (Cre+ VD+L, $P < 0.01$). Interestingly, VD appeared to mitigate the effects of L on delayed weight gain in both Cre+ and Cre+ mice. Although plasma 25-hydroxyvitamin D3 levels were 4- to 5-fold higher in the VD-supplemented groups, plasma calcium levels were normal in all mice (Fig. 1C; Supplementary Fig. S1). L was shown to increase plasma levels of Ang II and renin (40). As shown in Fig. 1D and E, Cre+ UN mice had significantly higher plasma renin and Ang II levels compared with Cre- UN mice, indicating that tumor development upregulates plasma renin and Ang II. Compared with Cre+ UN mice, the Cre+ VD group had significantly lower plasma renin and Ang II levels, whereas the Cre+ L group had significantly higher levels. Renin and Ang II were also increased in the Cre+ VD+L group compared with diet-matched Cre- mice. In contrast to Cre+ VD and Cre+ L, renin and Ang II in the Cre+ VD+L were comparable with Cre+ UN mice.

**Effects of VD and L on tumor development**

There were no tumors in the Cre- mice. In contrast, in the Cre+ groups, tumors were detected on colonoscopy at 5 months of age (Fig. 2A). By 6 months, tumors were present in all Cre+ mice (all Cre+ mice had adenomas and/or carcinomas). As shown in Fig. 2B however, cancer incidence was significantly different among the groups ($P < 0.015$, $\chi^2$ test). Among Cre+ mice, cancer incidence in the unsupplemented group was 66.7%, compared with 22.2% in the Cre+ VD group ($P < 0.01$) and 17.6% in the Cre+ L group ($P < 0.01$). In contrast, the difference in cancer incidence between the Cre+ VD+L group (31.3%) and the Cre+ UN group (66.7%) did not reach statistical significance after applying the Bonferroni correction ($P = 0.05$).

Tumor multiplicities (adenomas plus carcinomas) were also significantly different among the Cre+ groups as assessed by the KW test ($P < 0.0005$). As shown in Fig. 2C, compared with the Cre+ UN group (TM 4.3 ± 2.1), the Cre+ VD group was 1.7 ± 0.7 (VD, $P < 0.0005$); and Cre+ L group was 1.6 ± 0.6 (L, $P < 0.0005$). As in the case of cancer incidence, TM was numerically less in the Cre+ VD+L group (2.8 ± 1.2) compared with Cre+ UN mice, but the difference was not significant ($P = 0.07$). Tumor sizes were significantly lower in all Cre+ supplemented groups compared with the Cre+ UN group (Fig. 2D). Ki67 was increased in tumors compared with mucosa from diet-matched Cre- mice (Fig. 2E and F; $^*$, $P < 0.0001$). Ki67 was significantly lower in tumors from the Cre+ VD group compared with the Cre+ UN group (Fig. 2F; $\dagger$, $P < 0.01$).

**Effects of VD and L on colonic Cyp27B1 and VDR and on plasma 1α,25-dihydroxyvitamin D3**

Conversion of circulating 25-hydroxyvitamin D3 to active 1α,25-dihydroxyvitamin D3 requires Cyp27B1, and mostly occurs in the proximal renal tubule. Cyp27B1 was shown to be increased in human colon tumors (data accessible at NCBI Geo database, accession GSE10950; ref. 41). To assess the potential role of VDR signals, we measured plasma 1α,25-dihydroxyvitamin D3 and colonic Cyp27B1 and VDR. As shown in Fig. 3A, Cyp27B1 transcripts were significantly increased in tumors from all groups except Cre VD+L. Compared with the Cre+ UN group, Cre+ L group showed reduced colonic tumor Cyp27B1. Plasma 1α,25-dihydroxyvitamin D3 was significantly increased only in the Cre- VD group (Fig. 3B). As shown in Fig. 3C, colonic Vdr transcripts were increased in Cre- VD group, and downregulated in tumors in agreement with prior studies (data accessible at NCBI Geo database, accession GDS2947 and GDS389; refs. 25, 42). Vdr transcripts were higher in Cre+ VD and Cre+ L, but not Cre+ VD+L in adjacent (normal-appearing) mucosa compared with the Cre+ UN group. VDR receptors were significantly
elevated in the Cre-VD group and significantly decreased in tumors in the Cre+VD+L group (Fig. 3D).

Effects of VD and L on renin and Ang II receptors, AT1 and AT2

Renin transcripts and protein were increased in tumors from the Cre+UN group. Compared with the Cre+UN tumors, renin was downregulated in Cre+VD and Cre+VD+L tumors (Fig. 4A and B). It was of interest to examine the effects of VD and L on colonic AT1 and AT2 that mediate Ang II signals. As shown in Fig. 4C, Agtr1 transcripts were increased in tumors from Cre+UN group and Cre+L groups. VD significantly reduced Agtr1 upregulation in tumors from the Cre+VD group compared with the Cre+UN group. AT1 receptors were lower in tumors in Cre+VD groups compared with the Cre+UN group (Fig. 4D). As shown in Fig. 4E, Agtr2 transcripts were also increased in tumors from the Cre+UN group and the Cre+L group. As shown in Fig. 4F, AT2 receptors were increased in the Cre+UN group and significantly reduced in tumors from the Cre+VD+L group compared with tumors from the Cre+UN group.

Effects of VD and L on ADAM17, pAKT, and pERK

We showed that ADAM17-EGFR signals play important roles in genetic and carcinogen-induced colon cancer (13, 15, 43). In other tissues, Ang II was shown to transactivate EGFR by an ADAM17-dependent mechanism (44). We, therefore, examined colon tumors for ADAM17 and EGFR effectors. Although there were no differences in Cre- control groups, ADAM17 was significantly increased in tumors in the Cre+UN group compared to the Cre- groups (Fig. 5A and B). ADAM17 upregulation was significantly reduced in tumors in the Cre+VD group, but not in the Cre+VD+L group (Fig. 5A and B). We also investigated AKT and ERK, downstream effectors of ADAM17-EGFR signals. AKT and ERK are activated in Apc-mutant Min adenomas (45). In agreement with this, phospho-AKT (pAKT) and phospho-ERK (pERK) were increased in tumors in the Cre+UN group. Compared
with tumors in the Cre+ UN group, pAKT was significantly reduced in tumors by VD and/or L, whereas pERK was significantly reduced only in tumors from the Cre+ L-treated groups.

Effects of VD and L on β-catenin expression

Because β-catenin plays a key oncogenic role in this model and EGFR is required for β-catenin activation (46, 47), we examined the effects of VD and L on β-catenin. As shown in Fig. 5C, both nuclear and cytoplasmic β-catenin stained strongly in tumors from the Cre+ UN group. Quantitation of nuclear β-catenin is shown in Fig. 5D. As shown in Fig. 5E and F, Western blotting confirmed this upregulation and showed that β-catenin expression was significantly reduced (3.9 ± 0.5 vs. 2.0 ± 0.6, P < 0.01) in tumors from the Cre+ VD group, but not in the Cre+ L or Cre+ VD+L group compared with Cre+ UN tumors.

Effects of VD and L on notch signaling

Notch signaling is regulated by ADAM17 and β-catenin and implicated in colonic tumorigenesis (48–50). The Notch receptor, when ligand bound, is cleaved by gamma secretase, releasing the NICD that migrates to the nucleus as an active transcription factor. Because ADAM17 and β-catenin were altered by VD and/or L in this model, we examined VD and L effects on transcript levels of notch components: Notch 1 and Notch 2 receptors; Notch ligands, Jagged1 and Jagged2; and Notch effector, Hes1. As shown in Supplementary Table S1, transcripts for
Figure 4.
Renin and angiotensin II receptors, AT1 and AT2, are upregulated in colonic tumors. RNA was extracted from colonic mucosa from Cre- groups and from tumors from Cre+ mice in the indicated groups. Transcripts were measured by real-time PCR (n = 8 treatment-matched controls and n = 16 tumors per group). Control mucosal and tumor lysates were probed for indicated proteins by Western blotting. A, Ren1 transcripts are suppressed in Cre+VD and Cre+VD+L colonic tumors ( , P < 0.0001 and †, P < 0.02, compared with mucosa from diet-matched Cre- mice; ‡, P < 0.00001 and §, P < 0.001 compared with tumors from the Cre+ UN group). (Continued on the following page.)
Jagged1 and Jagged2 and Notch1 and Notch2 were significantly upregulated in colonic tumors in the Cre+ UN group. L and/or VD inhibited upregulations of Jagged2 and Notch1 and Notch2 transcripts. Jagged1 upregulation was reduced by these agents, but did not reach significance. Similarly, Notch target Hes1 was greater in tumors in the Cre+ UN group (P = 0.14), and decreased in the Cre+ VD, Cre+ L, and Cre+ VD+L, but differences were not significant after correction for multiple comparisons.

We next examined the protein expression levels of several Notch signaling components. As shown in Supplementary Fig. S2A and S2B, Jagged1 and NICD were increased in tumors in the Cre+ UN group. Interestingly, NICD was shown to activate renin promoter in rat (51). Jagged1 was reduced in tumors from the Cre+ L and Cre+ VD+L group. Levels of NICD and Hes1 were significantly reduced in tumors in all supplemented groups, compared with Cre+ UN group. We next examined the cellular localization of AT1 and Jagged1 by immunostaining. As shown in Supplementary Fig. S2C, AT1 was expressed predominantly in stromal cells of tumors. Jagged1 was expressed in a heterogeneous cytoplasmic pattern in tumor cells and appeared more restricted in the Cre+ supplemented groups.

AT1 and AT2 in human colon cancers and cell lines
To assess AT1 and AT2 expression in human colon cancer, we isolated stromal cells and colonocytes from tumors and adjacent normal-appearing colonic mucosa. As shown in Fig. 6A and B, and Supplementary Fig. S3, AT1 was expressed in stromal cells (VIM positive) and colonocytes (CK20 positive) in normal colon and increased in stroma cells from tumors. AT2 was expressed at low levels in normal colon, but was significantly upregulated in stromal cells and colonocytes from colon cancers (see also Supplementary Fig. S3).

To begin to assess the roles of AT1 and AT2 in tumor growth, we examined the effects of Ang II and L (AT1 inhibitor) and PD123319 (AT2 inhibitor) on proliferation of colon cancer cells and colonic fibroblasts. As shown in Fig. 6C, Ang II induced nearly a 50% increase in proliferation of colon cancer cells and 100% increase in proliferation of colonic fibroblasts. Colon cancer cell proliferation in the Ang II + L or Ang II + PD-treated cells was significantly lower than cells treated with Ang II alone. Although separately, L or PD had less effect on Ang II–induced colonic fibroblast proliferation, the combination of L+PD significantly reduced Ang II–induced proliferation of these cells. These results suggest that both AT1 and AT2 might drive proliferation in transformed colonocytes and colonic fibroblasts.

Discussion
VD is a prohormone synthesized in the skin from 7-dehydrocholesterol by solar UV radiation, and metabolically transformed in the liver by 25-hydroxylation followed by 1α-hydroxylation in the kidney catalyzed by Cyp27B1 to yield active 1α,25-dihydroxyvitamin D3. VDR is ubiquitous and regulates numerous processes, including cellular proliferation, differentiation, apoptosis, and immunity. Multiple mechanisms have been proposed for its putative chemopreventive actions, and several VDR SNPs suggested as protective (34, 52). AA genotype in rs7968585 or CC genotype in rs731236 decreased the risk of advanced adenomas in the presence of VD supplementation (10). In contrast, other studies have not supported VDR SNPs as modulators of VD effects on colorectal cancer risk (53). Interestingly, the TT genotype in rs731236, in association with an HLA-DRB1*15 allele, was associated with 7.9-fold higher VDR expression compared with the putative protective CC genotype (54).

A WD was shown to induce spontaneous colon tumors in mice that were suppressed by increasing calcium and VD (12). However, the low penetrance and long latency make this model challenging to test chemopreventative agents. Preclinical studies have demonstrated that 1α,25-dihydroxyvitamin D3 or its active analogs can inhibit tumor development in several models of colon cancer, including the AOM model, the AOM/DSS model of colitis-associated colon cancer, and the Apc-mutant Min mouse model (5, 29, 55). A major question remains whether the prohormone VD, when supplemented in the diet, can inhibit tumorigenesis in a sporadic model of colon cancer. Studies using carcinogenic and genetic models have provided conflicting results that have not resolved the controversy (25, 30, 31). The study by Pence and colleagues in DMH-treated rats compared a standard-fat and high-fat diet (30). The latter was similar to our WD, and VD supplementation inhibited tumor development in the high-fat diet, but interestingly not the standard-fat diet (30). In contrast, in a study in Apc-mutant Min mice by Giardina and colleagues, WD did not alter tumor...
development in the setting of standard- or high-fat diet, but the dose of VD in that study was 4-fold lower than the current study. Moreover, colon tumors are infrequent in the Apc-mutant Min mouse model (25). In a study by Irving and colleagues, using the Apc-mutant Pirc rat and the Min mouse, dietary supplemented 25-hydroxyvitamin D3 was also not chemopreventive, but that study only examined rodents fed a standard 5% fat diet (31).

To address the clinically important question of colon cancer prevention in mice on WD, we employed the
Apc<sup>+/−;LoxP</sup>; Cdx2P-Cre mouse model with a conditional Apc exon 14 deletion that closely mimics sporadic human colon cancer (35). We used a WD, rich in saturated and omega-6 fatty acids and relatively deficient in VD and calcium that mimics a high-risk WD (15). This diet is similar in fat composition and concentrations of VD and calcium used by Yang and colleagues, to induce spontaneous colon tumors in mice (12). In the current study, WD increased 1α,25-dihydroxyvitamin D3 and colonic VDR in the Cre+ group and inhibited colon cancer development in the Cre+ VD group. The VD dose of 3,000 IU/kg body weight per day compares with 20 IU/kg body weight per day in humans supplemented with 10,000 IU VD weekly, a standard repletion regiment. Although this dose was well tolerated, with mice showing normal plasma calciums and weight gain, a comparable dose in humans will require further study.

We previously showed that active VD is a negative regulator of renin gene transcription (24). Renin drives the RAS, which is implicated in colon cancer development (22, 56).
We postulated that renin suppression might mediate some chemopreventive effects of VD. Our prior studies in the AOM/DSS model showed that global Vdr deletion upregulated renin and other RAS components in the colon and increased tumor development (6). Studies by other investigators showed increased renin in human colonic adenomas (data accessible at NCBI Geo database, accession GDS2947; ref. 25). We also showed that supplemented VD suppressed colonic renin expression in Vdr wild-type mice treated with AOM/DSS (6). In those studies, however, we did not directly test the antitumor effects of RAS blockade. Furthermore, for the AOM/DSS tumor studies, we employed a Vdr-null mouse to dissect the role of VDR signals. Results of the global Vdr KO and the AOM/DSS model are not readily translatable to humans.

In the current study, in addition to VD supplementation, we blocked the RAS signals with L, a specific inhibitor of angiotensin II type 1A receptors (AT1) to directly address the role of RAS in colon cancer. In prior studies, we showed that L suppressed tumor development in the AOM model (57). We chose a L dose that was well tolerated in preliminary experiments (comparable weight gain at 2 weeks). In our longer-term studies, however, Cre- L and Cre+ L groups both showed delayed weight gain. This was unexpected as we found that a higher L dose of 10 mg/mL in the drinking water was protective in a colitis model (58). Although L blocked RAS as assessed by increased plasma renin and Ang II and concomitantly reduced tumor development, its effect on weight gain confounds a firm conclusion that L is chemopreventive in this model. In this regard, reduced weight gain by caloric restriction suppressed tumor burden in the small intestine of the Apc+/Min mouse, but interestingly not in the colon (59). Future studies with lower L doses will be needed to separate its effects on AT1 blockade from effects on growth. To assess renin-independent antitumor effects of VD, we also included the VD+L group. Unexpectedly, the combination was not chemopreventive, though intriguingly the addition of VD partially mitigated the inhibitory effects of L on weight gain.

To explore potential RAS- and VD-dependent mechanisms that might contribute to chemoprevention, we examined several plasma and colon markers implicated in VDR and RAS signaling. With respect to VDR signaling, we showed that plasma 1α,25-dihydroxyvitamin D3 and colonic VDR were increased in the Cre- VD group, but we showed that plasma 1α,25-dihydroxyvitamin D3 and colon-ic VDR in the Cre- VD group, in contrast to the Cre+ VD group. We speculate that failure to increase plasma 1α,25-dihydroxyvitamin D3 and colonic VDR in the Cre- VD+L group in contrast to effects of VD in the Cre- VD group contributed to the lack of chemopreventive efficacy of VD+L in the Cre+ group.

With respect to RAS signaling, we showed that plasma RAS markers, renin and Ang II, and colonic tumor renin were increased in the Cre+ UN group. Renin is also increased in human and mouse colonic adenomas (data accessible at NCBI Geo database, accession GDS2947, GSE8671, GSE5261; refs. 25–27). Upregulation of the RAS in colon cancer is likely linked to increased Wnt/β-catenin signaling driving expression of RAS components (64). L further increased RAS components, uncovering an inhibitory AT1-dependent feedback mechanism. Plasma renin and Ang II were decreased in the Cre+ VD group, presumably via inhibited renin gene transcription. Interestingly, in the Cre+ VD+L group, renin and Ang II levels were comparable with the Cre+ UN group. VD, by inhibiting renin expression and thereby reducing Ang II, and L, by blocking AT1, leading to counter regulatory feedback increases in plasma renin and Ang II, are predicted to differentially modulate AT1 and AT2 signals. Both AT1 and AT2 expression levels were increased in our model in all Cre+ groups. Increased AT1 and AT2 were previously noted in stromal cells from human colonic tumors (data accessible at NCBI Geo databases, accession GSE39397 and GSE70468; refs. 65, 66). In the AOM model, both AT1 and AT2 appear to promote tumor development (57, 67). In primary human colon cancers, we showed that AT1 and AT2 were upregulated, and in cell culture, these receptors mediated Ang II–induced colonic cell proliferation. Based on the observations that plasma 1α,25-dihydroxyvitamin D3 and colonic VDR did not increase in the Cre- VD+L group, together with failure of VD+L in Cre+ mice to

[Paper continues with further scientific content.]
suppress plasma renin and Ang II suggests that in CRE+VD+L group VDR signals will be lower, whereas RAS signals will be enhanced compared with the CRE+VD group that is expected to contribute to the lack of chemopreventive efficacy of VD+L in CRE+ mice.

ADAM17, an upstream activator of EGFR, is required for robust colon tumor development in this model (13). EGFR in turn is required for β-catenin activation, and both ADAM17 and β-catenin were increased in our model (47). Decreases in β-catenin expression in tumors from the CRE+VD group, compared with the CRE+VD+L group, are consistent with increased 1α,25-dihydroxyvitamin D3 in the CRE-VD group, but not in the CRE-VD+L group. This is noteworthy given the role of β-catenin in regulating both oncogenesis and transcription of RAS signaling components (64). Notch signals, while variably decreased in all supplemented groups, did not provide further insights to distinguish preventive (VD or L monotherapies) versus ineffective therapies (combination VD+L therapy).

Renin can also bind to the (pro)renin receptor (ATP6AP2) that is increased in colon tumors and promotes Wnt/β-catenin signals independent of RAS (68). Given the observed differences in plasma renin between the CRE+VD group and the CRE+VD+L group, increased renin signaling through (pro)renin receptor could also contribute to lack of chemopreventive efficacy seen in the CRE+VD+L group. Hedgehog signaling is also implicated in colon cancer development, and the active VD precursor, cholecalciferol, appears to directly suppress smoothened (SMO) in this pathway (69, 70). Circulating cholecalciferol is expected to be high in the CRE-VD group, but not in the CRE-VD+L group. This is import for the role of β-catenin in regulating both oncogenesis and transcription of RAS signaling components (64). Notch signals, while variably decreased in all supplemented groups, did not provide further insights to distinguish preventive (VD or L monotherapies) versus ineffective therapies (combination VD+L therapy).

In summary, we showed for the first time that RAS is upregulated in the ApCfl/fl Cdx2P-Cre sporadic model of colon cancer. These studies have highlighted the potential importance of systemic and colonic RAS in tumor development. Furthermore, VD or L inhibited development of colon tumors in this model, though the effect of L on weight gain complicates the interpretation of chemopreventive efficacy. These agents exert differential effects on circulating 1α,25-dihydroxyvitamin D3 in CRE mice, and on plasma renin and Ang II in CRE mice. Although L inhibits increases in 1α,25-dihydroxyvitamin D3 and increases proinflammatory plasma renin and Ang II, systemic and/or colonic AT1 blockade appears to play an essential role in L’s chemopreventive effects. Because in the CRE+VD group, Ang II and renin were significantly downregulated, this suggests RAS blockade plays an important role in VD chemopreventive effects. A schema summarizing WD-promoted RAS signals promoting ApC mutant colonic tumors and tumor suppression by VD or L is shown in Fig. 6D. Additional studies will be needed to dissect the potential roles of (pro)renin receptors and hedgehog signaling in colon cancer.

Future studies will be directed to uncover the underlying tumor-associated mechanisms that upregulate RAS signals and dissect the respective roles of AT1 and AT2 in colon cancer development. Although prior studies in humans failed to show that low-dose VD could prevent adenoma recurrence, this study suggests that higher VD doses, perhaps in individuals with VD-responsive VDR SNPs, might be required to protect against colon cancer (8, 10). Moreover, VD and L are widely available and well tolerated. These agents could potentially be rapidly advanced to clinical trials for colon cancer prevention, especially in high-risk individuals such as patients with a history of colon cancer or advanced adenomas. Further studies in humans would be needed to assess whether combination therapy might be antagonistic as suggested by our mouse studies.

Disclosure of Potential Conflicts of Interest
V.J. Konda reports receiving honoraria from the speakers’ bureau of Mauna Kea Technologies. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: U. Dougherty, R. Mustaﬁ, J. Pekow, M. Bissonnette
Development of methodology: U. Dougherty, R. Mustaﬁ, H.I. Haider, M. Bissonnette
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): U. Dougherty, R. Mustaﬁ, A. Khalil, J. Hart, V.J. Konda, J. Pekow, M. Bissonnette
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): U. Dougherty, R. Mustaﬁ, H.I. Haider, A. Khalil, J.S. Souris, L. Joseph, W. Zhang, J. Pekow, Y.C. Li, M. Bissonnette
Writing, review, and/or revision of the manuscript: U. Dougherty, R. Mustaﬁ, A. Khalil, J.S. Souris, L. Joseph, W. Zhang, J. Pekow, Y.C. Li, M. Bissonnette
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): U. Dougherty, R. Mustaﬁ, M. Bissonnette
Study supervision: U. Dougherty, R. Mustaﬁ, M. Bissonnette

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Losartan and Vitamin D Inhibit Colonic Tumor Development in a Conditional Apc-Deleted Mouse Model of Sporadic Colon Cancer

Urszula Dougherty, Reba Mustafi, Haider I. Haider, et al.


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