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# Extrarenal Vitamin D Hydroxylase Expression and Activity in Normal and Malignant Cells: Modification of Expression by Epigenetic Mechanisms and Dietary Substances

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Epidemiological studies have demonstrated an inverse correlation between risk of several cancers, sun exposure, and serum levels of 25-hydroxyvitamin $D_3$ (25-OH-$D_3$).[1,2] While 25-OH-$D_3$ is present in human serum at nanomolar concentrations, levels of the active vitamin D metabolite 1,25$(OH)_2D_3$ are in the picomolar range, i.e. a thousand-fold lower. These low 1,25$(OH)_2D_3$ levels are strictly regulated to maintain calcium and phosphate homeostasis. Renal 1,25$(OH)_2D_3$ synthesis is primarily stimulated by low serum calcium, and consequently by parathyroid hormone (PTH), which up-regulates 25-hydroxyvitamin $D_3$ 1$\alpha$-hydroxylase expression (CYP27B1). In contrast, 1,25$(OH)_2D_3$ itself down-regulates CYP27B1 by negative feedback.[3] Expression of the metabolizing hydroxylase CYP24A1 is under direct regulation of 1,25$(OH)_2D_3$, which, by binding to its vitamin D receptor (VDR), induces CYP24A1 levels rapidly and strongly.

In addition to maintaining mineral ion homeostasis, 1,25$(OH)_2D_3$ is a potent inhibitor of proliferation and promotes differentiation and apoptosis in a variety of cancer cells in vitro, including cells derived from the colon. However, this occurs only at nanomolar concentrations,[4] and it therefore seems unlikely that the picomolar levels present in serum could potentially protect against malignancies. Indeed, epidemiological studies demonstrated that normal to high levels of 1,25$(OH)_2D_3$ did not correlate inversely with tumor incidence.[5]

We demonstrated in human colonic cell lines, and other groups in cell lines derived from other organs, that non-renal cells can synthesize and degrade 1,25$(OH)_2D_3$[6] and that cells isolated from human colon tumors also possess this ability.[7] However, extrarenal 1,25$(OH)_2D_3$ synthesis heavily depends on serum 25-OH-$D_3$ levels for control of local cell proliferation. Expression of colonic CYP27B1 is not regulated by PTH, and regulation by calcium is in the opposite direction of that reported for renal hydroxylases (see section on calcium). Therefore, sufficiency of 25-OH-$D_3$ as the precursor is the major known limiting factor for extrarenal synthesis of 1,25$(OH)_2D$.

When evaluating expression of the vitamin D system in human colonic tumor and normal adjacent tissue from the same patients, we realized that, due to the high levels of the 1$\alpha$-hydroxylase during early tumor progression, sufficient 1,25$(OH)_2D_3$ could be synthesized by colonic mucosal cells to have protective action against further progression.[8] In contrast, in undifferentiated, well-advanced colon tumors, the synthesizing hydroxylase was barely detectable[9] but the metabolizing 24-hydroxylase was highly expressed.[10]

In order to better understand this differentiation-dependent expression pattern, we isolated primary cultures from moderately differentiated (G2, COGA-1) and undifferentiated (G3, COGA-13) human colon tumors. Comparing these with highly differentiated Caco-2 cells, as well as with tumor cells from the prostate and mammary gland, a distinct mRNA and activity pattern of CYP27B1 and of CYP24A1 became apparent: while all cells have at least some CYP27B1 expression (and differentiated cells have high CYP27B1), expression of CYP24A1 is low or nonexistent in many tumor cells unless stimulated by 1,25$(OH)_2D_3$. Stimulation with 1,25$(OH)_2D_3$ actually results in similar levels of CYP24A1 activity in mammary, colon, and prostate cells. The exceptions are cells isolated from tumors that are well advanced (biological grade G3, undifferentiated) and already in epithelial-mesenchymal transition with vimentin positivity; these express extremely high levels of basal CYP24A1 and cannot be stimulated further.[11]

Since the ability of cells to synthesize 1,25$(OH)_2D_3$ suggests a mechanism whereby the vitamin D system can protect against colonic malignancies, we investigated whether this protective system could be enhanced by

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modulation of vitamin D hydroxylases. Modulation of extrarenal expression would need to be independent of the renal system in order to maintain serum calcium homeostasis. In the digestive system, there is a considerable impact of nutritional substances on the molecular behaviour of mucosal cells. In addition, vitamin D hydroxylases are regulated by nutritional factors.

## CALCIUM

The epidemiology of colorectal cancer and calcium intake shows a fairly consistent inverse association: on average, risk reductions are in the range of 15% to 40% for the highest versus the lowest intake categories. Mechanisms underlying the chemopreventive effects of calcium are not completely understood. Several reports have implied that the beneficial effect of calcium supplementation was due to the ability of calcium to form insoluble salts with irritating and ultimately tumorigenic bile acids. However, calcium intake may also reduce hyperproliferation of the colonic mucosa directly, by binding to the calcium sensing receptor (CaR) and by activating antimitotic, pro-apoptotic signal transduction mechanisms.[12]

It has been assumed that vitamin D or calcium insufficiency would contribute to the development of colon cancer by different pathogenic mechanisms, implying that a nutritional calcium deficit or a compromised vitamin D status would constitute independent risk factors. However, there is increasing evidence that calcium and vitamin D status act largely together in the control of colon epithelial cell proliferation. Garland et al.[13] have shown that risk of colorectal cancer is inversely correlated with dietary vitamin D and calcium. Calcium supplementation was effective only in patients with physiologically normal 25-OH-$D_3$ concentrations. Conversely, 25-OH-$D_3$ sufficiency was associated with a reduced risk of adenoma recurrence only among subjects receiving calcium supplements.[14,15] Null findings after vitamin D and calcium supplementation (e.g., in women enrolled in the Women's Health Initiative) can be explained by the relatively short duration of supplementation and the healthy nutritional background of subjects.[16] We discovered a potential mechanism for an interaction of calcium and vitamin D in an animal model.

We demonstrated in a mouse model that ingestion of low nutritional calcium leads to significantly reduced calcium levels in colonic feces, whereas normal serum calcium levels are maintained. This need for serum calcium homeostasis results in enhanced renal vitamin D synthesis and reduced degradation. However, colonic mucosal cells that are in direct contact with low nutritional calcium in feces start to hyperproliferate. In addition, low levels of calcium in the feces affected cells lining the colonic lumen in a very unexpected manner: expression of the $1,25(OH)_2D_3$ metabolizing enzyme, the 24-hydroxylase, was significantly enhanced.[17] This could cause reduced colonic accumulation of $1,25(OH)_2D_3$ by stimulating its degradation, and could potentially lead to increased tumor incidence. The inflammatory marker COX-2, which is known to be elevated during colon tumor progression, has increased expression in colonic mucosa of mice fed a low-calcium diet.[18] Such a biological effect of low dietary calcium not only highlights its importance for prevention of hyperproliferation of the intestinal tract, but also could provide an experimental basis for the epidemiological observation that only when there is sufficiency of both vitamin D and dietary calcium are patients protected against tumor progression.[19] Sufficiency of 25-OH-$D_3$ provides the precursor for extrarenal synthesis of $1,25(OH)_2D_3$, and enough dietary calcium protects against colonic $1,25(OH)_2D_3$ degradation.

In another mouse model, we evaluated the effects of "normal" and low nutritional calcium and vitamin D on CYP24A1, CYP27B1, and VDR expression, and compared this with the effects of high levels comparable with nutrient-density equivalents of the maximum levels recommended in US diets.[20] Two very interesting facts became apparent. First, the VDR and CYP27B1 are regulated in the distal colon, whereas CYP24A1 is primarily regulated by vitamin D and calcium in the proximal colon. The second important observation was that maximum levels of calcium and vitamin D are needed to normalize expression of the vitamin D system.[21] This further supports numerous claims that the Dietary Reference Intake (DRI) for vitamin D and calcium for human consumption need to be re-evaluated.

## (PHYTO)ESTROGENS AND COLON CANCER PREVENTION

Until fairly recently, the colon was not considered a sex hormone-sensitive organ. However, considerable physiological evidence is accumulating for a protective effect of estrogenic substances against colorectal cancer incidence. At all ages, women are less likely than men to develop colon cancer, and postmenopausal hormone replacement therapy further reduces colon cancer risk by up to 25 %.[22] Potter et al.[22] demonstrated lower risk of adenomatous polyps of the large bowel with hormone replacement therapy. In addition, in several colon cancer animal models, male rodents were shown to have higher tumor load and increased aberrant crypt formation rates, the latter being a typical precursor lesion of colorectal cancer. The Women's Health Initiative demonstrated a reduction in incidence of colorectal cancer and of osteoporosis with hormone replacement therapy. In contrast,

most other parameters that had been assumed to be beneficially affected by hormone replacement therapy were either negatively affected, as in the case of breast cancer, or not at all affected.

Estrogens seem to have a protective effect against colorectal cancer through estrogen receptors (ER). There are at least two distinct estrogen receptors, ER-$\alpha$ and ER-$\beta$, and these have differential distribution in most organs of the human body, including the brain. In the normal human colon, ER-$\beta$ is widely regarded to be the predominant estrogen receptor.[23] It has been suggested that ER-$\beta$ may conceivably mediate signals that would protect the colon against tumorigenesis, because Foley et al.[24] found that malignant transformation of the human colon was associated with reduced expression of ER-$\beta$.

Recently we conducted a study among postmenopausal women who were given enough 17$\beta$-estradiol to increase their serum estradiol to premenopausal levels. Evaluation of rectal biopsies for markers of the vitamin D system indicated that both VDR and the synthesizing CYP27B1 were increasingly expressed following 17$\beta$-estradiol therapy, while COX-2 expression was reduced (Cross et al., unpublished observations). This suggests that the observed reduced incidence of colorectal cancer in women compared with age-matched males might be due to positive regulation of the vitamin D system by female sex hormones. It also suggests that lack of sufficient 17$\beta$-estradiol might be the reason for enhanced incidence of colorectal cancer in men. How could men profit from the protective action of estrogens as well?

A dramatically reduced incidence of hormone-related cancers such as mammary or prostate tumors has been linked to consumption of an Asian diet containing soy products. It is provocative that not only are there gender-related differences in colorectal cancer occurrence, but there is also reduction of colorectal cancer incidence in soy-consuming countries when comparing the male and female population.[25]

Soy products are rich in phytoestrogens, and these substances have a potential as selective estrogen receptor modulators that mimic or sometimes counteract estrogenic effects. Due to digestive processes and especially the differential efficiency of the intestinal flora, phytoestrogens and their metabolites could possess variable regulatory capacity in the digestive tract.

Phytoestrogens bind with high affinity to ER-$\beta$ and could therefore have a protective effect in the colon. We demonstrated in an in vivo mouse model that soy as well as genistein, a prominent phytoestrogen contained in soy, can induce CYP27B1 expression and reduce that of CYP24A1. The reduction of CYP24A1 expression by soy or genistein was even more pronounced if, by feeding low dietary calcium, proliferation of colonocytes and 24-hydroxylase expression was stimulated.[25] Comparison of effective doses in vitro for stimulation of the synthesizing 1$\alpha$-hydroxylase indicated that, whereas nanomolar concentrations of 17$\beta$-estradiol were sufficient, micromolar concentrations of genistein were needed. This concurs with previous serum measurements in Asian populations, who had high micromolar levels of genistein after habitual soy consumption.[27]

## FOLATE AND EPIGENETIC REGULATION OF VITAMIN D HYDROXYLASE EXPRESSION

Folate, a water-soluble vitamin of the B family, is essential for synthesis, repair, and methylation of DNA. Humans are unable to synthesize folate, so it must be provided in the diet. Important sources include citrus fruits, dark-green vegetables, and dried beans. Evidence is increasing that diminished folate status predisposes individuals to the development of several common cancers, and that habitual ingestion of folate in concentrations above present recommendations is cancer preventive. Giovannucci et al.,[28] among others, demonstrated that prolonged intake of folate significantly reduced the risk of colorectal cancer. Folate is crucial for normal DNA synthesis and can regulate DNA methylation. Decreased dietary folate intake may cause global hypomethylation, but also regional DNA hypermethylation and overexpression of DNA methyltransferase 1 (DNMT).[29]

DNA methylation is an epigenetic modification involving methylation of cytosine residues of CpG dinucleotides and is associated with transcriptional silencing of gene expression in mammalian cells, because CpG islands are localized predominantly in the promoter regions of genes. Aberrant DNA methylation has been linked to diverse human pathologies. Bariol et al.[30] have shown that proliferation in small adenomas and hyperplastic polyps correlated with the extent of DNA demethylation. Gene silencing also involves chromatin remodeling factors and histone deacetylases, which by chromatin condensation make it inaccessible to transcription factors.

DNA methylation could play a role in regulating the vitamin D system. Enhanced expression of the VDR for protection against chemically induced colon cancer in a mouse model was associated with decreased methylation of VDR CpG islands.[31] We have recently demonstrated in prostate tumor cells that the deacetylation inhibitor trichostatin A (TSA) can increase transcription of CYP27B1 and decrease that of CYP24A1.[32] To evaluate epigenetic regulation of vitamin D hydroxylase expression during prostatic malignant progression, we compared normal human prostate-derived PNT cells with prostate tumor DU-145 cells. A demethylation agent led to a much higher increase in catabolic CYP24A1 transcription in normal cells than in tumor cells, indicating

that in tumor cells the "bad" enzyme was largely constitutively expressed.[33]

CYP24A1 activity in colon cells derived from differentiated tumors is very low, whereas it is high in cells derived from undifferentiated tumors. Comparing the methylation status of CpG islands in the CYP24A1 promoter of undifferentiated cells with that of differentiated colon cells by three different methods (bisulfite sequencing, inhibition of methyltransferases, restriction enzyme PCR), it became evident that the CYP24A1 promoter of undifferentiated cells was totally devoid of methylation, whereas in cells with low levels of CYP24A1 activity, both methylated and unmethylated CpG islands were found in the CYP24A1 promoter.

To test in vivo whether nutritional folate indeed has regulatory potential for vitamin D hydroxylases, this was included as one of the nutritional variables using a mouse model. Elevated folate ingestion was the most effective means to down-regulate CYP24A1 expression in the ascending colon, even overriding the stimulatory action on CYP24A1 expression of low nutritional calcium.[21]

Figure 1 describes our present view of colonic vitamin D synthesis and its regulation by some nutrients. While it has to be recognized that, according to recent data, there is widespread insufficiency of vitamin D and calcium intake in European and North American populations,[34] a claim for action of nutritional folate is not so easy to substantiate. At least in the United States, there is folic acid fortification in grain. However, according to studies in Ireland and the United Kingdom, this folic acid fortification could be safely doubled up to 1 mg/d. A recent meta-analysis suggested a link between cancer, alcohol, and folate intake: the increased incidence of breast cancer found in women consuming high levels of alcohol was abrogated with high folate intake.[35] While hypomethylation of CYP24A1 in mammary tumors has not been investigated yet, it is known that folate metabolism is altered by alcohol,[36] and methylation of homocysteine to methionine is through a vitamin $B_{12}$- and folate-dependent pathway.

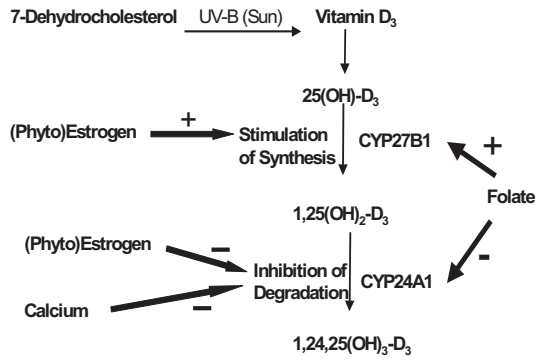


**Figure 1.** (Phyto)estrogen and folate stimulate expression and activity of CYP27B1, the 1,25-$(OH)_2$-$D_3$ synthesizing hydroxylase. Folate, (phyto)estrogens, and nutritional calcium sufficiency inhibit expression and activity of CYP24A1, the catabolic 1,25-$(OH)_2$-$D_3$ hydroxylase.


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