Effect of Dietary Vitamin D and Calcium on the Growth of Androgen-insensitive Human Prostate Tumor in a Murine Model

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Abstract. Vitamin D deficiency has been associated with increased risk of prostate cancer (PC) in epidemiologic and prospective studies. An association has also been made between high dietary calcium and increased PC risk. In this study, we evaluated the effect of dietary vitamin D and calcium on the growth of human androgen-insensitive prostate tumor in an athymic mouse model. We observed highest tumor growth in the normal calcium - vitamin D-deficient group, while tumor growth between the normal calcium - vitamin D-sufficient, high calcium - vitamin D-sufficient and high calcium - vitamin D-deficient diet-groups did not significantly differ but was significantly lower than that in the normal calcium - vitamin D-deficient group. Our results suggest an important role of dietary vitamin D as a preventive agent in androgen-insensitive PC.

Prostate Cancer (PC) is the most prevalent cancer among men, and the second leading cause of cancer death among men in the US (1). Although PC occurs more often in elderly men, the incidence of prostatic carcinoma is also significant among young men. Thus, as the average life expectancy continues to increase and as the elderly population grows, there is a greater need for effective preventative measures, such as dietary intervention, as well as nutritional strategies for PC, particularly androgen-insensitive and metastatic PC.

Vitamin D (vitamin D2 of plant origin and vitamin D3 of animal origin) is a naturally occurring molecule that is biosynthesized in the skin. In 1989, Garland et al. carried out an eight-year prospective study among 25,620 healthy adults to demonstrate that if the initial level of serum 25-hydroxyvitamin D is at least 20 ng/ml, there is a 50% reduced risk of developing colon cancer (2). Since this observation, other investigators have confirmed the effect of latitude and vitamin D intake on reducing risk of various types of cancer, including malignancy of the prostate (3-9).

Several epidemiological and dietary studies have suggested a correlation between calcium intake and PC risk. Because high levels of calcium reduce the renal production of 1,25-dihydroxyvitamin D (1,25(OH)2D, the dihydroxy metabolite of vitamin D, the active form of the vitamin D hormone) it was suggested that high calcium levels may diminish the beneficial effect of vitamin D on some types of cancer including PC (10, 11). Bao et al. hypothesized that vitamin D has an antioxidant effect via up-regulation of glucose-6-phosphate-dehydrogenase and glutathione levels (12). Guzey et al. found that vitamin D up-regulates pro-apoptotic genes in PC cells (13). Moreno et al. observed that vitamin D reduces cyclooxygenase-2 and this inhibition leads to a reduction of angiogenesis (14). Based on these studies, it was concluded that decreased production of 1,25(OH)2D in a high calcium diet and/or its higher rate of catabolism may contribute significantly towards PC risk. Thus it was suggested that high intake of milk and dairy products may increase the risk of developing PC (15, 16).

In contrast, Huncharek et al. carried out a meta-analysis of 45 observational studies and showed that there is no correlation between dairy, vitamin D, calcium intake and PC risks (17). Tseng et al. also failed to find significant correlation between vitamin D intake (dietary and supplemental) and PC, although a weak but significant correlation with low-fat milk intake and PC was observed in the same study (18). In another study, an association between...
calcium intake and PC risk was observed in cases where calcium intake exceeded 2,000 mg/day (19). In contrast, Allen et al., in a large prospective study consisting of 2,727 cases of PC, found a positive correlation between increase in PC incidence with an increased intake of calcium from dairy products, but not calcium from other foods (20). But Park et al. examined over 82,000 men via a detailed food frequency questionnaire and found no correlation between calcium intake and vitamin D levels and PC risk (21).

In essence, careful review of the available literature suggests that the relationship between increased calcium intake and an increased risk of PC is rather inconclusive. In order to address this controversy we chose an athymic mouse tumor model of human androgen-insensitive PC to study the effect of dietary vitamin D and calcium on the growth of PC. Results of this study and their implications in the dietary intake of calcium and vitamin D in the growth of prostate tumor, and possibly its prevention are discussed here.

Materials and Methods

**Diets.** Specialized animal diets were purchased from Dyets, Inc. (Bethlehem, PA, USA) with defined compositions (normal calcium, NCa2+; high calcium, HCa2+; vitamin D, D): I, NCa2+ +D: D (5000 IU/kg), Ca2+ (0.9%), phosphorus (P, 0.7%), II, NCa2+ +D: D (none), Ca2+ (0.9%), P (0.7%), III, HCa2+ +D: D (5000 IU/kg), Ca2+ (2.0%), P (0.7%), and IV, HCa2+ -D: D (none), Ca2+ (2.0%), P (1.25%).

**Animals.** Six week-old, male nu/nu athymic mice, weighing approximately 30 g (Charles River Laboratories Inc., Wilmington, MA, USA) were maintained under standard light and dark cycles with the specialized animal chow and water ad libitum unless specified otherwise. Approval from the Animal Safety Board of Boston University School of Medicine was obtained prior to animal experiments, which were carried out with strict adherence to established animal safety rules.

**Establishment of diets in mice.** Fifty-two mice were randomized into four groups of 13 each. Animals in each group were maintained on one of the specific diets listed above. Four weeks after the establishment of diets in mice, animals in each group were maintained on one of the specific diets listed above. Four weeks after the introduction of the custom diets, blood samples from all the mice were obtained via mandibular vein and sera were produced. The sera samples were subjected to 25-hydroxyvitamin D [25(OH)D] analysis by a method established in our laboratory (22).

**Cell culture and implantation of tumor.** DU-145 cells (ATCC, Manassas, VA, USA) were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum and other additives (according to manufacturer’s specification) until approximately 80% confluency. They were trypsinized, washed with phosphate saline buffer (PBS), re-suspended in PBS and counted in a hemocytometer. The cell density of the suspension was adjusted by re-suspension in equal parts of PBS and matrigel (BD Biosciences, Franklin Lakes, NJ, USA) so that there were approximately 4x10⁶ cells/100 μl of suspension. The cell suspension was injected under the skin (right flank) of the animals with a 25-gauge hypodermic needle. Animals were observed daily for tumor growth.

**Established animal safety rules.** For cell culture and implantation of tumor.

**Results of this study and their implications in the dietary intake of calcium and vitamin D in the growth of prostate tumor, and possibly its prevention are discussed here.**

**Tumor measurements.** Tumor volume was measured with a caliper using the formula: volume=length (longest measurement)/2 × length × width. Measurement started when the tumor size reached approximately 3.5 mm³ (approximately 29 days post-injection). Body weights and tumor measurements were made three times a week up to the 76 day post injection, when mice were sacrificed and blood samples were collected via cardiac puncture and sera made and stored to be assayed for calcium (QuantiChrom Calcium Assay Kit, BioAssays Systems, Hayward, CA, USA) and 25(OH)D (22).

**Results and Discussion**

A link between PC risk and dietary intake of calcium and vitamin D is confusing and inconclusive, as discussed earlier. Therefore, we set out to determine the effect of dietary calcium and vitamin D in a mouse model of human androgen-insensitive PC. Androgens have been positively implicated in the growth and progression of prostate tumor. As a result, anti-hormone treatment is one of the first-line therapies of prostate cancer. But, within a relatively short period of time, hormone-sensitive tumors become hormone insensitive. Therefore, we hypothesized that an androgen-insensitive PC model would be most appropriate to study the effect of dietary calcium and vitamin D on tumor growth, because in general, the effect of a dietary component needs a long time to take hold, mirroring slow development on prostate tumor and eventual androgen insensitivity.

In our studies, we first established a significant decline in the vitamin D status of animals fed vitamin D-deficient diets prior to implantation of tumor by measuring serum 25(OH)D levels. The serum 25(OH)D level in NCa2+ +D group (38.3±4.8 ng/ml) is significantly higher (p<0.05) than its vitamin D-deficient counterpart, NCa2+ -D (10.7±1.3 ng/ml). The serum 25(OH)D level of the HCa2+ +D group (27.0±4.0 ng/ml) was also significantly higher (p<0.05) than its vitamin D-deficient counterpart (HCa2+ -D=10.6±3.7 ng/ml). These results established that the mice fed a vitamin D deficient diet had a low serum 25(OH)D level, consistent with vitamin D deficiency, i.e. <20 ng/ml (23).

At the termination of the study, mice fed the normal-calcium-vitamin D-deficient diet had an undetectable level of serum 25(OH)D (<5 ng/ml), while the corresponding vitamin D-sufficient group (NCa2+ +D) had an average serum 25(OH)D value of 28.07±2.2 ng/ml. A similar trend existed in the high calcium diet groups (HCa2+ +D=18.67±4.0 ng/ml vs. HCa2+ -D=7.13±0.5 ng/ml). Our laboratory reported that LNCPa prostate cancer cells, transfected with 1,25-dihydroxyvitamin D-1α-hydroxylase are capable of converting 25(OH)D₃ to 1,25(OH)₂D₃, bypassing the renal route (24). This is a probable explanation, why mice fed the HCa2+ –D diet still had a detectable level of 25(OH)D at the end of the study.

Tumors from the NCa2+ –D group had an average volume of 381±59 mm³ compared to those of the NCa2+ +D group,
which had an average volume of 179±36 mm$^3$. Tumor volumes of HCa$^{2+}$+D and HCa$^{2+}$ -D diet groups averaged 199±25 mm$^3$ and 204±43 mm$^3$, respectively. Overall, tumor volume from the NCa$^{2+}$ -D group was significantly higher ($p<0.05$) than the tumor volumes of three other groups, which were not significantly different from one another.

Vitamin D is biologically inert, and it undergoes stepwise metabolic activation, first to 25(OH)D in the liver, and then to 1,25(OH)$_2$D in the kidney. 1,25(OH)$_2$D is the most active vitamin D metabolite in terms of inhibiting the growth of tumor cells in vitro, as well as reducing tumor growth in vivo (25). Therefore, the tumor inhibitory effect of dietary vitamin D is most likely due to its conversion to 1,25(OH)$_2$D. In our study, the NCa$^{2+}$ –D group, deprived of the beneficial effect of vitamin D in inhibiting tumor growth, showed highest tumor growth (Figure 1). Recent evidence suggests that calcium, particularly in high concentration, may cause cytotoxicity via necrosis or apoptosis (26). Therefore, in mice fed with the high calcium diet with or without vitamin D (HCa$^{2+}$ +D, HCa$^{2+}$ -D) cell growth inhibitory effect of high calcium may have primarily contributed to the reduction of tumor growth, because high calcium is known to reduce the renal production of 1,25(OH)$_2$D.

We chose androgen-insensitive DU-145 tumor as our model for reasons specified earlier. However, several studies have demonstrated that DU-145 cells are unresponsive towards 1,25(OH)$_2$D treatment due to up-regulation of 1,25-dihydroxyvitamin D-24-hydroxylase, the enzyme responsible for initiating the catabolic degradation of 1,25(OH)$_2$D and reducing its half-life (27, 28). Results delineated in this communication raise an interesting possibility that increased production of this enzyme observed in cell-culture models may not be operative in vivo.

A potential problem with the chemopreventive use of vitamin D includes its calcemic toxicity either via a cumulative path due to repeated/daily use at high doses, or a very large bolus due to accidental misuse (29, 30). Such
toxicity is often reflected in the rise of the serum calcium level, and/or a negative impact on body weight. At the termination of our study, HCa$^{2+}$ –D, HCa$^{2+}$ +D and NCa$^{2+}$ +D groups had serum calcium levels of 12.7±4 mg/dl, 12.0±0.9 and 12.6±1.1 mg/dl, respectively, slightly above the normal limit of 10-12 mg/dl. However, calcium level in the NCa$^{2+}$ –D group was 9.4±1.0 mg/ml ($p<0.001$) which was significantly lower than in other groups. As another indicator of the lack of toxicity, weights of the animals at the end of the experiment showed no significant difference among the groups (Figure 2).

1,25(OH)$_{2}$D has been shown to act through p21$^{CIP/WAF}$ and p27 pathways resulting in the inhibition of G1/S phase transition in the cell cycle (31-33). It has also been shown to induce p21 in a p53-dependent manner, that results in G0/G1 senescence in a variety of cell types, e.g. breast, prostate, kidney (34-36). On the other hand, growth inhibition of tumor cells by 1,25(OH)$_{2}$D has been shown to involve apoptosis, and modulation of BCL pathway markers (37). However, gene analysis for p21, p27, p53, BAX and BCL-2 in tumor samples as well as immunohistochemical analysis of tumor slices did not show any significant difference among the groups. Therefore, molecular basis for the results of our diet study remains an open question.

In summary, results of our study suggest that a normal calcium, vitamin D-deficient diet may be detrimental towards the growth of androgen-insensitive prostate tumor, further emphasizing the role of dietary vitamin D in the prevention of PC.

Acknowledgements

Elizabeth Genova is acknowledged for technical assistance. This work was supported by a grant from the National Dairy Council (DMI 1253).

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Received December 21, 2011
Revised January 30, 2012
Accepted February 1, 2012