Pilot Study on the Bioactivity of Vitamin D in the Skin after Oral Supplementation

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Abstract

Laboratory studies suggest that vitamin D (VD) supplementation inhibits skin carcinogenesis. However, epidemiologic studies report mixed findings in the association between circulating VD levels and skin cancer risk. We conducted a clinical study to determine whether oral cholecalciferol supplementation would exert direct bioactivity in human skin through modulation of the VD receptor (VDR). We enrolled 25 individuals with serum 25-hydroxyvitamin-D levels <30 ng/mL and with skin photodamage to take 50,000 IU of cholecalciferol biweekly for 8 to 9 weeks. Then, we obtained baseline and end-of-study skin biopsies from photodamaged (PD) and photoprotected (PP) skin, and from benign nevi (BN) and tested for mRNA expression of VDR and cytochrome P450-24 (CYP24), and markers of keratinocytic differentiation. High-dose cholecalciferol supplementation significantly elevated circulating levels of 25-hydroxyvitamin-D (P < 0.0001) and 1,25-dihydroxyvitamin-D (P < 0.0001). VDR expression in PD- and PP-skin showed minimum changes after supplementation. CYP24 expression in PD- and PP-skin was increased after supplementation by 186%, P = 0.08, and 134%, P = 0.07, respectively. In BNs from 11 participants, a trend for higher VDR and CYP24 expression was observed (average of 20%, P = 0.08, and 54%, P = 0.09, respectively). Caspase-14 expression at the basal layer in PD skin samples was the only epidermal differentiation marker that was significantly increased (49%, P < 0.0001). High-dose cholecalciferol supplementation raised serum VD metabolite levels concurrently with CYP24 mRNA and caspase-14 levels in the skin. Our findings of significant variability in the range of VDR and CYP24 expression across study samples represent an important consideration in studies evaluating the role of VD as a skin cancer chemopreventive agent. Cancer Prev Res; 8(6): 563–9. ©2015 AACR.
Experimental studies suggest a beneficial effect of VD mediated events in keratinocytes (13, 14) and in some instances in melanocyte's biology (15). Specifically, 1,25-dihydroxyvitamin D₃, the biologically active metabolite of VD, reduces keratinocytes growth and promotes differentiation, and consequently has already been successfully used in the therapy of hyperproliferative skin disorders (15). At high levels, 1,25(OH)₂D inhibits keratinocyte proliferation in vitro and interacts with calcium to regulate keratinocyte differentiation (16). Keratinocytes lacking VDR are hyperproliferative and exhibit decreased apoptosis (17). Genetically engineered mice lacking the VDR (VDR-knockout mice) demonstrate a variety of phenotypes according to the experimental model, including reduced alopecia, abnormal hair follicles, dermal cysts, and more skin tumors (primarily BCCs), when exposed to a carcinogen (oral 7,12-dimethylbenz[a]anthracene; ref. 18), indicating a role of VDR in keratinocyte differentiation. Mice lacking VDR are predisposed to SCC formation when exposed to high and prolonged doses of UVB (19).

Like keratinocytes, melanocytes also have the capacity for autonomous local production of 1,25(OH)₂D and harbor VDR (20). Such locally produced 1,25(OH)₂D may play a role in innate and acquired cutaneous immunity (21). In vitro, 1,25(OH)₂D2 stimulates melanocyte maturation, possibly through the stimulation of tyrosinase activity (22, 23). It also protects cells from apoptosis (24) and upregulates VDR expression (15).

To date a controversial association between low levels of serum 25(OH)D and risk of skin cancer development has been proposed by epidemiologic studies. In addition, experimental studies as the ones mentioned above, support the notion that VD plays an important role in keratinocyte and melanocyte differentiation state and proliferative capability. These studies combined have fueled the scientific community with the rationale to further consider interventional studies to assess the role of cholecalciferol supplementation for the prevention of primary skin cancers and further recurrences.

Before such studies can be undertaken, it is imperative that effective and reproducible biomarkers of VD bioactivity in the skin can be identified in the setting of oral supplementation. Understanding the net effect of oral VD in the biology of the targeted organ, in this case the skin, is critical to the justification and design of future interventional studies.

To determine the potential role of VD for skin cancer prevention, we performed a pilot clinical study of oral cholecalciferol supplementation to determine whether oral supplementation activates the VD pathway in the target skin tissue and whether serum 25(OH)D and 1,25(OH)₂D correlates with a modulatory effect in skin differentiation markers.

**Materials and Methods**

**Study design**

The study was an open label, single-arm intervention trial of high-dose cholecalciferol (vitamin D₃) in healthy individuals who were considered VD insufficient or deficient (serum 25-hydroxyvitamin D <30 ng/mL) with evidence of moderate to severe skin photodamage on the forearms. The study endpoints include intervention-induced change in the expression of VD receptor (VDR) and cytochrome P450 24 (CYP24) in keratinocytes, skin layers thickness, markers of keratinocyte differentiation (caspase-14 and loricrin), and serum levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D. The study also explored the intervention-induced changes in VDR and CYP24 expression in a subset of participants with qualifying benign melanocytic nevi. The University of Arizona Institutional Review Board approved the study and informed consent was obtained from all study participants.

**Study drug**

Study drug capsules were supplied by the National Cancer Institute, Division of Cancer Prevention. Each capsule contains 50,000 IU of cholecalciferol and inactive ingredients microcrystalline cellulose, silica, and gelatin. The study capsules were stored at room temperature and protected from environmental extreme.

**Study populations**

We recruited healthy men and women who had serum 25-hydroxyvitamin D levels <30 ng/mL, had moderate to severe sun damage on the forearm, were at least 40 years of age, had normal liver, renal, and marrow function, had normal serum calcium and parathyroid hormone (PTH) levels, and had skin phototype II or III. Participants were excluded if they had acne or chronic hyperparathyroidism or hypercalcemia, had a history of increased arterial calcification or atherosclerosis, sarcoidosis, histoplasmosis, hyperparathyroidism, lymphoma, or kidney disease, were taking digoxin, cholestyramine, colistin, oral steroids, and antacids that contain magnesium, were taking other investigational agents, had a history of allergic reactions to cholecalciferol, lidocaine, or xylocaine, had uncontrolled intercurrent illness, were pregnant or breastfeeding. Additional exclusion criteria were based on medical and drug history, including diagnosis of invasive cancer or cancer treatment within the past 5 years, except NMSC, were immunosuppressed by virtue of medication or disease, were unwilling or unable to refrain from taking herbal medicines or above-standard vitamin or mineral supplements during the study, had used tanning beds or other methods to promote sun-tanning within 6 months of study entry, were unwilling to minimize their exposure to sunlight, were treated with topical retinoids, steroids, 5-fluorouracil, Levulan, effomithine, diclofenac, or imiquimod within 30 days of study entry, had received treatment for basal cell carcinoma or squamous cell carcinoma at study sites within 6 months of study entry.

**Study procedures**

**Prescreening visit.** Participants received a skin exam and had a blood sample collected for serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D analysis. Participants were asked to return if their 25-hydroxyvitamin D serum levels were <30 ng/mL and they had evidence of at least moderate skin photodamage on the forearms. A blood sample was collected for complete blood count, comprehensive metabolic panel and intact PTH. A urine pregnancy test was done for women with childbearing potential. Photodamage of the right dorsal forearm was measured using a clinical assessment scale.

**Selection of benign melanocytic nevi.** Subjects were then evaluated for the presence of two benign melanocytic nevi, ≥4 mm, located on photoprotected areas of the body where biopsies would be appropriate. Epiluminescent microscopy (ELM; ref. 25) was used by the study physician aided by the pattern analysis (26) and modified ABCD (27) rule algorithms to exclude atypical/dysplastic nevi and exclusively select either intradermal or compound
nevi to maximize the population of melanocytes in the study samples.

**Baseline specimen collection.** Participants who met all eligibility criteria returned for baseline skin specimen collection. A urine pregnancy test was repeated at baseline, follow-up, and end of study if indicated. The clinician selected two areas on the mid-upper right dorsal forearm (photodamaged area) plus two areas on the left buttock (photoprotected area), for biopsy. If available, BN were photographed (standard and ELM photos) and a baseline lesion was biopsied. One set of biopsies were immediately fixed in 10% neutral buffered formalin for 24 hours then transferred to 70% ethanol before routine processing and paraffin embedding. Another set of biopsies were immediately separated from surrounding connective and fat tissue, placed in RNAlater overnight at 4°C, and stored at −80°C.

**Agent intervention.** Following baseline specimen collection, participants were instructed to take one cholecalciferol capsule (50,000 IU/capsule) twice each week for a period of 8 to 9 weeks. Participants returned to the clinic after 3 to 5 weeks of agent intervention for adherence and safety evaluation. A blood sample was drawn for assessment of serum calcium levels.

**Post-intervention evaluation.** Participants then returned after 8 to 9 weeks of study agent intervention for post-intervention evaluation. Photodamage of the right forearm was measured using a clinical assessment scale. Two skin biopsy samples were collected adjacent to the baseline biopsy sites on the right dorsal forearm and two skin samples collected from the left buttock. When applicable, the other BN identified at screening was reevaluated by ELM using the pattern analysis and Modified ABCD rules algorithms, photographed, and then collected in an excisional manner. The post-intervention biopsies were handled as described above for the baseline biopsies. A blood sample was collected for laboratory evaluation (CBC-diff, CMP, intact PTH, 25-hydroxyvitamin D, and 1,25-dihydroxyvitamin D serum level analysis). Participants were followed for approximately 10 to 14 days after the post-intervention evaluation and returned for a final visit for suture removal and examination of the biopsy sites.

**Measurement of CYP24 and VDR expression**

CYP24 and VDR expression was evaluated using real-time PCR. Total RNA was isolated from the frozen skin tissue (with RNAlater) using an RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer’s protocol. The RNA obtained was then quantified using A260/A280 spectrophotometry. DNase-treated RNA (2 μg) was reverse transcribed using the High Capacity RNA-to-cDNA kit (Life Technologies). The obtained cDNA was used in 20 μL PCR reactions containing 10 μL Maxima SYBR Green qPCR Master Mixes (Thermo Scientific) 0.4 μL primers, 0.86 μL of cDNA template sample, and 8.74 μL of molecular grade water. Reactions were performed in 284-well PCR plates and read on a ABI Prism 7900 HT Sequence Detection System. Data were analyzed using the comparative C<sub>t</sub> method as a means of relative quantitation, normalized to an endogenous reference (TBP cDNA) and relative to a calibrator (normalized C<sub>t</sub> value obtained from controls) and expressed as 2<sup>−ΔΔCt</sup> according to Applied Biosystems User Bulletin 2: Rev B, “Relative Quantitation of Gene Expression.”

**Measurement of skin layers thickness**

The thickness of skin layers was measured on 4-μm sections of formalin-fixed paraffin-embedded (FFPE) biopsies stained with hematoxylin and eosin. The measurement was accomplished using an imaging system composed of a Leica microscope equipped with a Sony DXC 9000 SSCD color video camera, a full complement of Leica plan app objectives and the Image-Pro Plus version 6.3 imaging capture and analysis software (Media Cybernetics). Three areas from each skin biopsy were imaged using ×40 magnification and the Image-Pro Plus line draw tool, which provides measurements in microns. The stratum corneum was measured from the outer edge of the layer to the base of the lucidum. Epidermis was measured from the base of the lucidum to the basal layer-dermis interface. The three measurements were averaged for analysis.

**Measurement of differentiation markers**

Loricrin and caspase-14 protein expression was determined by immunohistochemistry. FFPE tissue sections (4 μm) were deparaffinized and rehydrated. All tissue sections were subjected to antigen retrieval using a citrate buffer for 30 seconds. Immunohistochemical staining was performed using a Vectastain Elite Standard ABC kit immunoperoxidase detection system, a Vector Nova Red substrate for peroxidase (Vector Laboratories), and a hematoxylin counterstain (Surgipath). Antibodies included caspase-14 (mouse monoclonal, 1:100 dilution; Santa Cruz Biotechnology) and loricrin (rabbit polyclonal, 1:1,000 dilution; Covance), which were incubated 1 hour at room temperature. Skin from routine surgical procedures was used as a positive control. Image-Pro Plus image analysis software was used to capture and analyze three representative areas at ×40 magnification. A standardized intensity score (mean intensity divided by the SD) for each participant was derived at both baseline and post-intervention and used to calculate the change from baseline to post-intervention. For loricrin, the entire epidermis was imaged and an intensity score generated. In contrast, for caspase-14, the basal and suprabasal were measured separately and each was given an intensity score.

**Measurement of circulating concentrations of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D**

The analysis of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D was performed by validated radioimmunoassays at Heartland Assays (28, 29).

**Statistical analysis**

Descriptive statistics, for example, mean and SD, were calculated on each of the endpoints. A two-sided paired t test was performed to test whether the percentage of change from baseline to post-intervention in each of the endpoints is significantly different from zero. Each analysis was performed comparing results within each individual from baseline to post-intervention values. A two-sided paired t test was also performed to compare the baseline tissue biomarkers between photoprotected and photodamaged skin except a signed rank test was used to compare the baseline CYP24 expression between photoprotected and photodamaged skin because the data were not normally distributed. Pearson correlation coefficients between changes in serum levels of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and changes in each tissue biomarker were calculated to evaluate whether the changes are significantly correlated.
Table 1. Study participants characteristics

<table>
<thead>
<tr>
<th>Variables</th>
<th>Age, y (mean ± SD)</th>
<th>Weight, pounds (mean ± SD)</th>
<th>Height, inches (mean ± SD)</th>
<th>BMI, kg/m² (mean ± SD)</th>
<th>Sex, male/female</th>
<th>Race, White/Other</th>
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</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>57.8 ± 7.2</td>
<td>185.2 ± 40.8</td>
<td>66.2 ± 3.9</td>
<td>29.7 ± 5.9</td>
<td>8/17</td>
<td>23/2</td>
</tr>
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</table>

Results

The study prescreened 50 and accrued 25 participants between October 2011 and July 2012. Characteristics of study participants are summarized in Table 1. The mean age of the study participants was 57.8 years with a male:female distribution of 8:17. The mean BMI was 29.7 kg/m².

High-dose cholecalciferol (50,000 IU) was given twice a week for 8 to 9 weeks in our study participants. None of the patients reported adverse events. The two most frequently reported adverse events were headache (four grade 1 and two grade 2 reports) and flu-like symptoms (three grade 1 and one grade 2).

The intervention also did not result in clinically significant changes in hematology, blood chemistry, and PTH. As shown in Table 2, the study intervention significantly increased serum levels of 25-hydroxyvitamin D from 21.6 ± 5.2 to 70.5 ± 18.2 ng/mL (P < 0.0001) and 1,25-dihydroxyvitamin D from 31.1 ± 12.4 to 51.4 ± 13.5 pg/mL (P < 0.0001). The systemic increase in these two metabolites did not correlate with individual modulations in VDR and CYP expression.

Table 3 summarizes the changes in VDR and CYP24 expression. The baseline VDR expression was similar between photoprotected and photodamaged skin. The intervention induced minimal changes in VDR expression in both photoprotected and photodamaged skin. Baseline CYP24 expression was higher in photodamaged skin compared with photoprotected skin (37,698 ± 10,801 vs. 10,859 ± 21,767, P < 0.0001). The intervention induced a consistent upregulation trend of CYP24 expression (percentage of change: 134.4 ± 37.55; P = 0.07 in photoprotected skin and 185.92 ± 502.19; P = 0.08 in photodamaged skin). VDR and CYP24 expression was also evaluated in 11 subjects with eligible benign melanocytic nevi. This number of nevi was consistent with our anticipated recruitment rate of approximately 40% of subjects who would have eligible nevi for inclusion in this exploratory endpoint. An upregulation trend of VDR (percentage of change: 19.86 ± 33.61; P = 0.08) and CYP24 (percentage of change: 544.02 ± 955.27; P = 0.09) was observed in nevi following supplementation.

Table 4 summarizes the measurements of differenti- ation markers. Baseline loracrin and caspase-14 staining intensity was similar between photoprotected and photodamaged skin in different skin layers. The staining intensity of these markers did not change significantly after oral cholecalciferol supplementation with the exception of a significant increase (P < 0.0001) in caspase-14 staining intensity in the basal layer of the photodamaged skin (Fig. 1). In addition, VD supplementation did not result in significant changes in skin layer thickness (data not shown).

Discussion

Our study is one of the first to assess the levels of putative markers of VD bioactivity in human skin and to determine the changes in these markers after oral supplementation. We showed that supplementation with 50,000 IU of cholecalciferol biweekly for 8 weeks improved the D serum levels status of 25(OH)D and 1,25(OH)2D in individuals who were considered VD insufficient. Armas and colleagues (30) showed that a single dose of 50,000 IU of cholecalciferol resulted in persistent elevation of serum 25-hydroxyvitamin D levels over a 4-week period, which supported the significant increase in 25-hydroxyvitamin D with the dosing regimen used in our study. In addition, we showed that high-dose VD supplementation also resulted in a significant elevation in serum levels of 1,25-dihydroxyvitamin D, the bioactive form of VD that is generally considered to be tightly regulated.

We selected measurements of VDR mRNA expression as the primary measure of the bioactivity of VD in the skin because VD supplementation to VD-deficient rats has been shown to increase VD mRNA expression in skin keratinocytes (31). Despite showing a significant elevation in circulating levels of 25(OH)D and 1,25(OH)2D, we did not find a change in the expression of VDR mRNA after high-dose oral cholecalciferol supplementation in either photodamaged and photoprotected human skin. One explanation may be that the expression of VDR in skin is not suppressed in individuals with this level of VD insufficiency. Thus, VD supplementation may not be able to exert a significant modulatory effect in cells with normal VDR expression. Interestingly, the baseline VDR expression was similar between photodamaged and photoprotected skin, suggesting that VDR expression may not be sensitive to solar exposure and/or photodamage. Given the important role of VDR in cell function, it is plausible that the expression level is preserved despite broad changes in 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D. Studies evaluating the effect of oral supplementation in more profoundly depleted patients might provide additional information as to the possibility of modulating VDR expression in this clinical setting. However, VDR modulatory findings in such extreme circumstances and beneficial effect of supplementation might not be applicable to the vast majority of patients. An additional explanation to the significant variability in VDR expression observed across study participants and lack of reaching clinical significance in this pilot study include the range of VDR polymorphisms that might result in different binding capabilities to VD and further activation of downstream signaling a different serum levels (32, 33). Additional studies involving VDR and CYP24...
genotyping might provide further information on the role of this potential confounder and possibility to identify those individuals that will be more likely to respond to oral supplementation. In addition, changes in a larger array of gene-expression profiles or proteomics analysis in samples collected from our study may help identify more suitable biomarkers for future clinical research.

We also assessed the CYP24 mRNA expression as a measure of the VD bioactivity in the skin because multiple in vitro studies have shown an induction of CYP24 with VD treatment (34). We observed a consistent upregulation of CYP24 mRNA expression after high-dose cholecalciferol supplementation, although the observed a consistent upregulation of CYP24 mRNA expression added from a paired t test. A derived from a linear regression after adjusting for baseline value for change (% changes) from baseline.

We conclude that high-dose cholecalciferol supplementation resulted in significant elevation of circulating levels of both

| Table 3. Measurements of VDR and CYP24 expression | Baseline Mean ± SD | Post-intervention Mean ± SD | Change Mean ± SD | Percentage of change Mean ± SD | P
| VDR (24-34) | | | | | |
| Photoprotected skin (n = 25) | 4.993 ± 2.488 | 5.073 ± 3.525 | 0.08 ± 3.546 (0.91*) | 35.3 ± 159.2 (0.28) | 0.10 (0.01)
| Photodamaged skin (n = 25) | 5.771 ± 2.785 | 5.009 ± 2.678 | −16.3 ± 1.207 (0.51) | −0.99 ± 28.00 (0.86) | 0.31 (0.58)
| Benign Nev (n = 11) | 2.607 ± 1.180 | 2.993 ± 1.396 | 0.386 ± 7.12 (0.10) | 19.86 ± 33.61 (0.08) | 0.56 (0.09)
| CYP24 (24-34) | | | | | |
| Photoprotected skin (n = 25) | 10.859 ± 2.716 | 19.403 ± 4.194 | 8.544 ± 21.53 (0.06) | 134.43 ± 247.55 (0.07) | 0.87 (0.07)
| Photodamaged skin (n = 24) | 37.698 ± 10.801 | 48.502 ± 9.677 | 10.804 ± 102.72 (0.61) | 185.92 ± 502.19 (0.08) | 0.10 (0.08)
| Benign Nev (n = 11) | 92.472 ± 17.728 | 19.371 ± 36.018 | 0.063 ± 39.24 (0.42) | 544.02 ± 955.27 (0.09) | 0.19 (0.07)

* Derived from a paired t test for the percentage of change from baseline.
25-hydroxyvitamin D and 1,25-dihydroxyvitamin D in individuals who are considered VD insufficient and variable changes in selected markers of VD bioactivity in the skin. Our data suggest that CYP24 expression in the skin may be a sensitive marker for solar exposure, photodamage, and VD status and supplementation. Our study highlights the importance of evaluating the bioactivity of VD supplementation in the target tissue to optimize the development of VD supplementation for cancer prevention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: C. Curiel-Lewandrowski, J.Y. Tang, D.S. Alberts, H.-H.S. Chow
Development of methodology: C. Curiel-Lewandrowski, J.G. Einspahr, Y. Bermudez, H.L. Parnes
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Curiel-Lewandrowski, J.Y. Tang, J.G. Einspahr, M. Rezaee
Writing, review, and/or revision of the manuscript: C. Curiel-Lewandrowski, J.Y. Tang, J.G. Einspahr, Y. Bermudez, M. Rezaee, A.H. Lee, J. Tangrea, H.L. Parnes, H.-H.S. Chow
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