Vitamin D₃ promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling

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The β-catenin signaling pathway is deregulated in nearly all colon cancers. Nonhypercalcemic vitamin D₃ (1α,25-dehydroxyvitamin D₃) analogues are candidate drugs to treat this neoplasia. We show that these compounds promote the differentiation of human colon carcinoma SW480 cells expressing vitamin D receptors (VDRs) (SW480-ADH) but not that of a malignant subline (SW620) cells lacking VDR. 1α,25(OH₂)D₃ induced the expression of E-cadherin and other adhesion proteins (occludin, Zonula occludens [ZO]-1, ZO-2, vinculin) and promoted the translocation of β-catenin, plakoglobin, and ZO-1 from the nucleus to the plasma membrane. Ligand-activated VDR competed with T cell transcription factor (TCF)-4 for β-catenin binding. Accordingly, 1α,25(OH₂)D₃ repressed β-catenin–TCF-4 transcriptional activity. Moreover, VDR activity was enhanced by ectopic β-catenin and reduced by TCF-4. Also, 1α,25(OH₂)D₃ inhibited expression of β-catenin–TCF-4-responsive genes, c-myc, peroxisome proliferator-activated receptor β, Tcf-1, and CD44, whereas it induced expression of ZO-1. Our results show that 1α,25(OH₂)D₃ induces E-cadherin and modulates β-catenin–TCF-4 target genes in a manner opposite to that of β-catenin, promoting the differentiation of colon carcinoma cells.

Introduction

Colon cancer is the third cause of cancer death in Western countries and a major health problem worldwide (Parker et al., 1997). Colon carcinogenesis is the result of the progressive transformation of colonic epithelial cells, which accumulate mutations that increase their proliferation and alter their phenotype.

E-cadherin is a transmembrane linker protein of the intercellular adheren junctions that play a key role in the maintenance of the adhesive and polarized phenotype of epithelial cells (Takeichi, 1995; Gumbiner, 1996). Loss of E-cadherin expression is a common event during the transition from adenoma to carcinoma, which involves the alteration of the normal epithelial phenotype and the acquisition of invasive capacity (Birchmeier and Behrens, 1994; Perl et al., 1998; Christofori and Semb, 1999). E-cadherin is regarded as a tumor suppressor gene and its loss as a predictor of poor prognosis (Vleminckx, et al., 1991; Takeichi, 1993; Mareel et al., 1994; Guilford et al., 1998). β-Catenin is a protooncogene encoding a cytoskeleton-associated protein, which in normal epithelial cells is bound mostly to the cytoplasmic tail of E-cadherin at the adherens junctions (for review see Morin, 1999). Free cytosolic β-catenin levels are strictly controlled by phosphorylation of the NH₂-terminal region of the protein by glycogen synthase kinase (GSK)* 3β. This reaction requires association with axin/conductin and the product of the adenomatous polyposis coli (APC) tumor suppressor

*Abbreviations used in this paper: APC, adenomatous polyposis coli; DR, direct repeat; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK, glycogen synthase kinase; GST, glutathione S-transferase; LEF, lymphoid enhancer-binding factor; 1α,25(OH₂)D₃, 1α,25-dihydroxyvitamin D₃; PPAR, peroxisome proliferator-activated receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; TCF, T cell transcription factor; TER, transepithelial electrical resistance; VDR, vitamin D receptor;VDRE, vitamin D response element; ZO, Zonula occludens.
gene, and targets β-catenin for ubiquitin-mediated degradation by the proteasome (Rubinfeld et al., 1996; Behrens et al., 1998; Kishida et al., 1998). Interaction of Wnt ligands with their membrane receptors blocks GSK-3β, leading to the accumulation of free β-catenin (for reviews see Eastman and Grosschedl, 1999; Peifer and Polakos, 2000). In the cell nucleus, β-catenin binds members of the T cell transcription factor (TCF)/lymphoid enhancer-binding factor (LEF)-1 family and thus regulates gene expression (Behrens et al., 1996; Huber et al., 1996; Billin et al., 2000). Nearly all colon tumors present a deregulated β-catenin signaling pathway by mutation of either APC or β-catenin, which leads to the blockade of phosphorylation by GSK-3β, resulting in β-catenin stabilization (Inomata et al., 1996; Korinek et al., 1997; Morin et al., 1997), reduced APC-regulated nuclear export (Henderson, 2000; Rosin-Arbesfeld et al., 2000), and perhaps higher specific activity (Guger and Gumbiner, 2000).

As a result, β-catenin accumulates in the nucleus, leading to both the activation of genes involved in the control of cell proliferation and invasiveness such as c-myc, cyclin D1, peroxisome proliferator-activated receptor (PPAR) δ, matrilysin, c-jun, fra1, uPA receptor, fibronectin, CD44, TCF-1, Cdx-1 and gastrin, and the loss of expression of DRCRNB1A and differentiated epithelial markers such as Zonula occludens (ZO)-1 (He et al., 1998, 1999; Crawford et al., 1999; Gradl et al., 1999; Mann et al., 1999; Tetsu and McCormick, 1999), reduced APC-regulated nuclear export (Henderson, 2000; Rosin-Arbesfeld et al., 2000), and perhaps higher specific activity (Guger and Gumbiner, 2000).

Vitamin D, especially its most active metabolite, 25-hydroxyvitamin D3 (1α,25(OH)2D3), not only contributes to calcium homeostasis but also regulates cell proliferation and differentiation (Saez et al., 1993; Xi and Feldman, 1993; Buras et al., 1994; Kane et al., 1996). 1α,25(OH)2D3 and several synthetic vitamin D derivatives (deltanoids), which show reduced calcemic activity such as EB1089, MC903, and KH1060, inhibit the growth of epithelial, melanoma, soft tissue sarcoma, and leukemic cells by inducing cell cycle arrest or apoptosis (Diaz et al., 2000; Park et al., 2000). Furthermore, they inhibit the invasive capacity in vitro, the synthesis of several invasion-associated proteins (Hansen et al., 1994; Gonzalez-Sancho et al., 1998; Koli and Keskü-Oja, 2000), and the tumor-induced angiogenesis (Majewski et al., 1993) of breast cancer cells, and they show a chemopreventive activity in animal models of colorectal and breast cancer (Akhter et al., 1997; van Weelden et al., 1998).

Vitamin D and its analogues regulate gene expression by binding to specific vitamin D receptors (VDRs) of the nuclear receptor superfamily, which are ligand-modulated transcription factors (for review see McDonald et al., 2001). Upon ligand activation, VDR binds specific nucleotide sequences (vitamin D response elements, VDREs) in target genes to activate or repress their expression through multiple but ill-defined interactions with coactivator complexes and components of the basal transcription machinery (for review see McDonald et al., 2001). Several vitamin D target genes have been characterized in several tumor cell types such as c-myc, p21Waf1, p27Kip1, tenascin-C, fibronectin, laminin and its receptor, apolipoprotein D, insulin-like growth factor binding protein 3, cyclin C, and several members of the transforming growth factor family and their receptors (Freedman, 1999). Two studies have reported the regulation of epidermal growth factor receptor expression (Tong et al., 1998) or the cross-talk between vitamin D and tumor growth factor β signaling pathways (Yanagisawa et al., 1999), although the physiological relevance of these mechanisms remains unclear.

VDR is expressed in the normal colonic mucosa, slightly increases in the hyperplastic condition, and is clearly lower in late stages of carcinogenesis (Vandewalle et al., 1994). Accordingly, high VDR expression is associated with favorable prognosis in colorectal cancer patients, suggesting that these receptors are involved in this pathogenesis and their potential role as a predictive marker (Shahang et al., 1993; Evans et al., 1998). Several clinical trials are underway to assess the activity of several vitamin D derivatives in patients with colorectal carcinoma and other neoplasias (Gross et al., 1998; Gulliford et al., 1998; Smith et al., 1999). However, their molecular and cellular mechanisms of action in colon carcinoma cells remain mostly unknown.

The SW480 cell line is one of the best characterized human colorectal cancer lines, and it has been widely used as a model system for the study of this neoplasia. It was established in 1976 from a primary human Duke’s B colon adenocarcinoma of a 50-yr-old patient (Leibovitz et al., 1976). SW480 cells harbor most of the genetic abnormalities that characterize advanced colon cancers such as activation of K-ras oncogene, c-myc amplification, deletion of chromosome 18, and mutation of APC and p53 tumor suppressor genes (Tomita et al., 1992; Schwarte-Waldhoff et al., 1999). In addition, these cells are defective for E-cadherin and express high levels of nuclear β-catenin, transforming growth factor β, and epidermal growth factor receptors (Tomita et al., 1992). We used the SW480 cell line to examine the mechanism of action of 1α,25(OH)2D3 and several nonhypercalcemic analogues in colon cancer cells. Our results show that these compounds have a prodifferentiation phenotypic effect on VDR-positive SW480 cells parallel to the induction of E-cadherin, induce β-catenin nuclear export, and inhibit β-catenin gene regulatory activity. Moreover, 1α,25(OH)2D3 promotes a direct VDR–β-catenin interaction, which may decrease TCF-4–β-catenin complexes and may thus constitute another mechanism of inhibition of β-catenin signaling.

**Results**

1α,25(OH)2D3 induces the differentiation of a VDR-positive subpopulation of SW480 cells to an epithelial-like phenotype

To investigate its mechanism of action in human colon cancer cells, two cell lines from the same patient, SW480 cells established from a primary adenocarcinoma and SW620 from a lymph node metastasis, were treated with 1α,25(OH)2D3. Upon 1α,25(OH)2D3 addition, a proportion of SW480 cells...
changed in shape and properties to a more adhesive epithelial phenotype (Fig. 1 A, a and b), whereas the rest of the SW480 population and SW620 cells were unaffected (Fig. 1 A, a, b, g, and h). These two distinct responses in SW480 cultures correlated with two cell morphologies: flat, polygonal, and adherent to plastic dishes, which corresponded to 1α,25(OH)₂D₃-responsive cells, and rounded, refractile, and less adherent, which corresponded to nonresponsive cells (Fig. 1 A, a and b, arrows). This is consistent with previous reports of the existence of two populations in SW480 cell cultures (Tomita et al., 1992; Baulida et al., 1999) and led us to obtain clonal sublines of each cell type: SW480-ADH (adherent) and SW480-R (rounded). In agreement with the previous finding, the two established sublines retained their distinct morphology and hormonal response for >2 yr: upon addition of 1α,25(OH)₂D₃, the bulk of SW480-ADH cells acquired an epithelial-like phenotype, forming compact islands (Fig. 1 A, c and d), whereas SW480-R cells were unaffected (Fig. 1 A, e and f). Moreover, SW480-R cells were highly tumorigenic in athymic mice (eight of eight tumors were generated 9 wk after the subcutaneous injection of 10⁶ cells), whereas in contrast SW480-ADH cells were weakly tumorigenic (3 of 24). The distinct behavior of these cell types was attributable to differences in the expression of VDR, which was checked in Northern blot assays (Fig. 1 B). VDR mRNA levels were high in the SW480-ADH subline and three times lower in parental SW480 cells. In contrast, SW620 cells expressed a very low level of VDR mRNA, which was undetectable in SW480-R cells. Further, 1α,25(OH)₂D₃ increased the expression of a transfected luciferase reporter gene cloned under the control of four copies of a direct repeat (DR) VDRE (4 × VDRE) in SW480-ADH and to a lesser extent in parental SW480 cells but had no effect in SW480-R or SW620 cells (Fig. 1 C). In addition, cotransfection of an exogenous VDR clearly induced the luciferase gene in all cell types (Fig. 1 C). Thus, the VDR expression of SW480-ADH cells and to a lesser extent of SW480 cells, whereas SW480-R and SW620 cells did not respond to this agent, owing to the lack of endogenous VDR expression. In agreement with these results and its effect in other cell types (Buras et al., 1994; Kane et al., 1996), 1α,25(OH)₂D₃ inhibited the proliferation of SW480-ADH cells and to a lesser extent that of SW480 cells, whereas SW480-R and SW620 cells were totally unresponsive (Fig. 1 D). We tested whether the reexpression of VDR reverted the malignant phenotype of SW480-R cells. However, no clones of SW480-R cells stably expressing VDR were obtained in any of three experiments carried out using various vectors that were functional in other cell systems, suggesting that VDR, perhaps by inhibiting cell growth, counterselects these cells and thus hampers clonal expansion.

1α,25(OH)₂D₃ induces E-cadherin transcription and regulates the expression and localization of other adhesion proteins

To examine the effect of 1α,25(OH)₂D₃ on the phenotype of SW480-ADH cells, we performed immunofluorescence and confocal laser microscopy analyses of several epithelial markers. Although SW480 cells have been described as E-cadherin–defective (Sadot et al., 1998; Baulida et al., 1999; Orsulic et al., 1999), SW480-ADH cells exhibited low but detectable membrane-bound expression of this intercellular adhesion protein that was drastically increased upon treatment with 1α,25(OH)₂D₃ (Fig. 2 A, a–o). The induction of E-cadherin expression correlated with the phenotypic change: it was observed as soon as 16 h after treatment, was especially high at 48 h, and lasted 7 d (Fig. 2 A, a–c). Like parental SW480 and other colon cancer lines defective for APC and E-cadherin, SW480-ADH cells showed high β-catenin levels in the nucleus and cytoplasm (Fig. 2 A, d). Remarkably, 1α,25(OH)₂D₃ severely altered the subcellular location of β-catenin, from predominantly nuclear to a nearly exclusive plasma membrane distribution (Fig. 2 A, d–f). This paralleled the induction of E-cadherin, which was maximal 48 h after 1α,25(OH)₂D₃ addition and agreed with the reported sequestration of β-catenin at the plasma membrane compartment after transient transfection of SW480 cells with E-cadherin (Sadot et al., 1998; Orsulic et al., 1999).

1α,25(OH)₂D₃ also progressively increased the amount of occludin andZO-2, two components of the tight junctions that are located predominantly at the plasma membrane (Fig. 2 B, a–c and d–f, respectively). Zo-1, another component of these structures, was also enhanced (see Fig. 6). We also studied the effects of 1α,25(OH)₂D₃ on the expression of vinculin, a protein located in tight junctions, adherens junctions, and focal adhesion plaques (Watabe-Uchida et al., 1998). Untreated SW480-ADH cells showed a punctate staining of vinculin at the cell periphery, which probably corresponded to focal adhesion plaques (Fig. 2 B, g). 2 d after 1α,25(OH)₂D₃ addition, an increased dotted basal, but not apical, vinculin staining was observed (Fig. 2 B, h–i), whereas at 7 d after treatment both basal and apical surfaces were strongly stained, revealing the relocation of vinculin in intercellular junctional structures (Fig. 2 B, j–k). Altogether, these data indicated that 1α,25(OH)₂D₃ induces the expression of intercellular adhesion proteins in SW480-ADH cultures. The acquisition of the adhesive phenotype and the formation of compact islands increased with the duration of 1α,25(OH)₂D₃ treatment and depended on the presence of this agent in the medium, since its removal led to progressive cell disaggregation. Finally, 1α,25(OH)₂D₃ also relocated γ-catenin/plakoglobin from the nucleus and cytoplasm to the plasma membrane (unpublished data).

To determine whether the induction of occludin, ZO-1, and ZO-2 led to the formation of functional tight junctions, we measured the transepithelial electrical resistance (TER) of SW480-ADH cell monolayers grown on porous filters. As expected from their tumoral origin and spindle shape, untreated SW480-ADH cells showed no significant TER. In contrast, cells treated for 4 wk with 1α,25(OH)₂D₃ displayed low but detectable TER when seeded on filters (day 0), which progressively increased (fourfold) on the following days, revealing the formation of functional tight junctions (Table 1). The requirement for long 1α,25(OH)₂D₃ treatments to induce TER (1–2 wk were insufficient) suggests that additional unknown proteins must be induced with slow kinetics and/or that the establishment of adequate molecular interactions is a slow process.
Figure 1. Effect of 1α,25(OH)2D3 on human colon carcinoma cells expressing variable levels of VDR. (A) Phase-contrast micrographs of various cell lines upon 48-h treatment with 1α,25(OH)2D3 (10-7 M) or vehicle (control): SW480 (a and b); SW480-ADH (c and d); SW480-R (e and f); SW620 (g and h). The two distinct cell types found in SW480 cultures are indicated in a and b: flat, polygonal, and 1α,25(OH)2D3-sensitive (white arrows), and 1α,25(OH)2D3-unresponsive (black arrows). (B) Northern blot analysis of VDR and RXRα expression in SW480, SW480-ADH, SW480-R, and SW620 cells untreated or treated with 10-7 M 1α,25(OH)2D3 for 48 h. 10 μg of poly(A)+ RNA was loaded per lane. GAPDH was used as internal control. (C) 1α,25(OH)2D3 transcriptional responsiveness of each cell line. Cells were transfected with the 4VDRE–DR3-tk-luc construct and a human VDR expression vector as indicated. After 48-h incubation in the presence or absence of 1α,25(OH)2D3 (10-7 M), luciferase activity in total cell extracts was measured as described in Materials and methods. A β-galactosidase expression vector was also transfected as internal control. Mean values and standard deviations of the mean obtained in three experiments using triplicates are shown. (D) Effect of 1α,25(OH)2D3 on DNA synthesis. Cells were untreated or treated with the indicated 1α,25(OH)2D3 concentrations for 48 h, and the level of DNA synthesis was measured by estimating the incorporation of labeled thymidine in TCA-precipitable material as described in Materials and methods. Mean values and standard deviations of the mean obtained in three experiments using duplicates are shown. ○, SW480 cells; ●, SW480-R cells; □, SW480-ADH cells; ■, SW620 cells.
Figure 2. Induction of epithelial markers by 1\textalpha,25(OH)\textsubscript{2}D\textsubscript{3} in SW480-ADH cells. (A) Analysis by immunofluorescence and confocal laser scanning microscopy of the expression of various adhesion proteins in cells treated with 10\textsuperscript{-7} M 1\textalpha,25(OH)\textsubscript{2}D\textsubscript{3} for the indicated times or left untreated (control): E-cadherin (a–c); β-catenin (d–f). (B) Same as in A with longer treatments: a–c, occludin; d–f, ZO-2; g–k, vinculin. Vinculin expression was analyzed at two sections: basal (g, i, and k) and apical (h and j). Bars, 10 μm.
Given that adherens junctions are essential to intercellular adhesion and epithelial polarity and that E-cadherin plays a role in these structures, we explored the mechanism by which 1α,25(OH)2D3 induces this protein. Northern blotting revealed that a 48-h treatment with 1α,25(OH)2D3 substantially increased (five- to sixfold) the level of E-cadherin 4.6 kb mRNA in SW480-ADH cells but not in cells lacking VDR (SW480-R and SW620) (Fig. 3 A). In contrast, the amount of β-catenin 3.4 kb mRNA was unaffected, and comparable levels, slightly lower in SW480-R, were detected in the three cell lines. In agreement with these results, a strong increase in the level of E-cadherin protein but no change in that of β-catenin were found upon 1α,25(OH)2D3 treatment (Fig. 3 B). Other hormones acting through members of the nuclear receptor superfamily such as dexamethasone, thyroid hormone, and all-trans retinoic acid failed to induce E-cadherin expression, showing the specificity of 1α,25(OH)2D3 (Fig. 3 C; unpublished data). Likewise, 9-cis retinoic acid, whose retinoid X receptor (RXR) is the common partner of VDR, was ineffective by itself and it did not alter the induction of E-cadherin by 1α,25(OH)2D3 (Fig. 3 C; unpublished data). This strongly suggests that ligand activation of VDR is sufficient for E-cadherin induction by RXX-VDR heterodimers. We then studied the kinetics of the induction of E-cadherin mRNA in SW480-ADH cells by 1α,25(OH)2D3. The induction was abrupt with a rapid increase (10-fold) 4 h after addition and a peak at ~8 h (12.5-fold) followed by a slow decay (Fig. 3, D and F). Accordingly, the cellular content of E-cadherin protein increased by 7-fold at 16 h and by ~13-fold at 48 h after treatment (Fig. 3, E and F).

Transcription and translation inhibitors (actinomycin D and cycloheximide, respectively) were used to understand the E-cadherin induction. Actinomycin D completely blocked the effect of 1α,25(OH)2D3, whereas cycloheximide strongly inhibited it (Fig. 4 A). This indicates that 1α,25(OH)2D3 induces E-cadherin expression by a transcriptional mechanism that requires protein synthesis de novo. Since 1α,25(OH)2D3 interacts with the phosphoinositol 3-kinase and protein kinase C–signaling pathways in various cell types (Gniadecki et al., 1997; Hmama et al., 1999), we aimed to elucidate whether this also occurs in SW480-ADH cells. However, the induction of E-cadherin was unaffected by inhibitors of phosphoinositol 3-kinase (wortmannin, Ly294002), and it was only partially affected (30% reduction in RNA increase at 4 h) by a protein kinase C antagonist (bisindolylmaleimide), suggesting that these two signaling pathways are not relevant to the activity of 1α,25(OH)2D3 (unpublished data). The induction of E-cadherin transcription by 1α,25(OH)2D3 was tested in transient transfection experiments using two distinct constructs: either a short 270-bp (~178 to +92) or a long 1,072-bp (~987 to +92) fragment of the human gene promoter controlling the expression of the luciferase reporter gene. Consistently, a 1.8-fold induction of the large promoter construct but not of the small one was observed (Fig. 4 B). Further supporting the transcriptional effect of 1α,25(OH)2D3, no induction was detected when mutant forms of VDR (ΔAF2), lacking the COOH-terminal AF-2 domain responsible for transcriptional activation or harboring inactivating point mutations in this region (L417S and E420Q), were transfected (unpublished data). This discrepancy between the level of induction of the promoter construct used and the higher increase in E-cadherin mRNA content induced by 1α,25(OH)2D3 in SW480-ADH cells may be due to the lack of additional regulatory sequences in the available promoter region studied and/or effects on RNA stability. We ruled out the second hypothesis by analyzing whether 1α,25(OH)2D3 modulated the half-life of E-cadherin mRNA in SW480-ADH cells: the decay in E-cadherin mRNA content was the same in untreated and in 1α,25(OH)2D3-treated cells (Fig. 4 C).

1α,25(OH)2D3 causes β-catenin to translocate from the nucleus to E-cadherin complexes at the plasma membrane and inhibits its gene regulatory activity

Exogenous E-cadherin induces β-catenin relocation by nuclear export and the formation of E-cadherin–β-catenin complexes at the plasma membrane in SW480 and other cell lines lacking endogenous E-cadherin (Sadot et al., 1998; Orsulic et al., 1999). Since 1α,25(OH)2D3 induced E-cadherin expression in SW480-ADH cells, we determined whether it could promote a similar effect in these cells. 2-d treatments with 1α,25(OH)2D3 induced a nearly complete colocalization of both proteins at the region of the plasma membrane in contact with neighboring cells (Fig. 5, top). An identical effect was observed when untreated SW480-ADH cells were transfected with an E-cadherin expression plasmid (Fig. 5, top). As controls, we used VDR-negative SW480-R cells, which did not show any change in the strong nuclear β-catenin staining upon 1α,25(OH)2D3 addition (Fig. 5, bottom). As expected, exogenous E-cadherin caused the same change in β-catenin localization as in SW480-ADH cells (Fig. 5, bottom). To correlate the change in β-catenin localization induced by 1α,25(OH)2D3 with the effects on β-catenin–TCF/LEF-1 target genes as a function of time, we studied the kinetics of β-catenin nuclear export. To this end, we analyzed the immunofluorescence of SW480-ADH cells stained with an anti-β-catenin antibody by confocal laser microscopy and counted the cells displaying predominantly nuclear, a mixed nuclear-cytosolic, or an exclusively mem-
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branous β-catenin localization (Fig. 6A, insets). The number of nuclear-positive β-catenin cells clearly decreased (from 66 to 24%) as soon as 16 h after 1α,25(OH)2D3 addition, was further reduced (to 10%) at 48 h, and was extremely low (around 2%) 7 d later (Fig. 6A, white bars). Conversely, the proportion of cells with predominant membrane-bound β-catenin increased progressively from 11% in nontreated cultures to 70% after 7 d of 1α,25(OH)2D3 treatment (Fig. 6A, black bars).

Therefore, we aimed to examine the putative effects of 1α,25(OH)2D3 on β-catenin signaling. First, we checked whether TCF-4 RNA expression was modified by 1α,25(OH)2D3 or not as in the case of β-catenin. No changes were detected by Northern and Western blotting (unpublished data). To measure β-catenin–mediated transcription, we used a reporter gene placed under the control of a promoter constructed by fusing three copies of the TCF/LEF-1 consensus responsive sequence and a c-fos minimal promoter (TOP-flash). A mutated form of this plasmid (FOP-flash) was used as negative control. 1α,25(OH)2D3 effectively inhibited β-catenin–TCF/LEF-1 transcriptional activity in transfected SW480-ADH cells (Fig. 6B). Moreover, since these cells express a mutant APC protein and 1α,25(OH)2D3 did not modify β-catenin levels this result shows that wild-type APC is not required by 1α,25(OH)2D3 to inhibit β-catenin signaling.

Next, we analyzed the regulation of endogenous β-catenin–TCF/LEF-1 target genes. 1α,25(OH)2D3 reduced the cellular RNA levels of c-myc, Tcf-1, CD44, and PPARδ genes induced by β-catenin–TCF/LEF-1 (He et al., 1998, Figure 3. Induction of E-cadherin expression by 1α,25(OH)2D3. (A) Northern blot analysis of E-cadherin and β-catenin mRNA expression in SW480-ADH, SW480-R, and SW620 cells untreated or treated with 10−7 M 1α,25(OH)2D3 for 48 h. 10 μg of poly(A)+ RNA was loaded per lane. GAPDH was used as an internal control. (B) Western blot analysis of E-cadherin and β-catenin protein expression in the same conditions. (C) Specificity of 1α,25(OH)2D3 action. Northern blot analysis of E-cadherin mRNA expression in SW480-ADH cells untreated or treated with 10−7 M of the indicated agent or the corresponding vehicles for 48 h. Conditions were as above. (D) Northern blot analysis of the kinetics of induction of E-cadherin mRNA by 1α,25(OH)2D3 in SW480-ADH cells. Times of treatment are indicated. Conditions were as above. (E) Western blot analysis of the kinetics of induction of E-cadherin protein by 1α,25(OH)2D3 in SW480-ADH cells. Times of treatment are indicated. (F) Quantification of the induction by 1α,25(OH)2D3 in SW480-ADH cells of E-cadherin mRNA (●) and protein (○) and of β-catenin mRNA (◼) and protein (□). Fold increase with respect to expression in untreated cells (time 0) is represented. Mean values of three experiments are shown. Quantifications were performed using NIH image software.
1α,25(OH)2D3 (Act D, 2 μg/ml) and then incubated in the presence (•) or absence (○) of 1α,25(OH)2D3 (10−7 M), during the indicated times. Northern blot analysis of E-cadherin and GAPDH mRNA expression. Conditions were as above. Two independent experiments gave the same result.

Figure 4. Mechanism of E-cadherin gene induction by 1α,25(OH)2D3. (A) Northern blot analysis of E-cadherin mRNA expression in SW480-ADH cells untreated or treated with 10−7 M 1α,25(OH)2D3 for 4 h. Where indicated, cells were pretreated with actinomycin D (Act D, 2 μg/ml) or cycloheximide (CHX, 8 μg/ml) 30 min before 1α,25(OH)2D3 addition. 10 μg of poly(A)+ RNA was loaded per lane. GAPDH was used as an internal control. (B) Activation of the human E-cadherin gene promoter by 1α,25(OH)2D3. SW480-ADH cells were transfected with either −987-TK-Luc plasmid, which contains the genomic sequence from −92 to −987 bp, or −178-TK-Luc containing the sequence from −92 to −178 bp of the human E-cadherin gene. The empty TK-Luc vector was used as control. Transfections were performed as described in Materials and methods. White bars, untreated cells; black bars, cells treated with 10−7 M 1α,25(OH)2D3 during 48 h after transfection. Mean values correspond to five independent experiments done in triplicate are shown. (C) Lack of effect of 1α,25(OH)2D3 on E-cadherin mRNA stability. SW480-ADH cells were pretreated or not pretreated for 30 min with actinomycin D (2 μg/ml) and then incubated in the presence (○) or absence (●) of 1α,25(OH)2D3 (10−7 M), during the indicated times. Northern blot analysis of E-cadherin and GAPDH mRNA expression. Conditions were as above. Two independent experiments gave the same result.

VDR and β-catenin interact in a ligand-regulated manner

The rapid changes in the RNA levels of target genes after 1α,25(OH)2D3 treatment, which occurred earlier than the induction of β-catenin nuclear export, suggested additional mechanisms of action for 1α,25(OH)2D3. We tested whether VDR and β-catenin interacted, thus affecting the formation of β-catenin–TCF-4 complexes. To this end, in vitro–translated VDR was incubated with either glutathione S-transferase (GST)–β-catenin or GST proteins in the presence or absence of 1α,25(OH)2D3. After precipitation with GSH–Sepharose beads, the presence of VDR in the precipitates was tested by immunoblotting using an anti-VDR antibody, revealing a direct mostly ligand-independent VDR–β-catenin interaction (Fig. 8 A). To determine whether VDR and β-catenin form complexes in vivo, extracts from
Figure 5. **Induction of colocalization of E-cadherin and β-catenin at the plasma membrane by 1α,25(OH)2D3.** Analysis by immunofluorescence and confocal laser scanning microscopy of the expression of these two proteins in SW480-ADH or SW480-R cells at 48 h after either treatment with 1α,25(OH)2D3 (10^{-7} M) or transfection with an expression vector for human E-cadherin. Double immunofluorescence was performed using anti-E-cadherin and anti-β-catenin antibodies followed by the addition of the corresponding secondary TRICT-conjugated (E-cadherin, red) or FITC-conjugated (β-catenin, green) antibodies. The merge of both signals (yellow) indicates the areas of colocalization of both proteins. Bars, 10 μm.
Figure 6. Inhibition of the β-catenin–TCF-4 signaling by 1α,25(OH)2D3. (A) 1α,25(OH)2D3 induces nuclear export of β-catenin. Quantification of the percentage of SW480-ADH cells showing predominant nuclear (left inset, white bars), mixed nuclear-cytoplasmic (middle inset, gray bars), or exclusively membranous (right inset, black bars) β-catenin localization after treatment with 1α,25(OH)2D3 (10^{-7} M) for the indicated times. 500 cells were analyzed at each time point. (B) Inhibition of β-catenin–TCF-4 transcriptional activity by 1α,25(OH)2D3. SW480-ADH cells were transfected with the wild-type (TOP-flash) or mutated (FOP-flash) β-catenin–TCF4/LEF-1–sensitive reporter plasmids and then left untreated (white bars) or treated (black bars) with 1α,25(OH)2D3 (10^{-7} M) for 48 h. Mean values and standard deviation of the mean of triplicates obtained in three experiments are shown. (C) Effects of 1α,25(OH)2D3 on the expression of β-catenin–TCF-4 target genes. Northern blots analysis of mRNA expression in SW480-ADH cells untreated or treated with 10^{-7} M 1α,25(OH)2D3 for the indicated times. Conditions were as above. (D) Quantification of the change in ZO-1, PPARδ, CD44, Tcf-1, and c-myc mRNA levels induced by 1α,25(OH)2D3. Mean values and error bars corresponding to triplicates obtained in three experiments are shown. (E) Induction and redistribution of ZO-1 protein by 1α,25(OH)2D3 treatment. Analysis by immunofluorescence and confocal laser microscopy of ZO-1 expression in SW480-ADH cells after addition of 1α,25(OH)2D3 (10^{-7} M). Bar, 10 μm.
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Cells incubated with 1α,25(OH)2D3 for various periods were immunoprecipitated with either anti–β-catenin or anti-VDR antibodies and then subjected to immunoblotting with the opposite antibody. 1α,25(OH)2D3 rapidly (4 h) enhanced the VDR–β-catenin interaction (Fig. 8 B). Conversely, 1α,25(OH)2D3 decreased the amount of TCF-4 present in β-catenin immunoprecipitates (Fig. 8 B), suggesting that VDR and TCF-4 compete for β-catenin binding. 48 h after treatment, the level of VDR–β-catenin interaction was similar to that of untreated cells, probably because of the strong induction of E-cadherin, which leads to β-catenin sequestration at the cell junctions. In fact, coimmunoprecipitation using anti–E-cadherin and anti–β-catenin antibodies revealed the formation of E-cadherin–β-catenin complexes, which replaced VDR–β-catenin complexes as early as 16 h after 1α,25(OH)2D3 treatment in the same extracts (Fig. 8, B–F). We would like to point out that the change in complex formation was concomitant with E-cadherin protein induction and phenotypic modulation by 1α,25(OH)2D3. Similar results were obtained when the presence of β-catenin was determined in VDR immunoprecipitates. Differences in the amount of β-catenin–VDR complexes immunoprecipitated with one or the other antibodies at 16 h after 1α,25(OH)2D3 addition must be attributed to differences in affinity and/or epitope recognition. As stated above, 1α,25(OH)2D3 did not modify the cellular content of VDR, whereas in the case of β-catenin and TCF-4 the apparent changes were due to the weak extracting conditions used to preserve protein–protein interactions in these assays (Fig. 8 B). However, no changes in total β-catenin, VDR, or TCF-4 levels were detected under strong solubilization conditions as mentioned above (unpublished data). As expected, no VDR–β-catenin complexes were detected in SW480-R cells expressing only residual amounts of VDR (Fig. 8 G) that were unable to mediate VDRE activation (Fig. 1 C and Fig. 7).

Functional interplay between VDR, β-catenin, and TCF-4

Given that β-catenin is a transcriptional coactivator and interacts with VDR, we examined whether it enhances the activation of a VDRE by 1α,25(OH)2D3. To this end, we used a plasmid encoding a stable form of β-catenin resistant to APC-induced degradation in which the GSK-3β target Ser37 residue is mutated to Ala (S37A) (Easwaran et al., 1999). Exogenous β-catenin S37A expression did not modify VDRE activation in SW480-ADH or SW480-R cells, probably because of the high level of endogenous nuclear β-catenin (Fig. 9 A). To circumvent this problem, we transfected human MCF-7 mammary epithelial cells containing extremely low amounts of nuclear β-catenin. In these cells, β-catenin S37A fostered the induction by 1α,25(OH)2D3 of
a consensus VDRE from 6- to 49-fold (Fig. 9 B). In contrast, β-catenin S37A did not affect the basal VDRE levels in untreated cells (unpublished data). No such effect was observed on thyroid or glucocorticoid hormone response elements or on a β-catenin–dependent construct (unpublished data).

In contrast to β-catenin, exogenous TCF-4 caused a twofold reduction in VDRE activation in SW480-ADH (over endogenous VDR) and SW480-R (over exogenous VDR) cells (Fig. 9 A), further supporting the hypothesis that VDR and TCF-4 compete for β-catenin binding. A dose-response assay of transfection of increasing amounts of TCF-4 expression plasmid in SW480-ADH cells confirmed that TCF-4 hinders VDR activity (Fig. 9 C). In contrast, a mutant TCF-4 lacking the NH₂-terminal region required for β-catenin binding (ΔN-TCF-4) was unable to inhibit VDR activity (Fig. 9 C). Additionally, the inhibition of VDRE activation by TCF-4 was completely reversed by cotransfection of β-catenin S37A (Fig. 9 C).

Next, we examined whether ligand-activated VDR inhibits the transcriptional activity of β-catenin–TCF/LEF-1 complexes regardless of E-cadherin induction. To this end, we transfected β-catenin and TCF-4 in Pam212, a keratinocyte murine cell line expressing high amounts of E-cadherin and lacking nuclear β-catenin. In these cells, which express only residual levels of VDR, the activation of the TOP-flash reporter by β-catenin–TCF-4 was inhibited (greater than threefold) by 1α,25(OH)₂D₃ upon ectopic expression of VDR (Fig. 8 D) without concomitant induction of E-cadherin as assessed by Western blotting (unpublished data). This result is in line with those obtained in LS-174T cells in which 1α,25(OH)₂D₃ induces VDRE activation and inhibits β-catenin–TCF-4 transcriptional activity in spite of a total lack of E-cadherin induction (Fig. 7 B).

Nonhypercalcemic vitamin D₃ analogues are potent inhibitors of the β-catenin signaling pathway

Three synthetic vitamin D analogues with low calcemic properties, EB1089, KH1060, and MC903, used currently in clinical trials to treat several neoplasias (see Introduction for references) were studied for their effects on β-catenin signaling in SW480-ADH cells. Like 1α,25(OH)₂D₃, all three analogues displayed antiproliferative activity (unpublished data). We then studied whether these compounds induce the expression of E-cadherin mRNA and protein by North-
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Figure 9. Modulation of the transcriptional activity of VDR by β-catenin and TCF-4. (A) SW480-ADH and SW480-R cells were transfected with the 4 × VDRE–DR3-Tk-Luc construct in combination with expression vectors for VDR, β-catenin S37A, or TCF-4 as indicated. Luciferase activity was measured in extracts of cells untreated or treated with 1α,25(OH)₂D₃ (10⁻⁷ M) for 48 h. (B) β-Catenin enhances VDRE activation in MCF-7 cells. Cells were cotransfected with 4 × VDRE-DR3-Tk-Luc and increasing amounts of β-catenin S37A expression vector. (C) TCF-4 inhibits VDRE activation in SW480-ADH cells. Cells were cotransfected with 4 × VDRE-DR3-Tk-Luc construct and variable amounts of expression vectors for wild-type TCF-4, the mutant ΔN-TCF-4, or β-catenin S37A as indicated. Conditions and measurements were as above. In A–C, VDRE activation is represented as fold increase in treated versus untreated cells. (D) 1α,25(OH)₂D₃ inhibits β-catenin–TCF-4 transcriptional activity in Pam212 cells. Cells were co-transfected with TOP-flash or FOP-flash reporter constructs and with expression vectors for β-catenin, TCF-4, and VDR as indicated. 1α,25(OH)₂D₃ (10⁻⁷ M) was added 24 h after transfection, and cell extracts were prepared 24 h later. Fold increase values of luciferase activity (TOP/FOP) after normalization were calculated. In A–D, mean values and standard deviation of the mean obtained in duplicates of three independent experiments are shown.

ern and Western blotting. As 1α,25(OH)₂D₃, doses in the 10⁻¹¹–10⁻⁷ M range of each compound induced E-cadherin expression (Fig. 10 A). All three compounds reduced the expression of a β-catenin–TCF-4 reporter construct in SW480-ADH cells (Fig. 10 B). The effects observed in the assays show that EB1089 and KH1060 are more potent than 1α,25(OH)₂D₃, whereas MC903 is less active. In agreement with our previous results, all three analogues promoted the morphological differentiation and nuclear export of β-catenin in SW480-ADH cells but had no effect in VDR-defective SW480-R or SW620 cells as revealed by immunofluorescence (Fig. 10 C).

Discussion

Our results show that 1α,25(OH)₂D₃ and several nonhypercalcemic analogues used currently in clinical trials as potential anticancer drugs induce the differentiation of human colon carcinoma cells. They promote a phenotypic change involving the induction of E-cadherin expression and the blockade of the β-catenin gene regulatory activity in SW480-ADH cells.

1α,25(OH)₂D₃ inhibits the transcriptional activity of β-catenin by two mechanisms. On the one hand, it rapidly increases the amount of VDR bound to β-catenin, blocking the interaction of this catenin to TCF-4. Therefore, 1α,25(OH)₂D₃ modulates TCF/LEF-1 target genes in a manner opposite to β-catenin. In some cells, such as Pam212 and LS-174T, this effect is independent of changes in E-cadherin expression. Secondly, in SW480-ADH cells changes in β-catenin transcriptional activity caused by 1α,25(OH)₂D₃ are accompanied by the nuclear export of β-catenin and its relocalization to the plasma membrane that happens concomitantly to E-cadherin protein expres-
Figure 10. Nonhypercalcemic 1α,25(OH)₂D₃ derivatives induce E-cadherin and inhibit β-catenin-TCF-4 transcriptional activity in SW480-ADH cells, causing β-catenin nuclear export and morphological differentiation. (A) Northern and Western blot analyses of E-cadherin expression in cells treated for 24 h and 48 h, respectively, with various doses of 1α,25(OH)₂D₃, MC903, KH1060, or EB1089. Conditions were as above. (B) β-catenin-TCF-4 transcriptional activity in cells transfected with the TOP-flash and FOP-flash constructs and treated 48 h with 10⁻⁷ M of the indicated compound. Mean values and standard deviation of the mean obtained in duplicates of two independent experiments are shown. (C) Induction of differentiation and nuclear export of β-catenin. Immunofluorescence and confocal laser microscopy analysis of β-catenin expression was done as before. SW480-R and SW620 cells were used as negative control.
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1α,25(OH)₂D₃ might enhance the sequestration of β-catenin in the plasma membrane compartment by E-cadherin or alternatively might stimulate β-catenin nuclear export, a process mediated by APC (Henderson, 2000; Rosin-Arbesfeld et al., 2000). Alternatively, 1α,25(OH)₂D₃ may stimulate the APC-independent β-catenin export described recently (Eleftheriou et al., 2001). Although an estimation of the respective contribution of these activities to the inhibition of β-catenin signaling is very difficult, their respective timing supports the hypothesis that the primary effect is due to the formation of VDR–β-catenin complexes. By favoring this, 1α,25(OH)₂D₃ may indirectly regulate the transcription of β-catenin–TCF/LEF-1 target genes such as Tcf-1, CD44, PPARγ, and ZO-1. The changes in RNA content for these genes after 1α,25(OH)₂D₃ treatment occur earlier than those in β-catenin localization, which suggests that they are initiated as a result of the VDR–TCF-4 competition for β-catenin and later strengthened by the nuclear export of β-catenin.

The physical interaction of VDR with β-catenin adds to that reported previously for retinoic acid receptor (RAR) (Easwaran et al., 1999). However, the interaction of β-catenin with these two nuclear receptors differs. RAR strictly depends on ligand binding, whereas a certain amount of VDR–β-catenin complexes were found in vitro in the absence of 1α,25(OH)₂D₃. In contrast, this basal interaction augments in the presence of 1α,25(OH)₂D₃ in vivo, suggesting the participation of ligand-dependent nuclear mediator(s) or mechanism(s). However, given the high activity of 1α,25(OH)₂D₃ in our cell system, low amounts of metabolically active vitamin D derivatives in the culture medium may be sufficient to activate VDR in the absence of added agent. Likewise, the stimulation by β-catenin of the effect of 1α,25(OH)₂D₃ on a VDRE-dependent promoter agrees with that reported for RAR-responsive promoters (Easwaran et al., 1999). In vivo, the interaction between VDR and β-catenin may ameliorate β-catenin–TCF-4 signaling. Upon β-catenin stabilization due to its mutation or that of APC, binding to VDR may buffer its stimulatory action on TCF-4 target genes, a protective effect which can be lost along with VDR expression during malignant progression. Additionally, our data suggest that nuclear β-catenin might transiently potentiate VDR transcriptional activity before β-catenin moves out of the nucleus and/or VDR is extinguished.

The β-catenin homologue γ-catenin/plakoglobin is also regulated by APC and functions as an oncogene (Kolligs et al., 2000). Like β-catenin, it activates c-myc expression in APC-mutated cells, which together with mutations in its NH₂-region is thought to be critical for its oncogenic activity (Kolligs et al., 2000). γ-Catenin/plakoglobin indirectly activates TCF/LEF-1–regulated genes by increasing the levels of β-catenin and by inducing its nuclear translocation (Zhurinsky et al., 2000). These data show similarities but also differences in the mechanism of action of β- and γ-catenin. 1α,25(OH)₂D₃ induces the nuclear export of γ-catenin/plakoglobin to the plasma membrane, which may also contribute to the phenotypic change described in this study.

Our results show the competition between VDR, TCF-4, and E-cadherin for binding to β-catenin. Since the E-cadherin gene has been proposed to be down-regulated by β-catenin upon binding of β-catenin–TCF/LEF-1 to its promoter (Huber et al., 1996) and 1α,25(OH)₂D₃ induces a concomitant increase in VDR–β-catenin binding and E-cadherin mRNA, an effect on the E-cadherin promoter is plausible. The formation of VDR–β-catenin complexes may reduce the β-catenin binding to TCF-4 and so lead to a relief of promoter repression and/or have a direct activating effect. In disagreement with this hypothesis, VDR overexpression in SW480-ADH cells did not increase the basal activation of the 1.1-kb E-cadherin promoter construct, although potential regulatory sequences outside this region cannot be ruled out. The moderate induction by 1α,25(OH)₂D₃ of this E-cadherin gene promoter construct does not unambiguously demonstrate that E-cadherin gene is regulated transcriptionally by this agent. However, this effect is consistently observed, and together with the inhibitory action of actinomycin and the lack of regulation of E-cadherin RNA half-life suggests an effect at the transcriptional level.

Our data show that 1α,25(OH)₂D₃ increases E-cadherin gene transcription by a mechanism that is dependent on the AF-2 domain of VDR and mediated by the novo synthesis of short-lived proteins, although additional posttranscriptional routes cannot be discarded. E-cadherin expression is inhibited by the product of Snail gene, which is a transcriptional repressor on the E-boxes located near the transcription start site (Batlle et al., 2000; Cano et al., 2000). We show that 1α,25(OH)₂D₃ regulates the human E-cadherin promoter when a region of ~1.1 kb upstream of the +92 site is studied, but it has no effect on the activity of the proximal promoter region that contains the E-boxes bound by Snail. These data suggest that 1α,25(OH)₂D₃ and Snail have opposite effects on distinct sequences of the E-cadherin promoter. Though no changes in Snail mRNA expression were detected upon 1α,25(OH)₂D₃ treatment, further research is required to determine a putative interplay between these agents.

Whether the increase observed in the cellular content of other intercellular adhesion proteins such as occludin, ZO-1, and vinculin is a direct effect of 1α,25(OH)₂D₃ or a corollary of its primary effect on E-cadherin and/or β-catenin is unknown. Since the ZO-1 gene is repressed by β-catenin–TCF-4 (Mann et al., 1999) and ZO-1 mRNA increases before E-cadherin protein levels are significantly elevated, that is, when β-catenin is abundant in the nucleus, the effect of 1α,25(OH)₂D₃ on this gene is probably due to the rapid formation of VDR–β-catenin complexes and the subsequent reduction of β-catenin–TCF-4 complexes. Additionally, ZO-1 protein forms complexes with α-, β- and γ-catenins (Rajasekaran et al., 1996), and like β-catenin it can locate in the cell nucleus where it may function as a signaling molecule (Gottardi et al., 1996). In untreated SW480-ADH cells, ZO-1 is distributed diffusely in the cytoplasm and in intranuclear foci. Upon 1α,25(OH)₂D₃ treatment, ZO-1 is first increased and then redistributes to the cell surface as in MDCK cells after E-cadherin expression (Rajasekaran et al., 1996). The relevance of the effects of 1α,25(OH)₂D₃ on ZO-1 reported here is supported by a recent report describing the regulation of c-ErbB2/Neu expression by ZO-1 (Balda and Matter, 2000). It is also significant that other genes regulated by 1α,25(OH)₂D₃ in SW480-ADH cells
play important roles: occludin expression has been associated with tumor differentiation (Kimura et al., 1997), whereas vinculin is involved in the organization of tight junctions (Watabe-Uchida et al., 1998). These changes are consistent with the key role of E-cadherin in the maintenance of epithelial characteristics and account for the drastic change induced by 1α,25(OH)2D3 in the differentiation status of SW480-ADH cells.

The rapid inhibitory effect of 1α,25(OH)2D3 on c-myc expression, another TCF/LEF-1–β-catenin target gene, probably results from the combined effects of activities at various levels, which take place before nuclear export of β-catenin. First, 1α,25(OH)2D3 may induce the binding of regulatory proteins to the first intron of the gene (Pan and Simpson, 1999). Second, the formation of VDR–β-catenin complexes may inhibit the activation by β-catenin–TCF/LEF-1, as may the alteration of γ-catenin/plakoglobin localization (Kolligs et al., 2000).

The effects of 1α,25(OH)2D3 in SW480-ADH cells are transient and depend on its nuclear receptors. VDR content is low in normal colon epithelial cells, increases at the early stages of tumor progression, and is almost absent in the more malignant carcinoma cells (Vandevalle et al., 1994). This agrees with the finding that 1α,25(OH)2D3 inhibits proliferation in human rectal mucosa (Thomas et al., 1992) and with its activity in the weakly tumorigenic SW480-ADH cells and its inefficacy in the highly tumorigenic SW480-R cells. Our data are consistent with the proposed protective role of dietary vitamin D or sunlight exposure and with the predictive use of VDR expression in colon cancer biopsies. Our results suggest that liganded VDR may hinder the loss of differentiation and the increase in proliferation at the early stages of carcinogenesis. 1α,25(OH)2D3 has a pleiotropic biological activity with complex cell-specific tumour properties that include induction of apoptosis, growth arrest, inhibition of invasiveness, and stimulation of differentiation. The pattern of expression of the coactivators (SRC-1, CBP, GRIP-1/TIF-2, and others) and corepressors (NCOR, SMRT, Alien) (Polly et al., 2000; for review see McDonald et al., 2001) that interact with VDR and the activation of other signaling pathways such as that of TGF-β (Yanagisawa et al., 1999) may be responsible for the effects of 1α,25(OH)2D3 in a particular cell type. Our data reveal a complex network of interactions between cell junction proteins with signaling abilities such as β-catenin, γ-catenin/plakoglobin, and ZO-1 and nuclear hormone receptors such as VDR, which together with other signaling mediators regulate gene expression and the phenotype of epithelial cells in a combinatorial fashion.

In summary, we report here on a novel activity of 1α,25(OH)2D3 in human colon carcinoma cells, consisting of the induction of E-cadherin and the inhibition of β-catenin signaling, which has an antitumor effect in vivo. 1α,25(OH)2D3 exerts these protective effects in SW480-ADH cells carrying a panel of mutations in critical genes such as p53, ras, and APC and expressing negligible amounts of E-cadherin but overexpressing c-myc and in other human colon cell lines expressing functional VDR. These results point to the key role of VDR expression in colon carcinogenesis and support the use of nonhypercalcemic vitamin D derivatives for the treatment of this neoplasia.

Materials and methods

Cell culture

SW480, SW620, Caco-2, HT-29 M6, SW1417, LS-147T, MCF-7, and Panm212 cells were grown in DMEM supplemented with 10% FCS. SW480-ADH cells were grown in DMEM supplemented with 10% charcoal-treated FCS and cultured in the same medium. All experiments using 1α,25(OH)2D3, MC903, KH1060, and EB1089 (a gift from Dr. Lise Binderup, Leo Pharmaeuticals, Copenhagen, Denmark) were performed in DMEM supplemented with charcoal-treated FCS to remove liposoluble hormones. 1α,25(OH)2D3 and deltanoids were dissolved in isopropanol, and dexamethasone, all-trans-retinoic acid, and 9-cis-retinoic acid (all from Sigma-Aldrich) were dissolved in ethanol.

Cloning of the human E-cadherin gene promoter

A −178/+92 human E-cadherin promoter fragment was amplified by PCR using Pfu DNA polymerase (Stratagene) and 5′-GACTACCGGTTACCATGAGTGAGGTC-3′ and 5′-GATCGTATACGGGTGCTG-GGTCTCGGGCGCGCAA-3′ as sense and antisense oligonucleotides, respectively. The −987/+92 E-cadherin promoter fragment was amplified using the same antisense oligonucleotide and 5′-AGCTGGTACGAGTCCGTCACC-3′ as sense oligonucleotide. Fragments were digested with MluI/EcoRV or SacI/Ncol restriction enzymes and inserted in pGL3 reporter vector (Promega). Cloned fragments were sequenced in order to rule out differences with respect to the published sequence (sequence data available from GenBank/EMBL/DDBJ under accession no. L34545).

Antibodies

The following antibodies were used: rabbit polyclonal anti-VDR (sc-1008; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti–β-catenin (C19220; Transduction Laboratories), rat monoclonal anti-mouse E-cadherin (ECCD-2; a gift from Dr. M. Takeichi, Kyoto University, Kyoto, Japan), rabbit polyclonal antioccludin (71-1500; Zymed Laboratories), rabbit polyclonal anti-ZO-1 (61-7300; Zymed Laboratories), rabbit polyclonal anti-β-catenin (71-1400; Zymed Laboratories), rabbit polyclonal antivinulin IgG (sc-7649; Santa Cruz Biotechnology, Inc.), goat anti–TCF-4 (sc-8632; Santa Cruz Biotechnology, Inc.), FITC-conjugated goat anti-mouse IgG (115-095-003; Jackson Immunoresearch Laboratories), TRITC-conjugated anti-rat IgG (112-025-003; Jackson Immunoresearch Laboratories), TRITC-conjugated anti–rabbit IgG (Jackson Immunoresearch Laboratories), goat anti–rabbit IgG (H + L) HRP-conjugated (67437; ICN Biomedicals), goat anti–mouse IgG (H + L) HRP-conjugated (67428; ICN Biomedicals), goat anti–rat IgG (H + L) HRP-conjugated (31472; Pierce Chemical Co.), and rabbit anti–goat IgG (612762; ICN Biomedicals).

Immunostaining

Cells were rinsed four times in PBS, fixed in cold methanol for 30 s at −20°C and rinsed in PBS. The nonspecific sites were blocked by incubation with PBS containing 1% BSA at room temperature. Cells were then washed four times in PBS and incubated with the primary antibodies diluted in PBS containing 1% BSA for 1 h at room temperature or overnight at 4°C. After four washes with PBS, cells were incubated with secondary antibodies for 45 min at room temperature, washed, and mounted in VectaShield (Vector Laboratories). Confocal microscopy was performed with a Bio-Rad Laboratories MRC-1024 laser scanning microscope equipped with an Axiovert 100 invert microscope (ZEISS) at excitation wavelengths of 488 nm for FITC and 543 nm for TRITC. Each channel was recorded independently, and pseudocolor images were generated and superimposed. Images were processed by the Adobe Photoshop® 5.0 software (Adobe Systems, Inc.).

RNA preparation and Northern analysis

Purification of poly(A)+ RNA was carried out as reported elsewhere (Vennstrom and Bishop, 1982). Northern blots were performed on nylon membranes (Nytran; Schleicher & Schuell) using the random priming method (Feinberg and Vogelstein, 1983). Hybridizations were carried out overnight at 65°C in 7% SDS, 500 mM sodium phosphate buffer, pH 7.2, and 1 mM EDTA as described by Church and Gilbert (1984). Filters were washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate buffer, pH 7.2, at 65°C. The sizes of respective mRNAs were calculated using 92 human E-cadherin promoter fragment was amplified

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cDNA (donated by Dr. J. León, Facultad de Medicina, University of Cantabria, Santander, Spain); for CD44, the full-length human cDNA; for Tcf-1, the full-length TcF-1 (van de Wetering et al., 1991) (both donated by Drs. E. Sancho and H. Clevers, University Medical Center, Utrecht, Netherlands); for ZO-1, a 558-bp fragment of the human cDNA obtained by reverse transcriptase-PCR with the 5'–CTTATACATTGAGCTGG-3' and 5'-CTTCACTGGTGCAGCG-3' oligonucleotides as sense and antisense primers, respectively; for PPAR-β, a 407-bp fragment of the human cDNA obtained by reverse transcriptase-PCR with the 5'-CTACGGTTCTGATCAGCG-3' and 5'-CATAAGGTCGGTCTGCG-3' oligonucleotides as sense and antisense primers, respectively; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the complete human cDNA.

Immunoprecipitation and Western blotting

Immunoprecipitation of whole cell extracts with specific antibodies was carried out as described elsewhere (Lozano and Cano, 1998). Whole cell extracts were prepared by washing the monolayers twice in PBS, and the cells were lysed by incubation in RIPA buffer (150 mM NaCl, 1.5 mM MgCl₂, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 50 mM Hepes, pH 7.4, plus phosphatase- and protease-inhibitor mixture (PPIPM: 25 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotonin) for 15 min on ice followed by centrifugation at 13,000 rpm for 10 min at 4°C. Immunoprecipitated proteins were analyzed in 7.5% or 12% SDS-PAGE gels. Immunoblotting of cell lysates or immunoprecipitates was performed by protein transfer to Immobilon-P membranes (Millipore Corp.) and incubation with the appropriate specific antibody. Blots were developed using the ECL detection system (Amersham Pharmacia Biotech).

In vitro protein–protein interaction

100 ng each of bacterially produced GST–β-catenin protein and human VDR translated in vitro in rabbit reticulocyte lysates (TNT™ T7 Quick Kit; Promega) were mixed with 500 µl of immunoprecipitation buffer (1B: 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 5 mM CaCl₂, 5 mM MgCl₂, 1% NP-40, 1% Triton X-100) and incubated for 1 h at 4°C. GST–β-catenin complexes were collected by addition of glutathione-Sepharose 4B (Amersham Pharmacia Biotech), washed twice with IB, and resuspended in Laemmli sample buffer. Proteins in the complexes were analyzed in 7.5% or 10% SDS-PAGE gels, which was followed by immunoblotting with the indicated antibodies.

Transactivation assays

Nearly confluent cells were transfected in triplicate P-60 dishes using LipofectAMINE™ reagent (Life Technologies) following the manufacturer guidelines. The 4×10⁵ cells were seeded in 24-well dishes (Falcon) and incubated in normal growth medium in the presence of or absence of the indicated concentrations of 1α,25(OH)₂D₃. 48-h later, cells were pulsed with 1 µCi/ml [³H]-thymidine for 3 h. At the end of the labeling period, the medium was removed and the cells were rinsed twice with PBS and fixed with chilled 10% trichloracetic acid for 10 min. Trichloracetic acid was then removed and the monolayers were air-dried at room temperature for 20 min. Thereafter, precipitated cellular macromolecules were dissolved in 500 µl of 0.1 N NaOH, 0.1% SDS, and 450 µl of each sample was diluted in 5 ml of scintillation solution OptiPhase HighSafe (Wallac Scintillation Products). Radioactivity was measured using a 1209 RackBeta counter (LKB Wallac).

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References

Akhter, J., X. Chen, P. Bowrey, E.J. Bolton, and D.L. Morris. 1997. Vitamin D₃ analog, EB1089, inhibits growth of subcutaneous xenographs of the human colon cancer cell line, LoVo, in nude mouse model. Dis. Colon Rec. 40:317–321.

Balda, M.S., and K. Matter. 2000. The tight junction protein ZO-1 and an interacting transcription factor regulate E-cadherin expression. EMBO J. 19:2024–2033.

Baille, E., E. Sancho, C. Francí, D. Domínguez, M. Mondír, J. Baulida, and A. García de Herreros. 2000. The transcription factor Snail is a suppressor of E-cadherin gene expression in epithelial tumor cells. Nat. Cell Biol. 2:84–91.

Baulida, J., E. Baille, and A. García de Herreros. 1999. Adenomatous polyposis coli. proteins (APC)-independent regulation of β-catenin/Tcf-4 mediated transcription in intestinal cells. Biochem. J. 344:565–570.

Behrens, J., J.P. von Kries, M. Kühl, L. Brünn, D. Wedlich, R. Grosschedl, and W. Birchmeier. 1996. Functional interaction of β-catenin with the transcription factor LEF-1. Nature. 382:638–642.

Behrens, J., B.A. Jerchow, M. Württele, J. Grimm, C. Asbrand, R. Wirtz, M. Kühl, D. Wedlich, and W. Birchmeier. 1998. Functional interaction of an axin homolog, conductin, with β-catenin, APC, and GSK3β. Science. 280:596–599.

Billin, A.N., H. Thirwell, and D.E. Ayer. 2000. Beta-Catenin-histone deacetylase interactions regulate the transition of LEF-1 from a transcriptional repressor to an activator. Mol. Cell Biol. 20:6882–6890.

Birchmeier, W., and J. Behrens. 1994. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochem. Biophys. Acta. 1198:11–26.

Bursa, R.K., L.M. Schumaker, F. Davoodi, R.V. Brenner, M. Shabahang, R.J. Naura, and S.R.T. Evans. 1994. Vitamin D receptors in breast cancer cells. Breast Cancer Res. Treat. 31:191–202.

Cano, A., M.A. Pérez-Moreno, I. Rodríguez, A. Locasio, M.J. Blanco, M.G. del Barrio, F. Portillo, and M.A. Nieto. 2000. The transcription factor Snail controls the epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat. Cell Biol. 2:76–83.

Christofori, G., and H. Semb. 1999. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. Trends Biochem. Sci. 24:73–76.

Church, G.M., and W. Gilbert. 1984. Genome sequencing. Proc. Natl. Acad. Sci. USA. 81:1991–1995.

Crawford, H.C., B.M. Fingleton, L.A. Rudolph-Owen, K.J. Hepner-Goss, B. Rubinfeld, P. Polakis, and L.M. Matthiessen. 1999. The metalloproteinase matrilysin is a target of β-catenin transactivation in intestinal tumors. Oncogene. 18:2883–2891.

Diaz, G.D., C. Paraskeva, M.G. Thomas, L. Binderup, and A. Hague. 2000. Ap-
oprosis is induced by the active metabolite of vitamin D3 and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implication for prevention and therapy. Cancer Res. 60:2304–2312.

Duval, A., S. Rolland, E. Tübuchar, H. Bui, G. Thomas, and R. Hamelin. 2000. The human T-cell transcription factor-γ gene: structure, extensive characterization of alternative splicings, and mutational analysis in colorectal cancer cell lines. Cancer Res. 60:3872–3879.

Eastman, Q., and R. Groschedl. 1999. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr. Opin. Cell Biol. 11:233–240.

Eswaran, V., M. Pusiaquin, Salimuddin, and S. Byers. 1999. Cross-regulation of β-catenin-LEF/TCF and retinoid signalizing pathways. Curr. Biol. 9:1415–1418.

Elefteriou, A., M. Yoshida, and B.R. Henderson. 2001. Nuclear export of human β-catenin can occur independently of CRM1 and APC. J. Biol. Chem. M102566200, in press.

Evans, S.R.T., J. Nolla, J. Hanfelt, M. Shabahang, R.J. Nauta, and I.B. Schepoortin. 1999. Vitamin D receptor expression as a predictive marker of biological behavior in human colorectal cancer. Clin. Cancer Res. 4:1591–1595.

Feinberg, P.A., and B.A. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266–267.

Fodde, R., J. Kuipers, C. Rosenberg, R. Smits, M. Kielman, C. Gaspar, J.H. van Es, C. Breukel, J. Wiegant, R.H. Giles, et al. 2001. Mutations in the APC tumor suppressor gene cause chromosomal instability. Nat. Cell Biol. 3:433–438.

Freeman, L.P. 1999. Transcriptional targets of the vitamin D3 receptor-mediating cell cycle arrest and differentiation. J. Nutr. 129:5815–5863.

Garland, C.F., C.F. Gazlanted, E.K. Shaw, G.W. Comstock, K.J. Helsing, and E.D. Eastman. 1999. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr. Opin. Cell Biol. 11:233–240.

Gottardi, C.J., M. Arpin, A.S. Fanning, and D. Louvard. 1996. The junction-associated protein, in human digestive tract. Curr. Opin. Cell Biol. 8:345–357.

González-Sancho, J.M., M. Alvarez-Dolado, and A. Muñoz. 1998. 1,25-dihydroxyvitamin D3 and its analogues down-regulate cell invasion-associated proteases in cultured malignant cells. Cell Growth Differ. 11:221–229.

Kollias, F.T., B. Kollias, K.M. Hajra, G. Hu, M. Tani, K.R. Cho, and E.R. Fearon. 2000. γ-Catenin is regulated by the APC tumor suppressor and its oncogenic activity is distinct from that of β-catenin. Gene Dev. 14:1319–1331.

Korinek, V., N. Barker, P.J. Morin, D. van Wijchen, R. de Weeger, K.W. Kinzler, B. Vogelstein, and H. Clevers. 1997. Constitutive transcriptional activation by β-catenin-Tcf complex in APC−/− colon carcinoma. Science. 275:1784–1787.

Leibovitz, A., J.C. Stinton, W.B. McCombs, C.E. McCoy, K.C. Mazur, and N.D. Mabry. 1976. Classification of human colorectal adeno carcinoma cell lines. Cancer Res. 36:4562–4569.

Lickert, H., C. Domon, G. Hub, C. Weller, I. Duluc, H. Clevers, B. Meyer, J.N. Freund, and R. Kemler. 2000. Wnt/β-catenin signaling regulates the expression of the homeobox genes Cdx1 in embryonic tissue. Development. 127: 3805–3813.

Lozano, E., and A. Cano. 1998. Cadherin/catenin complexes in murine epidermal keratinocytes: E-cadherin complexes containing either β-catenin or plakoglobin contribute to stable cell-cell contacts. Cell Adh. Commun. 6:51–67.

Majewski, S., A. Szumurko, M. Marczak, S. Janowska, and W. Bollag. 1993. Inhibition of tumor cell-induced angiogenesis by retinoid, 1a,25-dihydroxyvitamin D3 and their combination. Cancer Letters. 75:35–39.

Mann, B., M. Gelos, A. Siedow, M.L. Hanski, A. Gratchev, M. Illyas, W.F. Bodmer, M.P. Moyer, E.O. Riecken, H.J. Buhr, et al. 1999. Target genes of β-catenin-T cell factor-lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc. Natl. Acad. Sci. USA. 96:1603–1608.

Marel, M., M. Brake, and F. van Roy. 1994. Inhibition promoter versus suppressor molecules: the paradigm of E-cadherin. Mol. Biol. Rep. 19:45–67.

McDonald, P.N., D.M. Kraichely, and A.J. Brown. 2001. The vitamin D receptor. In Nuclear Receptors and Genetic Disease. T.P. Burris and R.B. McCabe, editors. Academic Press, Inc., Orlando, FL. 197–243.

Morin, J.P. 1999. β-Catenin signaling in cancer. Bioessays. 21:1021–1030.

Morin, J.P., A.B. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, and K.W. Kinzler. 1997. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science. 275:1787–1790.

Newmark, H.L., and M. Lipkin. 1992. Calcium, vitamin D, and colon cancer. Cancer Res. 52:2067–2070.

Orsulic, S., O. Huber, H. Aberle, S. Arnold, and R. Kemler. 1999. E-cadherin binding prevents β-catenin nuclear localization and β-catenin/LEF-1-mediated transcription. J. Cell Sci. 112:1327–1345.
in patients with advanced malignancy. *Clin. Cancer Res.* 5:1339–1345.

Takeichi, M. 1993. Cadherins in cancer: implications for invasion and metastasis. *Curr. Opin. Cell Biol.* 5:806–811.

Takeichi, M. 1995. Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* 7:619–627.

Tetsu, O., and F. McCormick. 1999. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature.* 398:422–426.

Thomas, M.G., S. Tebbutt, and R.C. Williamson. 1992. Vitamin D and its metabolites inhibit cell proliferation in human rectal mucosa and a colon cancer cell line. *Gastroenterology.* 33:1660–1663.

Tomita, N., W. Jiang, H. Hibshoosh, D. Warburton, S.M. Kahn, and I.B. Weinstein. 1992. Isolation and characterization of a highly malignant variant of the SW480 human colon cancer cell lines. *Cancer Res.* 52:6840–6847.

Tong, W.M., E. Kallay, H. Hofer, W. Huilla, T. Manhardt, M. Peterlik, and H.S. Cross. 1998. Growth regulation of human colon cancer cells by epidermal growth factor and 1α,25-dihydroxyvitamin D3 is mediated by mutual modulation of receptor expression. *Eur. J. Cancer.* 34:2119–2125.

Vandewalle, B., A. Adenis, L. Hornes, F. Revillion, and J. Lefebvre. 1994. 1α,25-dihydroxyvitamin D3 receptors in normal and malignant human colorectal tissues. *Cancer Lett.* 86:67–73.

van de Wetering, M., M. Oosterwegel, D. Dooijes, and J. Clevers. 1991. Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. *EMBO J.* 10:123–132.

van Weelden, K., L. Flanagan, L. Binderup, M. Tenen, and J. Welsh. 1998. Apoptotic regulation of MCF-7 xenografts in nude mice treated with the vitamin D3 analog, EB1089. *Endocrinology.* 139:2102–2110.

Venstrom, B., and J.M. Bishop. 1982. Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. *Cell.* 28:135–143.

Vera, J., M. Wielenga, R. Smits, V. Kotinck, C. Smit, M. Kielman, R. Fodde, H. Clevers, and S.T. Pols. 1999. Expression of CD44 in APC/Tcf-1 mutant mice implies regulation by the WNT pathway. *Am. J. Pathol.* 154:515–523.

Vlemmix, K., L. Vakaet, M. Mareel, W. Fiers, and F. van Roy. 1991. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell.* 66:107–119.

Watake-Uchida, M., N. Uchida, Y. Imamura, A. Nagafuchi, K. Fujimoto, T. Uemura, S. Vermeulen, F. van Roy, E.D. Adamson, and M. Takeichi. 1998. α-Catenin–vinculin interaction functions to organize the apical junctional complex in epithelial cells. *J. Cell Biol.* 142:847–857.

Xi, Z., and D. Feldman. 1993. Regulation of vitamin D receptor abundance and responsiveness during differentiation of HT-29 colon cancer cells. *Endocrinology.* 132:1808–1814.

Yanagisawa, J., Y. Yanagi, Y. Masuhiro, M. Suzawa, M. Watanabe, K. Kashiwagi, T. Toriyabe, M. Kawabata, K. Miyazono, and S. Kato. 1999. Convergence of transforming growth factor-β and vitamin D signaling pathways on SMAD transcriptional coactivators. *Science.* 283:1377–1381.

Zhutovsky, J., M. Shtrum, and A. Ben-Ze’ev. 2000. Differential mechanisms of LEF/TCF family-dependent transcriptional activation by β-catenin and plakoglobin. *Mol. Cell. Biol.* 20:4238–4252.