Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier

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Departments of 1Medicine and 4Pathology, The University of Chicago, Chicago, Illinois; 2The Huck Institutes for Life Sciences, The Pennsylvania State University, University Park, Pennsylvania; and 3Gastroenterology and Hepatology Division, Department of Medicine, University of Rochester Medical Center, Rochester, New York

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Kong J, Zhang Z, Musch MW, Ning G, Sun J, Hart J, Bissonnette M, Li YC. Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. Am J Physiol Gastrointest Liver Physiol 294: G208–G216, 2008. First published October 25, 2007; doi:10.1152/ajpgi.00398.2007.—Emerging evidence supports a pathological link between vitamin D deficiency and the risk of inflammatory bowel disease (IBD). To explore the mechanism we used the dextran sulfate sodium (DSS)-induced colitis model to investigate the role of the vitamin D receptor (VDR) in mucosal barrier homeostasis. While VDR+/– mice were mostly resistant to 2.5% DSS, VDR−/− mice developed severe diarrhea, rectal bleeding, and marked body weight loss, leading to death in 2 wk. Histological examination revealed extensive ulceration and impaired wound healing in the colonic epithelium of DSS-treated VDR−/− mice. Severe ulceration in VDR+/− mice was preceded by a greater loss of intestinal transepithelial electric resistance (TER) compared with VDR+/+ mice. Confocal and electron microscopy (EM) revealed severe disruption in epithelial junctions in VDR−/− mice after 3-d DSS treatment. Therefore, VDR−/− mice were much more susceptible to DSS-induced mucosal injury than VDR+/+ mice. In cell cultures, 1,25-dihydroxy-vitamin D3 [1,25(OH)2D3] markedly enhanced tight junction protein expression and TER and preserved the structural integrity of tight junctions in the presence of DSS. VDR knockdown with small interfering (si)RNA reduced the junction proteins and TER in Caco-2 monolayers by increasing junction protein expression. Confocal and electron microscopy revealed severe disruption in epithelial junctions in VDR+/− mice after 3-d DSS treatment. Therefore, VDR−/− mice were much more susceptible to DSS-induced mucosal injury than VDR+/+ mice. In cell cultures, 1,25-dihydroxy-vitamin D3 [1,25(OH)2D3] markedly enhanced tight junction protein expression and TER and preserved the structural integrity of tight junctions in the presence of DSS. VDR knockdown with small interfering (si)RNA reduced the junction proteins and TER in Caco-2 monolayers. 1,25(OH)2D3 can also stimulate epithelial cell migration. These observations suggest that VDR plays a critical role in mucosal barrier homeostasis by preserving the integrity of junction complexes and the healing capacity of the colonic epithelium. Therefore, vitamin D deficiency may compromise the mucosal barrier, leading to increased susceptibility to mucosal damage and increased risk of IBD.

The integrity of the intestinal mucosal barrier is preserved by the enormous regenerating capacity of the mucosal epithelium. The intestinal stem cells, located at the base of the crypt, are responsible for replenishing the epithelium through cell division and differentiation. After extensive destruction, rapid resealing of the surface epithelium is accomplished by epithelial cell restitution, proliferation, and differentiation (6). Another important component of the mucosal barrier is the apical and subapical intercellular junctions between the epithelial cells, namely tight junctions and adherens junctions (18). These junction structures seal the paracellular space and regulate the permeability of the mucosal barrier.

IBD, including Crohn’s disease and ulcerative colitis, is a major chronic disorder affecting the gastrointestinal tract in humans. Although the etiopathogenesis of IBD has not been clearly elucidated, it is thought to involve a complex interplay among genetic, environmental, microbial, and immune factors (33). One potential pathogenic factor is impaired mucosal barrier function, and intestinal hyperpermeability is common in IBD patients (11). A relatively high number of first degree relatives of patients with Crohn’s disease have increased intestinal permeability in the absence of clinical symptoms (32, 42), suggesting barrier dysfunction precedes, or is at least a very early defect, in the disease process that might require genetic predisposition and environmental triggers. Indeed, previous studies have demonstrated decreased expression and differential localization of junction complex proteins in the mucosa of patients with IBD (10, 16, 29). Therefore, dysregulation of junction proteins is an important pathogenic mechanism underlying the increased permeability seen in the intestinal epithelium of IBD patients.

Previous studies have suggested a link between vitamin D deficiency and IBD risk (23). The prevalence of IBD exhibits a north-south gradient (24), paralleling sunlight exposure, an important source of vitamin D. Populations near the equator are at relatively lower risk for developing IBD. Seasonal variations in the onset and exacerbation of IBD have also been reported (27, 36) with high incidence in the winter. Early studies have reported a high prevalence of vitamin D deficiency in patients with established Crohn’s disease (12, 38). Decreased vitamin D levels have also been detected in patients with newly diagnosed IBD (17, 19, 35). In the IL-10−/− mouse model of intestinal inflammation, vitamin D deficiency or vitamin D receptor (VDR) deficiency exacerbates the symp-
toms of enterocolitis and increases morbidity and mortality due to increased T cell-mediated immune response in the intestine (1, 9), suggesting that vitamin D can prevent IBD by suppressing T cell-mediated immune response. However, the role of vitamin D and/or VDR in the regulation of intestinal barrier integrity has hardly been studied. Here we provide evidence that vitamin D and the VDR play important roles in maintaining the integrity of the intestinal mucosal barrier.

**MATERIALS AND METHODS**

**Animal studies.** VDR+/+ (wild-type) and VDR−/− mice in C57BL6 background were obtained by breeding VDR+/+ mice (22). Two- to 3-mo-old mice were fed 2% or 2.5% dextran sodium sulfate (DSS) dissolved in drinking water for indicated days, and the mice were either killed or fed regular water and killed at a later time point. Body weight and animal symptoms, including the extent of diarrhea and rectal bleeding, were closely monitored during and after DSS treatment. Symptom scores were determined by assessing the degree of body weight loss, stool consistency (diarrhea), and hemocult positivity or gross bleeding in each animal according to previously published methods (3). The Institutional Animal Care and Use Committee at the University of Chicago approved the protocol for the use of mice in the study.

**Histology and immunostaining.** Two hours before death, mice were injected with 50 mg/kg ip of 5-bromo-2-deoxyuridine (BrdU) to label the proliferating cells. After death, the colons were harvested, fixed in 10% formalin (pH 7.4), processed, and paraffin embedded. “Swiss rolls” were prepared as described (31). Sections (5 μm) were cut with Leica microtome 3030. Slides were stained with hematoxylin and eosin (H and E). For immunostaining, antigens were retrieved by 10-min boiling in 10 mM citrate (pH 6.0). The slides were stained with anti-VDR antibody (Santa Cruz) or anti-BrdU monoclonal antibody (Roche). After incubation with peroxidase-conjugated secondary antibody, signals were visualized with a diaminobenzidine (DAB) peroxidase substrate kit (Vector Laboratories). For tight junction staining, cell monolayers (fixed in 95% ethanol) or colonic sections were incubated with anti-zonula occludens (ZO)-1, anti-claudin-1, or anti-occludin antibody (Zymed), then with an FITC-conjugated secondary antibody (Sigma). Slides were examined with a Leica DMIRE2 scanning laser confocal microscope (37).

**Electron microscopy.** Colonic samples were fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature. The tissues were dehydrated in an ascending series of ethanol, infiltrated with Eponate 12 resin (Ted Pella, Redding, CA) and then embedded and polymerized at 70°C for 24 h. Resin-embedded blocks were sectioned at 70 nm and collected on 200-mesh, Formvar-coated copper grids. Grids were stained with uranyl acetate and lead citrate and examined with a JEOL 1200 EX II transmission electron microscope.

**Cell culture.** SW480 and Caco-2 cells were cultured in DMEM supplemented with 10% FBS. For transepithelial electric resistance (TER) measurement, Caco-2 cells were cultured on collagen-coated transwell polycarbonate membrane filter inserts (Corning). To knockdown VDR, Caco-2 cells were transfected with human (h)VDR-small interfering (si)RNA and scramble siRNA using Transfectamine 2000 (Invitrogen). VDR mRNA and protein levels were determined after 24–48 h. The hVDR-siRNA was a mixture of two oligoribonucleotides 5’CCACCUGGGCUAUGCUGACUUAA3’ and 5’AAUG-GCUUACAACCGCUUAGCAUC3’.

**Epithelial cell migration assay.** Cell migration assays were carried out according to methods described previously (39) with modifications. Caco-2 cells were grown in DMEM containing 10% FBS to monolayers in 6-well plates. After the cells were starved in serum-free media for 24 h, a linear “wound” was generated by scratching the monolayers with a sterile razor blade. Detached cells were washed off with PBS. The monolayers were continually incubated in DMEM containing 1% FBS in the presence or absence of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] (10−8 M) for 48 h. Cell migration across the wound line was assessed under a microscope.

**TER measurement.** TER of Caco-2 monolayers grown on collagen-coated filter inserts was measured using a Millicell-ERS VoltOhmter (Millipore). The TER of intestinal epithelia was measured using an Ussing chamber system (Physiologic Instruments). The full-thickness colonic strips freshly harvested from mice were mounted onto the chamber for TER measurement.

**Western blot analysis.** Proteins were separated by SDS-PAGE and transferred onto an Immobilon-P membrane. Western blot analysis was performed as described previously (21).

**RT-PCR.** Total cellular RNAs were extracted using TRizol reagent (Invitrogen). First-strand cDNA templates were synthesized using Moloney murine leukemia virus (MML-V) reverse transcriptase (Invitrogen) and hexanucleotide random primers. PCR amplification was carried out using specific primers designed based on cDNA sequences deposited in the GenBank database (Table 1).

**Statistical analysis.** Data were presented as means ± SD. Data were analyzed with Student’s t-test to assess significance. A P value of 0.05 or smaller was considered statistically significant.

**RESULTS**

**VDR null mutant mice develop more severe colitis.** The major effect of DSS is believed to damage the colonic mucosal barrier, leading to ulceration. Therefore, we used the acute DSS-colitis model to investigate the role of VDR in mucosal barrier homeostasis. We compared colitis development in VDR+/+ and VDR−/− mice treated with DSS. Figure 1 shows experiments in which VDR+/+ and VDR−/− mice were fed 2.5% DSS water for 7 days; the DSS water was then replaced with tap water, and the mice were allowed to recover until day 15. VDR−/− mice lost up to 15% of body weight within 8 days (Fig. 1A) and developed much more severe clinical symptom scores determined on days 3 and 7 (Fig. 1B), and up to 70% died within 2 wk, even after DSS treatment was stopped (Fig. 1C). The clinical symptoms included diarrhea, gross bleeding, and rectal prolapse. In contrast, VDR+/+ mice were relatively resistant at 2.5% DSS, with little body weight loss and no death seen within the 2-wk period (Fig. 1, A–C). These data indicate that in the absence of VDR, mice are much more susceptible to mucosal injury caused by DSS insult.

<table>
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<tr>
<th>Gene</th>
<th>Primer Nucleotide Sequences</th>
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<td></td>
<td>Reverse 5’ GCA TCG AGA AGA GCA TCA GC 3’</td>
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<td>Claudin-2</td>
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<td>Ocludin</td>
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<td>Reverse 5’ GAA GAC ATC TGC AGG GTT 3’</td>
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All primers were designed according to the cDNA sequence deposited in the GenBank database. ZO, zona occuludens; VDR, vitamin D receptor.
Fig. 1. Vitamin D receptor (VDR)−/− mice develop more severe dextran sodium sulfate (DSS)-induced colitis than VDR+/+ mice. Mice were fed 2.5% DSS water from days 1 to 7 and tap water from days 7 to 15. A: body weight (BW) curve. VDR−/− mice lost weight, whereas VDR+/+ mice were hardly affected at this DSS dose. B: VDR−/− mice develop more severe clinical symptoms, reflected by higher symptom scores on days 3 and 7. *P < 0.05 vs. VDR+/+. C: survival curve. Most VDR−/− mice were not able to recover and died during or following DSS treatment (n = 10–15 each genotype). D: histological assessment of colonic mucosa of VDR+/+ (a, c, and e) and VDR−/− (b, d, and f) mice. a and b: Hematoxylin and eosin (H and E) staining of “Swiss rolls” of the entire large intestine on day 7. Note the extensive ulceration with complete loss of crypts (arrows) in VDR−/− mice (b). c and d: A high magnification of the ulcer area on day 7. e and f: 5-bromo-2-deoxyuridine (BrdU) staining of day 10 colon showing intestinal wound healing. Ulcer healing and reepithelization (arrow) were apparent in VDR+/+ mice (e); note the strong BrdU staining (arrow) in the newly formed crypts. No signs of healing and reepithelization or crypt formation were seen over the broad ulcer area in VDR−/− mice (f). Magnification: a and b, ×40; c and d, ×200; e and f, ×100.

Severe ulceration and impaired mucosal wound healing in VDR−/− mice. Histological examination of the colon was performed on days 3, 7 (Fig. 1D, a–d), and 10 (Fig. 1D, e and f) after 2.5% DSS treatment. In VDR+/+ mice, the colon appeared intact at day 3 (not shown), and focal erosions were only occasionally seen by day 7 (Fig. 1D, a and c). In contrast, erosions were commonly seen in colons of VDR−/− mice on day 3 (not shown), and by day 7, substantial ulcerations were observed; in some segments, the ulceration was so severe that the entire crypt was lost (Fig. 1D, b and d). By day 10, 3–4 days after DSS water was stopped, healing and reepithelization of the ulcers were apparent in VDR+/+ mice, with strong BrdU labeling in newly formed crypts surrounding the ulcers (Fig. 1D, e). In contrast, in surviving VDR−/− mice, although there appeared to be some epithelial cells covering the surface of the ulcerated area, there was little BrdU labeling. No new crypts were formed around the ulcers, and no signs of effective wound healing were seen in VDR−/− mice (Fig. 1D, f). These observations suggest that the mucosal wound healing capacity is impaired in VDR−/− mice.

Severe clinical symptoms are preceded by a greater reduction in colonic TER. TER is an indication of epithelial barrier integrity, as decreased epithelial barrier function is accompanied by a reduction in TER. DSS has been shown to increase mucosal permeability in mice (14). To test the hypothesis that VDR is important for maintaining the mucosal barrier integrity, we compared the colonic TER of VDR+/+ and VDR−/− mice. In this experiment, to reduce mortality, mice were treated with 2% DSS for 3 days and killed at days 0 (untreated), 2, and 3. As shown in Fig. 2, in untreated VDR+/+ and VDR−/− mice, the TER was not significantly different. TER was relatively unchanged in VDR+/+ mice in all three colonic segments even after 3-day DSS treatment (Fig. 2, A–C). This is expected because VDR+/+ mice were resistant to this DSS dose (see Fig. 1). Interestingly, in all three colonic segments, the TER of VDR−/− mice was significantly reduced on day 3 compared with VDR+/+ mice or relative to the untreated VDR−/− mice (Fig. 2, A–C). At this time, clinical symptoms (such as diarrhea or bleeding) were not yet observed in VDR−/− mice. Therefore, the accentuated clinical symptoms

Fig. 2. Effect of DSS on colonic transepithelial electric resistance (TER). VDR+/+ and VDR−/− mice were fed 2% DSS water and killed on days 0 (no DSS), 2, and 3. Immediately after death, the colons were harvested and cut into 3 segments: distal (A), middle (B), and proximal (C). The TER in each colonic segment was measured using the Ussing chamber. Note the significantly greater reduction in TER in VDR−/− mice than in wild-type (WT) mice on day 3. At this time, no obvious clinical symptoms were seen yet. *P < 0.05 vs. VDR+/+, n = 3 or 4 of each genotype.
and epithelial ulceration seen in VDR\(^{-/-}\) mice were preceded by a great loss of colonic TER, suggesting a compromised or more susceptible mucosal barrier in VDR\(^{-/-}\) mice.

Epithelial junction disruption in DSS-treated VDR\(^{-/-}\) mice. To investigate whether the reduction in TER is due to impairment in the epithelial junctions, we immunostained the colonic epithelium from VDR\(^{+/+}\) and VDR\(^{-/-}\) mice that had been treated with 2% DSS for 3 days, using antibodies against tight junction proteins claudin-1, ZO-1, and occludin. As shown in Fig. 3, ZO-1 and occludin staining were seen on the luminal junction proteins claudin-1, ZO-1, and occludin. As shown in Fig. 3, ZO-1 and occludin staining were seen on the luminal surface of the colonic mucosa from untreated VDR\(^{+/+}\) and VDR\(^{-/-}\) mice (Fig. 3A, c and g; 3B, a and b). After DSS treatment, ZO-1 and occludin remained highly stained in the colonic epithelium of treated VDR\(^{+/+}\) mice (Fig. 3A, k; 3B, c), whereas the staining was markedly diminished in the treated VDR\(^{-/-}\) mucosa (Fig. 3A, o; 3B, d). These observations suggest some degree of structural disruption in DSS-treated VDR\(^{-/-}\) colonic mucosa.

Further examination of these colonic mucosa with electron microscopy (EM) revealed intact tight junctions, adherens junctions, and desmosomes in DSS-treated VDR\(^{+/+}\) colonic mucosa (Fig. 4A). In contrast, severely disrupted and opened tight junctions and desmosomes were seen in the treated VDR\(^{-/-}\) epithelia (Fig. 4B). These data confirm that the junctional complexes in the colonic epithelia appear to be much more susceptible to DSS damage in the absence of VDR.

Pattern of VDR expression changes in recovering colonic mucosa. VDR expression in the colon was assessed by immunostaining with anti-VDR antibody. Under normal conditions, VDR was highly expressed near the luminal surface of the crypt, whereas very little VDR staining was detected at the base of the crypt (Fig. 5A). Interestingly, 8 days after DSS (2.5%) treatment was stopped (i.e., day 15), when the crypts were in the recovery phase, VDR expression in the colon was markedly increased, and VDR positive cells were broadly distributed in the crypts from base to surface (Fig. 5B). As expected, no VDR staining was detected in VDR\(^{-/-}\) mice (not shown). The change in VDR localization in the crypt under these two conditions appears to be consistent with its role involved in intestinal mucosal barrier homeostasis.

Vitamin D induces junction proteins. The animal data prompted us to use in vitro culture systems to address the mechanism underlying the protective role of VDR in intestinal barrier function. We first assessed the effect of vitamin D on junction proteins in SW480 cells, a human colonic cancer cell line, because a previous study had demonstrated that 1,25(OH)\(_2\)D\(_3\) induced E-cadherin in these cells (30). Interestingly, treatment with 1,25(OH)\(_2\)D\(_3\) for 24 h increased levels of tight junction proteins ZO-1, claudin-1, claudin-2, as well as adherens junction protein E-cadherin; the maximal induction occurred at 10\(^{-9}\) M. 1,25(OH)\(_2\)D\(_3\) appeared to have little effect on two other tight junction proteins, claudin-5 and occludin (Fig. 6, A and B). Therefore, vitamin D may participate in preservation of the junctions by increasing the level of some key junction proteins. Although occludin protein was not directly induced by vitamin D, it was markedly diminished from VDR\(^{-/-}\) colonic mucosa after DSS insult, suggesting the importance of VDR to maintain the integrity of the junction complex.

Fig. 3. Effect of DSS on colonic epithelial junctions. VDR\(^{+/+}\) and VDR\(^{-/-}\) mice were treated with or without 2% DSS for 3 days as indicated, and the colonic sections were examined by immunostaining. A: colonic slides from untreated (a–h) and DSS-treated (i–p) VDR\(^{+/+}\) (a–d and i–l) and VDR\(^{-/-}\) (e–h and m–p) mice were stained with anti-claudin-1 (green) or anti-zonula occludens (ZO)-1 (red) antibody. d, h, i, and p show the merged images. B: colonic slides from untreated (a and b) and DSS-treated (c and d) VDR\(^{+/+}\) (a and c) and VDR\(^{-/-}\) (b and d) mice were stained with anti-occludin antibody (red). The nuclei were stained with DAPI (blue). The slides were observed under a confocal microscope. Note the intense ZO-1 and occludin staining on the mucosal luminal surface in untreated VDR\(^{+/+}\) and VDR\(^{-/-}\) mice, as well as in DSS-treated VDR\(^{+/+}\) mice (arrows); the staining in DSS-treated VDR\(^{-/-}\) mice was markedly decreased (e.g., A, a; B, d). Magnification: A, \(\times200\); B, \(\times400\).
Vitamin D strengthens tight junctions in Caco-2 monolayer. To further address the effect of vitamin D on the structure and function of the junction complex, we studied Caco-2 cells. Caco-2 cells form tight junctions when grown to monolayers on membrane filters. As in the case of SW480 cells, 1,25(OH)2D3 induced ZO-1 and E-cadherin in Caco-2 cells (Fig. 6C). Consistently, immunostaining with anti-ZO-1 antibody showed that 1,25(OH)2D3 (10−8 M) markedly enhanced ZO-1 expression in the Caco-2 monolayers, reflected by brighter staining of the membranes of the treated cells (Fig. 6D, compare a and b), indicating that 1,25(OH)2D3 enhances the formation of the tight junction. When the cells were incubated with 5% DSS for 2 h, the tight junction on the monolayer was markedly disrupted in the absence of 1,25(OH)2D3 (Fig. 6D, compare a and c); in contrast, in the presence of 1,25(OH)2D3, the tight junction was resistant to the DSS damage and remained intact, as the strong ZO-1 staining remained detectable on the membranes between the cells (Fig. 6D, compare b and d). When Caco-2 monolayers were incubated with 5% DSS, the TER gradually decreased over the following 3.5 h in the control cells; in contrast, in 1,25(OH)2D3-treated cells, the TER was moderately reduced in the beginning and fully recovered by 3.5 h (Fig. 6E). Therefore, 1,25(OH)2D3 treatment can strengthen the epithelial junction complexes in colonic epithelial cells and increase its resistance to DSS damage.

VDR knockdown reduces tight junction proteins and compromises tight junction functions. To further explore the role of VDR in the regulation of the tight junction, we evaluated the effect of VDR reduction on the monolayer formed by Caco-2 cells. The VDR level was reduced using hVDR-specific siRNA, with unrelated (scramble) siRNA as a control. As shown in Fig. 7, 2 days after siRNA transfection, the VDR mRNA level was markedly reduced, as were the levels of claudin-5, claudin-1, E-cadherin, and ZO-1; the levels of claudin-2 and occludin were unchanged (Fig. 7A). These results were generally consistent with the data presented in Fig. 6, which shows that claudin-1, ZO-1, and E-cadherin were induced by 1,25(OH)2D3 in SW480 and Caco-2 cells. The level of the VDR protein was markedly reduced for up to 4 days after siRNA transfection and recovered to the pretreatment level by day 9 (Fig. 7B). When the siRNA-transfected cells were treated with 1,25(OH)2D3, ZO-1 failed to be induced in the VDR-siRNA-transfected cells, in contrast to the scramble siRNA-treated cells (Fig. 7C). We also monitored the TER in the VDR-siRNA-transfected cells over the next 4 and 6 days after transfection. Interestingly, whereas the TER continued to rise in the control siRNA-transfected cells, no increase in the TER was observed in VDR-siRNA-transfected cells (Fig. 7D). Moreover, when the control siRNA- and VDR-siRNA-transfected cell monolayers were exposed to 5% DSS, the decrease in TER was more robust in VDR-siRNA-transfected cells than in the control cells within 3.5 h (Fig. 7E). These data demonstrate that VDR knockdown reduces the expression of junction proteins and diminishes the epithelial barrier integrity. These results were consistent with the in vivo observations that the colonic TER was decreased more in VDR−/− mice than in VDR+/+ mice after DSS damage (Fig. 2).

Vitamin D stimulates cell migration in culture. Epithelial cell restitution is a key step in mucosal repair that involves cell restitution.
migration (6); we therefore determined the effect of vitamin D on cell migration. We measured cell migration under a condition where cell proliferation was stopped with 24-h starvation with serum-free media, followed by low (1%)-serum media. In the cell migration assay, using Caco-2 cells, we found that 1,25(OH)₂D₃ treatment markedly stimulated cell migration after the monolayers were wounded (Fig. 8); in comparison, fewer cells migrated across the scratched line in the untreated control (Fig. 8A). This observation suggests that vitamin D may stimulate epithelial restitution in mucosal wound healing.

Fig. 6. Effect of vitamin D on tight junctions. A and B: induction of junction proteins by vitamin D in SW480 cells. SW480 cells were treated with indicated doses of 1,25-dihydroxy-vitamin D₃ [1,25(OH)₂D₃] for 24 h, and cell lysates were analyzed by Western blot analysis with antibodies against tight junction proteins (A) or adherens junction protein E-cadherin (B). C, D, and E: effect of vitamin D on tight junctions formed by Caco-2 cells. C: Western blot analysis showing induction of ZO-1 and E-cadherin protein after 24 h of 1,25(OH)₂D₃ (VD) treatment. D: immunostaining of Caco-2 monolayer with anti-ZO-1 antibody. Caco-2 cell monolayers were pretreated with or without 10⁻⁸ M 1,25(OH)₂D₃ for 48 h, and 5% DSS was then added to the media for 2 h before immunostaining. a, control; b, 1,25(OH)₂D₃; c, control + DSS; d, 1,25(OH)₂D₃ + DSS. E: effects of DSS on TER. Caco-2 monolayers grown on filters in the presence (VD) or absence (control) of 1,25(OH)₂D₃ (10⁻⁸ M) were treated with 3% DSS. TER was monitored from 0 to 210 min after the addition of DSS to the media. The TER is presented as % of the basal value in cells at 0 min not treated with DSS.

Fig. 7. Effect of VDR knockdown on tight junctions. A: Caco-2 cells grown at 50–70% confluency were transfected with 100 nM human (h)VDR-small interfering (si)RNA (+) or scramble siRNA (−). Total RNAs were extracted after 48 h and used for RT-PCR analysis of the junction proteins as indicated. B: VDR protein levels were monitored by Western blot analysis at 4, 7, and 9 days after hVDR-siRNA transfection. C: at 48 h after hVDR-siRNA transfection, Caco-2 cells were treated with 10⁻⁸ and 10⁻¹⁰ M 1,25(OH)₂D₃ for 24 h, and ZO-1 protein levels were measured by Western blot analysis. d9, Day 9. D: effect of VDR knockdown on TER. Caco-2 cells were transfected with VDR-siRNA or scramble control siRNA (C-siRNA) on day 0, and the TER was measured on days 0, 4 and 6 posttransfection. E: VDR knockdown increases the susceptibility of Caco-2 cell monolayers to DSS treatment. Caco-2 monolayers transfected with VDR-siRNA or C-siRNA were exposed to 5% DSS. TER was monitored from 0 to 210 min after the addition of DSS to the media. The TER is presented as % of the basal value at 0 min.
Fig. 8. Effect of vitamin D on cell migration. Caco-2 cells were grown to monolayers in DMEM supplemented with 10% FBS. After 24-h starvation in serum-free media, the monolayers were scratched with a sterile razor blade. The cells were continually incubated in DMEM supplemented with 1% FBS for 48 h in the absence (A) or presence (B) of 10^{-8} M 1,25(OH)2D3, and cell migration was assessed under a microscope. Note that 1,25(OH)2D3 stimulated more cell migration across the scratched wound line (arrows) compared with the untreated control. Magnification: ×100.

DISCUSSION

Epidemiological evidence has suggested an association between vitamin D deficiency and increased IBD risk (23); however, the underlying mechanisms remain to be elucidated. Animal studies using IL-10 knockout mice have pointed to an immunomodulating role of VDR in IBD development. Vitamin D may reduce IBD risk by suppressing T cell-mediated immune response in the colon (1). This notion is consistent with the well-known immunomodulating activity of vitamin D (26). However, regulation of the immune compartment may be only part of the mechanism linking vitamin D deficiency to IBD.

Our data obtained from the present study suggest that maintenance of the epithelial barrier integrity of the large intestine by vitamin D is also critical in preventing IBD development. To our knowledge the role of vitamin D/VDR in intestinal barrier homeostasis has not been reported.

The DSS-colitis model shares many clinical and pathological features of human ulcerative colitis with regard to ulceration and loss of barrier function (28). Although the exact action of DSS is not fully understood, it is believed that DSS causes mucosal injury and disrupts the barrier function, leading to inflammation. DSS has been shown to increase mucosal permeability in mice (14) and reduce TER in intestinal cell monolayers (39), and the DSS-colitis model has been widely used to study mucosal healing (20, 25, 41). Therefore, the DSS model is an appropriate model to investigate the effect of VDR on mucosal barrier function and repair. Using this model, we found that mice lacking VDR are much more susceptible to mucosal injury than wild-type mice. After DSS treatment, VDR−/− mice lost more weight and developed more severe clinical symptoms, including significant bleeding and dehydration, leading to early death. A more recent study reported similar findings in DSS-treated VDR−/− mice in terms of symptom development (8), but this study focused on the innate immune response of the colon. Under normal conditions, VDR−/− mice exhibit no colonic abnormalities, except for a degree of hyperpermeability reported earlier (13); without stress their colonic mucosa appear to function normally. Under the DSS insult, however, massive ulcerations developed. This is probably due to some compromise in the tight junction structure and impairment in mucosal wound healing in the absence of VDR.

The intestinal mucosal barrier is mainly composed of epithelial cells and intercellular junctions that seal the paracellular space. The integrity of the intestinal mucosal barrier is preserved by the enormous regenerating capacity of the mucosal epithelium. The pluripotent stem cells give rise to the major cell types in the colonic epithelium (4). The progeny of the dividing cells migrate upwards from the base to the luminal surface of the crypt (34). Repairing of damaged mucosa normally requires epithelial cell restitution, involving cell migration, proliferation, and differentiation, leading to new crypt formation (6). Indeed, in VDR+/− mice, after DSS treatment was stopped, epithelial restitution, proliferation, and new crypt formation were clearly seen in the ulcerated lesions. In contrast, no effective wound-healing process occurred in VDR−/− mice during the recovery phase. Therefore, the VDR appears to be required for mucosal repair. This notion is supported by the observation that VDR expression is markedly induced in the colonic mucosa during mucosal recovery after DSS insult. Accordingly, wound healing is impaired in VDR−/− mice because VDR upregulation is not possible. Consistently, in vitro data demonstrate that vitamin D stimulates epithelial cell migration, suggesting that vitamin D is involved in the regulation of epithelial restitution in wound healing. Multiple signaling pathways, such as those of BMP, Wnt, hedgehog, trefoil factors, and growth hormone, are involved in intestinal mucosal regeneration and wound healing (4, 20, 25, 41). VDR may regulate components of these and other pathways in mucosal regeneration. More in-depth investigations are required to elucidate the VDR-regulated downstream events involved in mucosal repair.

Another key component of the mucosal barrier is the apical and subapical intercellular junctions between the epithelial cells, namely tight junctions and adherens junctions (18). These junctions seal the paracellular space and regulate the permeability of the mucosal barrier. The junctions are highly ordered structures formed by multi-protein complexes consisting of transmembrane proteins and nonmembrane proteins. In the apical tight junction, neighboring cells are sealed by transmembrane proteins, including claudins, occludin, junctional adhesion molecules, and Crumb. These proteins interact with the cytosolic terminal plaques formed by zonula occludens (ZO) family members, which function as scaffolds to link the junction to the actin cytoskeleton and recruit signaling molecules (15). In the subapical adherens junction, the neighboring cell membranes are adhered by cadherins. The cytoplasmic domain of E-cadherin associates with catenins, which provide the anchorage to the actin cytoskeleton to form stable cell-to-cell contacts (5). In this study, we found that the severe mucosal ulceration in VDR−/− mice was preceded by a marked drop in TER in DSS-treated colons, suggesting that in the absence of VDR the paracellular junctions are less resistant to DSS. Weaker paracellular junctions and erosion on the colonic epithelium can all contribute to the steeper reduction in TER seen in VDR−/− mice. Confocal immunostaining and EM
data confirmed the disruption of the tight junction complex by DSS treatment in VDR−/− mice. The observations in mice are supported by in vitro cell studies, which showed that vitamin D increased the resistance of Caco-2 monolayers to DSS disruption by stimulating several tight junction proteins and enhancing tight junctions. The observation that VDR knockdown by siRNA led to downregulation of junction proteins and loss of TER confirms the importance of VDR-mediated vitamin D signaling in tight junction regulation. The junction proteins upregulated by 1,25(OH)2D3 include ZO-1, claudin-1, -2, and E-cadherin. One exception is occludin, which is not directly regulated by vitamin D in cell culture but diminished from VDR+/− colonic mucosa under DSS insult. These results suggest that the VDR-mediated action also involves stabilization of epithelial junction complex in vivo. However, the mechanism underlying these observations is unclear and remains to be elucidated. Of note is an early study and our present study is unknown. The reason for the discrepancy between that study and our present study is unknown.

Because the myeloid cells in the large intestine also express VDR, one limitation of this study is that the contribution of the myeloid compartment to the supersensitivity to DSS injury in VDR−/− mice cannot be ruled out. Future bone marrow transplant experiments will help address this issue. It remains to be determined whether the DSS susceptibility is entirely caused by the loss of VDR in the epithelial compartment. Furthermore, the fact that the colonic epithelial cells in VDR−/− mice are hyperproliferative (13) also complicates the mechanism underlying the sensitivity to DSS insult. More studies are needed to fully elucidate the mechanism.

In summary, in this report we presented evidence suggesting that the VDR plays a critical role in preserving the integrity of the intestinal mucosal barrier. VDR is able to enhance the intercellular junctions; it is also required for mucosal wound healing. Mice lacking VDR are much more susceptible to DSS-induced mucosal injury, leading to extensive ulceration and early death. In vitro experiments demonstrate that VDR mediates the activity of 1,25(OH)2D3 that induces junction protein expression and strengthens the tight junction complex. These data are consistent with, and explain at least in part, the observation reported in the literature that vitamin D deficiency is linked to increased incidence of IBD in human population.

GRANTS

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REFERENCES


