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Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis

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The inhibitory effects of vitamin D on colitis have been previously documented. Global vitamin D receptor (VDR) deletion exaggerates colitis, but the relative anticolitic contribution of epithelial and nonepithelial VDR signaling is unknown. Here, we showed that colonic epithelial VDR expression was substantially reduced in patients with Crohn’s disease or ulcerative colitis. Moreover, targeted expression of human VDR (hVDR) in intestinal epithelial cells (IECs) protected mice from developing colitis. In experimental colitis models induced by 2,4,6-trinitrobenzenesulfonic acid, dextran sulfate sodium, or CD4+CD45RBhi T cell transfer, transgenic mice expressing hVDR in IECs were highly resistant to colitis, as manifested by marked reductions in clinical colitis scores, colonic histological damage, and colonic inflammation compared with WT mice. Reconstitution of Vdr-deficient IECs with the hVDR transgene completely rescued Vdr-null mice from severe colitis and death, even though the mice still maintained a hyperresponsive Vdr-deficient immune system. Mechanistically, VDR signaling attenuated PUMA induction in IECs by blocking NF-κB activation, leading to a reduction in IEC apoptosis. Together, these results demonstrate that gut epithelial VDR signaling inhibits colitis by protecting the mucosal epithelial barrier, and this anticolitic activity is independent of nonepithelial immune VDR actions.

Introduction

Ulcerative colitis (UC) and Crohn’s disease (CD) are major chronic inflammatory bowel diseases (IBDs) in humans. Although the etiology and pathogenesis of IBD remain uncertain, it is believed that derangements in the complex interplay among genetic, environmental, microbial, and immune factors contribute to these disorders (1). Impaired gut mucosal barrier function is thought to be a significant pathogenic factor leading to intestinal hyperpermeability in IBD (2). The epithelial barrier consists of a monolayer of epithelial cells with intercellular junctions between adjacent cells that seal the paracellular space and regulate barrier permeability (3). This barrier prevents harmful solutes, microorganisms, toxins, and luminal antigens from entering the body (4). Compromised barrier function results in the invasion of luminal antigens and bacteria into the lamina propria, which triggers an immune response that leads to chronic colonic inflammation (5).

Aberrant apoptosis of intestinal epithelia cells (IECs) is thought to be a major pathogenic mechanism leading to increased mucosal permeability and colonic inflammation. Increased IEC apoptosis has been reported in patients with UC and CD (6–8) as well as in murine models of colitis (9, 10). Excess IEC apoptosis causes focal disruption of the mucosal barrier, leading to invasion of luminal antigens and bacteria, and proinflammatory cytokines induced in this process can cause more IEC apoptosis. This vicious cycle of events eventually results in clinical symptoms of IBD. Indeed, TNF-α and IFN-γ, two cytokines critical to IBD pathogenesis, induce IEC apoptosis (11). Recent studies demonstrated that p53-upregulated modulator of apoptosis (PUMA) is a key mediator of IEC apoptosis in IBD (12). PUMA is a BH3 domain proapoptotic BCL2 family member that interacts with antiapoptotic BCL2 family members to activate proapoptotic BAX and BAK and trigger mitochondrial dysfunction. This results in the release of several apoptogenic mitochondrial proteins, such as cytochrome C, leading to caspase activation and cell death (13). PUMA can promote apoptosis in various cell types by p53-dependent and -independent mechanisms. In the colon, PUMA is transcriptionally induced by NF-κB in a p53-independent manner to mediate TNF-α-induced apoptosis in IECs (12, 14).

Vitamin D hormone is a pleiotropic hormone with a broad range of biological activities (15). The majority of the body’s vitamin D content is derived from photosynthesis in the skin following UV light irradiation (16). Vitamin D is converted to the active hormone 1,25-dihydroxyvitamin D (1,25(OH)2D3) via 25-hydroxylation in the liver, followed by 1α-hydroxylation in the kidney; the latter is catalyzed by 25-hydroxyvitamin D 1α-hydroxylase (CYP27B1). CYP27B1 is also expressed in extrarenal tissues, including the intestine, to drive local production of 1,25(OH)2D3. Interestingly, colonic Cyp27B1 expression is influenced by Toll-like receptor activation and colonic inflammation (17, 18). The biological activity of 1,25(OH)2D3 is mediated by the vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily (19). A growing body of epidemiological data has documented an association between vitamin D deficiency and increased risk of IBD (20–22), including both CD and UC (23–30). A high prevalence of vitamin D deficiency was reported in patients with established as well as newly diagnosed IBD (31–33). Vitamin D deficiency was independently associated with lower quality of life and greater disease activity in IBD (34). Analyses of the large Nurses’
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Health Study database showed that high vitamin D intake lowered the risk of IBD (35). Moreover, VDR gene polymorphisms were reported to be associated with IBD (36–39). These observations suggest that vitamin D status might be an environmental determinant for IBD, whereas VDR status might be a key genetic factor influencing IBD development.

Animal studies have provided evidence for an inhibitory role of vitamin D in the development of IBD. Vitamin D deficiency exacerbated enterocolitis and increased mortality in Il10−/− mice, a model of spontaneous colitis, whereas dietary vitamin D supplementation ameliorated colitis and decreased mortality in this model (40). Vdr−/−/Il10−/− mice developed more severe colitis and had higher mortality than Vdr−/−/Il10+/− mice (41). These observations suggest that VDR signaling in the immune cells plays a protective role against IBD. We reported that global VDR deletion increased mucosal injury that led to high mortality in an experimental colitis model (42). In Vdr−/− mice, colonic transepithelial electrical resistance, an indicator of epithelial barrier integrity, was significantly reduced before clinical colitis symptoms and histological abnormalities were detected, suggesting that epithelial VDR signaling suppresses colonic inflammation by protecting the integrity of the mucosal barrier. The relative anti-inflammatory contribution of intestinal epithelial VDR signaling and epithelium-independent immune VDR signaling, however, could not be separated in these studies. Here, we used a transgenic approach to specifically address the role of gut epithelial VDR in the pathogenesis of colitis. Our results indicate that epithelial VDR signaling inhibits colitis in part by reducing IEC apoptosis in a manner independent of VDR actions in the non-epithelial immune compartment.

Results
Colonic epithelial VDR levels are markedly reduced in CD and UC patients. Given the potential importance of colonic VDR in IBD pathogenesis, we examined VDR status in colonic biopsies from both CD and UC patients compared with normal colon samples (Figure 1A). Immunostaining with anti-VDR antibodies showed that VDR was highly expressed in normal colon epithelial cells, but in both CD

Figure 1
Reduced VDR expression in patients with IBD. (A) Representative H&E histology of colonic biopsies obtained from normal subjects and CD and UC patients. Original magnification, ×100. (B) Representative immunostaining of colonic biopsies from normal control and CD and UC patients with anti-VDR antibodies. Arrows indicate VDR staining in the epithelial cells. Original magnification, ×100. (C) Microarray heatmap showing relative VDR transcript levels in normal and UC colonic biopsies. Red color indicates high transcript levels, and green color represents low levels. n ≥10 in each group. (D–F) Representative Western blots of colonic biopsies from the Chicago cohort (D) and the Shenyang cohort (E) with anti-VDR antibodies and respective densitometric quantitation (F) of VDR protein levels in each cohort (Full, uncut gels are shown in the supplemental material. Supplemental material available online with this article; doi:10.1172/JCI65842DS1). §P < 0.001 versus normal (n = 5–12). Patients are numbered in the Shenyang cohort. ac, active; qu, quiescent; D, diseased lesion tissues; N, normal tissue. (G) Serum 25-hydroxyvitamin D concentrations in normal controls and IBD patients from the Chicago and Shenyang cohorts as indicated. Average values are marked by horizontal lines.
and UC biopsies, epithelial VDR levels were markedly reduced (Figure 1B). Data from cDNA microarrays (43) confirmed that VDR transcript expression was downregulated in UC biopsies (n ≥ 10) relative to normal colon samples (Figure 1C). To further quantify VDR protein levels and serum vitamin D status in IBD patients, we recruited two cohorts of study subjects, one from Chicago (Illinois, USA), and the other from Shenyang (Liaoning, China). In the Chicago cohort, we found by Western blot analyses that colonic VDR protein levels were significantly reduced in both CD and UC patients compared with normal control subjects (Figure 1, D and F). In active CD biopsies, VDR was lower compared with the quiescent CD biopsies (Figure 1D), suggesting that inflammation may downregulate epithelial VDR expression. In the Shenyang cohort, we collected biopsies from the lesion as well as from the adjacent normal colon tissue in each UC patient (there were very few CD patients available there). In most patients, VDR protein levels were dramatically decreased (by >60% on average) in the lesion relative to the adjacent normal region within the same patient (Figure 1, E and F), whereas proinflammatory cytokine transcripts (TNFA and IL1B) were elevated in the lesion (not shown). This observation again suggests a repressive effect of local colonic inflammation on epithelial VDR expression.

In the Chicago cohort, the average serum 25-hydroxyvitamin D level in the IBD patients was above the normal range (>75 nmol/l) (Figure 1G), probably reflecting the fact that most of these patients received oral vitamin D supplementation. (When the patients were divided into CD and UC, the mean serum 25-hydroxyvitamin D levels remained normal in each group). In the Shenyang cohort, however, the average serum 25-hydroxyvitamin D level in the UC patients was significantly lower compared with the normal subjects and fell within a severe deficiency range (<50 nmol/l) (Figure 1G). None of the patients in this cohort took vitamin D supplements. These observations suggest that in these patients, the VDR status in the lesion is independent of the serum vitamin D status but is likely affected by the local inflammatory status. The reduction in epithelial VDR could be a causative factor promoting
colitis. Therefore, we used a genetic approach to specifically assess the role of epithelial VDR signaling in experimental colitis models.

**Generation of transgenic mice that overexpress VDR in intestinal epithelium.** To produce Tg mouse lines that express human VDR (hVDR) specifically in IECs, we used the 12.4-kb villin promoter to drive a FLAG-tagged hVDR transgene (Figure 2A). The villin promoter has been used to target various transgenes to the intestinal epithelium (44). The FLAG tag, added to the N terminus of hVDR, has no effects on the transactivating activity of hVDR (45), but can distinguish the human transgene from the endogenous mouse VDR. Pronuclear injection of the 14.5-kb PmeI construct (Figure 2A) resulted in four Tg-positive founders on a C57BL/6 background. Three founder lines were mated with WT C57BL/6 mice to obtain germline transmission. Tg lines 3 and 14 were used in this study with comparable results. There was no obvious morphological or growth difference between the WT and Tg mice.

Western blot analyses with anti-FLAG antibodies revealed high expression of FLAG-hVDR from the jejunum to the distal colon in Tg mice (Figure 2B). The transgene expression in the duodenum was very low as reported (44). The FLAG tag, added to the N terminus of hVDR, has no effects on the transactivating activity of hVDR (45), but can distinguish the human transgene from the endogenous mouse VDR. Pronuclear injection of the 14.5-kb PmeI construct (Figure 2A) resulted in four Tg-positive founders on a C57BL/6 background. Three founder lines were mated with WT C57BL/6 mice to obtain germline transmission. Tg lines 3 and 14 were used in this study with comparable results. There was no obvious morphological or growth difference between the WT and Tg mice.

Western blot analyses with anti-FLAG antibodies revealed high expression of FLAG-hVDR from the jejunum to the distal colon in Tg mice (Figure 2B). The transgene expression in the duodenum was very low as reported (44). As expected WT littermates were negative for the FLAG-tagged hVDR (Figure 2B). Immunostaining with anti-FLAG antibodies confirmed that hVDR was predominantly expressed in the intestinal epithelial cells in Tg mice, with the highest level observed in the luminal side of the crypt (Figure 2C). No differences were detected in crypt morphology or cellular proliferation rates between WT and Tg colons, as revealed by BrdU labeling (Figure 2D). Western blotting with anti-VDR antibodies revealed about a 2- to 3-fold increase in VDR levels throughout the intestine in Tg mice relative to the endogenous mouse VDR expression in WT mice (Figure 2E), and immunostaining with anti-VDR antibodies confirmed that this increase was predominately located in the epithelial cells (Figure 2F).

**Epithelial hVDR expression protects from developing colitis in a TNBS-induced colitis model.** To understand the role of epithelial VDR signaling in colitis development, we compared WT and Tg mice using different experimental models of colitis. First, we used the 2,4,6-trinitrobenzene sulfonic acid–induced (TNBS-induced) colitis model, which is believed to resemble CD because it involves TH1-mediated mucosal inflammation (46). We subjected WT and Tg littermates to TNBS treatment as described (47). Intrarectal instillation of TNBS resulted in gradual weight loss in WT mice during the following 6 days, whereas the weight loss was ameliorated in Tg mice in the first 2 days and quickly recovered in the following days (Figure 3A). By day 6, WT mice
were symptomatic with severe colitis disease activity, manifested by severe diarrhea and rectal bleeding, and the colon appeared markedly shortened and thickened (Figure 3B). In contrast, Tg mice developed almost no symptoms, and their large bowel showed few signs of colitis (Figure 3B). We performed histological examinations, which confirmed crypt hyperplasia, loss of crypts, severe focal ulceration, and inflammation in the WT colon, along with thickened colonic walls, whereas the Tg colon showed few abnormalities (Figure 3C). Compared with WT mice, Tg mice had significantly lower colon/body weight ratios (Figure 3D), lower colonic damage scores (Figure 3E), and lower histological inflammation scores (Figure 3F) on day 6.

To gain insights into the change in mucosal permeability after TNBS injury, we used an Ussing chamber to measure transepithelial electrical resistance (TER) in the colons obtained from WT and Tg mice 2 days after TNBS treatment. At this time point, we observed no clinical symptoms or histological damage in either genotype. While the TER was markedly reduced in both the distal (Figure 3G) and proximal (Figure 3H) colon from WT mice, the colon mucosal TER was preserved in Tg mice (Figure 3, G and H). These results indicate that epithelial hVDR signaling protects the integrity of the mucosal epithelial barrier.

Next, we examined the colonic epithelial tight junction, which directly affects mucosal permeability. Immunostaining revealed diminished ZO-1 in focal regions in the WT colon 3 days after TNBS treatment, whereas the Tg colon maintained a relatively normal ZO-1 pattern in the epithelium (Figure 4A). Real-time RT-PCR quantitation showed substantial reductions in tight junction protein transcripts ($Zo1$, occludin-1, claudin-1, claudin-2, and claudin-5) in the colonic mucosa of WT mice after TNBS treatment, whereas most of these transcripts were maintained at relatively normal levels in Tg mice (Figure 4B). Moreover, TNBS treatment dramatically induced mucosal proinflammatory cytokines and chemokines ($Tnfa$, $Il1b$, $Il6$, $Il12$, $Mcp1$, and $Mip1$) in WT mice, but the induction of these cytokines and chemokines was substantially attenuated in Tg mice (Figure 4C), confirming that the Tg colon was much less inflamed than the WT colon.

Colonic epithelial cells express Toll-like receptors and can exert an inflammatory response. Since the hVDR transgene is only expressed in IECs, we examined the expression of proinflammatory cytokines in colonic epithelial cells immediately purified from WT and Tg mice treated with TNBS for 8 hours and for 2 days, respectively. Relative to untreated control IECs, there was a dramatic induction of $Tnfa$, $Ifng$, $Il6$, $Il1b$, $Mcp1$, and $Mip1$ in the WT IECs after 8-hour TNBS treatment, and this induction subsided after 2 days (Figure 4D). In the IECs purified from Tg mice, however, the induction of these cytokines and chemokines was markedly and significantly attenuated at both 8 hours and at 2 days.
transgene specifically in the IECs, designated as RagKO Tg mice (Figure 6A). We then compared the development of colitis between RagKO and RagKO Tg mice after injection of CD4^+CD45RB^hi T cells purified from WT donor mice (50). Approximately 60% of the RagKO mice died of severe colitis by 7 weeks after T cell transfer, whereas only 10% of the RagKO Tg mice died during this period (Figure 6B). Histological examination revealed severe histological damage and colonic inflammation manifested by a large increase in bowel wall thickness, massive leukocyte infiltration, and crypt abscess in the RagKO mice (Figure 6, C and D). In the RagKO Tg mice, we found that colonic inflammation was markedly ameliorated, with a significant reduction in the histological score (Figure 6C). Although crypt hyperplasia was present, we barely detected immune cell infiltration in most of these mice (Figure 6D). We consistently found that colonic proinflammatory cytokine production was substantially attenuated in RagKO Tg mice (Figure 6E). These observations provide very compelling evidence demonstrating that T cell–mediated colonic inflammation can be substantially suppressed by enhancing VDR signaling in the epithelial compartment alone, suggesting that hVDR overexpression strengthens the epithelial barrier function.

**Reconstitution of VDR-null epithelial cells with hVDR rescues VDR-null mice from colitis and death.** To further assess the antiinflammatory activity of epithelial VDR signaling, we asked whether reconstituting the IECs of Vdr^−/− mice with the hVDR transgene would prevent Vdr-null mice from developing colitis. To this end, we generated, through breeding, Vdr^−/− (VDRKO) mice that expressed hVDR only in the IECs (designated as KO Tg mice). Western blot analyses confirmed the expression of FLAG-hVDR in the colonic mucosa of KO Tg mice, but not of VDRKO mice (Figure 7A). We then examined WT, Tg, VDRKO, and KO Tg mice in parallel using the TNBS and DSS colitis models. In the TNBS model, VDRKO mice developed dramatic weight loss, and all died by day 5 after TNBS treatment (Figure 7, B and C). We found that the colon of VDRKO mice was markedly shortened and swollen (Figure 7D). Histological examination revealed severe ulcerations with complete crypt depletion in the distal colon (Figure 7E, also see Supplemental Figure 1 for whole colon “Swiss roll” images; supplemental material, including full, uncut gels, available online with this article; doi:10.1172/JCI65842DS1). As expected, the colitis phenotype of VDRKO mice was more severe compared with that of WT mice (Figure 7E and Supplemental Figure 1). In contrast, KO Tg mice only developed mild colitis with little weight loss and no death during this period of time (Figure 7, B and C), and their colon showed largely normal gross and histological morphologies (Figure 7, D and E, and Supplemental Figure 1). We consistently found among these four genotypes that VDRKO mice had the highest colonic damage score (Figure 7F) and histological score (Figure 7G), and the highest myeloperoxidase (MPO) activity (Figure 7H) and proinflammatory cytokine production (Figure 7I), whereas KO Tg mice had the best protective effect compared with WT and VDRKO mice.
The epidermal expression of inflammatory cytokine in the colonic mucosa (Figure 7I), but these severe colonic injury and inflammation parameters were almost completely “corrected” in the KO Tg mice (Figure 7, F–I). Similar results were obtained in the DSS colitis model, in which the epithelial hVDR transgene was able to substantially reduce animal mortality and clinical scores (Supplemental Figure 2, A and B) and markedly attenuated colonic injury in KO Tg mice compared with VDRKO mice (Supplemental Figure 2C). Taken together, these observations demonstrate that reconstituting only the gut epithelial cells with hVDR is sufficient to rescue Vdr-null mice from developing severe colitis despite the presence of a VDR-deficient immune system, confirming a critical and predominant anticolitic role of epithelial VDR signaling in the prevention of colitis.

Epithelial VDR signaling inhibits IEC apoptosis by suppressing PUMA. Increased apoptosis in gut epithelial cells compromises the mucosal barrier, leading to colonic inflammation. By TUNEL staining of WT mice, we observed abundant apoptotic colonic epithelial cells that were further increased in VDRKO mice after TNBS insult, whereas apoptotic epithelial cells were markedly reduced in Tg and KO Tg mice (Figure 8, A and B). In agreement with these observations, we found that caspase 3 cleavage was increased in colonic mucosal lysates from WT and VDRKO mice compared with Tg and KO Tg mice following TNBS treatment (Figure 8, C and D). PUMA, a key mediator of IEC apoptosis, was upregulated in WT mice and even more so in VDRKO mice compared with Tg and KO Tg mice (Figure 8, C and D). In contrast, protein levels of p53, another important regulator of apoptosis, were not altered in these mice (Figure 8, C and D). In the DSS model, PUMA induction (Supplemental Figure 3A) and caspase 3 activation (Supplemental Figure 3B) were also attenuated in the Tg mice. Supporting the relevance of this apoptotic pathway in colitis, we also found that PUMA was upregulated in human CD biopsies (Supplemental Figure 3C). These observations suggest that at least part of the anticolitic mechanism of epithelial VDR signaling involves blocking colonic epithelial cell apoptosis by suppressing PUMA in a p53-independent manner.

Epithelial VDR signaling downregulates PUMA by blocking NF-κB activation in colonic epithelial cells. We used a cell culture system to explore the VDR-dependent mechanism involved in PUMA downregulation. In HCT116 cells, a human colonic cancer cell line, TNF-α markedly induced PUMA, and the induction was attenuated by 1,25(OH)2D3 (Figure 9A). PUMA is regulated by NF-κB, and a functional cis-κB site has been identified in the PUMA gene promoter (12, 14) (Figure 9B). ChIP assays showed that 1,25(OH)2D3 blocked TNF-α–induced p65 binding to this κB site in HCT116 cells (Figure 9C), and EMSA confirmed that 1,25(OH)2D3 attenuated TNF-α–induced NF-κB binding to the PUMA κB probe in HCT116 nuclear extracts (Figure 9D). We have
shown previously that 1,25(OH)₂D₃ suppresses TNF-α–induced NF-κB activity in luciferase reporter assays (52). In HCT116 cells transfected with a luciferase reporter that contains the PUMA κB site or its mutant (Figure 9B and ref. 14), 1,25(OH)₂D₃ treatment inhibited TNF-α–induced luciferase activity with the WT PUMA κB luciferase reporter; for the mutant reporter, however, neither TNF-α nor 1,25(OH)₂D₃ had effects on the luciferase activity (Figure 9E). To validate that the luciferase activity was indeed mediated by the PUMA κB element, we cotransfected HCT116 cells with the PUMA κB luciferase reporter or its mutant and an IKKβ-expressing plasmid or its control plasmid. Transfection of IKKβ dramatically induced the luciferase activity of the WT reporter, but not of the mutant reporter, and this induction was also markedly abrogated by 1,25(OH)₂D₃ (Figure 9F). Moreover, IKK kinase assays demonstrated that 1,25(OH)₂D₃ blocked TNF-α–induced IKK activity to phosphorylate IκBα in HCT116 cells (Figure 9G), confirming our previous finding that 1,25(OH)₂D₃/VDR signaling is able to block NF-κB activation (52–54). To verify that 1,25(OH)₂D₃ downregulates PUMA by targeting NF-κB activation, we transfected HCT116 cells with an HA-IKKβ–expressing plasmid. IKKβ overexpression induced PUMA, but we found that this PUMA induction could be attenuated by treatment of the cells with 1,25(OH)₂D₃ (Figure 9H). This result is consistent with our recent finding that liganded VDR blocks NF-κB activation by interacting with IKKβ (55).

To assess whether the epithelial VDR signaling indeed inhibits NF-κB activation in vivo, we measured colonic mucosal IKK kinase activity in WT, Tg, VDRKO, and KO Tg mice with or without TNBS
Two-day TNBS treatment markedly induced colonic mucosal IKK kinase activity in WT mice and even more so in VDRKO mice, and this was accompanied by strong PUMA induction, and caspase 3 activation in WT and VDRKO mice (Figure 9I). Remarkably, the mucosal IKK kinase activity, PUMA induction and caspase 3 activation were all substantially attenuated in Tg and VDRKO Tg mice (Figure 9I). Taken together, these in vitro and in vivo data provide compelling evidence that epithelial VDR signaling inhibits inflammation-induced PUMA expression by blocking NF-κB activation, thereby reducing gut epithelial cell apoptosis.

**Discussion**

In this study, we found that epithelial VDR levels are substantially reduced in patients with CD and UC and demonstrated in experimental colitis models that gut epithelial VDR signaling has potent anticolitic activity that is independent of nonepithelial immune VDR actions. At least part of the anticolitic mechanism of the epithelial VDR is to inhibit epithelial apoptosis through downregulation of PUMA, a key proapoptotic regulator, and this action results in the protection of the colonic mucosal barrier and hence the reduction of colonic inflammation.

Epidemiological studies have shown that low vitamin D status is common in IBD. Since the major source of vitamin D comes from UV light–driven photosynthesis and dietary components, vitamin D is thought to be an environmental factor that might affect the development of IBD. Studies have suggested that high vitamin D intake is associated with a reduced risk of CD (35), and there are also reports that vitamin D can inhibit colitis in some animal models (40–42); however, whether vitamin D status plays a causative role in the pathogenesis of human IBD is unclear. In this study, we observed very low vitamin D status in one cohort of IBD patients (Shenyang) that did not use vitamin D supplementation and observed relatively normal serum vitamin D levels in another cohort (Chicago) that was routinely supplemented with vitamin D. These results neither include nor exclude low vitamin D status as a causative factor in human IBD. In fact, high serum 1,25-dihydroxyvitamin D status has been reported in a subset of CD patients (56). Future prospective studies are needed to address this issue more conclusively. Despite different serum vitamin D levels, one common feature of these two cohorts is a marked reduction of mucosal epithelial VDR expression. Thus, reduced VDR status in the lesion appears to be an intrinsic characteristic of IBD that is independent of serum vitamin D status. What drives down VDR expression remains to be resolved, but our ongoing preliminary studies suggest that local inflammation is a critical factor in VDR downregulation. In fact, few studies have examined VDR status in the IBD population, but the colonic VDR appears to be directly linked to colonic inflammatory status.
VDR is expressed in both the epithelial and nonepithelial compartments of the colon. Given the distinct anatomic and physiological differences between the epithelial and immune compartments in colonic biology, epithelial and nonepithelial VDR signaling may play distinct roles in the pathogenesis of colitis. Our observation of epithelial VDR reduction in diseased colonic regions in both CD and UC patients provides a strong rationale to pursue the role of epithelial VDR signaling in colitis development. In the current study, we employed a genetic approach to address this question. We demonstrated that an approximately 2-fold elevation of VDR expression in gut epithelial cells could render the Tg mice highly resistant to colitis in several different experimental colitis models, including the TNBS and DSS models, in which acute colitis is induced chemically, and the adoptive T cell transfer model, in which chronic colitis is induced by the disruption of T cell homeostasis. TNBS is thought to haptenize colonic autologous or microbiota proteins, rendering them immunogenic to the host immune system, inducing T cell–mediated colonic inflammation (57). Thus, the TNBS model is believed to resemble CD in humans. DSS is toxic to the gut epithelial cells and induces colitis by disruption of the mucosal epithelial barrier. Mice treated with DSS develop colitis resembling UC in humans (48). The adoptive T cell transfer model closely recapitulates the clinical and histopathological features of human CD, including transmural inflammation, epithelial hyperplasia, leukocyte infiltration, crypt abscesses, and epithelial cell erosions (50). Therefore, the clinical relevance of our animal observations is sound. In all these models, Tg mice overexpressing hVDR showed a remarkable reduction in colonic inflammation and colonic injury compared with their WT counterparts, as assessed by clinical, morphological, pathohistological, and molecular parameters. At the early phase, WT mice exhibited increased mucosal permeability prior to detectable morphological and histological abnormalities, but Tg mice maintained relatively normal permeability as well as relatively normal levels of tight junction proteins. Proinflammatory cytokines in the gut epithelial cells were also suppressed. At late stages, the severe colitis phenotype seen in WT mice, such as ulceration and massive leukocyte infiltration, was mostly absent in Tg mice. Together, our data suggest that the potent anticolitic activity of epithelial VDR signaling is derived at least in part from reducing the inflammatory status of the epithelial cells and maintaining the integrity of the mucosal epithelial barrier.

We have previously reported that genetic Vdr deletion leads to severe colitis in the DSS model (42). Because the mutant mice carried global deletion of the Vdr gene, the relative contribution of epithelial and nonepithelial VDR signaling in the development of colitis was indistinguishable. We reasoned that if the anticolitic activity of the epithelial VDR is a primary and essential protective mechanism, then reconstitution of the Vdr-deficient gut
epithelial cells in Vdr−/− mice with the hVDR transgene should be able to prevent or attenuate the severe colitic phenotype seen in Vdr−/− mice, even though the immune system remains Vdr deficient. Indeed, we observed that while VDRKO mice developed very severe colitis leading to high mortality following either TNBS or DSS insult, the KO Tg mice were highly resistant to colitis, with marked attenuation of colonic inflammation and no death in both experimental models. Relatively normal crypt architecture was maintained in these mice. Given that the KO Tg mice still have a Vdr-deficient immune system that is hyperresponsive to immune stimuli, since immune VDR signaling suppresses inflammatory response (58), we conclude that enhanced epithelial VDR signaling is sufficient to inhibit colitis, regardless of the VDR status in the nonepithelial immune system.

Vitamin D has immune-modulatory activities (59, 60), and the VDR-deficient immune system was thought to be the main promoter for the robust colitis phenotype seen in Il10/Vdr double-mutant mice (41). IBD is a chronic inflammatory disorder, and there is little doubt that immune VDR signaling can exert anticolitic effects by downgrading proinflammatory reactions. As such, immune Vdr deficiency could render the colonic immune components hyperreactive to invading luminal antigens or bacteria, leading to hypercolonic inflammation. Our study suggests, however, that epithelial VDR signaling functions as a primary defense mechanism to suppress colonic inflammation by maintaining an intact and functional mucosal barrier, which prevents luminal antigens and bacteria from interacting with the immune components in the lamina propria. As such, when the Vdr-deficient gut epithelial cells were reconstituted with the hVDR transgene, the integrity of the mucosal barrier was restored to prevent the otherwise hyperinflammatory response in the VDR-deficient lamina propria immune cells. We believe these data demonstrate for the first time that the anticolitic activity of the epithelial VDR is independent of VDR actions in the nonepithelial compartment. Therefore, the reduction of epithelial VDR observed in IBD patients is predicted to compromise the epithelial barrier and thus likely contributes to the development of IBD. One limitation of this study is that the gain-of-function approach that we took cannot directly address whether the 50%–60% reduction in epithelial VDR levels we observed in IBD patients would have a major impact on colonic inflammation. It might be more appropriate to use an epithelium-specific loss-of-function approach to address this issue in the future.

How does the epithelial VDR protect the mucosal barrier? In this study, we showed that epithelial VDR signaling inhibits gut epithelial cell apoptosis by downregulating PUMA. It is well understood that increased gut epithelial apoptosis compromises the mucosal barrier and increases mucosal permeability. In our colitis models, extensive colonoocyte apoptosis was detected in WT mice and more so in Vdr-null mice, accompanied by robust PUMA induction and caspase 3 activation, but epithelial expression of hVDR in either a Vdr WT or a null background markedly suppressed PUMA upregulation and caspase 3 activation, leading to a reduction in IEC apoptosis. The increase in colonic epithelial apoptosis appeared to be p53 independent (12).

Since PUMA is a key inducer of gut epithelial apoptosis, we embarked on studies to dissect the molecular mechanism whereby 1,25(OH)2D3-VDR downregulates PUMA expression. In IECs, NF-κB mediates inflammation-induced PUMA upregulation (14). Here, we demonstrated that 1,25(OH)2D3 abrogates PUMA induction by blocking NF-κB activation. This conclusion is based on several lines of in vitro and in vivo evidence. In HCT116 cells, 1,25(OH)2D3 inhibited TNF-α-induced PUMA protein and PUMA promoter activity, and this inhibition was mediated by the κB cis-DNA element in the PUMA gene promoter. To directly link vitamin D actions to the blockade of NF-κB activation, we showed that 1,25(OH)2D3 attenuated IKKβ-induced PUMA promoter activity and PUMA protein expression, inhibited TNF-α-induced IKK kinase activity, and disrupted TNF-α-induced p65 binding to the PUMA κB site. Importantly, we further demonstrated that hVDR expression in gut epithelial cells substantially inhibited IKK kinase activity in colonic mucosa from either Tg or KO Tg mice, together with the inhibition of PUMA induction and caspase 3 activation. These data confirm that VDR signaling suppresses epithelial NF-κB activity in vivo, providing mechanistic insights into the function of epithelial VDR signaling in colonic homeostasis. Our recent studies showed that VDR is able to directly interact with IKKβ protein to attenuate NF-κB activation (55).

Colonic commensal bacteria play a key role in the development of IBD. Colonic epithelial cells influence the microbiota by secreting antimicrobial peptides (61). Vitamin D has been reported to induce cathelicidin and β2-defensins (62, 63). It is conceivable that epithelial VDR can regulate colonic inflammation by regulating antimicrobial peptide production in colonoocytes. Moreover, epithelial VDR signaling may also regulate autophagy, another molecular event that has been implicated in IBD (64, 65). Indeed, vitamin D/VDR signaling is known to regulate autophagy (66). In this study, we showed that VDR overexpression also inhibited epithelial inflammatory cytokine production in IBD models, which may have a significant impact on the course of colonic inflammation. The inflammatory cytokines produced by the epithelial cells have autocrine and paracrine effects in the colon. These potential anticolitic mechanisms warrant further investigation in the future. It is conceivable that the anticolitic mechanism of epithelial VDR signaling is multifactorial and not limited to the regulation of barrier function.

In conclusion, in this report we provide strong evidence that epithelial VDR signaling inhibits colitis by protecting the mucosal epithelial barrier, and this anticolitic activity is independent of VDR actions in the nonepithelial immune system. Given the relatively deficient VDR status observed in IBD patients, targeting VDR expression in epithelial cells might be a useful strategy for the treatment of IBD.

Methods

Human biopsies. Patients with IBD and non-IBD control subjects were recruited with written informed consent for this study at the University of Chicago Medical Center and at Shenyjing Hospital of China Medical University. The non-IBD controls were patients who underwent screening colonoscopies without active gastrointestinal pathology. The diagnosis of CD or UC was based on a standard combination of clinical, endoscopic, histological, and radiological criteria. The severity of macroscopic inflammation of the colon mucosa at colonoscopy was graded according to the Mayo score for UC (67) and the disease endoscopic index of severity (CDEIS) subscore for CD (68). In the Shenyang cohort, we collected colonic biopsies from the lesion and adjacent normal tissue from each patient. We subjected the biopsies to histological and anti-VDR immunohistochemical analyses. Tissue lysates were also prepared from the biopsies for Western blot analyses. Data for human VDR transcript levels in colonic mucosal biopsies of normal control subjects and UC patients
without dysplasia were obtained from previously published microaray data (43) deposited in the NCBI's Gene Expression Omnibus repository (GEO accession number GSE37283).

**Transgenic mice.** FLAG-hVDR was generated by adding the FLAG nucleotide sequence to the N terminus of the hVDR cDNA coding sequence as described (45). The FLAG-hVDR cDNA (1.3 kb) was placed under the control of the 12.4-kb mouse villin gene promoter (44) (provided by Deborah Gumucio, University of Michigan, Ann Arbor, Michigan, USA), followed by the SV40 T antigen poly(A) sequence. The 14.5-kb Pmel-Pmel DNA construct was transfected into the SV40 T antigen-expressing cell line H-HVDR. Serologically aneuploid plaques were screened by PCR-based genotyping using hVDR-specific primers. Embryonic stem cells were isolated from H-HVDR embryos and used to create transgenic mice expressing the FLAG-hVDR transgene. FLAG-hVDR transgenic mice were mated with mice expressing the E6/E7 human papillomavirus type 11 transgene (13) or the SV40 T antigen poly(A) sequence in the context of the E7 open reading frame (14).

**RT-PCR.** Total RNA was extracted using TRIzol reagents (Invitrogen). First-strand cDNAs were synthesized using a ThermoScript RT kit (Invitrogen). Conventional PCR was carried out using conventional PCR primers and SYBR green PCR reagent kits (Clontech) in the presence of 1,25(OH)2D3 or ethanol as described (59). The relative amount of transcripts was calculated using the 2−ΔΔCt formula as described previously (76). PCR primers are provided in Supplemental Table 1.

**Western blotting.** Proteins were separated by SDS-PAGE and electroblotted onto Immobilon-P membranes (Millipore). Western blotting analyses were carried out as previously described (77). The antibodies used in this study included: VDR, IkBα, IKKα, IKKβ (all from Santa Cruz Biotechnology Inc.), FLAG, β-actin (Sigma-Aldrich), PUMA (Abcam), caspase 3, and p53 (both from Cell Signaling Technology).

**Kinase assays.** IkB kinase (IKK) assays were performed as described (78). Briefly, lysates prepared from HCT116 cells or colonic mucosa were immunoprecipitated with anti-IKKα antibodies (Santa Cruz Biotechnology Inc.) and run on SDS-PAGE. Western blotting was performed using anti-phospho-IKKα antibodies. The assays were quantified by densitometry.

**ChIP assays.** ChIP assays were performed as described previously (79) using anti-p65 antibodies. The assays were quantified by real-time PCR using primers (Supplemental Table 1) flanking the κB site in the promoter of the PUMA gene as described (14).

**EMSA.** Nuclear extracts were prepared from HCT116 cells treated overnight with or without TNF-α (100 ng/ml) in the presence of 1,25(OH)2D3 (20 nM) or ethanol as described (79). The nuclear extracts were incubated with a 32P-labeled double-stranded PUMA κB probe (S′-AAGCTGAGGAGTTCCCCAATG-3′), and EMSA was performed as described previously (79).
Statistics. Data values were presented as the means ± SEM. Statistical comparisons were performed using an unpaired 2-tailed Student’s t-test or a 1-way ANOVA, as appropriate, with P < 0.05 considered statistically significant.

Study approval. The collection of human colonic biopsies and blood was approved by the IRBs of The University of Chicago (Chicago, Illinois, USA) and by the Institutional Ethical Committee of China Medical University (Shenyang, Liaoning, China), respectively. Study subjects were recruited with written informed consent obtained from the participants or their guardians. All animal studies were approved by the IACUC of The University of Chicago.

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