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Molecular mechanisms mediating the anti-proliferative effects of Vitamin D in prostate cancer

Jacqueline Moreno, Aruna V. Krishnan, David Feldman *

Department of Medicine, Division of Endocrinology, Stanford University School of Medicine, Stanford, CA 94305, USA

Abstract

Calcitriol (1,25-dihydroxyvitamin D_3) inhibits the growth and stimulates the differentiation of prostate cancer (PCa) cells. The effects of calcitriol are varied, appear to be cell-specific and result in growth arrest and stimulation of apoptosis. Our goal was to define the genes involved in the multiple pathways mediating the anti-proliferative effects of calcitriol in PCa. We used cDNA microarray analysis to identify calcitriol target genes involved in these pathways in both LNCaP human PCa cells and primary prostatic epithelial cells. Interestingly, two of the target genes that we identified play key roles in the metabolism of prostaglandins (PGs), which are known stimulators of PCa cell growth and progression. The expression of the PG synthesizing cyclooxygenase-2 (*COX-2*) gene was significantly decreased by calcitriol, while that of PG inactivating 15-prostaglandin dehydrogenase gene (*15-PGDH*) was increased. We postulate that this dual action of calcitriol would reduce the levels of biologically active PGs in PCa cells decreasing their proliferative stimulus and contribute to the growth inhibitory actions of calcitriol. In addition, we propose that calcitriol can be combined with non-steroidal anti-inflammatory drugs that inhibit COX activity, as a potential therapeutic strategy to improve the potency and efficacy of both drugs in the treatment of PCa.

Keywords: CYP24; CYP27B1; VDR; cDNA arrays; Target genes; Prostaglandins; 15-PGDH; COX-2; NSAIDs

1. Introduction

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death in North American men. Primary therapy to treat PCa involves the surgical removal of the prostate or radiation therapy. However, in many men the cancer progresses to advanced or metastatic disease. Androgens play a crucial role in the development, growth and maintenance of the prostate. Most patients with metastatic PCa who have failed the primary therapy, receive drugs that block the production of androgens [1]. Although most men have a good initial response to the androgen deprivation therapy, almost all of them will eventually relapse after an average of 2–3 years. This progression develops when the cancer has evolved from androgen-dependent to androgen-independent PCa (AIPC) with limited treatment options and becomes ultimately lethal. 1,25-Dihydroxyvitamin D_3 (calcitriol), the active metabolite of Vitamin D, has emerged in recent years as a promising therapeutic agent in the treatment of PCa [2–11]. Calcitriol is an important regulator of calcium homeostasis and bone metabolism through its actions in intestine, bone, kidney and the parathyroid glands [12]. In addition to these classical actions, calcitriol also exerts anti-proliferative and pro-differentiating effects in a number of tumors and malignant cells including PCa raising the possibility of its use as an anti-cancer agent.

2. Calcitriol and prostate cancer

2.1. Epidemiological and genetic studies

PCa development has been shown to be associated with age, genetic factors and race [1]. Various studies indicate that dietary [13] and environmental factors also play a role in PCa genesis. Epidemiological data provide a strong correlation between the exposure to sunlight and the prevalence of certain cancers, particularly prostate cancer [14]. Since UV light

* Corresponding author. Tel.: +1 650 725 2910; fax: +1 650 725 7085.
E-mail address: feldman@cmgm.stanford.edu (D. Feldman).

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is essential for Vitamin D synthesis in the skin, these studies have led to the suggestion that low circulating levels of calcitriol increase the probability of developing PCa. Moreover, polymorphisms in the gene encoding the Vitamin D receptor (VDR), which mediates the biological activity of calcitriol, may be involved in the development and or progression of PCa [2–11,13,15].

2.2. Prostate as a target for calcitriol

Many studies have demonstrated a beneficial effect of calcitriol to inhibit PCa growth and progression [2–11]. Calcitriol exerts anti-proliferative actions in several PCa cell lines [16–18], as well as in primary cultures of normal and cancer cells derived from surgical specimens taken from men with PCa [19]. The inhibition of PCa cell growth is seen in both androgen-dependent and AIPC cells [20]. Several in vivo studies on tumor xenografts established by transplanting clinical prostate tumors or cultured human PCa cells into immune-deficient mice have also demonstrated the tumor inhibitory effects of calcitriol [2–11]. The concentrations of calcitriol required for eliciting a significant growth inhibitory response in vivo causes hypercalcemia as a side effect. Therefore, investigators have used structural analogs of calcitriol that exhibit reduced calcemic effects in in vivo studies [2–7,16]. Recent findings suggest that large doses of calcitriol can be safely given to patients if administered intermittently [8,10].

3. The role of calcitriol metabolism in cellular responsiveness to the hormone

3.1. 25-Hydroxyvitamin D_3-1-α-hydroxylase (CYP27B1)

25-Hydroxyvitamin D_3-1-α-hydroxylase (1α-hydroxylase or CYP27B1) is the enzyme mediating the conversion of 25(OH)D_3 to calcitriol in the kidney, which is the major site of calcitriol synthesis [12]. Interestingly, it has been found that 1α-hydroxylase is also expressed in tissues other than the kidney including the prostate [21]. This suggests that prostate tissue is able to produce calcitriol locally and raises the possibility that hypercalcemia could be bypassed by treatment with 25(OH)D_3. However, we and others [22,23] demonstrated a substantial reduction in 1α-hydroxylase activity in human PCa cell lines and cancer derived primary prostate epithelial cells compared to the cells derived from normal prostate or benign prostatic hyperplasia (BPH). Based on these findings we speculate that administration of 25(OH)D_3 to patients is unlikely to be effective as treatment of established PCa. However, the fact that normal cells exhibit high 1α-hydroxylase activity indicates that 25(OH)D_3 may be useful as a chemopreventive agent due to its local conversion to the active hormone within the normal prostate. The cause for the decreased expression of 1α-hydroxylase in cancer cells remains to be determined.

3.2. Differential sensitivity to calcitriol action and 24-hydroxylase (CYP24) expression

24-Hydroxylase or CYP24 is the enzyme that initiates calcitriol catabolism in target cells [12]. Calcitriol induces the expression of CYP24 in target cells. Therefore, calcitriol initiates its own inactivation. CYP24 is widely expressed in calcitriol target tissues including the prostate [16]. We and others have demonstrated that the anti-proliferative action of calcitriol varies among PCa cells and is inversely related to the level of CYP24 [16,18]. For example, the DU145 PCa cells, which are resistant to the growth inhibitory action of calcitriol, exhibit a high expression of CYP24. Treatment of the DU145 cells with liarozole, an inhibitor of P450 enzymes, sensitizes the cells to the anti-proliferative actions of calcitriol [24]. Similarly, Peehl et al. showed that the combined treatment with the general P450 inhibitor, ketoconazole and calcitriol or its analog EB 1089, increased their growth inhibitory actions [25]. These results strongly suggest that the combination of calcitriol with P450 inhibitors is a useful strategy to enhance the anti-cancer activity of calcitriol in PCa treatment.

4. Clinical studies of calcitriol effects in PCa

A pilot clinical study from our lab provides evidence that calcitriol effectively slows the rate of rise of serum prostatic specific antigen (PSA) in patients with early recurrent PCa after radical prostatectomy or radiation therapy [26]. However, the amount of calcitriol administered was limited by the development of hypercalciuria. Recent advances in the design of calcitriol analogs have resulted in potential drugs with increased potency and less tendency to cause hypercalcemia [2–11]. The beneficial effects of calcitriol have been observed only at supra-physiological concentrations (>1 nmol/L). These high concentrations might be more safely achieved without causing persistent hypercalcemia if calcitriol is administered intermittently [8,10]. Recent trials using intermittent high doses of calcitriol in combination with chemotherapy drugs have shown a beneficial effect in advanced PCa [27] with calcitriol potentiating the anti-tumor effects of many cytotoxic agents [28]. Trump et al. completed a phase II study of calcitriol and dexamethasone in AIPC. This study showed a significant slowing in PSA rise in 80% of the patients with the stabilization or decrease in PSA in 34% [10]. We believe that calcitriol or a new analog will prove to be a very useful adjunct for the therapy of both androgen-dependent PCa and AIPC. It is also possible that calcitriol therapy will prove to be useful in PCa chemoprevention.

5. Molecular mechanisms mediating anti-cancer response to calcitriol in PCa

The mechanisms governing the anti-proliferative actions of calcitriol are not fully known [2–11]. A number of important pathways have been shown to have a role in

calcitriol-mediated growth inhibition. A primary mechanism of calcitriol action is to induce cell cycle arrest in the G_1/G_0 phase. The growth arrest appears to be due to an increase in the expression of cyclin-dependent kinase inhibitors $p21^{Waf/Cip1}$ and $p27^{Kip1}$, a decrease in cyclin-dependent kinase 2 (Cdk2) activity, accompanied by a reduction in the nuclear fraction of this molecule and the hyperphosphorylation of the retinoblastoma protein (pRb). In addition, calcitriol induces apoptosis in some cells and down-regulates some anti-apoptotic genes, like bcl-2. Loss of the expression of cell cycle regulators has been associated with a more aggressive cancer phenotype, decreased prognosis and poorer survival. This suggests that calcitriol may be a suitable therapy to inhibit PCa progression [2–11]. Studies from our laboratory have implicated the increased expression of insulin-like growth factor binding protein-3 (IGFBP-3) in the growth inhibition induced by calcitriol which in turn increases $p21^{Waf/Cip1}$ expression [29]. Other mechanisms of calcitriol actions in PCa cells include the stimulation of differentiation, modulation of growth factor actions and the inhibition of invasion and metastasis [2–11]. Some in vivo studies have also demonstrated that the inhibition of angiogenesis contributes to the anti-tumor effects of calcitriol [2–11].

6. Novel calcitriol target genes

We performed cDNA-microarray analyses of normal and cancer-derived primary prostatic epithelial cells and LNCaP human PCa cells [30,31] to identify the molecular targets by which calcitriol mediates its effects on PCa cells. Table 1 presents some of the genes regulated by calcitriol in prostate cells. Our studies have recently shown that calcitriol regulates the expression of the genes involved in prostaglandin (PG) metabolism which has led us to hypothesize a new pathway for the anti-cancer activity of calcitriol, namely the regulation of PG metabolism [30,31].

6.1. Calcitriol effects on prostaglandin metabolism

Our microarray analyses showed that calcitriol up-regulates the expression of NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in LNCaP and normal primary prostate epithelial cells (see Fig. 1) [30,31]. Calcitriol also suppresses the expression of prostaglandin-endoperoxide synthase gene, also known as cyclooxygenase-2 (COX-2) in LNCaP cells (see Fig. 1). We have confirmed calcitriol regulation of these two genes by real time RT-PCR analysis. These data suggest that the regulation of PG metabolism could be an important molecular pathway of calcitriol action in prostate cells.

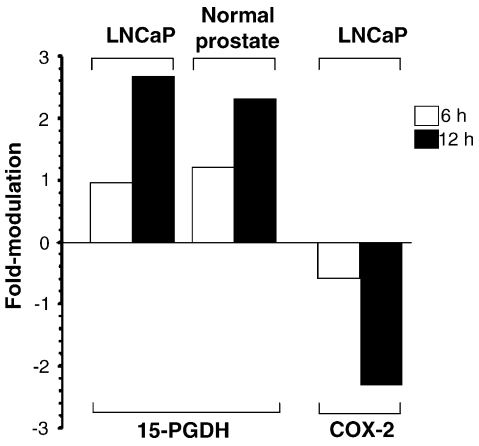

Fig. 1. Regulation of 15-PGDH and COX-2 by calcitriol in LNCaP cells and normal primary prostatic epithelial cells. LNCaP human prostate cancer cells or primary human prostatic epithelial cells derived from normal prostate were treated with calcitriol (50 nM) for 6 (□) or 24 h (■). $PolyA^+$ RNA was isolated and cDNAs were hybridized to a 24.192 element array. Data represent mean fold of increase or decrease of gene expression. (Adapted from Krishnan et al. [30] and Peehl et al. [31].)

6.1.1. COX-2

Prostaglandins play a role in the development and progression of PCa (for a review see Ref. [32]). Accumulating evidence implicates PGs as mediators of proliferation of PCa [33]. PG synthesis begins with the intracellular release of arachidonic acid (AA) from plasma membrane via the action of phospholipase A2. COX is the rate-limiting enzyme that catalyzes the conversion of AA to PGs [34]. The expression of COX-2 is rapidly induced by a variety of mitogens, cytokines, tumor promoters and growth factors, and therefore COX-2 is regarded as an immediate-early response gene [34]. Compelling evidence from genetic and clinical studies indicates that increased expression of COX-2 is one of the

Table 1
Genes modulated by calcitriol in prostate cells

Gene	Cell type	Fold change	
		6 h	24 h
Insulin-like growth factor binding protein-3	LNCaP	2.42	33.2
Vitamin D 24-hydroxylase	Normal prostate primary cells	79.1	82.5
Vitamin D 24-hydroxylase	PCa-derived epithelial cells	78.1	46.1
15-Hydroxyprostaglandin dehydrogenase	Normal prostate primary cells	1.2	2.3
15-Hydroxyprostaglandin dehydrogenase	LNCaP	0.95	2.66
Cyclooxygenase-2	LNCaP	−0.66	−2.13

Adapted from Krishnan et al. [30] and Peehl et al. [31].

key steps in carcinogenesis. Constitutive and high-levels of COX-2 activity have been detected in colon, lung, breast and prostate cancer [33,35]. It is known that high levels of PGE_2, one of the products of COX-2 activity, stimulate epithelial cell growth [33], promote cell survival and invasion [36]. Several studies have demonstrated COX-2 over-expression in prostate adenocarcinoma [32,37] suggesting a positive role for COX-2 in prostate tumorigenesis. COX-2 expression appears to be linked to cell survival. For example, COX-2 over-expression leads to the stabilization of survivin [38], a member of the inhibitor of apoptosis protein family that block caspase activation. The stabilization of survivin alters the balance between pro- and anti-apoptotic pathways leading to resistance to apoptosis. COX-2 expression has also been associated with angiogenesis and tumor metastasis [37] and the administration of a COX-2 inhibitor suppresses the growth of prostate tumor xenograft in mice by inhibiting angiogenesis [32]. We have found that calcitriol treatment decreases COX-2 gene expression in PCa cells (Fig. 1) [30]. Our data suggest that COX-2 suppression by calcitriol might contribute to the hormone's tumor inhibitory actions and anti-metastatic potential.

6.1.2. 15-PGDH

15-PGDH catalyzes the conversion of PGs into mostly inactive 15-keto derivatives [39]. A growing body of evidence has implicated 15-PGDH as a potential target for cancer therapy [40,41]. Various studies indicate that 15-PGDH acts as a putative tumor suppressor gene in lung cancer [41]. Backlund et al. [42] demonstrated a down-regulation of 15-PGDH expression in colorectal carcinoma. 15-PGDH has recently been described as an oncogene antagonist that functions as a tumor suppressor in colon cancer [43]. The study found that 15-PGDH, which physiologically antagonizes COX-2, was universally expressed in normal colon specimens but was routinely absent or severely reduced in cancer specimens. The study also showed that 15-PGDH expression was induced by transforming growth factor β (TGFβ) and that colon tumor cells exhibited mutations in TGFβ receptors or the genes involved in the SMAD signaling pathway explaining the decreased 15-PGDH levels in colon cancer. Most importantly, stable transfection of a 15-PGDH expression vector into cancer cells greatly reduced the ability of the cells to form tumors and/or slowed tumor growth in nude mice. The authors concluded that 15-PGDH suppressed the effects of the oncogene COX-2 and had an additional effect to inhibit angiogenesis in vivo [43]. Our studies show an up-regulation of 15-PGDH gene expression by calcitriol in normal and malignant prostate cells (Fig. 1) [30,31] and this regulation might mediate some of the anti-cancer effects of calcitriol. Interestingly, calcitriol has also been shown to induce the expression of 15-PGDH in human neonatal monocytes [44].

Based on our observations summarized in Table 1, we hypothesize that calcitriol treatment of PCa cells would reduce the levels of biologically active PGs due to its dual actions to reduce COX-2 expression and increase 15-PGDH expression. We further propose that the modulation of PG metabolism is an important molecular mechanism mediating the anti-proliferative and anti-metastatic activities of calcitriol.

7. Calcitriol and NSAIDs

Recent exciting findings show that non-steroidal anti-inflammatory drugs (NSAIDs) exert chemopreventive effects in several cancers [32,45]. NSAIDs have also been shown to suppress PCa development and progression [32,46]. The primary action of NSAIDs is to inhibit PG synthesis by directly inhibiting the enzymatic activity of COX-1 as well as COX-2. Many in vitro and in vivo studies have demonstrated that NSAIDs inhibit PCa growth and cause apoptosis of PCa cells [32,45]. However, many NSAIDs exhibit differences between their ability to inhibit COX-2 and to induce apoptosis suggesting that apoptosis induction may be independent of COX-2 inhibition [47]. The actions of NSAIDs on 15-PGDH are not clear. While many NSAIDs have been shown to inhibit the enzymatic activity of 15-PGDH [48], the non-selective NSAID indomethacin has been reported to enhance the expression and activity of 15-PGDH in thyroid carcinoma [49].

We hypothesize that the combination of calcitriol and NSAIDs would be additive/synergistic in their activity to inhibit PCa growth. The action of calcitriol at the genomic level to suppress COX-2 gene expression will decrease the levels of COX-2 protein and allow the use of lower concentrations of NSAIDs to inhibit COX-2 enzyme activity. The action of calcitriol to increase 15-PGDH expression would also complement NSAID action. The use of COX-2 selective NSAIDs has recently been shown to cause some serious cardiovascular side effects, such as increased risk of heart attacks, stroke, sudden death and blood clots [50]. The undesirable effect of calcitriol therapy is limited to hypercalcemia. We propose that a combination therapy of calcitriol with a NSAID would allow the use of lower concentrations of both drugs, thus reducing their individual side effects while increasing their anti-proliferative and pro-apoptotic activities.

8. Conclusions

Our research is directed at understanding the molecular mechanisms of the anti-proliferative activity of calcitriol in prostate cells with the goal of developing strategies to improve PCa treatment. Using cDNA microarrays we have recently found that calcitriol modulates the expression genes involved in PG metabolism. Calcitriol suppresses the expression of COX-2 gene, the enzyme that catalyzes PG synthesis and up-regulates the expression of 15-PGDH gene, the enzyme involved in PG inactivation. We hypothesize that

calcitriol treatment of PCa cells would reduce the levels of biologically active PGs due to its dual actions on COX-2 and 15-PGDH expression, and thereby decrease the PG-mediated proliferative stimulus. We further propose that the calcitriol effects would complement the action of NSAIDs, which are known inhibitors of both COX-1 and COX-2. The action of calcitriol at the genomic level to suppress COX-2 gene expression will decrease the levels of COX-2 protein and allow the use of lower the concentrations of NSAIDs needed to inhibit COX-2 enzyme activity. A combination of calcitriol with a NSAID would allow the use of lower concentrations of both drugs, thus reducing their individual side effects while increasing their anti-proliferative and pro-apoptotic activities. The combination approach is an attractive therapeutic strategy in the treatment of PCa and can be brought to clinical trials swiftly.

Note added in proof

Since this paper was originally written we have used real-time PCR and Western blot analyses to confirm that calcitriol inhibits PG actions in human PCa cell lines and primary prostatic epithelial cells. We found that calcitriol increased the expression of 15-PGDH, reduced the expression of COX-2, and reduced the expression of EP2 and FP PG receptors. These three calcitriol actions combine to decrease secreted PGE_2 and block cell growth stimulated by arachidonic acid and exogenous PGs. Moreover, calcitriol, in combination with various NSAIDs, produces synergistic inhibition of PCa cell growth at lower and safer drug concentrations. These data suggest that calcitriol and NSAIDs may be a useful combination for chemotherapy and/or chemoprevention of PCa. These new findings are now in press in Cancer Research 2005.

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