The Renin–Angiotensin System Mediates EGF Receptor–Vitamin D Receptor Cross-Talk in Colitis-Associated Colon Cancer

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Abstract

**Purpose:** We previously showed that EGF receptor (EGFR) promotes tumorigenesis in the azoxymethane/dextran sulfate sodium (AOM/DSS) model, whereas vitamin D suppresses tumorigenesis. EGFR–vitamin D receptor (VDR) interactions, however, are incompletely understood. Vitamin D inhibits the renin–angiotensin system (RAS), whereas RAS can activate EGFR. We aimed to elucidate EGFR–VDR cross-talk in colorectal carcinogenesis.

**Experimental Design:** To examine VDR–RAS interactions, we treated Vdr+/+ and Vdr−/− mice with AOM/DSS. Effects of VDR on RAS and EGFR were examined by Western blotting, immunostaining, and real-time PCR. We also examined the effect of vitamin D3 on colonic RAS in Vdr+/+ mice. EGFR regulation of VDR was examined in hypomorphic EgfrWa2 (Wa2) and Egfrwild-type (WT) mice. Angiotensin II (Ang II)–induced EGFR activation was studied in cell culture.

**Results:** Vdr deletion significantly increased tumorigenesis, activated EGFR and β-catenin signaling, and increased colonic RAS components, including renin and angiotensin II. Dietary VD3 supplementation suppressed colonic renin. Renin was increased in human colon cancers. In studies in vitro, Ang II activated EGFR and stimulated colon cancer cell proliferation by an EGFR-mediated mechanism. Ang II also activated macrophages and colonic fibroblasts. Compared with tumors from EgfrWa2 mice, tumors from Egfrwild-type mice showed upregulated Snail1, a suppressor of VDR, and downregulated VDR.

**Conclusions:** VDR suppresses the colonic RAS cascade, limits EGFR signals, and inhibits colitis-associated tumorigenesis, whereas EGFR increases Snail1 and downregulates VDR in colonic tumors. Taken together, these results uncover a RAS-dependent mechanism mediating EGFR and VDR cross-talk in colon cancer. Clin Cancer Res; 20(22); 5848–59. ©2014 AACR.

Introduction

Inflammation is recognized as an essential promoter of malignant transformation (1). Ulcerative colitis, an inflammatory bowel disease (IBD) of the colonic epithelium, is associated with increased colon cancer risk (2). The duration and severity of inflammation modulate this risk (2). Because diagnosis of early colon cancer in ulcerative colitis is challenging and the prognosis for invasive disease limited, increasing efforts have focused on chemoprevention. Vitamin D is a potential chemopreventive agent in IBD-associated colon cancer (3). This prohormone is converted to active 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3] by hepatic 25-hydroxylase and renal and extra-renal 1α-hydroxylase. 1,25(OH)2D3 binds the vitamin D receptor (VDR) to transduce biologic signals in diverse tissues, including the colon (4).

The azoxymethane/dextran sulfate sodium (AOM/DSS) model of inflammation-associated colon cancer mimics many features of IBD-associated colon cancer (5). Animals receiving AOM/DSS develop colitis followed by colon cancer. Colonocytes, initiated by the mutagen azoxymethane, are expanded by epithelial regeneration that follows DSS-induced colonic epithelial damage. In prior AOM/DSS studies using hypomorphic EgfrWa2 mice, we showed that EGFR receptor (EGFR) was required for Western diets to promote tumorigenesis, whereas others have shown that
EGFR inhibitors reduce stem cells in experimental colon cancer (6, 7). We also demonstrated that vitamin D suppresses dysplasia in this model (8), whereas VDR deletion increased DSS colitis (9). These studies suggest that decreased VDR signals exaggerate colonic proinflammatory cytokines (10). In addition, we recently demonstrated that colonic epithelial VDR maintains intestinal mucosal barrier integrity to prevent microbial inflammation (11). Thus, these data indicate that EGFR and VDR exert opposing effects on colonic inflammation and tumorigenesis. Furthermore, studies in cell culture have identified an important opposing VDR–EGFR cross-talk in colon cancer cells (12–14). Investigations to dissect mechanisms of this cross-talk in vivo in colon tumorigenesis, however, have not been reported.

The renin–angiotensin system (RAS) regulates systemic vascular tone and sodium balance (15). RAS is also mitogenic and angiogenic and contributes to neoplastic growth in breast, ovary, lung, prostate, and pancreatic cancers (16). Several RAS components, including renin, angiotensin-converting enzyme (ACE), and angiotensin II (Ang II), are locally upregulated in tumors. These components are also expressed in human colonic mucosa (17). Furthermore, epidemiologic studies suggest that inhibitors of the RAS reduce colonic tumorigenesis (18). In prior analyses, we demonstrated that vitamin D signals suppress renin transcription and that this limits macrophage-associated inflammation (19–21). The macrophage is implicated in DSS inflammation (22). In the current study, we therefore asked whether vitamin D and the VDR regulate colonic RAS signals modulated by Western diet or inflammation-associated colon cancer. We used Vdr−/− and Vdr+/+ mice and vitamin D supplementation to dissect VDR regulation of RAS signals. Because RAS can activate EGFR, we also examined VDR regulation of EGFR in colonic tumorigenesis. To examine the potential translational relevance of our findings, we measured renin expression in sporadic human colonic tumors.

Fibroblasts and macrophages are important stromal cells that drive cancer cell proliferation (23). As Ang II is a mitogen and can transactivate EGFR in noncolonic cells (24), we asked whether the RAS signaling could activate EGFR and stimulate proliferation of colon cancer cells and fibroblasts. Furthermore, as RAS can induce inflammation (25), we examined the effects of Ang II on TNFx in macrophages.

Finally, as studies in vitro suggest that EGFR can also regulate VDR (12, 13), we investigated potential EGFR regulation of VDR using archived tumors induced by AOM/DSS in EgfrWT and EgfrWaved2/Waved2 mice. The Waved2 Egfr mutation abrogates nearly 90% of receptor kinase activity in vitro (26). Furthermore, EGFR can upregulate Snail1 in vitro, and this transcription factor was shown to suppress VDR in colon cancer cells (27). We therefore also investigated EGFR regulation of Snail1 in AOM/DSS-induced tumors. Taken together, our findings uncover a functional VDR-regulated RAS pathway in vivo that controls EGFR signals in colonic carcinogenesis.

Materials and Methods

Materials

A defined Western style diet containing 20% fat was used for the experiments in Vdr−/− and Vdr+/+ mice. This diet, which included 2% calcium and 20% lactose to prevent hypocalcemia in Vdr-null mice, was modified from a previously described defined diet (6, 19). Azoxymethane was obtained from Midwest Research, the NCI Chemical Carcinogen Reference Standard Repository (Kansas City, MO). Tarceva was obtained from OSI Pharmaceuticals. Antibodies for immunostaining and Western blotting and molecular reagents for real-time PCR are provided in the Supplementary Data.

Methods

Experimental animal protocol for Vdr+/− and Vdr+/+ mice. We used 20 Vdr+/− and 20 Vdr+/+ mice (28), backcrossed 10 generations to CD-1 background, to dissect the role of VDR in colonic tumorigenesis. Mice were 6 to 10 weeks of age and included a comparable number of males and females in each genotype. For each genotype, 15 mice were treated with azoxymethane (7.5 mg/kg) and 5 mice received saline (azoxymethane vehicle) at days 1 and 14. After the second azoxymethane treatment, mice received saline (azoxymethane vehicle) at days 1 and 14.
treated mice received water only. The DSS concentration was chosen, as in preliminary studies, 2% DSS caused 80% mortality in Vdr−/− mice. Following 5 days, DSS mice received tap water for 2 weeks. The mice received 3 cycles of DSS, and colitis disease index was assessed for each cycle (29). Twenty-four weeks after the initial azoxymethane injection, mice were anesthetized and treated with 30% H2O2 and vanadate solution as described and sacrificed 20 minutes later (30). Tumors were measured with a micrometer, harvested, and fixed in 10% buffered formalin. Separate tumor aliquots were flash frozen in liquid nitrogen for RNA or protein. Tumors were classified according to histologic grade by the gastrointestinal pathologist (J. Hart; ref. 31). Distal colonic mucosa, cleared of any tumors, was scrape-isolated and aliquots frozen for protein and RNA. The remaining colons were fixed flat in 10% formalin for immunostaining. The Institutional Animal Care and Use Committee (IACUC) at the University of Chicago (Chicago, IL) approved all animal studies.

**Experimental animal protocol for Vdr wild-type CF-1 mice.** CF-1 female mice, age 4 to 6 weeks, were given azoxymethane (7.5 mg/kg body weight) or saline (azoxymethane vehicle) followed by one cycle of DSS (azoxymethane-treated) or water (saline-treated) for 5 days. Mice were then fed a Western diet (20% fat, n = 10) alone or Western diet supplemented with cholecalciferol (500 μg/kg diet, n = 10). The Western diet and cholecalciferol dose were previously shown to promote or inhibit AOM/DSS-induced colonic tumorigenesis, respectively (6, 32). Twelve weeks after Western diet initiation, mice were sacrificed and mucosa from left colon was harvested and RNA extracted.

** Archived colonic tissue.** **Mouse tissue** For some experiments, we used colonic tissue banked from a previous study (6). The prior study investigated the role of EGFR in Western diet–promoted colon cancer in the AOM/DSS model using Egfrwild-type and EgfrWa2 mice (6). The Wa2 mutation abrogates >80% receptor kinase activity in vitro (26).

**Human tissue** For studies involving sporadic human colon cancers, we obtained fresh flash-frozen tumors and adjacent normal-appearing mucosa dissected free from underlying muscle from the Human Tissue Resource Center according to histologic grade by the gastrointestinal pathologist. The Institutional Animal Care and Use Committee (IACUC) at the University of Chicago under an approved IRB protocol 10-209-A.

**Cell culture and proliferation.** Low-passage CCD-18Co colonic fibroblasts and HT29, HCT116, and DLD1 human colon cancer cells and RAW 264.7 murine macrophage cells were obtained from ATCC. These cell lines were authenticated by ATCC using short tandem repeat DNA fingerprinting. Cells were cultured at 37°C in a humidified atmosphere of 5%CO2, 95% air under conditions recommended by ATCC. Cells were treated with Ang II or vehicle or pretreated with losartan, gefitinib, or Tarceva at the indicated concentrations. For RNAi experiments, cells were pretreated for 24 hours with 20 nmol/L Egfr siRNA or a scrambled control. Cell proliferation was measured by WST-1 assay as suggested by the manufacturer (see Supplementary Methods).

**Real-time PCR.** RNA was extracted from snap-frozen tissue using Qiagen miRNeasy Mini Kit that captures total RNA including miRNA. Samples were homogenized with a Polytron and loaded onto an RNA-binding spin column, washed, digested with DNase I, and collected in 30 μL elution buffer. RNA samples were examined by Agilent chip for RNA purity and quantified by Ribogreen. Real-time PCR was performed as previously described (ref. 6; see Supplementary Methods for details).

**Immunohistochemistry.** Tumors and normal colon were immunostained as previously described (ref. 6; see Supplementary Methods for details). For semiquantitative analysis of immunostaining, we used a Leica DM2500 microscope equipped with a CCD camera (Q Imaging Retiga EXI Fast1394) and captured images with Image Pro Plus (V6.3) software. 3,3′-Diaminobenzidine (DAB) staining was analyzed using Fiji (ImageJ V1.48k) and the H DAB deconvolution plug-in (33, 34). Color-specific thresholds were adjusted to distinguish brown (DAB-positive) and blue (DAB-negative) cells and to calculate the ratio of positively stained cells. At least 5 fields per tumor and 3 tumors per group were scanned for quantitation. For nuclear β-catenin, Snail1, and VDR, we used Immunohistochemistry software (35).

**Western blotting.** Colonic mucosal lysates and lysates from tumors of comparable stage were used for Western blotting. Proteins were extracted in SDS-containing Laemmli buffer, quantified by RC-DC protein assay, and subjected to Western blotting as previously described (ref. 6; see Supplementary Methods for details).

**ELISA.** Ang II was assayed by ELISA in lysates prepared from colonic mucosa from left colon as suggested by the manufacturer. TNFz was assayed in RAW264.7 conditioned media by ELISA following the manufacturer’s directions. Amphiregulin was measured in conditioned media from HT29 cells by ELISA following the manufacturer’s directions.

**Statistical analysis.** Tumor incidence was defined as the percentage of mice with at least one tumor and compared between genotypes using the Fisher exact test. Western blotting densitometry and ELISA data were summarized as mean ± SD and compared by the unpaired Student t test. Reverse transcriptase reactions were run in duplicate and assayed in triplicate and Ct values averaged. Untransformed C Ct values were compared between groups (36). Relative abundance, expressed as 2−ΔΔCt, was calculated by exponentiating differences in Ct between mucosa from AOM/DSS-treated mice and mucosa from vehicle-treated mice with values normalized to β-actin mRNA as a reference gene. For all statistical analyses, P < 0.05 was considered statistically significant.

**Results**

**VDR suppresses inflammation and tumor development**

To examine the role of the VDR in colitis-associated cancer, we compared tumorigenesis in Vdr+/+ and Vdr−/− mice. Figure 1 summarizes the protocol (Fig. 1A) and clinical colitis score for the third DSS cycle (Fig. 1B). Clinical disease activity scores were low in Vdr+/+ mice, reflecting the low concentration of DSS chosen to prevent high mortality in Vdr−/− mice (80% mortality with 2% DSS). In agreement with previous studies, the prior study investigated the role of EGFR in Western diet–promoted colon cancer in the AOM/DSS model using Egfrwild-type and EgfrWa2 mice (6). The Wa2 mutation abrogates >80% receptor kinase activity in vitro (26).

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with prior studies, Vdr deletion increased colonic inflammation induced by DSS (9). All mice in the Vdr+/− group developed tumors (adenomas or cancers), compared with only 47% in Vdr+/+ group (n = 15 mice per genotype, P = 0.001; Fig. 1C). While tumor burdens were modest secondary to low DSS concentrations and calcium supplementation, Vdr-dependent differences in tumor incidence were significant, consistent with differences in inflammation (Fig. 1B; ref. 37). VDR deletion increased tumor incidence (n = 15 AOM/DSS-treated mice per genotype) and appeared to increase tumor progression to cancer (27% vs. 7%, P = 0.1). There were 11 adenomas and 4 cancers in the Vdr+/− group (n = 15 total) and 6 adenomas and 1 cancer in the Vdr+/+ group (n = 7 total). D, tumor size. Mean ± SD (+, P < 0.05, compared with Vdr+/−).

VDR negatively regulates EGFR signals

We next asked whether VDR modulates EGFR signals, as EGFR and VDR have opposing effects on tumorigenesis in this model. As shown in Fig. 2A and quantified in Fig. 2B, VDR deletion significantly increased activation of EGFR and ErbB2 and stimulated effectors AKT, ERK, and STAT3. While β-catenin plays a critical role in colonic tumorigenesis, in prior studies, we showed that EGFR controls β-catenin in AOM/DSS tumors in vivo, consistent with findings in colon cancer cells in vitro (6, 38). In agreement with these studies, we found that VDR deletion, which increases EGFR signals, also significantly enhanced nuclear β-catenin in malignant colonocytes, 49.2% ± 11.3% in tumors from Vdr−/− versus 28.8% ± 7.1% in tumors from Vdr+/− mice (Fig. 2C, *P < 0.05, n = 4 adenomas per genotype). Not surprisingly, β-catenin targets, Myc and cyclin D1 (6, 39, 40), were also increased in Vdr−/− tumors (Fig. 2A and B) consistent with reports that vitamin D signals suppress Myc and cyclin D1 in colon cancer cells (41, 42).

VDR negatively regulates RAS in AOM/DSS colonic tumors

The RAS is a potential link between VDR and EGFR signals, as vitamin D is a negative regulator of the RAS; and the RAS in turn can transactivate EGFR (19, 24). Furthermore, the RAS is mitogenic and angiogenic for many tumors, and RAS components are increased in other neoplastic tissue (16). We, therefore, examined the effect of Vdr deletion on colonic RAS by staining tumors for RAS components. Renin was greater in malignant colonocytes from Vdr−/− mice than in Vdr+/+ mice (Fig. 3A, top). AT1 receptor expression was also greater in tumors from Vdr−/− mice than in Vdr+/+ mice and was readily detectable in tumor stromal cells (Fig. 3A, middle). As the RAS is known to drive blood vessel development, we also examined nestin-1, a marker of angiogenesis. Nestin-1 was 2.6 ± 0.4-fold greater in tumors from Vdr−/− than in Vdr+/+ mice (Fig. 3A, bottom).

VDR regulation of colonic RAS—field effect

Molecular abnormalities in colons harboring tumors are frequently widespread, with derangements in normal-appearing mucosa (43). To investigate more generalized "field effects," we examined mRNA levels of several of the RAS components in distal colonic mucosa. In mice treated with saline alone (no AOM/DSS), ACE transcripts were elevated in Vdr−/− compared with Vdr+/+ mice (Fig. 3B).
With AOM/DSS treatment, angiotensinogen (Agt), renin (Ren), ACE, and Ang II receptor type 1A (Agtr1a) transcripts were upregulated in Vdr<sup>−/−</sup> mice compared with Vdr<sup>+/+</sup> mice (Fig. 3B). Protein levels were also significantly higher in Vdr<sup>−/−</sup> mice as shown in Fig. 3C and quantified in Fig. 3D. Levels of colonic mucosal Ang II, a major RAS effector, were significantly elevated in AOM/DSS-treated mice, compared with vehicle-treated mice matched for Vdr genotype. Increases were greater in Vdr<sup>−/−</sup> mice (Fig. 3E), consistent with greater increases in upstream RAS components in Vdr<sup>−/−</sup> mice. Colonic mucosal VEGF protein levels were also elevated in AOM/DSS-treated Vdr<sup>−/−</sup> mice compared with Vdr<sup>+/+</sup> mice (Fig. 3C and D). The latter results are consistent with differences in tumor nestin-1 levels by Vdr genotype (Fig. 3A) and with prior reports in other tissue of positive VEGF regulation by Ang II and negative regulation by VDR (44, 45). To assess the effects of supplemental vitamin D on colonic mucosal RAS, we measured transcripts of renin and angiotensinogen in colonic mucosa prepared from AOM/DSS- or saline-treated Vdr<sup>+/+</sup> CF-1 mice fed Western diet or Western diet supplemented with cholecalciferol. As shown in Fig. 3F, cholecalciferol significantly decreased expression of these genes in both control mice (no AOM/DSS) and AOM/DSS-treated mice. Thus, VDR gain of function inhibits RAS signaling, whereas VDR loss of function enhances colonic RAS signaling. With only 5 mice in the AOM/DSS alone group and 5 mice in the AOM/DSS + VD3 group, the study was not powered for tumor prevention. We noted, however, that there were 4 tumors in Western diet alone group versus 1 in the VD3-treated group (P = 0.1). To assess the translational relevance of these observations, we examined renin expression in human colon cancers. EGFR (pEGFR) activation and renin levels were increased in human colon cancers, emphasizing the potential relevance of upregulated RAS in sporadic colonic tumorigenesis (Fig. 3G and H). VDR levels were variable and not different in human tumors, suggesting that supplemental vitamin D by binding VDR might suppress tumor-associated RAS that we speculate promotes colonic tumorigenesis.

**EGFR mediates Ang II–induced colon cancer cell and colonic fibroblast proliferation**

We used cell culture to dissect Ang II–induced responses in malignant and nonmalignant colonic cells. Colon cancer cells, colonic fibroblasts, and macrophage cells express AT1 receptors (Fig. 4A). Ang II stimulated proliferation of HT29, HCT116, and DLD1 colon cancer cells and colonic fibroblasts (Fig. 4B). Losartan, a specific AT1 inhibitor, blocked Ang II–induced mitogenic effects (Fig. 4B). We infer that Ang II mitogenic effects are mediated by EGFR, as gefitinib blocked Ang II–induced proliferation (Fig. 4C). Similar results were obtained with Tarceva (Supplementary Fig. S1). Receptor knockdown with EGFR siRNA also blocked Ang II–induced proliferation (Fig. 4C). Basal proliferation was also controlled by EGFR, as treatment with gefitinib, Tarceva, or EGFR siRNA alone also reduced HT29 cell

![Figure 2. VDR deletion stimulates EGFR signals and increases nuclear β-catenin accumulation in tumors.](image-url)
proliferation (Fig. 4C and Supplementary Fig. S1). Ang II was shown previously to activate EGFR in noncolonic cells (24). In this study, we showed that Ang II activated EGFR signals in HT29 cells (Fig. 4D and E). In data not shown, Ang II also transactivated EGFR in HCT116 and DLD1 cells.

RAS signals (Ang II) induce inflammation in macrophage cells

The macrophage is implicated in DSS inflammation (22). We observed that macrophages were more abundant in tumors from Vdr−/− mice (Fig. 5A and B).
shown in Fig. 5C, colonic TNFα was increased in vehicle-treated $Vdr^{-/-}$ animals, compared with $Vdr^{+/+}$ mice. Following AOM/DSS treatment, colonic mucosal IL1β, IL6, and TNFα were upregulated, with significantly greater increases in $Vdr^{-/-}$ mice (Fig. 5C). Macrophage RAW264.7 cells express AT1 receptors (Fig. 4A). To directly examine the effect of the RAS on macrophage function, we treated RAW264.7 cells with Ang II. As expected, Ang II significantly increased TNFα secretion and the AT1 inhibitor losartan blocked this increase (Fig. 5D).

**EGFR signals suppress VDR in AOM/DSS colonic tumors**

While we demonstrated that VDR sufficiency inhibits EGFR signals in the AOM/DSS model (Fig. 2), we next asked the converse: does EGFR control VDR in this model? To address this question, we examined tumors from $Egfr^{+/+}$ and hypomorphic $Egfr^{Wa2/Wa2}$ mice. Nuclear VDR levels in malignant colonocytes were reduced in $Egfr^{+/+}$ mice, whereas nuclear VDR levels were maintained in malignant colonocytes from $Egfr^{Wa2/Wa2}$ mice, with positive nuclei in 17.1% ± 3.0% versus 30.2% ± 11.5%, respectively.
tumors from Vdr+/− (left) and Vdr−/− mice (right) were stained with anti-CDC antibodies. B, macrophage quantification, †, P < 0.05 compared with Vdr+/− mice, n = 3 tumors per group. C, proinflammatory cytokine levels. Colonic mucosal TNFα is increased in vehicle-treated (control, no AOM/DSS) Vdr−/− compared with Vdr+/− mice. IL1β, IL6, and TNFα are further increased in colonic mucosa from AOM/DSS-treated mice, with greater increases in Vdr−/− mice compared with Vdr+/− mice (†, P < 0.001, compared with vehicle-treated Vdr+/−; ††, P < 0.05; † ††, P < 0.005, compared with vehicle-treated Vdr+/− mice; † † † †, P < 0.0001, compared with AOM/DSS-treated Vdr+/−; † † † † †, P < 0.0001, compared with AOM/DSS-treated Vdr+/−; n = 4 control mucosa per genotype or 4 tumors per genotype). D, Ang II induces TNFα in macrophage cells. RAW264.7 cells were pretreated with 1 μmol/L losartan or vehicle for 2 hours and then treated with indicated concentrations of Ang II or vehicle and TNFα assayed by ELISA (†, P < 0.05 compared with untreated cells). Cell culture results were replicated in 3 independent platings.

Figure 5. VDR deletion increases macrophage infiltration and inflammation in vivo; Ang II induces macrophage TNFα in vitro. A, macrophage staining. Tumors from Vdr+/− (left) and Vdr−/− mice (right) were stained with anti-CDC antibodies. B, macrophage quantification, †, P < 0.05 compared with Vdr+/− mice, n = 3 tumors per group. C, proinflammatory cytokine levels. Colonic mucosal TNFα is increased in vehicle-treated (control, no AOM/DSS) Vdr−/− compared with Vdr+/− mice. IL1β, IL6, and TNFα are further increased in colonic mucosa from AOM/DSS-treated mice, with greater increases in Vdr−/− mice compared with Vdr+/− mice (†, P < 0.001, compared with vehicle-treated Vdr+/−; ††, P < 0.05; † ††, P < 0.005, compared with vehicle-treated Vdr+/− mice; † † † †, P < 0.0001, compared with AOM/DSS-treated Vdr+/−; † † † † †, P < 0.0001, compared with AOM/DSS-treated Vdr+/−; n = 4 control mucosa per genotype or 4 tumors per genotype). D, Ang II induces TNFα in macrophage cells. RAW264.7 cells were pretreated with 1 μmol/L losartan or vehicle for 2 hours and then treated with indicated concentrations of Ang II or vehicle and TNFα assayed by ELISA (†, P < 0.05 compared with untreated cells). Cell culture results were replicated in 3 independent platings.

EGFR signals in AOM/DSS colonic tumors induce Snail1, a negative regulator of VDR expression

To investigate potential EGFR-dependent mechanisms that might suppress VDR in colon tumors, we examined the transcription factor Snail1. Other investigators have shown that EGFR can upregulate Snail1 and that Snail1 in turn can suppress VDR (27, 46). As shown in Fig. 6C and D, Snail1 was increased in tumors from Egfrwild-type mice compared with EgfrWa2/Wa2 mice. EGFR signals also increased Snail1 in HT29 colon cancer cells (Supplementary Fig. S2). Thus, EGFR induction of Snail1 is a potential mechanism by which EGFR suppresses VDR in colonic tumorigenesis (Fig. 6E).

Discussion

Prior studies showed that EGFR promotes colonic tumor development, whereas vitamin D inhibits tumorigenesis in models of colon cancer (6, 8, 32, 47–49). To examine how VDR alters colonic tumorigenesis and EGFR signals, we treated Vdr−/− and Vdr+/+ mice with AOM/DSS. VDR signals suppressed colonic tumorigenesis, whereas VDR deletion increased tumor development, enhanced EGFR and β-catenin signals, and upregulated the colonic RAS. The effects of VDR deletion on nuclear β-catenin levels are in agreement with prior investigations by our laboratory and others (47, 50). Because β-catenin plays a critical role in colonic tumorigenesis, increased nuclear β-catenin is likely a key factor in enhanced tumorigenesis that occurs in VDR-null mice. The effects of VDR deletion on renin in the colon are consistent with prior reports that vitamin D is a negative transcriptional regulator of renin (20). The potential translational relevance of these studies is emphasized by our finding that renin is upregulated in human colon cancers. Mechanistically, Ang II transactivated EGFR and stimulated colon cancer cell proliferation by an AT1-mediated EGFR-dependent mechanism. In preliminary studies, Ang II caused a 25% increase in amphiregulin (AREG) secretion in HT29 cells (P < 0.05). RAS signals also activated fibroblasts and macrophages, key cellular components of tumor stroma (23). Thus, in colitis-associated colon cancer, the RAS and EGFR pathways are upregulated and their signals are negatively controlled by VDR (Fig. 6E). Taken together, these findings highlight a potentially important VDR-dependent mechanism that suppresses EGFR and RAS.
signaling and likely contributes to chemoprevention by vitamin D.

RAS components, including renin, ACE, Ang II, and AT1, have been detected in many tissues, including colon (17) and implicated in the development of breast, ovary, lung, and prostate cancer (16). Antihypertensive agents that block the RAS signals may inhibit colonic or pancreatic tumorigenesis in humans (18, 51). In the current report, we showed that colonic RAS components were upregulated in AOM/DSS-treated Vdr−/− mice. These changes reflected...
generalized field effects that we predict promote growth of mutated colonocytes. Interestingly, renin upregulation was detected in transforming colonocytes, whereas AT1 receptors were increased in tumor stroma. These findings uncover potentially important paracrine mechanisms in the microenvironment that drive stromal cell–cancer cell cross-talk. Presumably, release of angiotensinogen, renin, and ACE into the extracellular space in colonic mucosa would increase Ang II to stimulate malignant colonocytes and stromal cells. These local RAS paracrine networks are still little understood (52). In contrast to Vdr deletion, dietary supplementation with cholecalciferol suppressed colonic mucosal renin and angiotensinogen in both control and AOM/DSS-treated Vdr−/− mice fed a Western diet. This dose of cholecalciferol was previously shown to inhibit AOM/DSS tumorigenesis (32). In agreement with these results, in prior studies, we showed that 1α,25 dihydroxyvitamin D3 inhibited increases in inflammation-induced angiotensinogen in other tissues (33).

AOM/DSS colonic tumorigenesis is promoted by inflammation and TNFα plays a pathogenic role (54). We showed that Ang II increased TNFα secretion from the macrophage by an AT1-mediated mechanism. The macrophage is a major source of TNFα in tumor stroma and contributes to inflammation (22). We also showed that VDR suppresses accumulation of tumor-associated macrophages and reduces proinflammatory cytokine release as both were increased in Vdr−/− mice. In prior studies, we showed that vitamin D hormone inhibited TNFα release from macrophage (10). We speculate that suppression of macrophage recruitment and activation are likely essential for the antineoplastic effects of VDR in this model.

Endothelial cells and fibroblasts also express AT1 receptors and are important in tumor progression (23, 55, 56). Increases in tumor angiogenesis (detected by nestin-1 staining) in Vdr−/− mice are consistent with the upregulated RAS in these mice, as RAS is a known driver of angiogenesis. Ang II also stimulates colonic fibroblast proliferation by a losartan-sensitive mechanism. Thus, VDR inhibition of the RAS likely contributes to many of the antiproliferative, anti-angiogenic, and anti-inflammatory effects of VDR. Further supporting the importance of the RAS in this model, AT1 deletion mitigates DSS colitis (57). While the RAS signals promoting proliferation could also contribute to healing DSS colitis, presumably enhanced inflammation and proliferation of transforming cells are dominant over healing DSS colitis. In addition to the current report showing VDR suppression of nuclear β-catenin, EGFR signals, and colonic renin transcription, VDR signals have been shown to inhibit cell cycling and increase apoptosis in colon cancer cells (58, 59). Other potential chemopreventive mechanisms involving VDR warrant future investigation. In this regard in preliminary studies, Vdr deletion increased Notch and Hedgehog signaling, two other oncogenic pathways in colon cancer.

In prior studies, we demonstrated that EGFR signals play a critical role in colonic tumorigenesis (6, 48, 49). The AT1 receptor can transactivate EGFR (24). Here, we established that Ang II transactivates EGFR in colon cancer cells and increases proliferation by an EGFR-dependent mechanism. Thus, by suppressing colonic renin, we predict that VDR signals would inhibit EGFR activation by the RAS. We also demonstrated that EGFR signals suppressed VDR expression in tumors, confirming in vivo a novel antagonistic cross-talk between EGFR and VDR in colon cancer development. Interestingly, in the azoxymethane rat model (49), VDR downregulation was mitigated in tumors from animals supplemented with gefitinib (Supplementary Fig. S3). Thus, EGFR signals downregulate VDR in a model inflammation-associated colon cancer and perhaps also in azoxymethane-induced tumors, a model of sporadic colon cancer. In sporadic human colon cancers, we found that renin and phospho-active EGFR were increased, whereas VDR expression was variable in agreement with other studies (60). In addition, we showed that EGFR signals upregulated Snail1, a transcription factor important in tumor epithelial-to-mesenchymal transition, consistent with our prior studies (61). Other investigators showed that Snail1 was upregulated in the AOM/DSS model (62). Because Snail1 can suppress VDR transcription (27), we speculate that Snail1 upregulation may contribute to VDR downregulation by EGFR in AOM/DSS tumorigenesis (Fig. 6E; refs. 27, 62). In preliminary studies, we determined that EGF induced Snail1 in colon cancer cells in vitro (Supplementary Fig. S2). Snail1 upregulation by EGFR signals was not accompanied by reductions in VDR (Supplementary Fig. S2) in cell culture, however, suggesting that our in vivo conditions in human colon cancer cells were insufficient to mimic EGF-induced VDR downregulation that we observed in vivo in mouse model of inflammation-associated colon cancer. This may reflect differences between human colon cancer and the mouse model. In addition to our findings of EGFR and Snail1, several other mechanisms have been proposed to inhibit VDR signaling (63).

In summary, using genetic approaches and animal models of colon cancer, we have experimentally identified a novel mechanism involving RAS that may mediate the colon cancer chemopreventive effects of vitamin D. These in vivo results extend prior findings in cell culture, demonstrating an important cross-talk between VDR and EGFR in colonic tumorigenesis (12–14). Future studies to quantify the magnitude of RAS inhibition to the anti-inflammatory and chemopreventive effects of vitamin D are warranted. We speculate that the RAS may play a critical role in human IBD-associated colon cancer and that vitamin D, together with RAS inhibitors, might provide a useful chemopreventive strategy for this high-risk group.

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

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www.aacrjournals.org Clin Cancer Res; 20(22) November 15, 2014 5857

Published OnlineFirst September 11, 2014; DOI: 10.1158/1078-0432.CCR-14-0209

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Grant Support

These studies were funded, in part, by the following grants: P30DK42086 (Digestive Diseases Research Core Center), CA036745, CA141092 (M. Bissonnette), CA097540 (G.S. Karczmar, A. Wyrwicz).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked this fact.

Received January 26, 2014; revised July 1, 2014; accepted July 19, 2014; published OnlineFirst September 11, 2014.

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Clinical Cancer Research

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