Treatment With 1-Alpha,25-Dihydroxyvitamin D$_3$ (Vitamin D$_3$) to Inhibit Carcinogenesis in the Hamster Buccal Pouch Model

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Objective: To investigate whether systemic therapy with 1-alpha,25-dihydroxyvitamin D$_3$ (vitamin D$_3$ [hereinafter, VD$_3$]) prevents tumor formation in a hamster buccal pouch model of carcinogenesis.

Design: Randomized trial in which a known carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA), was applied to the buccal pouch of 40 hamsters. Animals were randomized to receive systemic VD$_3$ or no treatment and killed at 2, 6, and 14 weeks after the initiation of DMBA exposure.

Setting: Academic medical center.

Subjects: Forty male golden Syrian hamsters, aged 5 to 6 weeks, were used.

Interventions: A dose of 0.25 µg/kg of VD$_3$ via intraperitoneal injection was given to 20 animals 3 times per week. Of the remaining 20 control animals, 5 received placebo vehicle injection, and 15 received no further treatment.

Main Outcome Measures: Timing, size, and number of tumors that developed in the 2 groups.

Results: Only 1 hamster treated with VD$_3$ developed a confirmed neoplasm compared with 7 of the control animals ($P < .01$). The mean±SD total diameter of gross lesions per animal in the VD$_3$-treated group was 1.2±1.9 mm compared with 6.8±6.6 mm in the control group ($P = .03$). The time to onset of lesion formation was significantly delayed in those animals treated with VD$_3$, with a mean±SD time to development of 13.4±0.9 weeks, while the control animals developed lesions at 11.2±1.7 weeks ($P = .02$).

Conclusions: Systemic VD$_3$ therapy delays carcinogenesis in the hamster buccal pouch model. Further investigation into the mechanisms through which VD$_3$ inhibits carcinogenesis may lead to development of effective chemopreventive agents to combat head and neck cancer.


Chemoprevention, first described by Sporn and colleagues$^1$ in 1976, involves the use of either natural or synthetic compounds to disrupt carcinogenesis. The concept of chemoprevention in head and neck squamous cell carcinoma (HNSCC) is especially appealing for multiple reasons. First, patients with head and neck cancer frequently present with premalignant disease. Second, these patients have a high risk for developing a second primary tumor (3%-7% annually).$^2$ Finally, specific risk factors, namely, tobacco and alcohol abuse, make the identification of high-risk patients easier to target. The ideal chemopreventive agent would either prevent malignant transformation or reduce the risk of developing a second primary cancer without causing the substantial adverse effects encountered in traditional chemotherapy. Hundreds of compounds have been investigated in both human and animal studies in the hope of discovering the ideal substance. The most studied agent for chemoprevention of HNSCC has been retinoic acid.$^3$ However, toxic adverse effects limit its usefulness.

More recently, cholecalciferol (hereinafter, vitamin D) has been investigated as a chemopreventive agent. In addition to its well-known role in calcium homeostasis, vitamin D has been shown to regulate the proliferation and differentiation of many cell types.$^4$ The exact mechanisms for the chemopreventive properties of vitamin D are unclear. After hydroxylation in the liver and kidney, vitamin D is converted to 1-alpha,25-dihydroxyvitamin D$_3$ (vitamin D$_3$ [hereinafter, VD$_3$], which acts as a steroid hormone that regulates several cellular pathways by binding to the vitamin D receptor (VDR) and forming heterodimers with the retinoid X receptor (RXR).$^5$ The VDR-RXR heterodimer binds to DNA sequences called vitamin D response elements (VDREs) thus regulating transcription.$^6$
Lesions first developed in the control animals at week 9, while the first lesion did not appear in the VD3-treated animals until week 12. By 14 weeks, 9 of the 10 control animals had lesions compared with only 5 of the 10 VD3-treated animals.

The hamster buccal pouch carcinogenesis model provides a means for studying chemopreventive agents. In this model, a known carcinogen, 7,12-dimethylbenz(a) anthracene (DMBA; Sigma Chemicals Co, St Louis, Missouri) is applied to the buccal pouch of immunocompetent animals 3 times per week. With repeated exposure to the carcinogen, the animals display consistent temporal development of precancerous lesions followed by cancerous growths. While many animal models involve injecting or implanting tumor cells into the animals, the hamster cheek pouch model allows study of the sequence of events from normal epithelium to carcinoma. Additionally, immunocompetent animals are used, and the progression from normal epithelium to carcinoma closely resembles those changes involved in the carcinogenesis of upper aerodigestive tract carcinoma in humans. This study was performed to evaluate the role of VD3 in chemoprevention for HNSCC.

**METHODS**

**ANIMAL CARE**

Forty male Syrian golden hamsters, aged 5 to 6 weeks, were obtained from Harlan Laboratories, Indianapolis, Indiana. To ensure humane treatment, the experiment was approved by the Institution for Animal Care and Use Committee at the University of California Davis, and strict adherence to the protocol was observed. The animals were housed 2 to 3 per cage under controlled conditions with a 12-hour light-and-dark cycle and given water and standard laboratory chow ad libitum.

**INTERVENTION**

The 40 animals were randomly divided into 2 groups. The right buccal pouch of all animals was painted with a No. 4 camel hair paintbrush with 0.5% DMBA dissolved in mineral oil 3 times per week. Beginning on the first day of DMBA application and continuing on each day of DMBA application, 20 of the hamsters received intraperitoneal injections of 0.25 µg/kg of VD3 (Sigma Chemicals Co), while the remaining 20 animals (controls) received DMBA only. Five of the control animals received a concurrent intraperitoneal placebo injection of 5% ethanol in phosphate buffered saline, and these animals were killed at 14 weeks. At 2 and 6 weeks after the initiation of DMBA exposure, 3 animals in each group (VD3 and control) were killed. The remaining 20 animals were killed at week 14.

Because this was a pilot study, the number of animals needed to achieve statistical power was difficult to predict. If all of the control animals developed tumors and there was a 50% reduction in tumor formation in the VD3-treated animals, then only 9 animals in each group would be necessary to achieve a P value lower than .05 and statistical power of 80%. To allow for a 10% unexpected mortality rate, 10 animals were used. Only 5 animals in each group were killed at weeks 2 and 6 because data from these time points were intended to be more descriptive in nature.

The initial weight of each animal was recorded, and animals were weighed weekly. Calcium levels were measured from serum collected at the time of death. Beginning at week 9, all animals received weekly examinations of the buccal pouch, with manual eversion of the pouch using a Send retractor. At the time of death, photographs were taken. The mucosa was inspected for tumors or lesions, and any suspicious lesions were individually biopsied. The remaining cheek pouch was dissected, with half of the tissue snap frozen in liquid nitrogen and the remaining tissue fixed in 10% formalin.

**HISTOLOGIC ANALYSIS**

Formalin-fixed tissue was processed, embedded in paraffin, and cut into 4-µm-thick sections using a microtome. Standard hematoxylin-eosin staining was performed, and slides from each tumor or suspect lesion plus 3 random sections of the buccal mucosa were evaluated by a pathologist blinded to the study.

| Figure 1 | Graph depicting the temporal development of visible lesions in controls and animals treated with 1-alpha,25-dihydroxyvitamin D3 (vitamin D3 [hereinafter, VD3]). Lesions first developed in the control animals at week 9, while the first lesion did not appear in the VD3-treated animals until week 12. By 14 weeks, 9 of the 10 control animals had lesions compared with only 5 of the 10 VD3-treated animals. |

**RESULTS**

No gross lesions were identified in any of the animals killed at 2 or 6 weeks. In the animals killed at 14 weeks, suspect lesions were found in 9 of the 10 control animals, while only 5 of the 10 animals treated with VD3 exhibited suspect lesions (P = .05). The mean ± SD diameter of the lesions per hamster was 6.8 ± 6.6 mm in the control group, which was significantly larger than the 1.2 ± 1.9-mm diameter of the lesions per hamster in the VD3-treated group (P = .03). The earliest gross tumor was evident in a control animal at 9 weeks, while the earliest lesion in the VD3 group was not seen until week 12. At 11 weeks, 6 of the 10 control animals had developed significant lesions, while no detectable lesions were found in those animals treated with VD3. Figure 1 depicts the number of animals found to have visible lesions in weeks 8 through 14. The time to lesion formation was significantly delayed in those animals treated with VD3; the mean ± SD time to development was 11.2 ± 1.7 weeks in the control animals compared with 13.4 ± 0.9 weeks in the VD3-treated animals (P = .02). By 14 weeks, both endophytic lesions (Figure 2A) and exophytic masses (Figure 2B) were found.

After histologic examination of all suspect lesions, in the animals killed at 14 weeks, 7 of the 10 animals in the control group revealed histologic evidence of tumors, while only 1 of the 10 animals treated with VD3 revealed...
evidence of a tumor (P<.01). Microscopic examination of the remaining suspect lesions did not show evidence of tumors. All of these remaining lesions displayed significant inflammation and were believed to be either microabscesses or dysplastic lesions. The Table summarizes the analysis of the lesions by pathologic diagnosis.

Histologic premalignant changes were found at both 2 and 6 weeks after DMBA application. At 2 weeks, hyperkeratosis and focal hyperplasia were evident in random samples of the mucosa in animals from both groups (data not shown). By 6 weeks, dysplastic lesions were found. However, there was not a significant difference in the amount of dysplasia between the VD₃-treated animals and the controls. Analysis of 15 representative biopsy specimens from 5 control animals revealed that 9 of the samples were normal, 2 contained hyperplasia, and 2 contained dysplasia. In the animals treated with VD₃, at 6 weeks, 11 of the specimens were normal, 2 showed hyperplasia, and 2 contained dysplasia. In random biopsy specimens of the buccal pouches taken at 14 weeks, 14 of the 30 specimens from control animals revealed dysplasia compared with only 9 of the 30 specimens from the VD₃-treated animals (not significant). Of the 9 VD₃-treated animals at 14 weeks that did not contain tumors, 6 of these showed evidence of dysplasia on random biopsy specimen analysis.

No significant differences were observed in the animal growth rates between the 2 groups (Figure 3). Analysis of serum calcium levels showed no difference between the control and VD₃ groups (mean±SD serum calcium levels: controls, 11.7±0.3 mg/dL; VD₃, 11.5±0.5 mg/dL; to convert serum calcium to millimoles per liter, multiply by 0.25).

In spite of the advancements in surgical, medical, and radiation therapy, the overall survival rate for HNSCC has not improved significantly in recent years. A variety of factors have contributed to this failure to improve outcomes. One of these contributors is the high incidence of second primary tumors. In a retrospective review of 851 patients with HNSCC, Schwartz et al²² reported that 162 developed second carcinomas (19%).²² From their results, they estimated the incidence of a second metachronous cancer in 5 years to be 22%. They found the survival rate from the second cancer to be only 8%. Therefore, even if the initial carcinoma is successfully eradi-
cated, the risk of a patient succumbing to cancer remains relatively high. If nontoxic, natural compounds could prevent or delay recurrences or the formation of second primary tumors, the disease-free and overall survival rates could be improved.

In the present study, systemic VD₃ treatment significantly delayed carcinogenesis in the hamster cheek pouch. Topical VD₃ treatment has been shown to reduce carcinoma in this model as well. Other animal models have also demonstrated a chemopreventive effect of vitamin D in both prostate and colon tissue. The results of these studies suggest significant potential for vitamin D in chemoprevention. However, adverse effects may limit its efficacy. In previous animal studies, the dose needed to be effective often caused cachexia and hypercalcemia. These adverse effects have spurred the creation of many vitamin D analogues, which purport to be as or more effective in chemoprevention while avoiding the severe hypercalcemic adverse effects. In the present study, higher levels of serum calcium were not detected in the animals treated with VD₃ compared with the controls. Several phase I trials using high-dose, intermittent VD₃ as an adjunctive chemotherapeutic agent for treatment of advanced malignant neoplasms have shown potential without significant toxic effects.

Unfortunately, these high-dose, intermittent dosing schedules would likely be too short-term and ineffective in chemoprevention. Finding a mechanism for a lower-dose, long-term delivery of VD₃ would be promising for chemoprevention in HNSCC.

By the time a head and neck oncologic surgeon first sees a patient with HNSCC, the patient has usually a several-year history of exposure to alcohol and tobacco, and the premalignant changes throughout the mucosa are well under way. In the present study, VD₃ therapy was started concurrently with exposure to the carcinogen, not after several weeks of DMBA painting. After 6 weeks of DMBA exposure, we did not detect a significant difference in the amount of dysplasia. No conclusion can be drawn from this experiment as to which stages of carcinogenesis are inhibited by VD₃. Other authors have evaluated VD₃ inhibition of carcinogenesis using Nkx3.1; Pten mutant mice. These mice have deletions of the Nkx3.1 homeobox and Pten tumor suppressor genes, which are both important in the development of prostate carcinoma. In this study of prostate carcinoma, VD₃ was more effective in the early stages of disease than in the later stages.

The precise mechanism through which VD₃ prevents carcinogenesis is unclear. Extensive research elucidating the prodifferentiating, antiproliferative, and proapoptotic effects of VD₃ is ongoing. Ideally, better understanding of how VD₃ regulates these intracellular pathways may lead to a chemopreventive strategy that is specifically directed against novel molecular targets.

In conclusion, we found that systemic VD₃ treatment significantly inhibited neoplastic transformation in the hamster buccal pouch. These results reveal the potential of VD₃ as a chemopreventive agent and justify further research to investigate the intracellular mechanisms of VD₃-mediated anticarcinogenic effects.

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Author Contributions: Dr Farwell had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Meier, Enepekides, Bradley, and Farwell. Acquisition of data: Meier, Enepekides, Poirier, Albala, and Farwell. Analysis and interpretation of data: Meier, Enepekides, and Farwell. Drafting of the manuscript: Meier, Enepekides, and Farwell. Critical revision of the manuscript for important intellectual content: Meier, Poirier, Bradley, Albala, and Farwell. Statistical analysis: Meier. Obtained funding: Farwell. Administrative, technical, and material support: Meier, Enepekides, Poirier, Bradley, and Farwell. Study supervision: Enepekides, Albala, and Farwell.

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REFERENCES