The Pleiotropic Effects of Vitamin D on the Immune System and Cancer

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Abstract

The active form of vitamin D_3, calcitriol, has been implicated in numerous health areas. Included in these are immunity and cancer. Calcitriol plays a role in both innate and adaptive immunity. It also has been extensively studied in cancer through population-based studies, genome-wide associations and mechanistic work defining its role in differentiation and proliferation. We have studied calcitriol's role in the prodifferentiation and antiproliferation of canine cancer cells. As the defense against oxidative stress is an important element of the immune system, we also have studied the ability of calcitriol to regulate endogenous antioxidant enzymes in canine cancer cells.

Glossary of Abbreviations
cbTCC: Canine Bladder Transitional Cell Carcinoma
GWAS: Genome-Wide Association Studies
MAP: Mitogen-Activated Protein
ROS: Reactive Oxygen Species

Pleiotropic Effects: Historical Relevance

Vitamin D has gained much attention in recent years. While research has increased, primarily describing mechanisms of action, there has been continued controversy regarding vitamin D-associated health benefits as well as appropriate circulating levels. Aside from vitamin D's classical calcium homeostasis role, multiple pleiotropic actions have been described. These include immunity, cancer, depression, and cardiovascular disease. Many of these actions have been based on association studies using clinical data, circulating levels of vitamin D, as well as genetics. The exact role vitamin D plays in the above listed areas is not always well defined. To gain a better understanding of how these associations and controversies arose, it is important to consider the history of this fascinating molecule.

Vitamin D_3, also referred to as the "sunshine vitamin," is produced by most mammals via photolysis of the provitamin 7-dehydrocholesterol to its eventual form vitamin D_3. Vitamin D also can be absorbed from the diet. The lack of this understanding led to the first controversies, and also to the first major discoveries, of the actions of vitamin D. Mellanby first attributed the ability to cure the calcification of bone disorder, rickets, to vitamin A.[1] McCollum was later able to cure rickets using cod liver oil void of vitamin A activity.[2] This was attributed to an essential nutrient, vitamin D. Sunlight was then discovered to play a role in curing rickets, as well as restoring calcium balance and increasing the activity of vitamin D.[3-6] This clarified the issue regarding vitamin D as being essential, as it is only essential in the absence of sunlight, at least in most mammals. It turns out that dogs and cats cannot synthesize vitamin D and must obtain it from their diet.[7] In other words, it is always essential in dogs and cats. Understanding how vitamin D is obtained was a major step forward; however, the identification of the active metabolites would end up being just as crucial, as it would show vitamin D's role in actions beyond calcium and rickets.

Vitamin D_3 is hydroxylated by the liver to form 25-hydroxy vitamin D_3 (calcidiol). Calcidiol is then hydroxylated (via the 1α-hydroxylase), mostly by the kidney, to form the active, hormonal form, 1α,25-dihydroxy vitamin D_3 (calcitriol). Calcidiol was first identified as the active form of vitamin D_3.[8] However, calcitriol was later identified as the biologically active, hormonal form.[9,10] In addition, the location and enzymes responsible for catalyzing the hydroxylation steps as well as the nuclear receptor for the active, hormonal form were identified. With this knowledge, research began showing that not only were the 1α-hydroxylase and nuclear receptor present in tissues involved in calcium homeostasis, but they also were present in other tissues not attributed to calcium homeostasis. These results were crucial for increasing our understanding that calcitriol's biological actions went well beyond calcium homeostasis and thus had pleiotropic actions.

New advances in molecular technologies, in particular genome-wide association studies (GWAS) and the ability to identify receptor-binding sites on the genome, have and continue to increase our knowledge of vitamin D's involvement in various areas of biology. GWAS studies have shown associations between vitamin D intake or circulating levels with multiple diseases,[11] including asthma, congestive heart failure, depression,[12] diabetes, insulin secretion,[13] Parkinson's disease, multiple autoimmune diseases, and

From the Purina Companion Animal Nutrition Summit: *Pet Nutrition: Beyond Essential*; Fort Lauderdale, FL; March 31-April 2, 2016.

multiple cancers. Techniques showing the locations of the vitamin D receptor bound to the genome, with and/or without calcitriol, have shown significant enrichment of binding in areas involved in immune processes, autoimmune disease and cancer.[14-17] Although these studies were done with immune cells, the areas identified as being enriched align well with other studies.

Immunity

There is a multitude of evidence that vitamin D plays an active role in immunity. A few examples are described here. Some of the cells of both innate and adaptive immunity possess the ability to convert calcidiol into the active form, calcitriol.[18] Additionally, these and other cells of the immune system contain the vitamin D receptor allows the cell to respond to calcitriol signaling. Monocytes exposed to *Mycobacterium tuberculosis* are able to upregulate both the vitamin D receptor and the 1α-hydroxylase, which converts calcidiol to calcitriol.[19,20] This leads to an increased expression of the antimicrobial peptide, cathelicidin. Calcitriol has also been shown to increase the expression of defensin β2, another antimicrobial peptide, in multiple cell types.[20]

In both T and B cells, vitamin D is able to affect the expression of the vitamin D receptor leading to changes in the differentiation and proliferation of these cells. In T helper cells, calcitriol can modulate cytokine production and suppress proliferation and differentiation. The secretion of proinflammatory cytokines are inhibited, while anti-inflammatory cytokines are decreased.[21] In B cells, calcitriol is able to affect B cell homeostasis via the regulation of activated B cells and their differentiation.[22] Many of these processes are involved in autoimmune regulation. Lending more credibility to vitamin D's role in autoimmunity are the experiments showing locations on the genome where the vitamin D receptor binds in immune cells (described above).

Another area involved in the immune system is the ability to neutralize and/or maintain redox balance. Reactive oxygen species (ROS) are produced during metabolism, but immune cells, such as macrophages, can produce and use ROS to kill pathogens. ROS are also used by the immune system as signaling molecules through Toll-like receptor and mitogen-activated protein (MAP) kinase pathways.[23] The utilization of ROS by the immune system is closely monitored as many immune cell types, such as T cells, are sensitive to redox balance.[24] When ROS are not maintained properly, they can lead to oxidative stress. Oxidative stress has been implicated in a multitude of diseases including cardiovascular, neurodegenerative and cancer, as well as aging. Maintaining a proper redox balance involves antioxidants and antioxidant enzymes. We have shown that calcitriol can induce the expression of antioxidants, such as catalase, in canine cancer cells[25] (described below). Oxidative stress causes damage to biomolecules, such as DNA, but it also can lead to chronic inflammation. Chronic inflammation has been implicated in many diseases and degenerative states. Vitamin D has been shown to have anti-inflammatory effects. In macrophages and prostate cancer cells, calcitriol decreases the production of the inflammatory cytokine tumor necrosis factor alpha.[26,27] This mechanism involves the upregulation of the inhibitor of NFκB,[28] a major regulator of inflammatory responses. Other mediators of inflammation, such as prostaglandins, can also be regulated by calcitriol via the expression of the metabolic enzymes used in their production.[29]

Cancer

Aside from research describing its classical calcium homeostasis role, vitamin D's role in cancer has been extensively investigated. A few examples are described here. Vitamin D, though not directly associated, was first reported as a cancer preventive due to the inverse correlation of sunlight exposure and cancer mortality.[30] Since then, population-based studies have identified associations of circulating levels of vitamin D, and more specifically, calcidiol, with many cancers such as colon, prostate and breast, among others. Even in canines, an association has been shown between cancer risk and circulating calcidiol concentration.[31] Investigations into the mechanisms underlying the involvement of vitamin D and cancer have yielded insights into this potential preventive action. These include calcitriol signaling through genomic actions and alterations in vitamin D metabolic enzymes, usually through expression, function and genetic polymorphisms.

There have been multiple population-based studies describing vitamin D's role in cancer prevention.[32] In a study correlating serum vitamin D levels and stage III cancer in cancer patients, suboptimal or deficient levels of calcidiol were predictive of the advanced stage of the disease.[33] In a large study looking at over 26,000 individuals, there were associations of low calcidiol concentrations with all-cause mortality, cardiovascular mortality and cancer mortality in patients with a history of cancer.[3] In a meta-analysis of studies associating calcidiol and colorectal cancer, high-circulating calcidiol concentrations were shown to be preventive.[35] While the predominance of evidence supports a positive correlation of vitamin D and cancer prevention, there are reports that have shown little to no correlation.[36,37] Some of this may be attributed to a lack of proper controls in which subjects have a below-normal circulating level of calcidiol or the studies have been too small.

Studies have shown, many *in vitro*, that calcitriol elicits its anticancer effects by decreasing proliferation (with or without apoptotic events) and by inducing differentiation.[38] The first study showing calcitriol's ability to differentiate cells was in mouse myeloid leukemia cells where these cells were

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induced to differentiate into macrophages.[39] Phagocytic activity was used as the marker of differentiation. Prostate progenitor cells were shown to differentiate into androgen receptor-positive luminal epithelial cells, and growth was suppressed by calcitriol.[40] The upregulation of interleukin-1α, a proinflammatory cytokine, was required for the growth suppression contrary to calcitriol's other known anti-inflammatory effects. Calcitriol has also been shown to have antiproliferative actions in colon cancer cells.[41]

The molecular action of calcitriol is mediated via the nuclear binding of the vitamin D receptor to target genes, increasing or decreasing their expression. It is via this process that the biological responses to calcitriol, such as apoptosis, differentiation and proliferation, occur.[42] However, in order for these processes to occur, calcitriol needs to be available to the cell. The enzymes, such as the 1α-hydroxylase, need to function appropriately. There is widespread evidence that the dysregulation of these enzymes are associated with cancer.

There is an altered expression and regulation of vitamin D metabolic enzymes in a host of cancers. Studies have shown altered gene expression in cancers of the breast, colon, cervix, ovaries, skin (basal cell and squamous cell), and others. In most cases, the levels of enzymes responsible for the production of calcitriol are decreased where the enzymes involved in the degradation are increased. However, there are cases where the levels are not consistent.[43] In breast cancer, the vitamin D receptor and the 1α-hydroxylase are decreased, while the 24-hydroxylase (used in the catabolism of calcitriol) are increased.[44] In prostate cancer cells, 1α-hydroxylase activity is significantly decreased in adenocarcinoma cells as compared to normal or benign cells.[45] An increased expression or activity of the 24-hydroxylase has also been noted in cancer cells, including prostate, making them resistant to calcitriol.[46]

Identifying polymorphisms within genes of vitamin D metabolism is another area exemplifying the importance of proper functionality of these enzymes and cancer risk. These polymorphisms can affect the expression, activity or interaction with other proteins. In breast cancer, polymorphisms in the vitamin D receptor are associated with an increased risk of disease.[47] Two polymorphisms in the 24-hydroxylase are also associated with an increased risk of breast cancer.[48] Polymorphisms in the vitamin D receptor have also been associated with increased risk of thyroid, ovarian, melanoma, colorectal, and prostate cancers.[49] Additionally, multiple other examples exist describing polymorphisms in vitamin D metabolic enzymes associating with cancer.[4]

Figure 1. Summary of calcitriol's anticancer mechanisms through the upregulation of catalase.

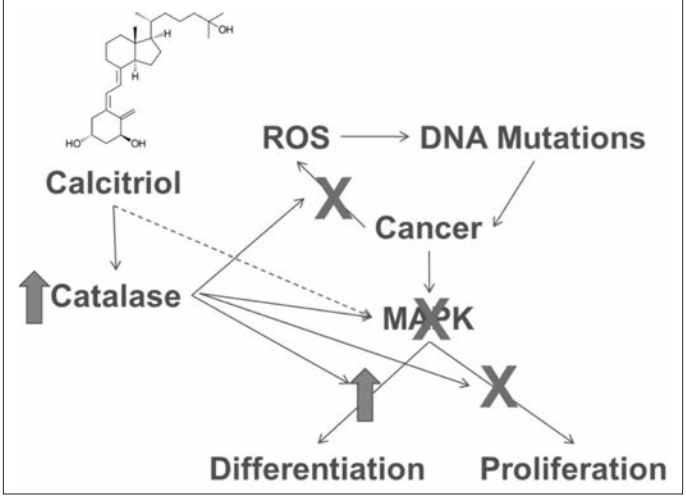
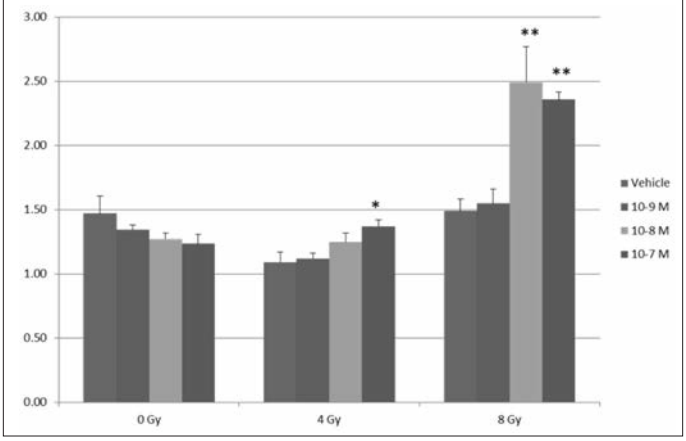


Figure 2. Catalase gene expression at 96 hours. Lymph cells were grown with varying concentrations of calcitriol (10-9 M, 10-8 M, 10-7 M) and ethanol (vehicle), and exposed to 0, 4 or 8 gray (Gy) units of radiation. Catalase gene expression is expressed as relative expression + SD. * = P<0.05; ** = P<0.01 as compared to control (vehicle).

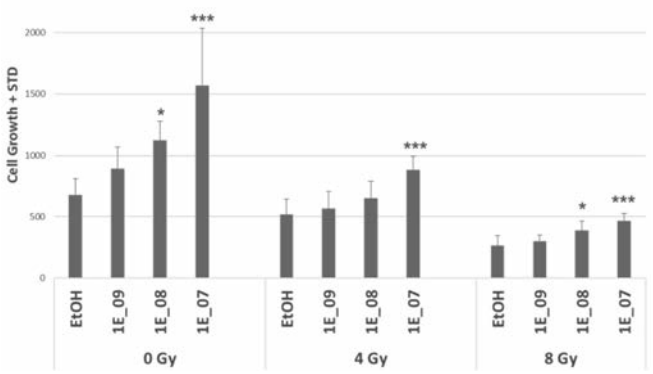


In Vitro Responses to Calcitriol

Considering vitamin D's role in cancer and the immune system, we have studied calcitriol's role *in vitro* in canine cancer cells as well as in normal cells undergoing insult from radiation. Calcitriol was able to decrease proliferation in canine squamous carcinoma cells (SCC 2/88).[50] These cells were also shown to express the vitamin D receptor. In order to further investigate the mechanism of calcitriol's actions in canine cancer cells, as well as the involvement of ROS and oxidative stress, we investigated the ability of calcitriol to modulate endogenous antioxidants.

One of the main lines of defense against ROS and the prevention of oxidative stress are endogenous antioxidant enzymes, such as catalase and the superoxide dismutases. Using canine bladder transitional cell carcinoma (cbTCC) cells, we were able to show that calcitriol upregulates catalase gene expression, protein expression and activity.[25] Catalase is a very efficient enzyme capable of converting hydrogen peroxide to water and oxygen. The overexpression of catalase has also been shown to reduce migration and proliferation in breast cancer cells *in vitro*[51] and to lower

Figure 3. Cell growth at 96 hours. Lymph cells were grown with varying concentrations of calcitriol (10-9 M, 10-8 M, 10-7 M) and ethanol (EtOH, used as vehicle), and exposed to 0, 4 or 8 gray (Gy) units of radiation. Catalase gene expression is expressed as relative expression + SD. * = P<0.05; *** = P<0.001 as compared to control (EtOH).



tumor grade and reduce metastatic tumor burden in mice.[52] It is well established that cancer cells typically produce large amounts of ROS and can have reduced levels of antioxidant enzymes. Therefore, increasing the expression of antioxidants should be able to reduce cancer progression, and possibly aid in its prevention. In our study using cbTCC cells, we were also able to show that the cell's response to calcitriol involves mitogen-activated protein (MAP) kinase/JUN signaling. MAP kinases are well known to play a role in proliferation, differentiation and cancer. All these actions are depicted in Figure 1.

Recently, we have been investigating calcitriol as a chemopreventive agent. Using radiation as a mechanism to produce ROS and create an oxidative stress environment, we have looked at the ability of calcitriol to protect lymph cells *in vitro*. Catalase expression increases in response to calcitriol (Figure 2). This is mediated by an increase in the expression of the vitamin D receptor. Additionally, cell growth is protected by calcitriol even though overall cell growth decreases in response to radiation (Figure 3). These and future results will aid in defining calcitriol's role in decreasing cancer progression as well as its role in prevention.

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