

ORIGINAL ARTICLE

# Enhancement of Vitamin D Metabolites in the Eye Following Vitamin D3 Supplementation and UV-B Irradiation

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## ABSTRACT

**Purpose:** This study was designed to measure vitamin D metabolites in the aqueous and vitreous humor and in tear fluid, and to determine if dietary vitamin D3 supplementation affects these levels. We also determined if the corneal epithelium can synthesize vitamin D following UV-B exposure.

**Methods:** Rabbits were fed a control or vitamin D3 supplemented diet. Pilocarpine-stimulated tear fluid was collected and aqueous and vitreous humor were drawn from enucleated eyes. Plasma vitamin D was also measured. To test for epithelial vitamin D synthesis, a human corneal limbal epithelial cell line was irradiated with two doses of UV-B (10 and 20 mJ/cm<sup>2</sup>/day for 3 days) and vitamin D was measured in control or 7-dehydrocholesterol treated culture medium. Measurements were made using mass spectroscopy.

**Results:** 25(OH)-vitamin D3 and 24,25(OH)<sub>2</sub>-vitamin D3 increased significantly following D3 supplementation in all samples except vitreous humor. Tear fluid and aqueous humor had small but detectable 1,25(OH)<sub>2</sub>-vitamin D3 levels. Vitamin D2 metabolites were observed in all samples. Vitamin D3 levels were below the detection limit for all samples. Minimal vitamin D3 metabolites were observed in control and UV-B-irradiated epithelial culture medium except following 7-dehydrocholesterol treatment, which resulted in a UV-B-dose dependent increase in vitamin D3, 25(OH)-vitamin D3 and 24,25(OH)<sub>2</sub>-vitamin D3.

**Conclusions:** There are measurable concentrations of vitamin D metabolites in tear fluid and aqueous and vitreous humor, and oral vitamin D supplementation affects vitamin D metabolite concentrations in the anterior segment of the eye. In addition, the UV exposure results lead us to conclude that corneal epithelial cells are likely capable of synthesizing vitamin D3 metabolites in the presence of 7-dehydrocholesterol following UV-B exposure.

**KEYWORDS:** Vitamin D, Cornea, Aqueous humor, Vitreous humor, Tear fluid

## INTRODUCTION

The eye is the only major organ, other than skin, that is directly exposed to sun light. The primary source of vitamin D3 (Vit D3) in humans is through sunlight-mediated ultraviolet B (UV-B) conversion of 7-dehydrocholesterol to Vit D3. Vit D3 is hydroxylated in the 25 position by cytochrome P-450 containing enzymes resulting in the production of 25(OH)-vitamin D3 (25(OH)D3). 25(OH)D3 is then converted to the active 1 $\alpha$ ,25-dihydroxyvitamin D3

(1,25(OH)<sub>2</sub>D3) by 1 $\alpha$ -hydroxylase. 25(OH)D3 can also be converted to 24R,25-dihydroxyvitamin D3 (24,25(OH)<sub>2</sub>D3) by the enzyme 24-hydroxylase. In individuals who are not regularly exposed to sun light, dietary and/or supplemental Vit D3 are the primary sources of vitamin D. Vitamin D2 (ergocalciferol; Vit D2) is not produced *de novo* by humans, but it is found in plants, yeast, and supplements and thus can be a dietary source of vitamin D. It is metabolized by the same pathways as Vit D3, and its active form,

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1,25(OH)<sub>2</sub>D<sub>2</sub>, is an effective agonist for the vitamin D receptor.

A recent report from our group determined that there are significant Vit D<sub>3</sub> metabolite levels in the aqueous and vitreous humor of rabbits, that the corneal epithelium contains mRNA for 1 $\alpha$ -hydroxylase and that Vit D<sub>3</sub> can affect tight junctions in corneal epithelial cells.<sup>1</sup> High plasma Vit D<sub>3</sub> levels may be protective against early age-related macular degeneration<sup>2</sup> and may also be protective for glaucoma.<sup>3</sup> The current study examined the two potential pathways for Vit D<sub>3</sub> to enter the eye, either via the circulation or through UV-B induced synthesis. The influence of circulating Vit D<sub>3</sub> was examined by measuring Vit D<sub>3</sub> metabolite levels in the aqueous and vitreous humor and in tear fluid following dietary Vit D<sub>3</sub> supplementation. The influence of UV-B on corneal epithelial Vit D<sub>3</sub> metabolite production was examined using a cell culture model.

## MATERIALS AND METHODS

### Diet

New Zealand White (NZW) rabbits were fed a control (1.1 IU/g vitamin D, *n* = 6) or a specially formulated Vit D<sub>3</sub> (cholecalciferol) supplemented diet (7 IU/g, *n* = 6) for 8 weeks (Harlan Laboratories, Madison, WI, USA). Animals were allowed to eat *ad libitum* and intake was not measured. All animal studies were approved by the University of Tennessee Health Science Center IACUC, and animals were treated in accordance with the ARVO statement for the use of animals in ophthalmic and vision research.

### Sample Collection

For intraocular Vit D<sub>3</sub> metabolite measurements, rabbits were killed by ear vein injection (IV) of Euthasol (390 mg/mL pentobarbital; Virbac Corporation, Ft. Worth, TX, USA) and eyes were enucleated. Aqueous humor was drawn from the anterior chamber and the anterior segment was dissected from the globe to collect vitreous humor. Blood was collected in EDTA tubes for plasma measurements.

### Tear Collection

For Vit D<sub>3</sub> metabolite measurements in tear fluid, pilocarpine-stimulated (200  $\mu$ g/kg) tear fluid was collected from NZW rabbits using microcapillary tubes. Rabbits were anesthetized with ketamine/xylazine (i.m., 35/5 mg/kg) and a single dose of IV pilocarpine was administered. Samples were collected immediately after pilocarpine injection, placed on ice, and then frozen at  $-80^{\circ}\text{C}$  until analysis.

## Cell Culture Study

A human corneal limbal epithelial cell line (HCLE)<sup>4</sup> was grown to confluence. To confirm the presence of vitamin D receptor (VDR) and 1 $\alpha$ -hydroxylase mRNA in this specific cell line, reverse transcriptase PCR was utilized as described previously.<sup>1</sup> Briefly, cells were grown on standard culture plates as described above. Total RNA was extracted using Trizol and samples were dissolved in diethylpyrocarbonate (DEPC) water. The concentration and purity of total RNA were determined by measuring the optical density at 260 and 280 nm. Samples were digested using RNase-free DNase I (Invitrogen, Carlsbad, CA, USA) to eliminate genomic DNA contamination. Primers for human VDR and 1 $\alpha$ -hydroxylase were copied from previously published reports.<sup>5,6</sup> The SuperScript<sup>TM</sup> III One-Step RT-PCR System (Invitrogen) was used to perform the RT-PCR protocol.

For measurement of Vit D<sub>3</sub> synthesis in response to UV-B, the cells were incubated in control medium or in medium containing 10  $\mu$ M 7-dehydrocholesterol for 24 h. Cells were exposed to UV-B (302 nm) using a previously described method.<sup>7</sup> Cells were replenished with fresh medium immediately prior to UV-B exposure, and the medium was not changed after the initial UV-B exposure to allow for accumulation of synthesized vitamin D metabolites. Cells were exposed to UV-B twice a day, at a total dose of 10 or 20 mJ/cm<sup>2</sup>/day in divided doses, administered 6 h apart for 3 days. This UV-B dose range was calculated to be significantly less than would be expected following mid-day sun exposure for 1 h in the Midwestern USA.<sup>7</sup> The medium was collected 1 h after the final UV-B exposure and stored at  $-80^{\circ}\text{C}$  until mass spectroscopy analysis for vitamin D metabolites.

## Vitamin D Measurements

A UPLC-MS/MS method was developed and used to detect the vitamin D metabolites in ocular fluids.<sup>1,8</sup> This method allows for a stable and sensitive quantification of Vit D<sub>2</sub>, Vit D<sub>3</sub>, 25(OH) D<sub>2</sub>, 25(OH)D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>2</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, and 24,25(OH)<sub>2</sub>D<sub>3</sub>. The vitamin D analytes were derivatized utilizing the Diels-Alder reaction to achieve a minimum quantification limit of 25 pg/mL. Liquid-liquid extraction was performed on all samples. Before extraction, pure organic solvent was used to clear out the proteins and release free analytes. Extracted residues were derivatized by 4-phenyl-1,2,4-triazo-3,5-dione (PTAD). The resolved solution was injected onto a UPLC column followed by detection in tandem quadrupole mass spectrometry under positive electrospray ionization.

Two kinds of internal standards were utilized to correct for loss during the extraction, Diels-Alder

reaction, and analysis of the samples. One is termed the surrogate internal standard, and included 6,19,19-duterated-vitamin D<sub>3</sub> (D3-Vit D<sub>3</sub>), 26,26,26,27,27,27-duterated-25-hydroxy vitamin D<sub>3</sub> (D6-25(OH)D<sub>3</sub>), 26,26,26,27,27,27-duterated-25-hydroxy vitamin D<sub>2</sub> (D6-25(OH)D<sub>2</sub>), 26,26,26,27,27,27-duterated-1,25-dihydroxy vitamin D<sub>3</sub> (D6-1,25(OH)<sub>2</sub>D<sub>3</sub>), and 26,26,26,27,27,27-duterated-1,25-dihydroxy vitamin D<sub>2</sub> (D6-1,25(OH)<sub>2</sub>D<sub>2</sub>). The other internal standard was selected to monitor the actual LC and MS analytical procedure. CUDA (12- [[(cyclohexylamino)carbonyl] amino]-dodecanoic acid) was selected due to its availability in the laboratory, its resistance to the derivatization agent, and its strong signal on MS analysis. A 10 µL aliquot of mixed surrogate standard solution (in acetonitrile) was added into each sample before liquid-liquid extraction (LLE), sealed under nitrogen and held at room temperature for 15 min. An equal volume of acetonitrile was then added to precipitate the protein. The supernatant was removed and extracted with twice the sample volume of peroxide free methyl tert-butyl ether (MTBE). After two extractions, the combined organic layer was dried down under vacuum. The residue was derivatized in 100 µL of a solution of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, 0.5 mg/mL) with a fixed concentration of CUDA (10 ng/mL) in the same acetonitrile solution. The area under the chromatographic peak was integrated by Masslynx 4.1 (Waters, Milford, MA, USA) to calculate the concentration. The recovery during sample preparation including LLE and derivatization was the ratio of the calculated concentration of surrogates to their theoretical concentration. The actual concentrations of analytes in each sample were further corrected by the recovery and concentration factor during sample preparation. Surrogates to correct the concentration of vitamin D<sub>2</sub> and D<sub>3</sub>, 25(OH)D<sub>2</sub>/D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>2</sub>/D<sub>3</sub> were D3-Vit D<sub>3</sub>, D6-25(OH)D<sub>2</sub>/D<sub>3</sub> and D6-1,25(OH)<sub>2</sub>D<sub>2</sub>/D<sub>3</sub>, respectively. The difference of CUDA concentration during analysis represented the fluctuation of LC/MS/MS system from injection to injection. The use of separate standards to monitor extraction, formation of derivatives and instrument performance was valuable for trouble shooting problems.

## Statistics

Comparisons between two groups were made using Student's *t*-test. Comparisons between more than two groups (plasma concentration comparisons) were made using the Student Newman Kuels test (SNK).

## RESULTS

### Plasma Measurements

All metabolites except for Vit D2 were elevated in plasma compared to baseline values at some point during the trial in either the control and/or the Vit D3 supplemented group (Table 1). As expected, Vit D3 was significantly elevated in the supplemented group, although only at 6 and 8 weeks. 25(OH)D2 was elevated in both groups, also at 6 and 8 weeks; possibly as the result of a diet change after entering the University animal facility. 25(OH)D3 levels also increased in both the control and supplemented groups during the time course of the trial. The increase in 25(OH)D3 was four- to five-fold greater in the supplemented group than the control group. 1,25(OH)<sub>2</sub>D2 was elevated at 6 and 8 weeks in the supplemented group, and at 6 weeks in the control group. 1,25(OH)<sub>2</sub>D3 was elevated in both groups at 6 weeks, being eightfold higher in the supplemented group, and also at 4 weeks in the supplemented group. No changes were detected in either group at 8 weeks. 24,25(OH)<sub>2</sub>D3 was significantly elevated in both groups at 6 and 8 weeks.

### Tears, Aqueous and Vitreous Humor Measurements

In both tear fluid and aqueous humor, 25(OH)D3 and 24,25(OH)<sub>2</sub>D3 were significantly elevated in the supplemented group. No other metabolites were significantly elevated in tear fluid, aqueous humor, or vitreous humor (Tables 2–4). Plasma versus ocular 25(OH)D3 and 24,25(OH)<sub>2</sub>D3 from Vit D3 supplemented rabbits are shown in Figures 1 and 2, respectively. It is apparent that both metabolites were elevated in the tear

TABLE 1 Rabbit plasma vitamin D concentrations: 8 weeks Vit D3 supplemented.

Mean ± SE (nM)	4 Weeks			6 Weeks		8 Weeks	
	Baseline (n = 12)	Control (n = 6)	High Vit D (n = 6)	Control (n = 6)	High Vit D (n = 6)	Control (n = 6)	High Vit D (n = 6)
Vit D2	0.44 ± 0.28	0.85 ± 0.83	0.44 ± 0.41	0.00	0.00	0.17 ± 0.03	0.16 ± 0.03
Vit D3	0.00	0.00	0.06 ± 0.04	0.00	0.73 ± 0.42	0.00	1.46 ± 0.31*
25(OH)D2	16.4 ± 6.2	14.9 ± 4.5	16.8 ± 3.5	164 ± 49	139 ± 21	314 ± 34	248 ± 9
25(OH)D3	27 ± 6	24 ± 2	105 ± 13*	209 ± 62	899 ± 172*	217 ± 17	924 ± 56*
1,25(OH) <sub>2</sub> D2	0.04 ± 0.01	0.09 ± 0.02	0.07 ± 0.02	12.20 ± 3.88	8.89 ± 1.20	2.29 ± 0.36	1.88 ± 0.22
1,25(OH) <sub>2</sub> D3	0.04 ± 0.01	0.04 ± 0.01	0.11 ± 0.02*	0.27 ± 0.09	1.65 ± 0.34*	0.08 ± 0.02	0.25 ± 0.08
24,25(OH) <sub>2</sub> D3	7.2 ± 0.5	8.4 ± 2.0	33.6 ± 4.9*	48.8 ± 13.8	260 ± 33*	27.4 ± 5.5	104 ± 20*

\**p* < 0.01 compared to control at same time point (Student's *t*-test).

TABLE 2 Rabbit tear vitamin D concentrations: 8 weeks Vit D3 supplemented.

Vitamin D metabolite	Control tear ( <i>n</i> = 6)		8 Week Vit D3 tear ( <i>n</i> = 6)	
	Mean (nM)	SE	Mean (nM)	SE
Vit D2	9.59	3.48	12.85	3.95
Vit D3	0	0	0	0
25(OH)D2	4.49	0.28	4.30	1.08
25(OH)D3	0.95	0.26	3.06*	0.36
1,25(OH) <sub>2</sub> D2	0.73	0.04	0.12	0.08
1,25(OH) <sub>2</sub> D3	0.01	0.01	0.01	0.01
24,25(OH) <sub>2</sub> D3	0.17	0.03	0.34*	0.06

\*Significantly different than control ( $p < 0.05$ , Student's *t*-test).

TABLE 3 Rabbit aqueous humor vitamin D concentrations: 8 weeks Vit D3 supplemented.

Vitamin D metabolite	Control aqueous humor ( <i>n</i> = 6)		8 week Vit D3 aqueous humor ( <i>n</i> = 12)	
	Mean (nM)	SE	Mean (nM)	SE
Vit D2	0.04	0.04	0.00	0.00
Vit D3	0.00	0.00	0.00	0.00
25(OH)D2	3.86	0.73	3.12	0.30
25(OH)D3	2.81	0.78	8.87*	1.67
1,25(OH) <sub>2</sub> D2	0.04	0.01	0.06	0.01
1,25(OH) <sub>2</sub> D3	0.01	0.01	0.01	0.01
24,25(OH) <sub>2</sub> D3	0.27	0.07	1.11*	0.22

\*Significantly different than control ( $p < 0.05$ , Student's *t*-test).

TABLE 4 Rabbit vitreous humor vitamin D concentrations: 8 weeks Vit D3 supplemented.

Vitamin D Metabolite	Control vitreous humor ( <i>n</i> = 6)		8 week Vit D3 vitreous humor ( <i>n</i> = 12)	
	Mean (nM)	SE	Mean (nM)	SE
Vit D2	0.00	0.00	0.00	0.00
Vit D3	0.00	0.00	0.00	0.00
25(OH)D2	1.31	0.12	1.01	0.10
25(OH)D3	0.50	0.15	0.79	0.11
1,25(OH) <sub>2</sub> D2	0.04	0.01	0.02	0.01
1,25(OH) <sub>2</sub> D3	0.00	0.00	0.00	0.00
24,25(OH) <sub>2</sub> D3	0.09	0.04	0.16	0.03

fluid and aqueous humor in parallel with the increase in plasma concentrations.

### Corneal Epithelial PCR and Vit D3 Production in Response to UV-B

Two UV-B doses were used to determine if HCLE cells can produce Vit D3 metabolites. These relatively low levels of UV-B exposure had no apparent effect on cell morphology or viability. At both UV-B doses there was a requirement that 7-dehydrocholesterol be present in the culture medium for there to be any significant increases in vitamin D metabolite concentrations. In the presence of exogenous 7-dehydrocholesterol, Vit D3, 25(OH)D3 and 24,25(OH)<sub>2</sub>D3 were significantly elevated in both UV-B dose groups (Tables 5 and 6). Doubling the UV-B dose resulted in a near doubling of the concentration of

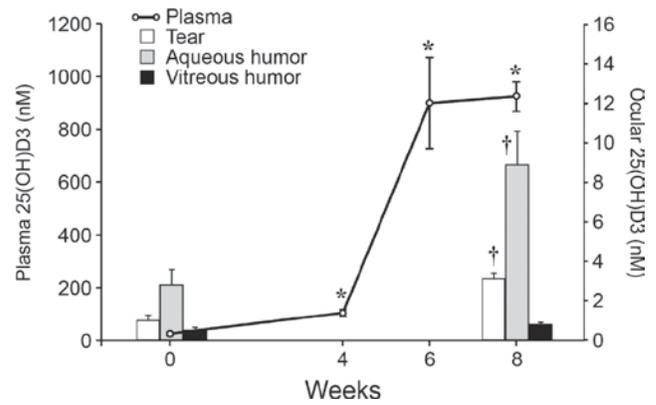


FIGURE 1 Plasma, tear, aqueous and vitreous humor concentrations of 25(OH)D3 in Vit D3 supplemented rabbits. Plasma was measured in the same rabbits at 0, 4, 6, and 8 weeks and ocular fluids measured at 0 and 8 weeks in different rabbits. \* $p < 0.001$  compared to time matched control (no supplement) and † $p < 0.001$  compared to no supplement.

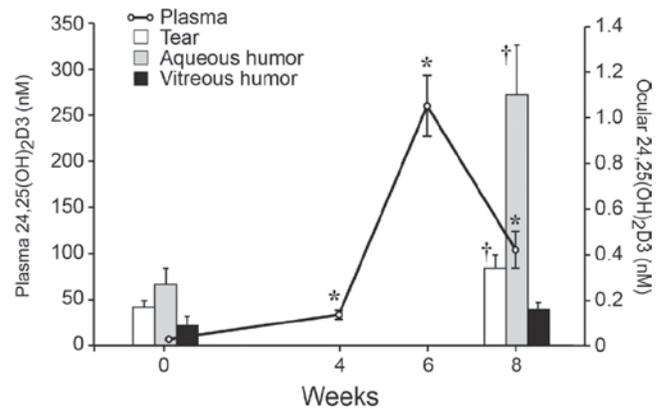


FIGURE 2 Plasma, tear, aqueous and vitreous humor concentrations of 24,25(OH)<sub>2</sub>D3 in Vit D3 supplemented rabbits. Plasma was measured in the same rabbits at 0, 4, 6, and 8 weeks and ocular fluids measured at 0 and 8 weeks in different rabbits. \* $p < 0.001$  compared to time matched control (no supplement) and † $p < 0.001$  compared to no supplement.

each metabolite. No significant changes were observed in the D2 metabolites or 1,25(OH)<sub>2</sub>D3. Reverse transcriptase PCR was positive for both VDR and 1 $\alpha$ -hydroxylase (Figure 3).

## DISCUSSION

The name vitamin D is a misnomer in that Vit D3 is both a hormone and a vitamin. It is a hormone because it can be synthesized in the skin from 7-dehydrocholesterol and circulates to act at a target different than its source, and a vitamin because most populations do not synthesize enough vitamin D by the *de novo* pathway. This study examined whether endogenous production of Vit D and its metabolites is possible in the eye, as well as the effects of dietary supplementation on Vit D metabolite levels in the eye.

TABLE 5 Human corneal limbal epithelial cell vitamin D production (nM) after 10 mJ/cm<sup>2</sup>/day UV-B exposure (*n* = 4).

Vitamin D metabolite	Culture medium-control	Culture medium UV	Culture Medium UV + 7-dehydrocholesterol
Vit D3	0.00	0.00	79 ± 14*
Vit D2	0.00	0.00	0.00
25(OH)D3	0.10 ± 0.02	0.10 ± 0.00	3.24 ± 0.60*
25(OH)D2	0.82 ± 0.04	0.77 ± 0.05	0.91 ± 0.01
1,25(OH) <sub>2</sub> D3	0.00	0.00	0.00
1,25(OH) <sub>2</sub> D2	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.00
24,25(OH) <sub>2</sub> D3	0.00	0.00	9.85 ± 2.21*

\*Significantly different than control and UV-B (*p* < 0.05, ANOVA).

TABLE 6 Human corneal limbal epithelial cell vitamin D production (nM) after 20 mJ/cm<sup>2</sup>/day UV-B exposure (*n* = 4).

Vitamin D metabolite	Culture medium-control	Culture medium UV	Culture medium UV + 7-dehydrocholesterol
Vit D3	0.00	0.00	142 ± 10*
Vit D2	0.00	0.00	0.00
25(OH)D3	0.10 ± 0.02	0.90 ± 0.01	5.78 ± 0.39*
25(OH)D2	0.82 ± 0.04	0.77 ± 0.05	0.74 ± 0.05
1,25(OH) <sub>2</sub> D3	0.00	0.00	0.00
1,25(OH) <sub>2</sub> D2	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
24,25(OH) <sub>2</sub> D3	0.00	0.00	13.4 ± 0.6*

\*Significantly different than control and UV-B (*p* < 0.05, ANOVA).

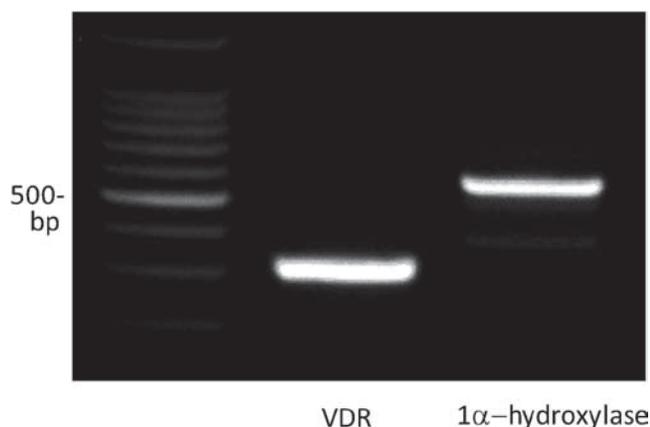


FIGURE 3 Reverse transcriptase PCR of cells from the human corneal limbal epithelium cell line showed positive bands for VDR and 1 $\alpha$ -hydroxylase mRNA.

Endogenous Vit D3 (cholecalciferol) is synthesized from 7-dehydrocholesterol following UV-B (270-315 nm) radiation exposure. 7-Dehydrocholesterol is a lipid incorporated into the bilayer of most cell membranes. The traditional systemic route for activation of Vit D3 is conversion to 25(OH)D3 in the liver followed by renal conversion to 1,25-(OH)<sub>2</sub>D3 by 1 $\alpha$ -hydroxylase. 25(OH)D3 can also be metabolized to 24,25(OH)<sub>2</sub>D3, which is found in target cells throughout the body and

its production is increased by 1,25(OH)<sub>2</sub>D3.<sup>9,10</sup> More recently, 1 $\alpha$ -hydroxylase activity has been found in such diverse tissues as the colon,<sup>11</sup> vascular smooth muscle,<sup>12</sup> and breast tissue.<sup>13</sup> A recent study from our laboratory measured significant Vit D3 metabolite concentrations in the aqueous humor and demonstrated the presence of both 1 $\alpha$ -hydroxylase and vitamin D receptor mRNA in the corneal epithelium.<sup>1</sup>

Rabbits have physiologically significant Vit D3 metabolism, and it is clear that sunlight can significantly elevate their serum 1,25(OH)<sub>2</sub>D3 levels.<sup>14</sup> Free range rabbits have a 1,25(OH)<sub>2</sub>D3 serum level of 34.3 pmol/L, whereas hutch raised rabbits fed similar diets have a serum value of 7.83 pmol/L. Free range rabbits also have significantly lower 1,25(OH)<sub>2</sub>D3 levels in the spring versus summer, while hutch rabbits have minimal seasonal variation. This is not unexpected, in that other fur-bearing animals, including horses<sup>15</sup> and sheep,<sup>16</sup> have shown similar variations. Laboratory NZW rabbits have control 1,25(OH)<sub>2</sub>D3 serum levels between 60 and 70 pg/mL,<sup>17,18</sup> while serum 25(OH)D3 levels have been measured at 34–68 ng/mL (85–170 nmol/L).<sup>17,19</sup> Plasma values from the current study are in agreement with these previously reported serum values. Vit D3 supplementation significantly increased all serum metabolite concentrations measured at 6 weeks as compared to baseline, and all but the 1,25(OH)<sub>2</sub>D3 concentration at 8 weeks. At 8 weeks 1,25(OH)<sub>2</sub>D3 was elevated, but that value was not significant. It is interesting that most 25(OH)D2 and 1,25(OH)<sub>2</sub>D2 levels were elevated in both control and supplemented rabbits, while Vit D2 was not elevated in any instance. This is likely the result of hydroxylated D2 being the most stable metabolite in the blood, as is 25(OH)D3, due to its association with vitamin D binding protein. The source of the Vit D2 in the rabbits was likely the rabbit chow. While the manufacturer (Harlon Laboratories, Inc., Indianapolis, IN, USA) does not routinely measure Vit D2 concentrations in its rabbit chow, isolated batch measurements have allowed them to estimate that the alfalfa meal component of the diet contributes Vit D2 at a level approximately one half that of Vit D3 (personal communication).

25(OH)D3 was elevated in tear and aqueous humor following Vit D3 supplementation. Interestingly, 25(OH)D2 was not elevated despite the almost 20-fold increase we measured in plasma. This points to possible selective transport of 25(OH)D3 in the anterior segment of the eye. Nearly all Vit D circulates in the bound form, primarily to Vit D binding protein (VDBP). In order to interact with the VDR, 25(OH)D2 and D3 must first enter the cell and be converted to 1,25(OH)<sub>2</sub>D2 and D3. It has become apparent that VDBP-bound 25(OH)D is taken up into target cells through endocytosis by the low density lipoprotein receptor family member megalin, along with its co-receptor cubilin.<sup>20,21</sup> It is known that VDBP has a higher binding affinity for Vit D3 and its metabolites as compared to those of Vit D2,<sup>22</sup> and this

may play a role in its selective transport. In addition, this weaker binding affinity would lead to a shorter half-life for Vit D2 metabolites,<sup>23</sup> decreasing their availability for transport into the anterior segment of the eye.

No correlation was found between 1,25(OH)<sub>2</sub>D3 levels and the levels of its substrate, 25(OH)D3. This is not surprising in that it is known that 1 $\alpha$ -hydroxylase activity is tightly regulated by parathyroid hormone (PTH) levels, cyclic adenosine monophosphate (cAMP), and phosphate deprivation,<sup>24</sup> and not as much by 25(OH)D3 levels.<sup>25,26</sup> A possible explanation for the relatively low 1,25(OH)<sub>2</sub>D3 values found in this study is that Vit D supplementation may have affected PTH, cAMP, and phosphate levels leading to low 1 $\alpha$ -hydroxylase activity.

Aqueous humor and tear 24,25(OH)<sub>2</sub>D3 levels were significantly elevated following Vit D supplementation, as were plasma 24,25(OH)<sub>2</sub>D3 levels. In the 8 week supplementation period plasma 24,25(OH)<sub>2</sub>D3 levels increased 15-fold, while tear and aqueous humor levels increased 2- and 4-fold, respectively. For many years after its discovery 24,25(OH)<sub>2</sub>D3 was considered to simply be a catabolite of 25(OH)D3, with catabolism of 25(OH)D3 to 24,25(OH)<sub>2</sub>D3 thought to only be involved in regulating 25(OH)D3 levels.<sup>27</sup> It is now clear that 24,25(OH)<sub>2</sub>D3 does indeed play physiological roles, including promotion of cell differentiation, injury repair, and bone mineralization.<sup>28,29</sup> Some of these effects are likely mediated through 24,25(OH)<sub>2</sub>D3-dependent activation of PKC.<sup>30,31</sup> While there is currently no evidence (pro or con) for physiological activity of 24,25(OH)<sub>2</sub>D3 in the eye, its presence in the aqueous humor, vitreous humor and tear fluid, as well as its apparently regulated increase in tears and aqueous humor following Vit D supplementation make this an interesting possibility.

HCLE cells exposed to UV-B light were able to synthesize 25(OH)D3 and 24,25(OH)<sub>2</sub>D3, although only when 7-dehydrocholesterol was added to the culture medium. No 1,25(OH)<sub>2</sub>D3 was present following UV-B exposure, nor were any Vit D2 metabolites. The lack of 1,25(OH)<sub>2</sub>D3 is interesting although not unexpected given our PCR results demonstrating 1 $\alpha$ -hydroxylase mRNA in the corneal limbal epithelial cell line used in this study. Despite the demonstrated presence and activity of 1 $\alpha$ -hydroxylase in a number of extrarenal cells, keratinocytes are the only cell type where synthesis of 1,25(OH)<sub>2</sub>D3 has been demonstrated.<sup>32,33</sup> A potential reason for the lack of 1,25(OH)<sub>2</sub>D3 in the current study is the extremely short half-life of this compound. While the serum half-life of 25(OH)D3 is approximately 3 weeks,<sup>34,35</sup> that for 1,25(OH)<sub>2</sub>D3 is on the order of 4-6 hours.<sup>36</sup> In part, this is the reason that 25(OH)D3 is the clinically accepted measurement for Vit D levels. It is also possible that 1 $\alpha$ -hydroxylase protein was not expressed in these cells or that its activity was altered by UV-induced inactive splice variants. Such variants have been observed following 20 mJ/cm<sup>2</sup> UV-B treatment of keratinocytes.<sup>33</sup>

Based on an earlier study by Ubels' group,<sup>7</sup> we expect that the low UV-B doses used in this study would result in little if any damage to the cells. The dose range is well below a typical ambient daily dose from 1 h sun exposure, and in fact the maximum daily UV-B dose used in the current study, 20 mJ/cm<sup>2</sup>/day, is equal to approximately 2 min direct sun exposure. In addition, only 10% of the cells in the Ubels study were apoptotic 6 h after exposure to a single 100 mJ/cm<sup>2</sup> UV-B dose compared to about 2% in controls, and UV-B at 50 mJ/cm<sup>2</sup> had no effect on mitochondrial potential after 6 h and only a minimal effect on caspase-8 activity.<sup>7</sup>

The requirement for exogenous 7-dehydrocholesterol for 25(OH)D3 synthesis by the corneal limbal epithelial cell line was unexpected. 7-Dehydrocholesterol is the immediate precursor of cholesterol in the cholesterol synthesis pathway, and corneas have been shown to be capable of synthesizing cholesterol.<sup>37</sup> On the other hand, it has been stated that 7-dehydrocholesterol is typically not detectable in corneas except for those with RSH/Smith-Lemli-Opitz syndrome,<sup>38</sup> a disease characterized by the loss of the enzyme required to convert 7-dehydrocholesterol to cholesterol. The cell line used for the current study apparently lacked the critical concentration of 7-dehydrocholesterol required to produce measurable Vit D3. The Vit D3 produced in the presence of 7-dehydrocholesterol was expected given that it is a simple chemical product of UV-B reacting with 7-dehydrocholesterol. Production of 25(OH)D3, on the other hand, required the presence of an active 25-hydroxylase, which has not been reported previously in the cornea or anterior segment of the eye. This 25(OH)D3 would be an additional source of 24,25(OH)<sub>2</sub>D3 if 24-hydroxylase is present in the anterior segment.

In summary, there are measurable concentrations of vitamin D metabolites in tear fluid and in aqueous and vitreous humor. Dietary Vit D supplementation leads to increases in tear and aqueous humor 25(OH)D3 and 24,25(OH)<sub>2</sub>D3 levels, with no measurable changes in 1,25(OH)<sub>2</sub>D3 levels or any levels in vitreous humor. In addition, the corneal limbal epithelium is capable of synthesizing 25(OH)D3 following UV-B exposure.

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