Mechanisms of action of vitamin D in colon cancer

Gemma Ferrer-Mayorga[1], María Jesús Larriba[1], Piero Crespo[2], Alberto Muñoz[1*]

[1]Instituto de Investigaciones Biomédicas "Alberto Sols", Consejo Superior de Investigaciones Científicas - Universidad Autónoma de Madrid, IdiPAZ and CIBERONC, Arturo Duperier, 4, E-28029 Madrid, Spain

[2]Instituto de Biomedicina y Biotecnología de Cantabria and CIBERONC, E-39011 Santander, Spain

*Corresponding author

E-mail addresses:

gferrer@iib.uam.es (G. Ferrer-Mayorga), mjlarriba@iib.uam.es (M.J. Larriba), crespop@unican.es (P. Crespo), amunoz@iib.uam.es (A. Muñoz)

Highlights

- **Colorectal cancer is the neoplasia that is most closely linked to vitamin D deficiency in epidemiological studies.**
- **Calcitriol inhibits the proliferation, migration, invasiveness and angiogenesis of colon carcinoma cells, and promotes their differentiation and sensitizes them to apoptosis.**
- **Calcitriol reduces the protumoral effects of colon cancer-associated fibroblasts.**
- **Calcitriol also regulates the biology of intestinal immune cells and affects the intestinal microbiota.**

Abstract

Colorectal cancer (CRC) is the neoplasia that is most frequently associated with vitamin D deficiency in epidemiological and observational studies in terms of incidence and mortality. Many mechanistic studies show that the active vitamin D metabolite (1α,25-dihydroxyvitamin D_3 or calcitriol) inhibits proliferation and promotes epithelial differentiation of human colon carcinoma cell lines that express vitamin D receptor (VDR) *via* the regulation of a high number of genes. A key action underlining this effect is the multilevel inhibition of the Wnt/β-catenin signaling pathway, whose abnormal activation in colon epithelial cells initiates and promotes CRC. Recently, our group has shown that calcitriol modulates gene expression and inhibits protumoral properties of patient-derived colon cancer-associated fibroblasts (CAFs). Accordingly, high VDR expression in tumor stromal fibroblasts is associated with longer survival of CRC patients. Moreover, many types of immune cells express VDR and are regulated by calcitriol, which probably contributes to its action against CRC. Given the role attributed to the intestinal microbiota in CRC and the finding that it is altered by vitamin D deficiency, an indirect antitumoral effect of calcitriol is also plausible at this level. In summary, calcitriol has an array of potential protective effects against CRC by acting on carcinoma cells, CAFs, immune cells and probably also the gut microbiota.

Keywords

Vitamin D; Vitamin D receptor; Calcitriol; Colorectal cancer; Wnt/β-catenin; Cancer-associated fibroblasts; Gut microbiota

1. Introduction

Colorectal cancer (CRC) results from malignant transformation of the epithelium of the large intestine. It is a major health problem and a leading cause of cancer-related mortality worldwide [1, 2]. Intestine is the organ with the highest expression of vitamin D receptor (VDR) [3, 4]. In addition, 1α,25-dihydroxyvitamin D_3 or calcitriol, the most active vitamin D metabolite, has many important homeostatic actions in this organ [5].

Many epidemiological and observational studies reported in recent decades have proposed CRC as the neoplasia that is preferentially associated with vitamin D deficiency in terms of incidence and/or mortality [6, 7]. However, controversy exists regarding the design, analysis and interpretation of data from some epidemiological and prospective interventional clinical studies [8, 9]. Interestingly, some studies have linked polymorphisms of genes of the vitamin D system (*VDR*, *CYP27B1*, *CYP24A1*, *GC*, *DHCR7* and *CYP2R1*) to CRC prognosis or response to therapy [10-13]. Various studies using animals, including some that analyze the effect of diets with high or low vitamin D (and calcium) and carcinogen content, and others with genetically-modified or xenotransplanted mice, support a beneficial effect of vitamin D, calcitriol or its analogues on colorectal tumorigenesis (reviewed in [14]). Altogether, these findings suggest a protective action of vitamin D against CRC by either reducing risk and/or attenuating the tumorigenic process. However, according to Koch's postulates of causality in biological systems [15], to accept a causal role and not a mere association of vitamin D/calcitriol in CRC, a mechanism of action must be elucidated.

2. Genetic basis of colorectal cancer

CRC is the best known solid cancer in terms of underlying genetic alterations [16]. The first genetic model for CRC tumorigenesis, based on the progressive accumulation of

mutations in tumor suppressor genes (*APC*, *TP53*) and oncogenes (*RAS*), was proposed by Fearon and Vogelstein in 1990 [17]. The initial key event in this model, known today as the Suppressor Pathway, is activation of the Wnt/β-catenin signaling pathway, which is the consequence of the mutually exclusive mutation of *APC* or *AXIN2* tumor suppressor genes or *CTNNB1*/β-catenin oncogene (for review see [18, 19]. A second, less frequent, mutational process in CRC is the Mutator Pathway first proposed by Perucho and cols. in 1991 [20] which is based on loss of expression (by mutation or silencing) of genes (*MLH1*, *MSH2*, etc.) of the mismatch repair (or base substitution: *POLE*, *POLD1*) systems, and is associated with microsatellite instability (MSI). Of note, this pathway can affect genes such as *RNF43* or *ZNRF3* in a way that potentiates Wnt/β-catenin signaling.

Thus, abnormal activation of the Wnt/β-catenin pathway is crucial in a substantial proportion of CRC. This abnormal activation implies the accumulation within the cell nucleus of β-catenin protein, which is usually bound to E-cadherin in the subcortical cytoplasmic area. Nuclear β-catenin binds DNA-bound T-cell factor (TCF) family members and acts as a transcriptional co-activator that derepresses a large number of genes whose expression is inhibited by TCF in the absence of β-catenin [18]. Remarkably, many β-catenin-TCF target genes promote cell proliferation and migration, whereas a minor proportion of targets that are induced by TCF alone and become repressed by β-catenin-TCF maintain the differentiated phenotype of intestinal epithelial cells [18]. Sequencing of hundreds of human colorectal tumors has confirmed that over 94% of them contain one or more mutations in genes of the Wnt/β-catenin pathway, which confirms its importance in CRC [21].

3. Mechanisms of action of calcitriol in colorectal cancer

In line with the wide expression of VDR, calcitriol has effects on most intestinal cell types and on many colon carcinoma cell lines that contain a sufficient level of VDR.

3.1. Effects on colon carcinoma cells. Antagonism of the Wnt/β-catenin pathway

Similarly to its action in other tumor cell types, calcitriol inhibits proliferation, sensitizes to apoptosis, and promotes differentiation of colon carcinoma cells through the regulation of genes and the modulation of signaling pathways implicated in these processes.

- Proliferation. Calcitriol reduces cell proliferation by several mechanisms: downregulation of cyclin-dependent kinases (CDKs), induction of CDK inhibitors ($p21^{CIP1}$ and $p27^{KIP1}$) and growth arrest and DNA damage 45 α (*GADD45A*) gene, which leads to cell cycle arrest in G_0/G_1, the direct and indirect repression of c-*MYC*, and the modulation of *JUN* and *FOS* genes [22-25]. In addition, calcitriol interferes with the proliferative signaling pathways triggered by epidermal growth factor (EGF) and insulin-like growth factor II [26-28], and sensitizes cells to the growth-inhibitory effects of transforming growth factor (TGF)-β [29].

Importantly, calcitriol antagonizes the Wnt/β-catenin signaling pathway, whose aberrant activation, as previously mentioned, is the initial event and a major contributor to the progression of CRC [30]. Calcitriol exerts this antagonism by acting at several levels: a) disruption of TCF/β-catenin transcriptional complexes due to the induction of VDR binding to β-catenin, b) diminution of nuclear β-catenin content by boosting the binding at the *adherens junctions* of the newly synthesized β-catenin to E-cadherin, which is induced by calcitriol, and c) upregulation of the extracellular Wnt inhibitor Dickkopf (DKK)-1 [30-32]. Additionally, the Wnt/β-catenin pathway is attenuated by calcitriol in carcinoma cells paracrinally *via* inhibition of interleukin (IL)-1β synthesis

and secretion by neighboring macrophages that stabilized β-catenin in carcinoma cells, resulting in an increase of TCF-dependent gene activation of the Wnt/β-catenin pathway target genes [33]. *Vdr* deficiency increases the tumor load and the accumulation of β-catenin protein within the nucleus of colon carcinoma cells in mice harboring a mutated *Apc* gene, which supports the antagonism of this pathway by calcitriol *in vivo* [34, 35]. Remarkably, vitamin D supplementation decreases β-catenin levels and increases those of E-cadherin, APC and p21 in the normal-appearing rectal mucosa of colorectal adenoma patients [36, 37].

- Differentiation. As a result of direct gene regulatory effects and interference with the Wnt/β-catenin signaling pathway, calcitriol promotes epithelial differentiation of colon carcinoma cells. Among the differentiation genes upregulated by calcitriol are several encoding intestinal epithelial markers (alkaline phosphatase, maltase, calcium-sensing receptor) and intercellular adhesion and cytoskeleton proteins (E-cadherin, occludin, claudins, plectin, filamin A, *Zonula occludens* (ZO)-1/2) [24, 38]. The increase in the cellular content of several of these proteins of the tight junctions, *adherens junctions* and hemidesmosomes contributes to maintenance of the intestinal epithelial barrier that protects against infection.

In addition, calcitriol modulates the expression of wide regulators of cell differentiation. Calcitriol induces the expression of *CST5*/cystatin D, classically known to encode an inhibitor of endosomal cysteine proteases of the cathepsin family [39], which partially locates within the nucleus and regulates the expression of several genes involved in a variety of cellular functions [40]. Moreover, cystatin D overexpression in CRC cells inhibits proliferation and migration, but increases cell-cell adhesion. In human CRC biopsies, cystatin D and VDR protein levels directly correlate, suggesting *in vivo* regulation [39]. Calcitriol inhibits the expression of Sprouty-2, an oncogenic

repressor of the epithelial phenotype, in CRC cells [41]. Sprouty-2 deregulates E-cadherin and tight junctions and epithelial polarity genes through the upregulation of ZEB1, promoting epithelial to mesenchymal transition (EMT) and proliferation. Concordantly, overexpression of Sprouty-2 is associated with poor clinical outcome of CRC patients [41-43]. Notably, calcitriol increases expression of the histone H3 lysine-27 demethylase Jumonji C domain-containing protein 3 (*JMJD3* or *KDM6B*) and modulates that of other epigenetic regulators that have profound effects on the biology of CRC cells [44-47]. Importantly, JMJD3 partially mediates some effects of calcitriol in CRC cells: prodifferentiation, antiproliferation, gene regulation, and antagonism of the Wnt/β-catenin pathway. In addition, *JMJD3* repression upregulates several EMT inducers and mesenchymal markers but downregulates the expression of epithelial proteins. Accordingly, the level of *JMJD3* RNA correlates directly with that of *VDR* in human CRC tumors [44, 46]. Altogether, these data show that calcitriol is a potent inducer of gut epithelial differentiation that prevents EMT by regulating genes involved in several mechanisms and signaling pathways [48].

- Apoptosis. Calcitriol sensitizes colon carcinoma cells to the induction of apoptosis by several agents through the upregulation of pro-apoptotic genes (*BAK1*, *BAG1* and *G0S2*), the downregulation of survival genes (survivin and thymidylate synthase) and, paracrinally, *via* interference of IL-1β secretion by macrophages [33]. Concordantly, VDR agonists potentiate the effect of chemotherapeutic agents in cultured cells and animal models of CRC (reviewed in [14]).

- Migration, invasiveness and angiogenesis. Calcitriol represses the expression of *DKK-4*, which promotes invasion, angiogenesis, and chemoresistance in colon carcinoma cells and is upregulated in human colon tumors correlating inversely with *VDR* RNA expression [49, 50]. In addition, calcitriol regulates the angiogenic

phenotype of colon carcinoma cells by controlling the expression of several genes that are responsible for it: vascular endothelial growth factor (VEGF), inhibitor of differentiation (ID)-1/2 and thrombospondin (TSP)-1 [51, 52]. Likewise, calcitriol decreases VEGF and angiogenesis in a rat model of chemically-induced colon tumorigenesis [53].

- MicroRNAs. Our group described that calcitriol regulates the expression of several microRNAs (miRs) in SW480-ADH human colon carcinoma cells [54]. One of them is *miR*-22, which is induced by calcitriol in a time-, dose- and VDR-dependent manner and mediates partially the antiproliferative and antimigratory effects of calcitriol in CRC cells. Subsequently, supporting the relevance of *miR*-22, other groups have shown that *miR*-22 inhibits proliferation, migration, invasion, EMT, and xenograft tumor growth in several cancer systems including CRC [55-57]. Notably, we found that *miR*-22 expression is lower in tumor than in the matched normal tissue in 78% of CRC patients, further suggesting that *miR*-22 contributes to the antitumoral action of calcitriol in human colon tissue [54].

3.2. Effects on colon normal and cancer-associated fibroblasts

The tumor stroma or tumor microenvironment is composed of several cells types and the extracellular matrix (ECM), and exerts a major influence on carcinoma behavior. Fibroblasts are the main cellular component of tumor stroma that, following a phenotype change ("activation") triggered by signals received from carcinoma cells, are thought to promote the tumorigenic process. Fibroblasts remodel the ECM and secrete factors that increase the malignancy and chemoresistance of cancer cells, induce the recruitment of bone marrow cells, and alter the behavior of immune cells [58-61].

Our group has recently described that primary cultures of human colon normal fibroblasts (NFs) and cancer-associated fibroblasts (CAFs) derived from fresh biopsies

of CRC patients express *VDR* and respond to calcitriol [62]. Importantly, calcitriol drastically changes the gene expression profile of NFs and CAFs regulating around one thousand genes with a 21% overlap between both types of fibroblasts. Interestingly, the gene signature imposed by calcitriol in CAFs correlates with a favorable CRC patient outcome. Accordingly, calcitriol inhibits two protumoral properties of activated fibroblasts: the capacity to alter the ECM (estimated by the contraction of collagen gels) and the ability to induce the migration of colon carcinoma cells [62]. These effects of calcitriol regulating gene expression and reprogramming activated CAFs add to its protective action on colon carcinoma cells. Concordantly, our study of a large cohort of metastatic CRC patients has shown that VDR is variably expressed in carcinoma cells and in tumor fibroblasts, and that a high level of VDR in any of the two compartments, and particularly in both of them, is associated with longer overall survival of these patients [62].

3.3. Effects on the immune system

Calcitriol has wide immunomodulatory effects by acting on a variety of immune cell types. Classically, calcitriol has been considered an inducer of the innate and an inhibitor of the adaptive immune response and an anti-inflammatory agent. However, it seems more rational that calcitriol has a global regulatory homeostatic role as an initial potentiator of the immune system against insults, and as a repressor of the immune reaction at later stages and a repressor of self-reactivity to prevent over-activation leading to undesirable consequences (inflammation, autoimmunity) [63]. The accepted influence of chronic inflammation in CRC, as seen by the increase in CRC risk in patients with inflammatory bowel diseases (ulcerative colitis, Crohn disease), and the important role of immune surveillance against this and other neoplasias, make it highly probable that calcitriol affects CRC through the regulation of immune cells [64].

Remarkably, vitamin D supplementation in colorectal adenoma patients decreases an inflammation score calculated from plasma levels of the pro-inflammatory markers C-reactive protein, TNF-α, IL-6, -1β and -8, and the anti-inflammatory marker IL-10 [65]. The effects of calcitriol on these and other cytokines (IL-12, TGF-β) that are overexpressed in CRC patients are mediated at least in part by multilevel inhibition of the transcription factor nuclear factor κ B (NFκB) [66-68]. Interestingly, two groups have recently shown that myeloid cell-specific or non-intestinal epithelial cell-specific *Vdr* deletion aggravates clinical symptoms in a mouse experimental colitis model and increases the expression of pro-inflammatory cytokines in the colon [69]. In a mouse experimental colitis model, gut epithelial *Vdr* deletion aggravates epithelial cell apoptosis, which alters mucosal barrier permeability and promotes inflammation [70].

Another beneficial immune mechanism of calcitriol in CRC may be potentiation of antibody-dependent cell cytotoxicity in patients receiving therapy with monoclonal antibodies (anti-EGF receptor, anti-VEGF). This is based on reports indicating that vitamin D deficiency diminishes the response to this type of antitumor therapy in breast cancer and lymphoma patients [71-73].

3.4. Indirect effects: detoxification and modulation of the gut microbiota

Calcitriol contributes to the detoxification process in the intestine by controlling the expression of antioxidant, phase I, and phase II enzymes involved in the catabolism of xenobiotics, steroids, bile acids and other compounds that promote CRC development [74]. Thus, several members of the cytochrome P450 (CYP) and multi-drug resistance-associated protein (MRP) families (CYP3A4, MRP3) and sulfotransferases such as SULT2A1, which are implicated in the elimination of the secondary bile acid lithocholic acid (LCA), are induced by calcitriol in CRC cells [75, 76].

There is increasing evidence of a link between alteration of the gut microbiota (dysbiosis) and many diseases including CRC [77]. Chronic inflammation-associated immune response and bacterial/viral infection influence each other and can alter the epithelial barrier. In addition, the microbiota metabolizes xenobiotics and can also regulate the response to some types of cancer chemotherapy [78, 79]. Interestingly, *Fusobacterium nucleatum* promotes colorectal carcinogenesis by activating the Wnt/β-catenin signaling pathway [80].

In line with this scenario, a low vitamin D diet and *Vdr* deficiency cause dysbiosis in mice that, as bacteria do not express VDR, must be mediated by the host [81-85]. In turn, bacterial infection downregulates *Vdr* expression [86]. Although only a few studies have been performed in humans, both experimental and genome-wide association analysis support mutual regulation between VDR/vitamin D status and the gut microbiota [87].

4. Conclusion

Calcitriol exerts a complex array of effects on colon carcinoma cells and on several types of normal cells present in the colon (Figure 1). Overall, these effects strongly suggest a beneficial, preventive action of calcitriol against CRC that perhaps also potentiates anticancer therapies. This result is in agreement with epidemiological data that associate vitamin D deficiency with higher risk of CRC. Calcitriol targets both tumor cells and the tumor microenvironment through a series of mechanisms that argue against reverse causation, which suggests that vitamin D deficiency is a consequence and not a contributing cause of the tumorigenic process. The specific effects of calcitriol most probably reflect a homeostatic role in the colon that protects against the development of devastating colorectal neoplasia.

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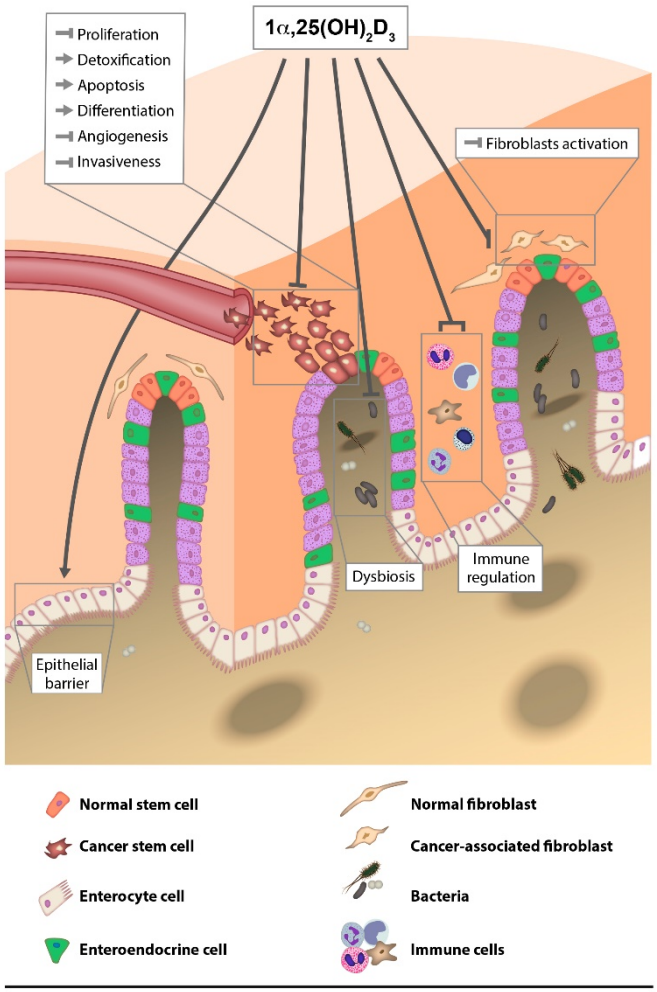

Figure 1. Schematic representation of the effects of calcitriol on the cell types present in the colon.

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