Published in final edited form as:
Mol Cell Endocrinol. 2011 December 5; 347(1-2): 61–69. doi:10.1016/j.mce.2011.05.010.

Vitamin D Metabolism and Action in the Prostate: Implications for Health and Disease

Srilatha Swami, **Aruna V. Krishnan**, and **David Feldman**
Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305

Abstract

Prostate cancer (PCa) is the second most common cancer in men worldwide. Epidemiological, molecular, and cellular studies have implicated vitamin D deficiency as a risk factor for the development and/or progression of PCa. Studies using cell culture systems and animal models suggest that vitamin D acts to reduce the growth of PCa through regulation of cellular proliferation and differentiation. However, although pre-clinical studies provide a strong indication for anti-cancer activity, proof of therapeutic benefits in men is still lacking. The anti-proliferative and pro-differentiating properties of vitamin D have been attributed to calcitriol [1,25$(OH)_2D_3$], the hormonally active form of vitamin D, acting through the vitamin D receptor (VDR). Metabolism of vitamin D in target tissues is mediated by two key enzymes: 1α-hydroxylase (CYP27B1), which catalyzes the synthesis of calcitriol from 25(OH)D and 24-hydroxylase (CYP24), which catalyzes the initial step in the conversion of calcitriol to less active metabolites. Many factors affect the balance of calcitriol synthesis and catabolism and several maneuvers, like combination therapy of calcitriol with other drugs, have been explored to treat PCa and reduce its risk. The current paper is an overview addressing some of the key factors that influence the biological actions of vitamin D and its metabolites in the treatment and/or prevention of PCa.

Keywords

Prostate cancer; vitamin D; calcitriol; vitamin D receptor (VDR); 1α-hydroxylase; 24-hydroxylase; combination therapy

1. INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed malignancy in American men, excluding skin cancer (Jemal et al., 2010). In men over the age of 50, sub-clinical PCa is found in as many as 40% of individuals (Stamey et al., 1993). Although PCa is generally a slowly progressing malignancy, mortality from this disease is nonetheless considerable. In contrast to many other malignancies, the incidence of PCa has continued to rise each year and currently PCa is the second leading cause of cancer in men. Its prevention and treatment has remained an ongoing challenge for clinicians the worldwide

Corresponding author David Feldman, M.D., Professor of Medicine, Department of Medicine/Endocrinology, Stanford University School of Medicine, 300 Pasteur Drive, Room S-025, Stanford, CA 94305-5103, Phone: 650-725-2910, Fax: 650-725-7085, dfeldman@stanford.edu.

This review will discuss the potential benefits of vitamin D and its active metabolites in preventing and treating PCa. The dietary forms of vitamin D include ergocalciferol (vitamin D_2) from plants, and vitamin D_3 from animal products, which is the human form of the hormone. Vitamin D_3 is approximately 87% more potent in raising and maintaining serum 25-hydroxyvitamin D [25(OH)D] concentrations and produces 2 to 3-fold greater storage of vitamin D than does equimolar vitamin D_2 (Heaney et al., 2011). This review will subsequently focus exclusively on vitamin D_3 and refer to it simply as vitamin D.

Vitamin D, a fat-soluble pro-hormone is synthesized in the body in response to sunlight and is converted to the active hormone 1,25-dihydroxyvitmin D_3 (calcitriol). Considerable preclinical and epidemiologic data suggest that vitamin D deficiency may play a role in the pathogenesis and progression of PCa, and that vitamin D or its active form, calcitriol, may be useful in the prevention or treatment of the disease. The anticancer properties of vitamin D have been attributed primarily to calcitriol, the hormonally active form of vitamin D. Measurement of vitamin D levels in many studies, including both surrogate estimates of vitamin D (e.g. sunlight exposure and diet) as well as assay of serum 25(OH)D, the circulating precursor for calcitriol, indicate that vitamin D deficiency increases the risk of PCa, although not all studies agree.

Several recent reviews discuss the role of vitamin D in PCa (Beer and Myrthue, 2006; Deeb et al., 2007; Fleet, 2008; Gombart et al., 2006; Kahraman et al., 2004; Krishnan and Feldman, 2010; Krishnan and Feldman, 2010; Krishnan et al., 2010; Stewart and Weigel, 2004). Based upon epidemiological studies, several risk factors such as age and genetics have been identified for PCa (Kozlowski and Grayhack, 1995; Ruijter et al., 1999). Studies published by Garland and Garland (Garland and Garland, 1980) and Schwartz and colleagues (Hanchette and Schwartz, 1992; Schwartz and Hulka, 1990) suggest a role for sunlight (a surrogate for vitamin D) in decreasing the risk of developing different cancers in humans. It has been shown that African American individuals have lower serum 25(OH)D levels as a result of their darker skin pigmentation. Melanin in the darkly pigmented skin of an African American individual absorbs UV radiation, inhibiting the formation of vitamin D_3 in the basal layer of the epidermis (Bell et al., 1985). This offers a plausible but unproven explanation for the higher incidence of PCa in African Americans when compared to their Caucasian counterparts (Studzinski and Moore, 1995). Many epidemiologic studies have examined whether vitamin D deficiency increases the risk of PCa. Although some demonstrate increased risk of PCa associated with deficiency, the various studies have not shown consistent results and some fail to show an inverse correlation. A recent review by Giovannucci discusses vitamin D deficiency in several cancers (Giovannucci, 2009).

Many preclinical studies indicate that exposing cancer cells to high concentrations of calcitriol arrests progression through the cell cycle, induces apoptosis and slows or stops tumor growth in vivo (Blutt et al., 2000; Yang and Burnstein, 2003). Calcitriol also potentiates the antitumor activity of a number of types of cytotoxic anticancer agents in preclinical models (Hershberger et al., 2001). Thus, studies of vitamin D and PCa have advanced from the hypothesis that vitamin D deficiency increases the risk of PCa to intervention trials of vitamin D in PCa patients. This review is aimed at addressing some of the key factors that influence the biological actions of vitamin D and its metabolites in the treatment and/or prevention of PCa.

2. VITAMIN D ACTIONS

Although the role of calcitriol in maintaining calcium homeostasis has been known for a long time, it is only recently that investigators have begun to understand the broader scope of calcitriol actions (Feldman et al., 2001). In studying the role of calcitriol in cancer it is

imperative to study the various factors that influence the cell- or tissue-specific actions of calcitriol. These include vitamin D receptor (VDR) expression and ligand interactions, as well as local synthesis, metabolism and transport of vitamin D and its metabolites.

2.1. Vitamin D receptor and prostate cancer

Although the initial description of the vitamin D receptor (VDR) in the prostate and most of the subsequent investigation of calcitriol actions have centered around PCa cell lines, calcitriol also appears to play an important role in normal prostate tissue. Peehl et al. (Peehl et al., 1994) reported the presence of VDR in freshly obtained surgical prostate specimens as well as primary cultures of epithelial and stromal cells of the prostate (Peehl, 2004). Primary cultures from surgical specimens of benign prostatic hyperplasia (BPH) also express VDR (Peehl et al., 1994). Although VDR is present in both epithelial and stromal cells cultured separately, lower levels of VDR were seen in the stromal fibroblasts compared to cells of the glandular epithelium. The region of origin within the prostate tissue did not influence the abundance of VDR, as both the peripheral zone and central zone cultures had similar VDR content (Peehl et al., 1994). Krill et al. (Krill et al., 2001) studied VDR expression in normal prostate glands from donors of various age groups and found that VDR expression changed with age with peak levels in the fifth decade and a decline thereafter. Calcitriol exerts anti-proliferative effects on rat neonatal prostatic epithelial cells (Konety et al., 2000) and human prostatic epithelial cells (Krill et al., 1999). Konety et al. (Konety et al., 2000; Konety et al., 1999) showed that exposure of rat pups *in utero* to i.p injections of calcitriol (1.25 μgs every other day) influenced prostatic growth and differentiation throughout the lives of the animals. In general, all these studies support a role for vitamin D in normal prostate physiology and growth.

The finding by Miller et al. (Miller et al., 1992) of the presence of VDR in LNCaP human PCa cells was an important early finding. The demonstration of VDR and the anti-proliferative actions of calcitriol (1–100 nM) in three different PCa cell lines including LNCaP, PC-3 and DU 145 cells by Skowronski et al. (Skowronski et al., 1993) initiated the subsequent research into the investigation of the direct role that vitamin D plays in prostate biology and how the hormone might be beneficial for therapy of PCa.

2.2. Vitamin D metabolism in prostate

Metabolism of vitamin D in target tissues is mediated by two key enzymes: 1α-hydroxylase (CYP27B1), which catalyzes the synthesis of calcitriol from 25(OH)D and 24-hydroxylase (CYP24), which catalyzes the initial step in the conversion of calcitriol to less active metabolites. Thus, the ratios of these two enzymes play an important role in determining the intracellular concentration of vitamin D metabolites and their biological activities.

2.2.1. 25-hydroxyvitamin D_3 1α-hydroxylase (CYP27B1)—The kidneys are the major source of circulating calcitriol in the body. The active hormone calcitriol is formed in the kidney by the hydroxylation of 25(OH)D at the C-1 position by the enzyme 1α-hydroxylase. In recent years, however, the presence of extra-renal 1α-hydroxylase has been demonstrated which contributes to the local production of calcitriol within various tissues. Schwartz et al. (Schwartz et al., 1998) showed that normal human prostatic epithelial cells express 1α-hydroxylase. They raised the possibility that treatment with vitamin D or 25(OH)D could potentially inhibit the growth of PCa, due to local production of calcitriol within the prostate. The ability of 25(OH)D to cause hypercalcemia is reduced when compared to calcitriol because of its much lower affinity for the VDR, thus avoiding the systemic side effect of hypercalcemia due to calcitriol administration. A study by Barreto et al. (Barreto et al., 2000) supports this hypothesis by demonstrating the growth inhibitory

effect of 25(OH)D in primary epithelial cell strains derived from normal human prostatic peripheral zone.

Hsu et al. (Hsu et al., 2001) showed that epithelial cells from normal prostate had more 1α-hydroxylase activity than those derived from BPH or cancer. The anti-proliferative effect of 25(OH)D correlated with the endogenous 1α-hydroxylase activity in these cells. The growth of primary epithelial cells from normal tissue or BPH was inhibited by 25(OH)D to an extent similar to calcitriol as it could be converted to calcitriol by endogenous 1α-hydroxylase activity. In contrast, in primary epithelial cells from cancer or in the LNCaP human PCa cell line, with very low endogenous 1α-hydroxylase activity, the anti-proliferative action of 25(OH)D was much less pronounced compared to calcitriol. Similarly, Whitlatch et al. (Whitlatch et al., 2002) found reduced 1α-hydroxylase activity in PCa cells compared to normal prostatic cells. Although these studies reported that 1α-hydroxylase activity was reduced in malignant prostate cells, it was still measurable and the biological effect of 25(OH)D in a reporter assay at concentrations similar to calcitriol was demonstrated in PC-3 cells transfected with the VDR (Chen et al., 2000). Later studies found equivalent levels of 1α-hydroxylase mRNA and protein but not enzyme activity in normal *vs*. malignant primary prostate cells (Ma et al., 2004). Chen et al suggested that the reduced enzyme activity might result from decreased 1α-hydroxylase promoter activity in the cancer cells (Chen et al., 2003). The finding of reduced 1α-hydroxylase activity in cancer-derived prostatic epithelial cells suggests that this difference may endow the malignant cells with an intrinsic growth advantage because of the resultant decrease in the local production of calcitriol allowing cellular de-differentiation and invasion that are the hallmarks of malignancy. Dwivedi and co-workers have identified a repressive region in the human CYP27B1 promoter following transient transfections in the PCa cell lines LNCaP, PC-3 and DU 145 as well as osteoblast like cells (Dwivedi et al., 2005; Dwivedi et al., 2007; Turner et al., 2007). These studies suggest a role for PTH, IGF-1, TGF-beta and GFI1 but not vitamin D in the down-regulation of 1α-hydroxylase expression in osteoblast like cells and provide the basis for a more detailed understanding of the differential regulation of CYP27B1 gene in various tissues.

The presence of 1α-hydroxylase in prostate cells raises the possibility that treatment with vitamin D could potentially inhibit PCa growth due to its conversion to 25(OH)D in the liver and than to calcitriol within the prostate. Calcitriol would then act in an autocrine/paracrine manner to exert anti-proliferative effects within the prostate. The presence of 1α-hydroxylase in the prostate therefore is potentially the critical factor resolving the conundrum of why circulating 25(OH)D concentration and not calcitriol concentration is the major factor correlated with reducing PCa risk. Unlike the renal enzyme, or other extra-renal tissue like the osteoblasts, the prostate 1α-hydroxylase is not regulated by PTH and serum calcium concentration (Young et al., 2004). Therefore the magnitude of local paracrine actions in the prostate appears to be mostly dependent on the circulating substrate levels of 25(OH)D that determine the extent of its conversion to calcitriol within the prostate. These paracrine actions would likely occur without increasing the concentration of circulating calcitriol. Thus, this hypothesis provides the basis for the protective effect of sunlight and dietary vitamin D in PCa, where serum concentrations of 25(OH)D and not calcitriol are most relevant to reduced risk and benefit. This hypothesis also raises the possibility that dietary vitamin D will be useful in the chemoprevention and/or treatment of PCa.

2.2.2. 25-hydroxyvitamin D_3 24-hydroxylase (CYP24)—Calcitriol induces the expression of 24-hydroxylase in target cells, which catalyzes the initial step in the conversion of the active molecule calcitriol into less active metabolites. The enzyme also converts 25(OH)D substrate to 24,25$(OH)_2$D preventing its activation to calcitriol. In prostate cells, the degree of growth inhibition by calcitriol appears to be inversely

proportional to the activity of 24-hydroxylase in the cells. Among the well-studied human PCa cell lines, DU 145 cells exhibit the highest level of 24-hydoxylase and are the least responsive to calcitriol in terms of growth inhibition (Miller et al., 1995; Skowronski et al., 1993). The enhanced 24-hydroxylase activity results in the rapid degradation of calcitriol, reducing its ability to regulate genes that control cellular proliferation and differentiation. On the other hand, the basal and induced expression of 24-hydroxylase is very low in the LNCaP cells and growth inhibition by 1 to 100 nM of calcitriol is substantial (50–70%) in these cells. Skowronski et al. (Skowronski et al., 1993) demonstrated growth inhibition by calcitriol in human PCa cells such as LNCaP, PC-3 and DU 145. The magnitude of growth inhibition was less in PC-3 cells (~40–50%) when compared to LNCaP cells, and the growth inhibition seen in DU 145 cells was minimal. Interestingly, 24-hydroxylase was induced by calcitriol maximally in DU 145 and moderately in PC-3 cells while the induction of 24-hydroxylase mRNA was not detected in LNCaP cells (Skowronski et al., 1993). Miller et al. (Miller et al., 1995) demonstrated that the differences in calcitriol-mediated growth inhibition between various PCa cell lines correlated inversely to levels of 24-hydroxylase expression in these cells. Numerous studies have indicated that antagonists of 24-hydroxylase enhance the anti-tumor effects of calcitriol. Combination of calcitriol with azole inhibitors of 24-hydroxylase such as liarozole (Ly et al., 1999) or ketoconazole (Peehl et al., 2002) increases the half-life of calcitriol and sensitize the cells to the biological actions of calcitriol. These studies raise the possibility of novel combination treatment regimens for PCa patients as discussed below.

2.2.3. Vitamin D receptor (VDR)—It is clear that the ultimate concentration of calcitriol hormone achieved in the cell, as a consequence of the interplay of sunlight, diet, transport in the circulation and metabolism, still requires the receptor and post-receptor signaling pathway to be intact for the stimulated or inhibited actions of the active hormone to be transmitted. For example the absence of intact VDR or loss of function mutations in the VDR would decrease or eliminate responses to any levels of calcitriol. Many factors regulate the concentration of VDR in target cells altering the magnitude of the response to calcitriol (Krishnan and Feldman, 1997). A recent study examining the expression of VDR protein by immunohistochemistry in 841 patients with PCa concluded that high expression of VDR in prostate tumors is associated with a reduced risk of lethal cancer (Hendrickson et al., 2011) suggesting a role for the VDR pathway in its ability to inhibit PCa progression that is dependent upon VDR content and not just 25(OH)D blood levels.

3. COMBINATION THERAPY OF CALCITRIOL WITH OTHER DRUGS

The efficacy of calcitriol in PCa therapy is thought to be dose related (for a more detailed discussion see Section 5). Hypercalcemia becomes more frequent at higher concentrations of calcitriol, which limits the maximum dose that can be given safely. One way to increase efficacy and decrease toxicity is to use a combination of agents that act by different mechanisms, at doses in the combination being less than the maximal doses of the individual agents. This drug combination strategy has the advantage of limiting the toxicity associated with the individual drugs while obtaining additive and potentially synergistic therapeutic effects. Investigators have been studying the anti-proliferative effect of calcitriol in PCa in combination with other agents (Krishnan et al., 2010; Peehl et al., 2004; Trump et al., 2010). This section discusses some of the agents that can be used in combination with calcitriol to improve its therapeutic utility.

3.1. 24-hydroxylase inhibitors

Azole antagonists of the primary vitamin D catabolic enzyme 24-hydroxylase enhance the anti-tumor effects of calcitriol. In DU 145 cells, addition of liarozole (an imidazole

derivative that inhibits P450 hydroxylases) causes significant inhibition of 24-hydroxylase activity. This action leads to an increase in calcitriol half-life in DU 145 cells and thereby allows a substantial calcitriol anti-proliferative effect (Ly et al., 1999). In many target cells, including PCa cells (Swami et al., 2005), calcitriol causes homologous up-regulation of VDR levels (Costa et al., 1985; Krishnan and Feldman, 1997). Prolongation of calcitriol half-life (due to the inhibition of 24-hydroxylase by liarozole) also leads to an enhanced up-regulation of VDR in DU 145 cells making them more responsive to calcitriol (Ly et al., 1999). Thus the combination of calcitriol and liarazole resulted in a substantial inhibition of DU 145 cell growth as a result of both an extension of the calcitriol half-life and enhanced VDR up-regulation. Ketoconazole, the more readily available imidazole derivative that inhibits mammalian P-450 enzymes (Feldman, 1986), exhibits growth inhibitory activity by itself in PCa cells (Blagosklonny et al., 2000) as well as in primary prostatic epithelial cells (Peehl et al., 2002). In primary human PCa cells, the use of the P450 inhibitor ketoconazole (Feldman, 1986) potentiates the growth inhibitory effects of calcitriol or its structural analog EB 1089 by inhibiting the 24-hydroxylase activity in these cells (Peehl et al., 2002). Ketoconazole enhances the inhibitory effects of calcitriol on the growth of PC-3 cells in culture as well as their growth as xenografts in nude mice (Muindi et al., 2010). The combination of calcitriol with 24-hydroxylase inhibitors, therefore, would allow the use of lower doses of calcitriol to achieve significant anti-proliferative effects. However, increased biological activity of calcitriol may not be selective for anti-proliferative effects alone. For example, ketoconazole, in addition to inhibiting 24-hydroxylase (Loose et al., 1983) also inhibits the activity of 1α-hydroxylase leading to low levels of calcitriol, creating a major risk for the development of osteoporosis and osteomalacia (Feldman et al., 2001). Indeed, administration of ketoconazole to normal men has been shown to result in a dose-dependent decrease in serum levels of calcitriol (Glass and Eil, 1986). Patients receiving ketoconazole-based androgen ablation therapy would therefore be at a greater risk for metabolic bone disease and other side effects of vitamin D deficiency. Adding calcitriol or analogs to therapeutic regimens that include ketoconazole would be beneficial since it would restore circulating levels of active vitamin D (Peehl et al., 2002). However, the clinical use of ketoconazole and calcitriol combination needs to be approached with caution. Ketoconazole inhibits multiple P-450 enzymes including those in the steroidogenic pathways leading to the synthesis of testosterone, cortisol and aldosterone (Feldman, 1986). Adrenal insufficiency is a health risk in its own right and it would also exacerbate hypercalcemia. Therefore, patients undergoing this combination therapy require careful monitoring for adrenal insufficiency and hypercalcemia in addition to the assessment of PCa progression. In fact, glucocorticoid replacement is usually given. Trump and colleagues have added dexamethasone to a combination of ketoconazole and calcitriol in experimental models of PCa (Muindi et al., 2010). The rationale for this approach is that dexamethasone serves to minimize calcitriol-induced hypercalcemia due possibly to a down-regulation of VDR in the intestine (Hidalgo et al., 2010; Krishnan and Feldman, 1997) as well as to serve as a glucocorticoid replacement for ketoconazole inhibition of adrenal steroidogenesis. Currently more specific inhibitors of 24-hydroxylase, both azoles and cholecalciferol analogs, are being developed. For example, 2-(4-hydroxybenzyl)-6-methoxy-3,4-dihydro-2H-naphthalen-1-one (tetralone) is a potent 24-hydroxylase inhibitor and its co-addition sensitizes DU145 cells to calcitriol (Yee et al., 2006). 24-sulfoximine (Kahraman et al., 2004) and 24-phenylsulfone (Lechner et al., 2007), analogs of calcitriol, are potent inhibitors of 24-hydroxylase and inhibit the degradation of calcitriol in many tumor cells including PCa cells, suggesting their combination with calcitriol might exhibit enhanced anti-tumor activity. But the approach requires some caution since amplification of calcitriol activity by inhibition of 24-hydroxylase would also increase its hypercalcemic effects since thus far there are no methods to specifically enhance the antiproliferative actions of calcitriol.

3.2. Calcitriol and soy isoflavones

Soy is a rich source of isoflavones such as genistein and daidzein. A prospective study of diet and prostate cancer has shown an inverse correlation between consumption of tofu in the diet and PCa incidence among men of Japanese ancestry (Severson et al., 1989). Soy-derived phytoestrogens, at concentrations ranging from 0.1 to 10 μM, inhibit PCa growth in cell culture models (Hempstock et al., 1998; Mitchell et al., 2000) and in animal models (Mentor-Marcel et al., 2001; Pollard and Wolter, 2000). Genistein decreases the expression of androgen receptor (AR) mRNA and protein and inhibits the transcriptional activation of the *PSA* gene in LNCaP cells (Davis et al., 2002). Genistein also decreases AR expression in the dorsolateral prostate of rats (Fritz et al., 2002).

Several studies have shown interactions between phytoestrogens and the vitamin D axis in prostate cells. Calcitriol and genistein synergistically inhibit the growth of human primary prostatic epithelial cells and LNCaP cells, causing the cells to arrest in the G_0/G_1 phase as well as the G_2M phase of the cell cycle (Rao et al., 2002). Farhan et al (Farhan et al., 2003) have reported that genistein and other isoflavanoids modulate the availability of calcitriol in DU 145 cells by reducing the expression of 24-hydroxylase and 1α-hydroxylase. The suppression of 24-hydroxylase appears to be due to regulation at the transcriptional level while that of 1α-hydroxylase involves deacetylation. Swami et al have shown that genistein causes a direct non-competitive inhibition of the enzymatic activity of 24-hydroxylase and a prolongation of calcitriol half-life in DU 145 cells, thereby rendering these cells sensitive to growth inhibition by calcitriol (Swami et al., 2005). In addition to inhibiting CYP24 enzyme activity, genistein has its own independent actions to inhibit the growth of PCa cells. In a recent study Swami et al showed that, similar to calcitriol, genistein decreased *COX-2* expression, leading to decreased synthesis of the prostaglandin (PG) PGE_2. In addition, genistein also induced down-regulation of PG receptors, EP2 and 4 and FP, thereby reducing the biological activity of PGE_2 (Swami et al., 2009). Since PGs exert an array of activities that stimulate proliferation, inhibit apoptosis and increase angiogenesis, invasion and metastasis (Markowitz and Bertagnolli, 2009) inhibition of PG synthesis and signaling is an important anti-cancer action. Taken together, the combination of calcitriol and genistein acted additively to inhibit the PG pathway in PCa cells (Swami et al., 2009). Thus, a combination of vitamin D compounds and soy or phytoestrogens such as genistein may be beneficial in PCa treatment as genistein can potentiate the growth inhibitory activity of vitamin D by the various mechanisms mentioned above (Swami et al., 2007; Swami et al., 2009). However, as in the case of the azole compounds, combination with genistein by inhibiting 24-hydroxylase and extending calcitriol half-life might also enhance the calcemic effects and toxicity of calcitriol.

3.3. Calcitriol and non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce inflammation and prevent PCa development in a rat model of prostate carcinogenesis (Narayanan et al., 2009). The use of NSAIDs such as aspirin lowers serum PSA levels in men with latent PCa (Fowke et al., 2009; Singer et al., 2008). In PCa patients a strong association between the levels of serum C-reactive protein (CRP), a non-specific marker of inflammation, and serum PSA has been reported (Lehrer et al., 2005). Recent studies also show that an elevated serum CRP level (> 8 mg/L) is a strong predictor of poor prognosis in patients with metastatic castration-resistant PCa (Beer et al., 2008; McArdle et al., 2006). NSAIDs are a class of drugs that decrease PG synthesis by inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymatic activities. Several NSAIDs non-selectively inhibit both the constitutively expressed COX-1 and the inducible COX-2, while others have been developed to more selectively inhibit COX-2.

Moreno et al showed that the combinations of calcitriol with the COX-2-selective NSAIDs NS398 and SC-58125 as well as the non-selective NSAIDs, naproxen and ibuprofen, caused a synergistic enhancement of the inhibition of PCa cell proliferation compared to the individual agents (Moreno et al., 2005a; Moreno et al., 2005). These results raised the possibility that the combination of calcitriol and NSAIDs may be clinically useful in PCa therapy (Moreno et al., 2005a). The combination strategy allows the use of lower concentrations of NSAIDs thereby minimizing their undesirable side effects. It has recently become clear that the long-term use of COX-2-selective inhibitors such as rofecoxib (Vioxx) causes an increase in cardiovascular complications in patients (Antman et al., 2005; Graham et al., 2005; Ray et al., 2002; Solomon et al., 2006). Even the use of non-selective NSAIDs has been shown to increase cardiovascular risk in patients with heart disease (Gislason et al., 2009). However, in comparison to some COX-2-selective inhibitors, non-selective NSAIDs such as naproxen may be associated with fewer cardiovascular adverse effects (Bombardier et al., 2000; Gislason et al., 2009). Preclinical data (Moreno et al., 2005) suggest that the combination of calcitriol with a non-selective NSAID would be a useful therapeutic approach in PCa that would allow both drugs to be used at reduced dosages leading to increased efficacy as well as improved cardiovascular safety (Krishnan et al., 2009; Moreno et al., 2005). A phase II trial evaluating the combination of the non-selective NSAID naproxen and high dose calcitriol in patients with early recurrent PCa demonstrated some benefit in terms of reduction in PSA doubling time (Srinivas and Feldman, 2009).

3.4. Calcitriol and chemotherapy drugs

Several studies in cell cultures and animal models as well as clinical trials have demonstrated the potential utility of calcitriol and its analogs as agents that can enhance the anti-proliferative and cytotoxic effects of conventional chemotherapeutic drugs. Combined administration of calcitriol or its analogs with platinum compounds such as carboplatin or cisplatin has been shown to result in a marked enhancement of growth inhibition in PCa cell lines (Moffatt et al., 1999) over that seen with the platinum compound alone. Hershberger et al. (Hershberger et al., 2001) showed that treatment of murine squamous carcinoma cells (SCC) and PC-3 PCa cells *in vitro* with calcitriol prior to taxane (paclitaxel) administration caused significantly greater growth inhibition than either agent alone. In mice bearing PC-3 xenograft tumors, calcitriol treatment in advance of engraftment, similarly enhanced the tumor inhibitory effect of paclitaxel (Hershberger et al., 2001). The molecular basis for the enhanced antitumor activity of this combination appears to be the increase in the expression of the cell cycle inhibitor *p2*1 by calcitriol, rendering the cells more sensitive to chemotherapeutic agents such as paclitaxel (Hershberger et al., 2001). Paclitaxel cytotoxicity has been similarly shown to be increased in breast and colon cancer cells when p21 expression is specifically perturbed (Barboule et al., 1997; Stewart et al., 1999).

Calcitriol up-regulates the expression of the pro-apoptotic signaling molecule mitogen-activated protein kinase kinase kinase 1 (MEKK1) in SCC cells (McGuire et al., 2001). This up-regulation is potentiated by co-treatment with cisplatin, suggesting that calcitriol pretreatment commits the cells to undergo apoptosis through specific molecular pathways, which is enhanced by the treatment with an additional genotoxic agent (Hershberger et al., 2002). Another molecular mechanism underlying the enhanced effect of the combination therapy involves an increase in the expression of the p53 homolog p73 by calcitriol in many cell types, which in turn makes these cells more susceptible to the cytotoxic effects of platinum compounds (Ma et al., 2008).

Docetaxel, another taxane, is known to induce apoptosis in PC-3 cells. It has been shown that pre-treatment of PC3 cells with 100 nM calcitriol followed by 0.5 nM docetaxel increased the percentage of apoptotic cells from 7 to 13%. (Ting et al., 2007). Thus, the ability of calcitriol to reduce cell survival signals and activate pro-apoptotic signals may

explain why it can enhance the anti-tumor activity of mechanically diverse cytotoxic agents such as platinum compounds and taxanes. These data clearly support the combined use of vitamin D compounds and cytotoxic drugs in the treatment of solid tumors such as PCa.

4. PROSTATE CANCER CHEMOPREVENTION IN ANIMAL MODELS

Although several rodent models of PCa have been developed (Getzenberg et al., 1997; Gingrich et al., 1997; Lucia et al., 1998; Navone et al., 1998), there is still a lack of a perfect model for human PCa. Several researchers have developed human PCa xenograft models by transplanting clinical specimens of human PCa or cultured human PCa cells into immuno-deficient mice (van Weerden and Romijn, 2000). Using these animal models investigators have attempted to validate the *in vitro* observations on the growth inhibitory actions of vitamin D compounds in PCa cells (Beer and Myrthue, 2006; Chen and Holick, 2003; Fleet, 2008; Gombart et al., 2006; Konety et al., 1999; Krishnan et al., 2007; Krishnan et al., 2003; Miller, 1999; Stewart and Weigel, 2004; Thorne and Campbell, 2008; Trump et al., 2010). Thus *in vivo* models provide a valuable tool to demonstrate the anti-tumor activity of calcitriol and its analogs while monitoring their tendency to elevate serum calcium levels and validate their use in clinical trials.

Investigators also have attempted to explore the chemopreventive or tumor- delaying activity of vitamin D compounds in animal models. Transgenic models of PCa have been developed in mice to explore molecular processes underlying PCa initiation, progression and metastasis and to evaluate the effects of potential therapeutic and chemopreventive agents [reviewed in (Kasper and Smith, 2004; Navone et al., 1998). The development of PCa is a multi-step process that transforms normal glandular epithelium to pre-neoplastic lesions that progress on to invasive carcinoma. This transformation generally progresses very slowly, likely over decades, before symptoms become clinically detectable and a diagnosis is made (Fang et al., 2001; Loeb et al., 2006; Steyerberg et al., 2007; Whittemore et al., 2005). The observed latency in PCa development provides a long window of opportunity for intervention by chemopreventive agents that would reverse, suppress or prevent the carcinogenic process (Syed et al., 2007). Inflammation in the prostate is proposed to be an etiological factor in the development of PCa. De Marzo et al (De Marzo et al., 2007) postulate that injury to the prostate epithelium caused by exposure to infectious agents, hormonal alterations and dietary carcinogens leads to inflammation and the formation of proliferative inflammatory atrophy (PIA) lesions, which are the precursors of prostate intraepithelial neoplasia (PIN). Progression to high grade PIN is the most likely precursor of prostate carcinoma (Montironi et al., 2004). The epithelial cells in PIA lesions have been shown to exhibit many molecular signs of stress including elevated expression of COX-2 (De Marzo et al., 1999; Wang et al., 2007; Zha et al., 2001). Thus mediators of inflammation appear to be involved in triggering the process of prostate carcinogenesis. Treatment with COX-2 selective NSAIDs such as celecoxib has been shown to suppress prostate carcinogenesis in the TRAMP model (Gupta et al., 2004). Calcitriol exhibits significant anti-inflammatory effects in experimental models of PCa. Overproduction of reactive oxygen species (ROS) causes the induction and accumulation of DNA damage contributing to carcinogenesis. Calcitriol demonstrates anti-oxidant activity by protecting non-malignant human prostate epithelial cells from oxidative stress-induced cell death (Bao et al., 2008). Therefore, calcitriol has the potential to be useful as a chemopreventive agent in PCa (Banach-Petrosky et al., 2006; Chen and Holick, 2003; Krishnan and Feldman, 2010; Krishnan et al., 2007).

The efficacy of calcitriol as a chemopreventive agent has been examined in Nkx3.1; PTEN mutant mice, which recapitulate stages of prostate carcinogenesis from PIN lesions to adenocarcinoma (Banach-Petrosky et al., 2006). As mentioned above, the data reveal that

calcitriol significantly reduces the progression of PIN from low grade to high-grade lesions. Calcitriol is more effective when administered before, rather than subsequent to, the initial occurrence of PIN. These animal studies as well as the *in vitro* observations described above suggest that clinical trials in PCa patients with PIN or early disease are warranted to evaluate whether calcitriol and its analogs can prevent PCa development and/or delay PCa progression.

5. CLINICAL TRIALS OF CALCITRIOL IN PROSTATE CANCER

It is generally accepted that the higher the dose of calcitriol used, the greater the anti-cancer effects that will be achieved (Beer et al., 2003; Krishnan and Feldman, 2010; Krishnan et al., 2010). The major side-effect of calcitriol when used therapeutically at higher than physiologic doses is the risk of inducing hypercalcemia and renal stones (Gross et al., 1998). There is also the theoretical risk of causing vascular calcification and thereby cardiovascular disease (Mizobuchi et al., 2009). Several alternatives to reduce these risks have been explored in Phase I-III trials. The following is a discussion of the clinical trials in patients with PCa.

5.1. Vitamin D_3 (cholecalciferol) in clinical trials

The presence of 1a-hydroxylase in prostate cells and the paracrine anti-proliferative actions of locally synthesized calcitriol (Hewison and Adams, 2005; Townsend et al., 2005) raise the possibility that increasing the circulating concentrations of 25(OH)D by dietary administration of precursor vitamin D will exert an anti-cancer effect due to its local conversion to calcitriol within the cancer cells. Eliciting an anti-cancer effect by treating with vitamin D and allowing the local production of calcitriol in the tumor site to generate paracrine actions of calcitriol might be safer than raising circulating calcitriol levels with calcitriol administration, which can be associated with hypercalcemia. An alternate and complementary hypothesis is that very high doses of vitamin D_3 will generate increased concentrations of circulating 25(OH)D that achieve high enough levels to directly bind to and activate the VDR (Peng et al., 2009). However, a recent assessment of the use of vitamin D supplementation in clinical trials indicates that although vitamin D supplementation does not cause hypercalcemia, it causes modest increases in serum calcium levels resulting in high normocalcemia (serum calcium levels that are high but fall within the normal reference range) (Skinner et al., 2010). High normocalcemia may represent a more sensitive index of potential vitamin D-related toxicity, which theoretically may be associated with increased risk of mortality due to cardiovascular disease (Skinner et al., 2010).

In a small pilot clinical study vitamin D was given to PCa patients at 2000 IU per day and monitored prospectively every 2–3 months over a period of 21 months. The results showed a beneficial effect of prolongation of serum PSA doubling time in 14 out of 15 patients (Woo et al., 2005). The serum PSA level is a useful biomarker of prostate cell abundance and its rate of rise is often used as a surrogate for tumor growth.

5.2. Calcitriol in clinical trials

The therapeutic utility of calcitriol has been evaluated in clinical studies in PCa patients. A decrease in the rate of rise of serum PSA levels was observed in PCa patients following modest but supra-physiological daily doses (2–2.5 micrograms/day) of calcitriol suggesting a beneficial effect in slowing the progression of the disease (Beer et al., 2003; Gross et al., 1998). However, in addition to decreasing or stabilizing the rate of rise of PSA, the objective benefits were small and the risk of renal stones was significant (Gross et al., 1998). To minimize these toxicities some investigations have followed the approach of administering

very high doses of calcitriol intermittently in an attempt to reach higher peak concentrations of calcitriol to achieve greater efficacy while causing only transient hypercalcemia. Schedules have been developed using high dose calcitriol 3 times a week (Trump et al., 2006) or very high doses once a week (Beer et al., 2003; Beer and Myrthue, 2004), where it apparently can still elicit its anti-proliferative effects but results in only transient hypercalcemia and infrequently causes renal stones.

5.3. Calcitriol analogs in clinical trials

An alternate approach is the development and use of calcitriol analogs that exhibit equal or even increased anti-proliferative activity while exhibiting reduced tendency to cause hypercalcemia. Preclinical and clinical studies have demonstrated the arrest of prostate growth using analogs of calcitriol (Maggi et al., 2006). The primary endpoint of a sustained 50% decrease in serum PSA was not met. However, the calcitriol analog paricalcitol significantly reduced the elevated serum PTH levels seen in these patients, suggesting that the analog may be beneficial in reducing skeletal morbidity in patients with advanced PCa (Schwartz et al., 2005).

5.4. Calcitriol in combination therapy with other drugs

Calcitriol is also being used in combination therapy with other agents to achieve enhanced anti-cancer effects (Beer et al., 2007; Trump et al., 2006). Administration of escalating intermittent doses of calcitriol along with the bisphosphonate zoledronic acid in men with progressive PCa did not demonstrate an anti-tumor effect (Morris et al., 2004). There have been several Phase II trials using calcitriol in combination with dexamethasone with conflicting results (Chadha et al., 2010; Flaig et al., 2006; Trump et al., 2006).

The results of the ASCENT I clinical trial in advanced castration-resistant prostate cancer (CRPC) patients, who failed other therapies, demonstrated that extremely high doses (45 micrograms) of a formulation of calcitriol (DN-101, Novacea) administered orally once a week along with the usual regimen of the chemotherapy drug docetaxel caused a statistically significant improvement in overall survival and time to progression of the disease (Beer et al., 2007). These findings suggested that calcitriol might enhance the efficacy of active drugs in cancer patients and provide a survival advantage. Based on the promising survival results, a larger phase III trial (ASCENT II) with survival as an endpoint was initiated. A new, improved docetaxel regimen (every 3 week dosing) was used in the control arm of the ASCENT II trial and compared to the older, weekly docetaxel regimen used in ASCENT I. Unfortunately, the improved survival due to the combination demonstrated in the ASCENT I trial could not be confirmed in the asymmetrically designed ASCENT II trial (Scher et al., 2011) and the trial was prematurely stopped by the data safety monitoring committee. After the trial was stopped, further analysis suggested that the increased deaths in the treatment arm compared to the control arm were not due to calcitriol toxicity but due to better survival in the control arm that received the new and improved docetaxel regimen (Scher et al., 2011).

In a randomized, double-blind, phase II study in patients with metastatic castrate resistant PCa (CRPC) without previous chemotherapy, the addition of daily doses of doxercalciferol (1α-hydroxyvitamin D_2) to weekly docetaxel did not enhance the PSA response rate or survival (Attia et al., 2008). Interestingly, another study testing the effect of the combination of weekly high dose calcitriol and docetaxel in PCa patients, whose disease progressed after resistance developed to first line chemotherapy using docetaxel alone, showed that high dose calcitriol restored the sensitivity to chemotherapy with docetaxel (Petrioli et al., 2007).

Based on preclinical observations in PCa cells (Moreno et al., 2005), a single arm, open label phase II study evaluating the combination of the non-selective NSAID naproxen (375 mg naproxen twice a day) and high dose calcitriol (45 micrograms of calcitriol (DN101) orally once a week) was carried out in patients with early recurrent PCa (Srinivas and Feldman, 2009). The trial was prematurely stopped after 21 patients had been enrolled when the FDA put a temporary hold on DN-101 based on the data from the ASCENT II trial described above. The therapy was well tolerated by most patients. A prolongation of the PSA doubling time was achieved in 75% of the patients, suggesting a beneficial effect of the combination therapy (Krishnan et al., 2009; Srinivas and Feldman, 2009).

6. CONLUSIONS

The preclinical data demonstrating a benefit of vitamin D on health and PCa is increasing but is not yet compelling in human studies according to the recent Institute of Medicine report (Report, November 2010 http://www.iom.edu/vitamind). Many epidemiologic studies, but not all, indicate that vitamin D deficiency increases the risk of PCa and that higher levels of vitamin D are associated with better prognosis and improved outcomes. Cellular and animal studies highlighting the antiproliferative role of vitamin D in PCa have stressed the importance of VDR and the roles played by the two enzymes CYP27B1 and CYP24 in extra-renal tissues. Recent data also suggest that high concentrations of the VDR are protective against aggressive disease indicating that both serum 25(OH)D or calcitriol concentration may only be part of the benefit and the signaling pathway in the PCa is also important. Although the preclinical data are persuasive and increasingly compelling, and they provide a credible rationale for benefit in PCa, the outcome of clinical trials using vitamin D, calcitriol or various vitamin D analogs in men with PCa have thus far been disappointing. It is our belief that avoidance of vitamin D deficiency should be an important goal in reducing cancer incidence in addition to its clear-cut benefits on skeletal homeostasis. Although calcitriol does induce apoptosis in some cancer cells, its major actions relate to its anti-proliferative, anti-inflammatory and pro-differentiating effects. Thus, it does not seem likely that calcitriol administered alone would be therapeutically beneficial in cases of far-advanced cancer unresponsive to other therapies. We believe that vitamin D or calcitriol would be most effectively used either in chemoprevention prior to clinical cancer development or as an adjunct to other treatments, delaying the progression of cancer in patients with early disease (Krishnan et al., 2010). While it is difficult to pinpoint a safe dose for calcitriol we conclude that administered at the highest dose that can be tolerated without serious side effects and probably in combination with other drugs, vitamin D, calcitriol or its analogs will augment other therapies and be a useful addition for the treatment of PCa. Although the benefits are not yet proven in men, the preclinical data are so compelling that further trials in men are warranted.

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