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Vitamin D and breast cancer: Inhibition of estrogen synthesis and signaling☆,☆☆

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ABSTRACT

Calcitriol (1,25-dihydroxyvitamin D_3), the hormonally active metabolite of vitamin D, inhibits the growth and induces the differentiation of many malignant cells including breast cancer (BCa) cells. Calcitriol exerts its anti-proliferative activity in BCa cells by inducing cell cycle arrest and stimulating apoptosis. Calcitriol also inhibits invasion, metastasis and tumor angiogenesis in experimental models of BCa. Our recent studies show additional newly discovered pathways of calcitriol action to inhibit the growth of BCa cells. Calcitriol suppresses COX-2 expression and increases that of 15-PGDH thereby reducing the levels and biological activity of prostaglandins (PGs). Calcitriol decreases the expression of aromatase, the enzyme that catalyzes estrogen synthesis selectively in BCa cells and the breast adipose tissue surrounding BCa, by a direct repression of aromatase transcription *via* promoter II as well as an indirect effect due to the reduction in the levels and biological activity of PGE_2, which is a major stimulator of aromatase transcription through promoter II in BCa. Calcitriol down-regulates the expression of estrogen receptor α and thereby attenuates estrogen signaling in BCa cells including the proliferative stimulus provided by estrogens. We hypothesize that the inhibition of estrogen synthesis and signaling by calcitriol and its anti-inflammatory actions will play an important role in the use of calcitriol for the prevention and/or treatment of BCa.

1. Introduction

Breast cancer (BCa) is the most common cancer in women in the United States with over 180,000 new cases in 2008 [1]. Estrogens drive the proliferation of mammary epithelial cells and therefore promote the growth of estrogen receptor positive (ER+) BCa. Approximately 70% of BCa is ER+ and is therefore amenable to hormonal treatments that ablate estrogen production or block estrogen action [2]. In spite of the available treatments, the incidence of BCa continues to rise and increasing emphasis is being placed on BCa chemoprevention, including approaches to reduce exposure to carcinogens and the use of nutritional agents to prevent and/or delay the development of BCa.

Calcitriol, the hormonally active form of vitamin D (1,25-dihydroxyvitamin D_3), plays an important role in calcium homeostasis through its actions in intestine, kidney and bone [3]. In the recent years it has been recognized that in addition to its actions on calcium and bone homeostasis, calcitriol also exhibits anti-proliferative and pro-differentiation activities indicating its potential use in the prevention and treatment of several cancers including BCa [4–11].

2. Vitamin D and BCa

2.1. Epidemiology

Epidemiological studies report lower incidence and mortality rates from BCa in regions with greater solar UV-B exposure [12]. The potential benefit from sunlight is attributed to vitamin D, since UV light is essential for the cutaneous synthesis of vitamin D [3]. Women who develop BCa are predominantly postmenopausal and estrogen deficiency and aging are associated with vitamin D deficiency [13]. There appears to be an inverse association between BCa risk and the levels of serum 25-hydroxyvitamin D [25(OH)D], the circulating precursor to calcitriol, which reflects both sun exposure and dietary vitamin D intake [14]. A serum 25(OH)D level of approximately 52 ng/ml has been shown to be associated with a reduction by 50% in the incidence of BCa [12]. Polymorphisms in the vitamin D receptor (VDR) gene have also been shown to correlate with BCa susceptibility (reviewed in [5,15]).

2.2. Vitamin D and normal mammary gland development

The role of vitamin D signaling in the development of normal mammary gland has been extensively investigated (reviewed

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in [5,15]). The data show that calcitriol acting through the VDR regulates gene expression resulting in the suppression of proliferation and the stimulation of differentiation of mammary epithelial cells [16], suggesting that vitamin D might be useful in preventing the development of BCa. Studies in VDR knock out mice also provide evidence that calcitriol signaling through the VDR opposes estrogen driven proliferation of mammary epithelial cells and maintains differentiation [15]. Several studies have examined the effect of vitamin D on mammary carcinogenesis in animal models and the data are supportive of a protective role for vitamin D in BCa development [5,15]. VDR is present in all the major cell types in the normal mammary gland and the regulation of receptor levels correlates with the developmental changes associated with puberty, pregnancy, lactation and weaning [15,16].

2.3. Preclinical and clinical studies

Calcitriol inhibits the proliferation of both non-transformed mammary epithelial cells as well as BCa cells in culture [15,17]. Calcitriol also induces the expression of differentiation markers in non-transformed mammary epithelial cells [17,18]. *In vivo* studies in animal models have examined the ability of calcitriol and its analogs to retard the growth of human BCa established as xenograft tumors as well as their effect to delay or prevent carcinogen-induced mammary tumor development (reviewed in [5,6]). The data from these preclinical studies support the use of calcitriol as a therapeutic agent in BCa.

Calcitriol is an FDA approved drug (for other indications) and its therapeutic utility has been evaluated in clinical studies in cancer patients. However, the anti-proliferative effects of calcitriol in cultured cells have been observed at high concentrations, which when administered *in vivo* in equivalent concentrations may cause hypercalcemia and hypercalciuria leading to renal stone formation [19]. Many academic investigators and pharmaceutical companies therefore have undertaken intense research to develop calcitriol analogs that exhibit increased anti-proliferative activity and reduced tendency to cause hypercalcemia. Many of the clinical studies evaluating calcitriol and its structural analogs have been conducted in prostate cancer patients and a relatively smaller number of studies have been carried out in patients with colorectal cancer (CRC) and BCa. In a small clinical study in women with BCa the efficacy of topical treatment of cutaneous nodules with the calcitriol analog MC903 was investigated. Of the 14 patients who completed the treatment, 3 showed a partial response and one showed a minimal response [20]. A phase I trial evaluated the calcitriol analog EB1089 (seocalcitol) in 36 patients with advanced BCa and CRC [21]. Although this study did not demonstrate an anti-tumor effect, as determined by a reduction in tumor volume, stabilization of the disease was seen in 6 patients for over 3 months [21]. The efficacy and safety of the calcitriol analog EB1089 was also tested in a study of 22 patients with hepatocellular carcinoma and partial to complete remission was seen in 2 of the patients [22]. The WHI clinical trial and observational study evaluated the effect of supplementation with calcium and vitamin D (the inactive precursor to calcitriol) primarily to prevent hip and other fractures and secondarily to prevent CRC and BCa [23]. Initial published results of this study did not find a protective effect of calcium and vitamin D against CRC [24]. However, a recent reanalysis of the data concluded that concurrent estrogen therapy modified the effect of calcium and vitamin D supplementation on CRC risk, and in the women concurrently assigned to placebo arms of the estrogen trials, the supplementation was beneficial [25].

3. Mechanisms by which calcitriol inhibits the growth of BCa cells

3.1. Growth arrest and differentiation

Several studies have shown that calcitriol inhibits the growth of human BCa cell lines (reviewed in [5,6,26]). In general ER+ BCa cell lines appear to be more sensitive to the growth inhibitory effects of calcitriol than ER-negative cell lines [16]. In ER+ cells such as MCF-7, calcitriol induces cell cycle arrest in the G0/G1 phase of the cell cycle by increasing the expression of cyclin-dependent kinase inhibitors such as $p21^{Waf/Cip1}$, decreasing cyclin-dependent kinase activity and causing the dephosphorylation of the retinoblastoma protein [27–29]. Calcitriol and its analogs also inhibit the growth of BCa cells by regulating the expression of oncogenes such as c-myc and c-fos and modulating the actions of several growth factors including epidermal growth factor (EGF), transforming growth factor β (TGF β) and insulin-like growth factor-I (IGF-I) (reviewed in [5,6]). Further, calcitriol and its analogs have been shown to induce a more differentiated phenotype in some BCa cells reversing the myoepithelial features associated with more aggressive forms of BCa [30,31].

3.2. Apoptosis

In addition to causing growth arrest, calcitriol and its analogs induce morphological and biochemical changes associated with apoptosis in BCa cells such as chromatin condensation and DNA fragmentation [32,33]. The induction of apoptosis involves the generation of reactive oxygen species, mitochondrial disruption and the release of cytochrome C [34]. These changes might be related to the regulation of the expression of bcl-2 family of genes by calcitriol resulting in a decrease in the relative expression of anti-apoptotic proteins such as bcl-2 and bcl-XL to pro-apoptotic proteins such as bax and bak [6,16]. Calcitriol has also been shown to potentiate tumor necrosis factor α (TNF α)-induced apoptosis of some BCa cells through the death receptor pathway, which is linked to the activation of caspases [35,36] and phospholipase A_2 [6].

3.3. Invasion and metastasis

Several ER-negative BCa cells are invasive *in vitro* and are highly metastatic *in vivo* and calcitriol reduces the invasive potential of these cells [37–39]. In some ER-negative BCa cells, calcitriol stimulates the expression of E-cadherin which is inversely associated with invasion and metastasis [31]. Other mechanisms of the suppressive effects of calcitriol on invasion and metastasis include decreases in the activities of matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator and increases in the expression of PA inhibitor 1 (PAI1) and MMP inhibitor 1 [39]. Calcitriol also has potent anti-angiogenic activity that might contribute to its actions to inhibit invasion and metastasis [5,6].

4. New mechanisms underlying the inhibitory effects of calcitriol on BCa

4.1. Anti-inflammatory effects

A variety of stimuli trigger chronic inflammation, which has been recognized as a risk factor for cancer development [40,41]. Cancer-related inflammation is characterized by the presence of inflammatory cells at the tumor sites and the over-expression of inflammatory mediators such as cytokines, chemokines, prostaglandins (PGs) and reactive oxygen and nitrogen species in tumor tissue [40–43]. Many of these pro-inflammatory mediators

activate angiogenic switches usually under the control of vascular endothelial growth factor (VEGF) and thereby promote tumor progression, metastasis and invasion [44,45]. Studies from our laboratory and others have shown that calcitriol exhibits significant anti-inflammatory actions in prostate cancer (reviewed in [8,46]). Our studies show that calcitriol inhibits the synthesis and biological activity of PGs in prostate cancer cells [47] and suppresses the activation of stress activated kinase and downstream production of pro-inflammatory cytokines such as interleukin-6 (IL-6) by increasing the expression of mitogen-activated protein kinase phosphatase 5 (MKP5) [48].

Our recent studies reveal that similar anti-inflammatory effects of calcitriol are seen in BCa cells as well. In both ER+ and ER-negative human BCa cells, calcitriol down-regulates the expression of cyclooxygenase-2 (COX-2), the rate-limiting enzyme catalyzing PG synthesis [49]. Calcitriol also increases the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which catalyzes the conversion of PGs to biologically inactive keto-derivatives [49]. As a result, calcitriol treatment significantly reduces both the synthesis and the biological activity of PGs in BCa cells [49]. PGs play an important role in the development and progression of BCa. PGs released from BCa cells or surrounding tissues mediate autocrine/paracrine stimulation of tumor progression by promoting cell proliferation and resistance to apoptosis and stimulating tumor cell migration, metastasis and angiogenesis [50]. Elevated expression of COX-2 in BCa is associated with larger tumor size, high histological grade and poor prognosis [51]. COX-2 may be an important factor in promoting tumor progression in ER-negative tumors and is a potential drug target in BCa therapy [50]. Low levels of expression of 15-PGDH are seen in ~40% of primary breast tumors [52] and *in vitro* studies in BCa cells reveal a tumor suppressive role for this enzyme in BCa [52]. Therefore, we propose that the down-regulation of COX-2 and the resultant decrease in the synthesis of pro-inflammatory PGs, as well as the increase in the expression of the putative tumor suppressor 15-PGDH by calcitriol, play an important role in its anti-proliferative and anti-inflammatory actions in BCa. The calcitriol-mediated decrease in COX-2 expression in BCa cells is especially interesting, because it has been shown that there is a tight coupling between the expression levels of COX-2 and aromatase, the enzyme that catalyzes estrogen synthesis, in tumor samples from BCa patients [53,54]. In the following section, we discuss these and other new findings from our laboratory [49] that demonstrate the regulatory effects of calcitriol on aromatase gene expression in BCa cells and the mechanism of this regulation in relation to COX-2 down-regulation (illustrated in Fig. 1).

4.2. Inhibition of estrogen synthesis and signaling

Our studies in experimental models of BCa have revealed that calcitriol inhibits both the synthesis and the biological actions of estrogens, the major stimulators of BCa growth (Fig. 1). As discussed below, calcitriol suppresses the estrogen pathway by exerting repressive effects on the expression of the gene encoding aromatase (CYP19A1), the enzyme that catalyzes estrogen synthesis from androgenic precursors. Calcitriol also down-regulates ERα, the nuclear receptor that mediates estrogen actions. These combined actions reduce the levels of the estrogenic hormones and the receptor that mediates their signaling (Fig. 1).

4.3. Direct repression of aromatase transcription by calcitriol

Aromatase is a cytochrome P450 enzyme that catalyzes the synthesis of estrogens from androgenic precursors. The ovaries are the principal source of circulating estrogens in premenopausal women. In humans a number of other tissues including the breast express aromatase and hence have the capacity to synthesize estrogens locally. Importantly, estrogens synthesized locally become the major source of the hormone in the breast after menopause when circulating estrogen levels from the ovaries dramatically decline. Aromatase expression is higher in human BCa than in normal breast tissue [55]. In postmenopausal women with BCa, estrogen levels within the breast tissue are several-fold higher than the serum levels indicating tumor accumulation or local synthesis of estrogens that can drive BCa growth [56]. Therefore aromatase is critical for the progression of ER+ BCa in postmenopausal women. While BCa cells express aromatase and have the capacity to synthesize estrogens, in the normal breast, aromatase is primarily expressed in the stromal mesenchymal cells of the breast adipose tissue (breast adipose fibroblasts (BAFs) or preadipocytes) and the levels are higher in the undifferentiated preadipocytes rather than mature lipid laden adipocytes [57]. Aromatase transcription is primarily driven by the tissue specific promoter I.4 in normal breast adipose tissue and bone [57]. However, in the presence of BCa, the transcription switches from promoter I.4 to predominantly promoter I.3 and promoter II both in the cancerous epithelial cells and in the surrounding BAFs [57].

Calcitriol has been shown to be a regulator of aromatase expression in bone and it causes the up-regulation of aromatase mRNA and activity in osteoblasts and fibroblasts [58,59]. Other studies in keratinocytes [60] and prostate cancer cells [61] found no effect of calcitriol on aromatase expression. Interestingly, recent *in vitro* and *in vivo* studies from our laboratory demonstrate that calcitriol regulates the expression of aromatase in a tissue-selective manner. Our findings reveal that calcitriol significantly decreases aromatase expression in both ER+ and ER-negative human BCa cells and a cell culture model of preadipocytes [49]. Further calcitriol decreases aromatase expression in xenografts of human BCa cells established in immunocompromised mice as well as in the mammary adipose tissue surrounding the xenograft tumors in these mice [49]. The mechanism of aromatase down-regulation in BCa cells appears to be a direct repression by calcitriol of aromatase transcription via promoter II through the vitamin D response elements (VDREs) that we have identified in this promoter [49]. In contrast, as reported previously [58,59] calcitriol substantially increases aromatase expression in human osteosarcoma cells with osteoblastic features while causing a modest increase or no change in ovarian aromatase [49], demonstrating the tissue selectivity of aromatase regulation by calcitriol.

4.4. Indirect suppression of aromatase expression by calcitriol through the inhibition of PG synthesis and signaling

Further, as discussed above, calcitriol also significantly reduces the levels of PGs through the suppression of COX-2 and induction of 15-PGDH expression in BCa cells and mammary adipose tissue (Fig. 1). Both promoters I.3 and II predominantly used in malignant breast epithelial cells and the BAFs surrounding a breast tumor are responsive to cAMP [62] and are significantly stimulated by PGE_2 [63,64]. PGs secreted by BCa cells or other infiltrating inflammatory cells at the tumor sites stimulate local estrogen synthesis within the breast and thus promote cancer cell proliferation by autocrine/paracrine actions [57,65]. A positive association between COX-2 expression and aromatase expression and activity in BCa specimens strongly supports this hypothesis [53,54]. Thus the calcitriol-mediated reduction of biologically active PG levels provides an important second, indirect mechanism for its down-regulatory effect on aromatase expression in BCa cells and the tumor adjacent BAFs (Fig. 1, [49]).

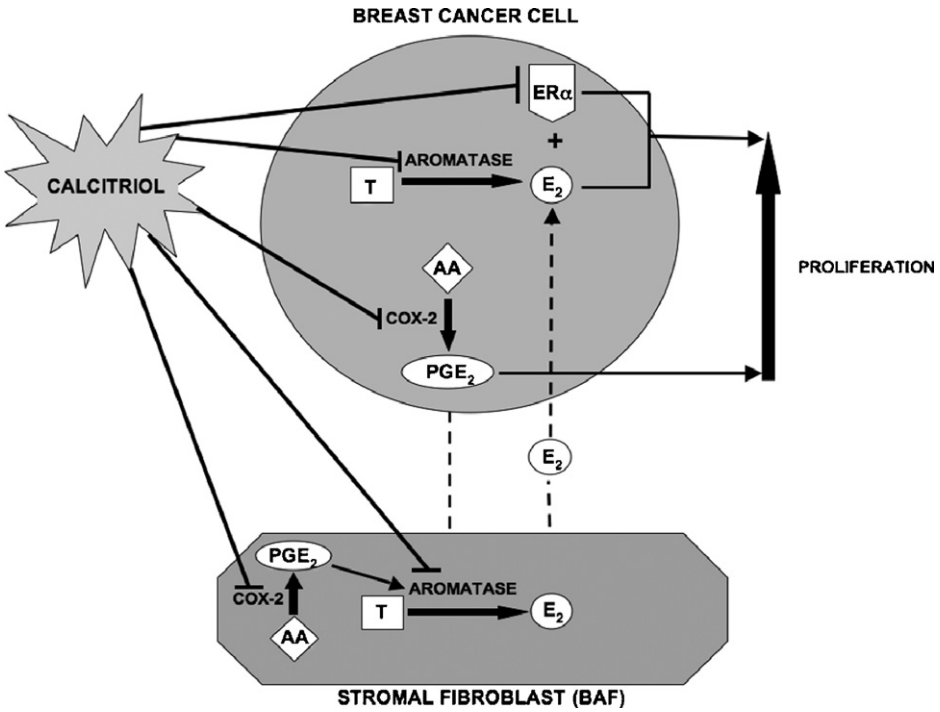

Fig. 1. Inhibition of estrogen synthesis and signaling by calcitriol. Calcitriol decreases the expression of aromatase, the enzyme that converts androgenic precursors to estrogens both in the cancerous breast epithelial cells (breast cancer, BCa cell) and in the breast adipose fibroblasts (BAF) in the stroma surrounding the tumor by a direct transcriptional repression of the aromatase promoter II. Calcitriol also suppresses the expression of COX-2 in the BCa cells and BAFs, thereby reducing the levels of PGE_2. PGE_2 stimulates proliferation, angiogenesis and other pro-carcinogenic pathways and inhibits apoptosis. PGE_2 is also a major stimulator of aromatase transcription via promoter II. The decrease in PGE_2 therefore provides a second, indirect mechanism for aromatase repression by calcitriol both in the BCa cells and in the surrounding BAFs leading to a decrease in estrogen synthesis in the BCa microenvironment. Calcitriol also down-regulates ERα levels by the direct transcriptional repression of the ERα promoter. The down-regulation of both the hormone (E_2) and the receptor (ERα) levels by calcitriol thus significantly reduces the important proliferative stimulus of estrogens on ER+ BCa cells. AA = arachidonic acid, E_2 = estradiol, ERα = estrogen receptor α, PGE_2 = prostaglandin E_2, T = testosterone.

4.5. Calcitriol and aromatase inhibitors

Aromatase inhibitors (AIs) inhibit the enzymatic activity of aromatase, while calcitriol reduces aromatase expression. Therefore we observed enhanced growth inhibitory effects of the combinations of calcitriol and AIs in BCa cell cultures [49]. AIs have become the major therapeutic agents to prevent ER+ BCa progression or recurrence in postmenopausal women after primary therapy [66–68]. However, AIs inhibit estrogen synthesis globally and therefore have a detrimental effect at sites such as bone [69,70] where normal estrogen function is required for the maintenance of bone homeostasis. The development of selective aromatase modulators (SAMs) that inhibit aromatase expression in breast, but allow unimpaired estrogen synthesis at other desirable sites such as bone, would have great utility in BCa therapy [57]. We postulate that calcitriol acts as a SAM, decreasing aromatase expression in BCa cells and breast adipose tissue surrounding BCa, while increasing aromatase expression in bone cells and thus it has the potential to ameliorate the AI-induced side effect of osteoporosis when used in combination with an AI in BCa patients.

4.6. Down-regulation of ERα by calcitriol

The growth stimulating actions of estrogen require the presence of ERα, the specific nuclear receptor that mediates proliferation in response to estrogens [71]. We [72] and others [29,73,74] have shown that calcitriol down-regulates ERα expression in BCa cells. As a result calcitriol and its analogs suppress estrogenic bioresponses in BCa cells including the induction of the expression of estrogen responsive genes such as the progesterone receptor and pS2 and attenuate the stimulation of the growth of BCa cells by estradiol (E_2) [72,73]. The mechanism of ER down-regulation appears to be a direct transcriptional repression of the ERα gene by calcitriol [72,74]. Further characterization of the ERα promoter by us (unpublished observations) and others [74] has identified negative vitamin D response elements (nVDREs) in the ERα promoter that mediate the repressive effects of calcitriol on the ERα gene. Combinations of calcitriol or its analogs with ER antagonists such as tamoxifen or ICI 182, 780 also exhibit enhanced inhibition of the growth of BCa cells [73,75,76].

We postulate that the cumulative actions of calcitriol cause a decrease both in the level of locally produced hormone (estrogens) by the BCa epithelial cells and the surrounding breast adipose tissue and in the levels of receptors through which they act (ERα) in BCa cells. Thus, the down-regulation of both the hormone and the receptor levels significantly reduces the important proliferative stimulus of estrogens on ER+ BCa cells (Fig. 1).

5. Summary and conclusions

Calcitriol exhibits anti-proliferative effects in BCa cells through a variety of mechanisms and retards tumor growth in animal models of BCa. Our recent research has identified several new calcitriol target genes revealing additional molecular pathways of calcitriol action. Calcitriol suppresses the expression of COX-2 and stimulates 15-PGDH expression thereby reducing the levels of biologically active PGs in BCa cells. We propose that calcitriol inhibition of the PG pathway contributes significantly to its anti-inflammatory actions in BCa. Acting as a SAM, calcitriol decreases aromatase expression in BCa cells and the breast adipose tissue surrounding BCa, while increasing aromatase expression in bone cells. The mechanism of the down-regulatory effect of calcitriol on BCa aromatase expression is two fold: a direct repression of aromatase transcription *via* promoter II through the VDREs present in this

promoter and an indirect effect due to the reduction in the levels and biological activity of PGE_2, which is a major stimulator of aromatase transcription through promoter II in BCa. Further, in addition to suppressing estrogen synthesis, calcitriol inhibits estrogen actions by down-regulating ERα expression. The regulation of PG and estrogen signaling pathways by calcitriol in BCa indicates that calcitriol has potent anti-inflammatory and anti-proliferative actions in addition to its other established anti-cancer effects. We hypothesize that these combined anti-proliferative actions of calcitriol will play an important role in the use of calcitriol for the prevention and/or treatment of BCa.

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