

# Vitamin D Enhances Corneal Epithelial Barrier Function

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**PURPOSE.** The purpose of this study was to determine whether 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) and/or its active metabolite, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), can enhance corneal epithelial barrier function. The authors also determined if corneas contain mRNA for the vitamin D receptor (VDR) and 1 $\alpha$ -hydroxylase, the enzyme required to convert 25(OH)D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub>, and measured vitamin D metabolite concentrations in aqueous and vitreous humor.

**METHODS.** RT-PCR was used to examine mouse, rabbit, and human corneal epithelial VDR and 1 $\alpha$ -hydroxylase mRNA. Vitamin D metabolites were measured using a selective vitamin D derivatizing agent and mass spectroscopy. Barrier function experiments were performed by measuring inulin permeability (IP) and/or transepithelial resistance (TER) in control, 25(OH)D<sub>3</sub>, and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated human and rabbit corneal epithelial monolayers cultured on permeable inserts. Ca<sup>2+</sup> was removed, then reintroduced to the culture medium while IP and TER readings were taken. Occludin levels were examined using Western blotting.

**RESULTS.** All corneal samples were positive for both VDR and 1 $\alpha$ -hydroxylase mRNA. All vitamin D metabolites except for unhydroxylated vitamin D<sub>3</sub> were detected in aqueous and vitreous humor. Epithelial cells showed increased TER, decreased IP, and increased occludin levels when cultured with 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>.

**CONCLUSIONS.** We conclude that corneas contain mRNA for VDR and 1 $\alpha$ -hydroxylase as well as significant vitamin D concentrations. 25(OH)D<sub>3</sub> and its active metabolite 1,25(OH)<sub>2</sub>D<sub>3</sub>, both enhance corneal epithelial barrier function. (*Invest Ophthalmol Vis Sci.* 2011;52:7359–7364) DOI:10.1167/iovs.11-7605

There are no published clinical or basic science studies examining the presence, metabolism, or physiological role of native vitamin D (Vit D) in the anterior segment of the eye. Given that >50% of the population may be suffering from Vit D insufficiency,<sup>1–3</sup> these are critically important topics for investigation. Low circulating Vit D levels could affect the levels of Vit D and its active metabolite, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), in the vitreous humor, aqueous humor,

and cornea. Anterior segment hypovitaminosis D could be particularly important after corneal injury, where Vit D may play a role in cell redifferentiation of the different cell layers during the wound healing process.

The classical systemic route for activation of Vit D is initial 25-hydroxylation to 25-dihydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) in the liver via cytochrome P-450 containing enzymes, followed by renal conversion to the active 1,25(OH)<sub>2</sub>D by 1 $\alpha$ -hydroxylase. 1,25(OH)<sub>2</sub>D can be subsequently degraded to 24R,25-dihydroxyvitamin D by 25-hydroxyvitamin D-24-hydroxylase, which is found in target cells throughout the body and is strongly induced by 1,25(OH)<sub>2</sub>D.<sup>4</sup> More recently, 1 $\alpha$ -hydroxylase activity has been found in such diverse tissue as the colon,<sup>5</sup> vascular smooth muscle,<sup>6</sup> and breast tissue.<sup>7</sup> Since the discovery that 1 $\alpha$ -hydroxylase is active in tissues outside of the kidney, it has become clear that Vit D acts as much more than a regulator of intestinal Ca<sup>2+</sup> absorption.

While the most thoroughly studied actions of Vit D are related to its management of calcium concentration in the plasma, 1,25(OH)<sub>2</sub>D has been found to induce keratinocyte differentiation and proliferation in the skin.<sup>8</sup> Studies in Vit D receptor (VDR) knockout mice determined that VDR is essential for normal keratinocyte stem cell function.<sup>9</sup> Vit D has also been found to affect extracellular matrix turnover and regulate growth factor release, in part through regulation of metalloproteinases.<sup>10</sup> In addition, the VDR signaling partner, retinoid X receptor (RXR), has been shown to play a significant role in the differentiation and maintenance of epithelial cells, as well as in the mediation of tight junction proteins and barrier function.<sup>11,12</sup> This barrier function mediation may be the result of the role Vit D plays in cell differentiation. Here we show that both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> are present in the eye, as are the VDR and 1 $\alpha$ -hydroxylase, and we demonstrate that 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> can both enhance the barrier function of corneal epithelial cells.

## MATERIALS AND METHODS

### Cell Culture

Primary New Zealand White rabbit corneal epithelium and a human cornea epithelium cell line (HCEC)<sup>13</sup> were grown on standard culture plates or on polycarbonate membranes in transwell plates (12 mm diameter insert, 0.4  $\mu$ M pore size; Costar, Cambridge, MA) until confluent. All cells were grown in Dulbecco's modified Eagle's medium/F12 medium (Gibco; Invitrogen, Carlsbad, CA) supplemented with FBS (20%), 1% ITS (BD Biosciences, Bedford, MA), and 40  $\mu$ g/mL gentamicin (Gibco). Primary mouse cornea epithelium was cultured by placing six corneas epithelium side up in a 60-mm dish (also containing 100 ng/mL cholera toxin) for 10 days, after which the corneas were removed and epithelial cells that had grown off the corneas were allowed to grow to confluence. The serum level was reduced to 10% after cells were observed to be growing off of the explant onto the culture dish. The corneal endothelium was removed with a cotton swab before placing corneas on the culture dishes. Primary epithelial cells had a cobblestone morphologic appearance in confluent cultures. Serum was

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removed from all cultures 24 hours before the start and for the duration of all Vit D protocols. 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 Visual Research.

### RT-PCR

Reverse-transcriptase-PCR was used to examine mouse, rabbit, and HCEC VDR and  $1\alpha$ -hydroxylase mRNA. Fresh rabbit corneal epithelium was removed from the eyes of euthanized rabbits using a Gil knife. For cultured rabbit, mouse, and human epithelium, cells were grown on standard culture plates as describe above. Total RNA was extracted using Trizol. All 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) to eliminate genomic DNA contamination. Rabbit VDR and  $1\alpha$ -hydroxylase primers (Table 1) were designed based on the partial National Center for Biotechnical Information (NCBI) Reference Sequence: XM\_002723443.1 and homogenomic sequences with other species. Mouse VDR and  $1\alpha$ -hydroxylase primers were designed based on sequences NCBI D<sub>3</sub>1969 and AF235021, with the VDR primer spanning introns 8-9 and 9-10, while the  $1\alpha$ -hydroxylase primer spans introns 6-7, 7-8, and 8-9, respectively. Primers are listed in Table 1. Primers for HCEC VDR and  $1\alpha$ -hydroxylase (Table 1) were copied from previously published reports.<sup>14,15</sup> An RT-PCR system (SuperScript III One-Step RT-PCR System; Invitrogen) was used to perform the RT-PCR protocol. The conditions of amplification were: cDNA synthesis at 55°C for 90 minutes, pre-denaturation at 94°C for 90 seconds followed by 38 cycles at 94°C for 35 seconds, 55°C for 40 seconds, 72°C for 30 seconds, followed by maintenance at 72°C for 10 minutes and storage at 4°C. Aliquots of the amplified products and 1 kb or 100 base pair DNA ladders were separated on a 1.2% agarose gel and visualized by ethidium bromide staining.

### Mass Spectroscopy

Mass spectroscopy was used to confirm the presence of and to quantify Vit D metabolites in the aqueous and vitreous humor. New Zealand White rabbits were killed and eyes were enucleated. Aqueous humor was drawn from the anterior chamber and immediately put on ice in the dark, and the anterior segment was dissected from the globe to collect vitreous humor. The low volume of aqueous humor necessitated pooling of samples from both eyes of each rabbit ( $n = 7$ ), while vitreous humor samples did not need to be pooled ( $n = 16$ ). Samples were frozen at -80°C before mass spectroscopy analysis. Assessment of vitamin D status was performed utilizing an ultra performance liquid chromatography (UPLC)-MS/MS method using the Diels-Alder reaction.<sup>16</sup> This method uses a selective derivative that dramatically improves the sensitivity of LC-MS for Vit D metabolites. This method

allowed for quantification of vitamin D<sub>2</sub> and 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 24R,25-dihydroxyvitamin D<sub>3</sub>. Vitamin D<sub>2</sub> and D<sub>3</sub> forms were distinguished by different transitions in UPLC-MS/MS detection. A benefit of the Diels-Alder reaction is a detection limit for most of the vitamin D metabolites of 25 pg/mL. As previously described, samples were pretreated before conducting the LC-MS analysis by precipitating proteins, liquid-liquid extraction of analytes and selective chemical derivatization of analytes with 4-phenyl-1,2,4-triazolo-3,5-dione (PTAD) using Diels Alder chemistry. This was followed by chromatographic separation using ultra performance liquid chromatography (UPLC) with detection by tandem quatropole mass spectrometry using positive electrospray ionization.<sup>17</sup>

### Transepithelial Resistance Measurements

Rabbit corneal epithelial cells (passage 2) and HCEC were grown on transwell plates until confluent. Transepithelial resistance (TER) measurements were made (World Precision Instruments, Sarasota, FL; EVOM instrument). Measurements were made after supplementing the culture medium with 25(OH)D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub>, or after removing and then replacing Ca<sup>2+</sup> in the culture medium. For the supplement study, three groups were analyzed (four wells per group): control, 25(OH)D<sub>3</sub> (100 nM), and 1,25(OH)<sub>2</sub>D<sub>3</sub> (2 nM). For the Ca<sup>2+</sup> reversal studies, when cells reached TER values greater or equal to 300 ohm × cm<sup>2</sup>, cells were serum-starved and 25(OH)D<sub>3</sub> (100 nM) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (2 nM) was added to the wells ( $n = 3$  each) 24 hours before removing Ca<sup>2+</sup>. Control cells had no Vit D. EGTA (5 mM) was then added to apical and basal wells for 10 minutes, and another TER measurement was taken. Wells were then washed twice and serum-free plus an identical Vit D medium was returned to the wells with the specified test compounds and additional TER measurements were taken.

### Inulin Permeability

Inulin permeability was measured simultaneously with TER. HCEC were grown until confluent and FITC-inulin was used as a tracer. Cells were exposed to a Ca<sup>2+</sup> switch as detailed above while monitoring TER. Inulin was added to the basal well 1 hour before the calcium switch. When TER reached a level no lower than 35% of that before the Ca<sup>2+</sup> switch, test solutions were added to both wells, with inulin still included in the basal well. Inulin flux was measured at 1 hour increments in parallel with TER for a total of 6 hours.

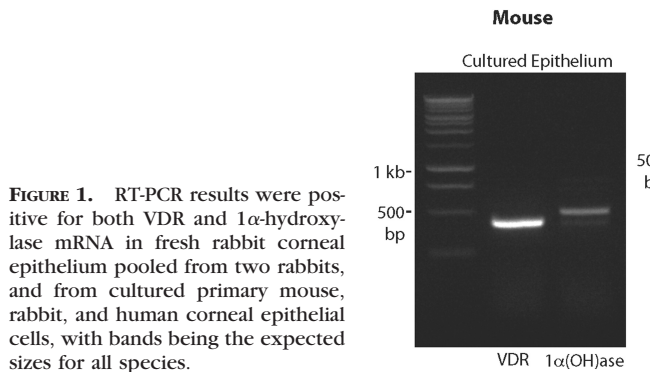
### Western Blot Analysis

Western blot studies were carried out on HCEC. HCEC cells were grown on six-well plates until confluent. Cells were serum-starved and 25(OH)D<sub>3</sub> (10 nM) or 1,25(OH)<sub>2</sub>D<sub>3</sub> (10 nM) was added to the wells ( $n = 3$ ) 24 hours before adding EGTA (5 mM) to the wells (10 minutes). Wells were washed twice with PBS and serum-free media, and Vit D medium was returned to the wells. After 22 hours, cells were

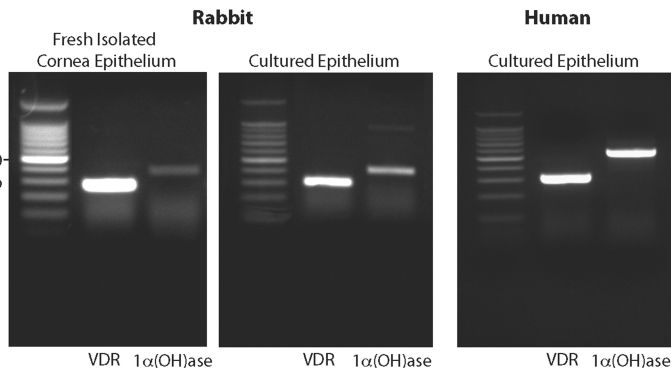
TABLE 1. Vitamin D Receptor and  $1\alpha$ -hydroxylase PCR Primer Sequences

| Species | Target                         | Primer Sequence                        |
|---------|--------------------------------|--|
| Mouse   | VDR forward                    | (917)-5'-CCAACCAAGTCTTTTACCATTGGATG-3' |
|         | VDR reverse                    | (1336)-5'-TGAGCTTCATGCTGTTCTCCG-3'     |
|         | $1\alpha$ -hydroxylase forward | (1024)-5'-GCACTCCACTCTGAGATCACAG-3'    |
|         | $1\alpha$ -hydroxylase reverse | (1486)-5'-CTGTAGATTGATGCTCCTCTCAGG-3'  |
| Rabbit  | VDR forward                    | 5'-ACC CTA CCT ATG CCG ACT TCT-3'      |
|         | VDR reverse                    | 5'-TTC TGG ATG CTG TAG CTG ACC-3'      |
|         | $1\alpha$ -hydroxylase forward | 5'-GGC ACT CCA CTC TGA GAT CAC-3'      |
|         | $1\alpha$ -hydroxylase reverse | 5'-ATC TGC AGC TCT AGC TCT GCT-3'      |
| Human*  | VDR forward                    | 5'-ATGCCATCTGCATCGTCTC-3'              |
|         | VDR reverse                    | 5'-GCACCCGACAGGCTGTCCTA-3'             |
|         | $1\alpha$ -hydroxylase forward | 5'-ACGCTGTTGACCATGGC-3'                |
|         | $1\alpha$ -hydroxylase reverse | 5'-GTGACACAGAGTGACCAGCGTAT-3'          |

\* Sequences obtained from References 14 and 15.



**FIGURE 1.** RT-PCR results were positive for both VDR and 1 $\alpha$ -hydroxylase mRNA in fresh rabbit corneal epithelium pooled from two rabbits, and from cultured primary mouse, rabbit, and human corneal epithelial cells, with bands being the expected sizes for all species.



harvested using 95°C lysis buffer D (0.3% sodium dodecyl sulfate, 10 mM Tris/HCL, 10  $\mu$ M sodium orthovanadate, 100  $\mu$ M sodium fluoride and protease inhibitor). The mixture was sonicated in ice water then heated to 95°C for 10 minutes, centrifuged at 14,000 rpm (4°C) for 10 minutes and the supernatant was collected.

Sample protein concentrations were measured using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (20  $\mu$ g) were loaded onto an 8% gel and separated by SDS-PAGE. Mouse anti-occludin, (Zymed, Invitrogen), and a horse-radish peroxidase-conjugated goat anti-mouse secondary antibody along with enhanced chemiluminescence were used for detection of these tight junction-associated proteins. For loading controls, membranes were stripped and reprobed with  $\beta$ -actin antibody (CP01; Calbiochem). Western blot analyses were digitally photographed, and blot density was determined by using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

**Statistical Analysis**

Analyses between individual samples were performed using a Student's *t*-test. Analyses between multiple groups were performed using Student-Newman-Keuls (SNK) ANOVA analysis. *P* values <0.05 were considered significant.

**RESULTS**

**RT-PCR**

RT-PCR results were positive for both VDR and 1 $\alpha$ -hydroxylase mRNA in all cells and tissue examined, with bands being the expected sizes for all species (Fig. 1).

**Vitamin D Concentrations**

Aqueous and vitreous humor Vit D concentrations are listed in Table 2. The quantitation limit (LOQ) for the instrument is 25 pg/mL, thus any readings below this are listed as 0 nM.

**TABLE 2.** Rabbit Aqueous and Vitreous Humor Vitamin D Concentrations

|                                       | Aqueous Humor<br>(n = 7) |      | Vitreous Humor<br>(n = 16) |      |
|---------------------------------------|--------------------------|------|----------------------------|------|
|                                       | Mean (nM)                | SE   | Mean (nM)                  | SE   |
| Vit D <sub>2</sub>                    | 0.06                     | 0.01 | 0.06                       | 0.01 |
| Vit D <sub>3</sub>                    | 0.00                     | 0.00 | 0.00                       | 0.00 |
| 25(OH)D <sub>2</sub>                  | 1.37                     | 0.28 | 0.12                       | 0.02 |
| 25(OH)D <sub>3</sub>                  | 1.60                     | 0.32 | 0.12                       | 0.02 |
| 1,25(OH) <sub>2</sub> D <sub>2</sub>  | 0.07                     | 0.01 | 0.10                       | 0.05 |
| 1,25(OH) <sub>2</sub> D <sub>3</sub>  | 0.04                     | 0.01 | 0.07                       | 0.07 |
| 24,25(OH) <sub>2</sub> D <sub>3</sub> | 1.64                     | 0.38 | 0.01                       | 0.01 |

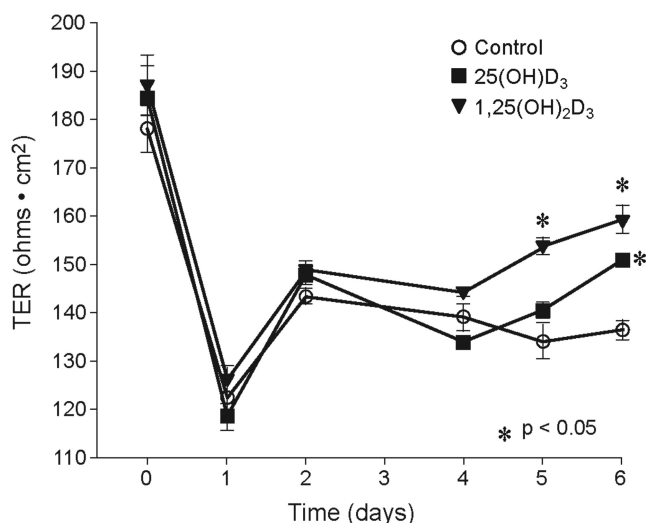
**Barrier Function**

Significant increases in TER were observed in cultured rabbit epithelial cells after addition of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> to the culture medium (Fig. 2). For all groups in this experiment, TER dropped immediately after adding the new serum-free Vit D supplemented medium, and then increased steadily over time. 1,25(OH)<sub>2</sub>D<sub>3</sub> TER was significantly greater than control at 5 and 6 days, while 25(OH)D<sub>3</sub> TER was significantly greater than control only at Day 6 (ANOVA + SNK test).

In experiments where Ca<sup>2+</sup> was removed from the culture medium and then replaced to allow for tight junction disruption and reformation, inulin flux was decreased in human cornea epithelial cells treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> at all treatment times except 5 hours, while 25(OH)D<sub>3</sub>-treated cells had lower flux values at 2 and 6 hours (Fig. 3A; SNK). Simultaneous TER results are shown in Figure 3B. 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> had significantly higher TER values at all time points after 10 minutes, and 1,25(OH)<sub>2</sub>D<sub>3</sub> TER was significantly higher than 25(OH)D<sub>3</sub> at those same time points (SNK).

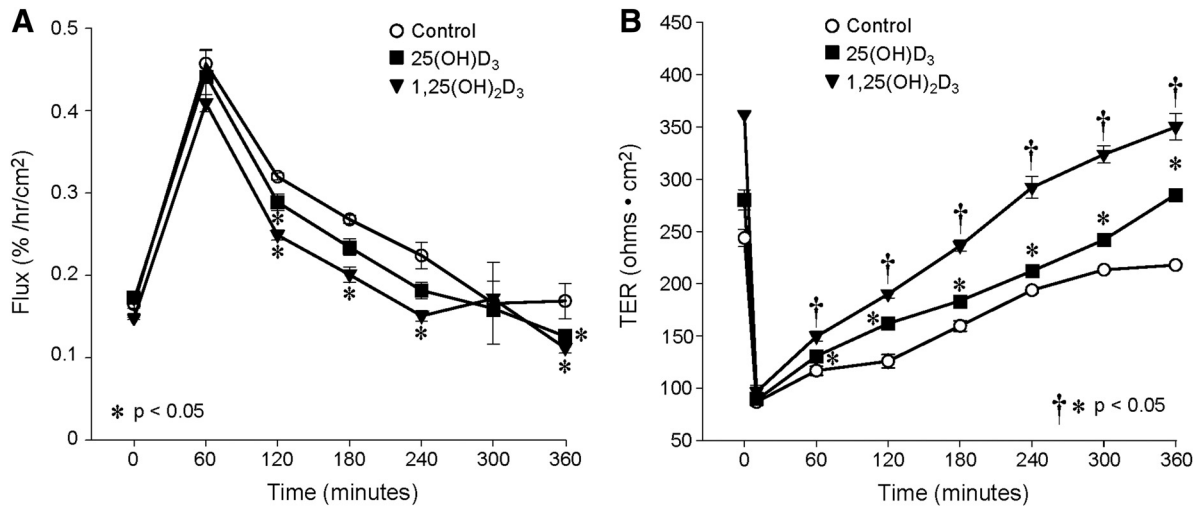
**Western Blot Analysis**

The tight junction-associated protein occludin was examined in cultured HCEC. HCEC exposed to both 25(OH)D<sub>3</sub>,



**FIGURE 2.** Changes in TER of cultured rabbit epithelial cells after addition of 25(OH)D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> to the culture medium. For all groups, TER dropped after adding the new medium, and then increased steadily over time. 1,25(OH)<sub>2</sub>D<sub>3</sub> TER was significantly greater than control at 5 and 6 days, while 25(OH)D<sub>3</sub> TER was significantly greater than control only at Day 6 (ANOVA and SNK comparison test).





**FIGURE 3.** (A) Inulin flux is decreased after  $\text{Ca}^{2+}$  reversal in human corneal epithelial cells treated with  $1,25(\text{OH})_2\text{D}_3$  at all treatment times except 5 hours, while  $25(\text{OH})\text{D}_3$ -treated cells had lower flux values at 2 and 6 hours (ANOVA and SNK comparison test). (B) TER results run in tandem with the inulin experiment shown in (a).  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  had significantly higher TER values at all time points after 10 minutes, and  $1,25(\text{OH})_2\text{D}_3$  TER was significantly higher than  $25(\text{OH})\text{D}_3$  at those same time points (ANOVA and SNK comparison test).

and  $1,25(\text{OH})_2\text{D}_3$  showed significantly elevated occludin expression (Fig. 4; Student's *t*-test).

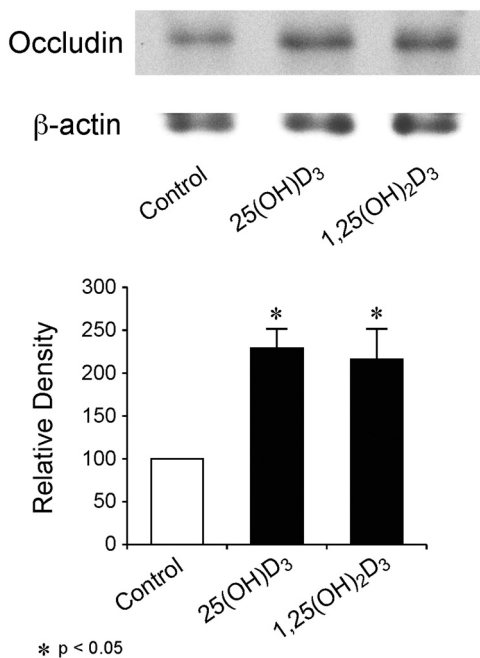
## DISCUSSION

In this study we show that cultured corneal epithelial cells from mouse, rabbit, and human contain both VDR and  $1\alpha$ -hydroxylase. Freshly isolated rabbit epithelium was also positive for VDR and  $1\alpha$ -hydroxylase, demonstrating that these findings are not just a culture artifact. These results add to the increasing number of cell types found to have the capability of activating and binding active Vit D. We also provide measure-

ments of Vit D<sub>2</sub> and D<sub>3</sub> metabolites in aqueous and vitreous humor and demonstrate that both  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  enhance barrier function in these cells.

To date, only a handful of studies have examined Vit D and/or its receptors in the cornea and anterior segment. All of these corneal studies limited their focus to the VDR and immune responses from externally applied  $1,25(\text{OH})_2\text{D}_3$ . In the rat eye, the VDR was immunolocalized to the inner and outer layers of the ciliary body epithelium (CBE) and was also present in the corneal epithelium. There was also sparse distribution in the corneal stroma.<sup>18</sup> In the human eye, VDR was immunolocalized to the CBE and lens epithelium, with weak focal staining in the corneal endothelium and basal epithelium.<sup>19</sup> In addition,  $1,25(\text{OH})_2\text{D}_3$  binding has been observed in the lacrimal gland epithelium, Harderian gland, nonpigmented CBE, and corneal epithelium of the lizard *Anolis carolinensis*.<sup>20</sup> Our study shows VDR mRNA in whole mouse cornea as well as cultured mouse, rabbit, and human corneal epithelium, and importantly  $1\alpha$ -hydroxylase mRNA in these same cells.

From a functional viewpoint, topical  $1,25(\text{OH})_2\text{D}_3$  administration was previously found to suppress ocular surface inflammation, and to a lesser extent neovascularization, by inhibiting Langerhan cell migration into mouse corneas.<sup>21</sup> This effect was attributed to the inhibition of the production of several cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-8) after addition of  $1,25(\text{OH})_2\text{D}_3$  to cultured corneal epithelial cells.<sup>22</sup> A similar study determined that suppression of *Pseudomonas aeruginosa* induced IL-1 $\beta$ , IL-6, and IL-8 production in cultured human corneal epithelial cells.<sup>23</sup> Gipson's group studied VDR-ablated mice and found altered conjunctival goblet cell mucin packaging that could be restored to normal by restoring depleted ionized calcium levels in these mice.<sup>24</sup> Most recently, the results of an ancillary study of the Woman's Health Initiative suggested that high  $25(\text{OH})\text{D}_3$  concentrations may protect against early age-related macular edema in women younger than 75 years.<sup>25</sup> In addition, a provocative study in the zebrafish model indicated that nonsense mutations in low density lipoprotein receptor-related protein 2 (Lrp2), also called Megalin, can result in high intraocular pressures and severe myopia with obviously enlarged eye globes and retinal ganglion cell damage. Lipoprotein receptor-related protein 2 participates in receptor-mediated endocytosis and is thought to be one of the primary proteins



**FIGURE 4.** (Top) Representative occludin Western blot analysis from HCEC exposed to both  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  show elevated occludin levels compared with control. (Bottom) Bar graph indicates blot densities ( $n = 3$ , mean  $\pm$  SEM, \* $P < 0.05$ ).

responsible for the transport of Vit D and/or the Vit D binding protein complex into cells.<sup>26</sup> The present study is the first to demonstrate direct functional activity of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> on corneal cells.

1,25(OH)<sub>2</sub>D<sub>3</sub> has previously been shown to enhance barrier function in a small intestinal epithelial cell line (Caco-2),<sup>27,28</sup> while on the other hand it decreased transepithelial resistance in a duodenal epithelium model.<sup>29</sup> Here we show that both 1,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub> enhance barrier function, leading to increased TER and decreased inulin permeability in human corneal epithelial monolayers as well as increased expression of the tight junction-associated protein occludin. TER was also elevated in cultured rabbit epithelial cells supplemented with 1,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub>. This is the first study demonstrating any 25(OH)D<sub>3</sub> effect on barrier function. Because of the demonstrated presence of 1 $\alpha$ -hydroxylase in the corneal epithelium, we hypothesize that the 25(OH)D<sub>3</sub> effect is being mediated through its active metabolite, 1,25(OH)<sub>2</sub>D<sub>3</sub>. These results may be the result of Vit D influencing the differentiation state on the epithelium and with it the degree of tight junctional integrity, or Vit D directly affecting tight junction protein control; the current results cannot differentiate between these two possibilities. In addition, all functional experiments carried out in this study were performed in serum-starved conditions to optimize sensitivity to Vit D.<sup>30</sup> It is possible that the observed barrier response represents Vit D being protective of the stress introduced by serum starving the cells. The overall results of these studies indicate that dietary Vit D can influence the physiological functions of the cornea.

The aqueous humor supplies the cornea with nutrients and bathes the entire anterior segment of the eye, while the vitreous humor bathes the posterior segment of the eye and acts as a reservoir for numerous substances, possibly including vitamin D metabolites. The measurements of vitamin D metabolites in the aqueous and vitreous humor in this study are the first such measurements in any species. We were unable to detect any unhydroxylated vitamin D<sub>3</sub> (Vit D<sub>3</sub>) in either aqueous or vitreous humor. This is not unexpected, because Vit D<sub>3</sub> is the byproduct of 7-dehydrocholesterol exposure to UV-B radiation, and these rabbits never receive any significant UV-B exposure. Given that fact, the vast majority of vitamin D metabolites measured in the aqueous and vitreous humor must originate from dietary intake and transport from the blood stream. This would be particularly true for Vit D<sub>2</sub> and its metabolites, which are only obtained through the diet. It is interesting that aqueous humor 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> levels are so much higher in aqueous humor compared with vitreous humor, while 1,25(OH)<sub>2</sub>D<sub>2</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> levels are comparable in the two compartments. It is possible that there is a specialized transporter directing 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> into the aqueous humor, although these species may move into the vitreous humor only by diffusion, possibly from the aqueous humor. On the other hand, the vitreous compartment may have more active 1 $\alpha$ -hydroxylase activity, resulting in similar, if not higher 1,25(OH)<sub>2</sub>D<sub>2</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> levels than the aqueous compartment. The high level of 24R,25-dihydroxyvitamin D<sub>3</sub> in the anterior chamber is likely the direct result of 25-hydroxyvitamin D-24-hydroxylase activity, which might in turn be another explanation for the a relatively low 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration in the aqueous humor. Because the primary epithelial barrier is located on the apical portion of the multilayered corneal epithelium,<sup>31</sup> Vit D metabolites should be accessible from both the aqueous compartment via the transcellular route and directly from the tear compartment if they are indeed also in tear. Endothelial cells on the other hand, which were not examined in this study, would be accessible primarily from the aqueous compartment

in intact corneas. This could change after damage or breakdown of the epithelial barrier.

25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> are commonly used analytical surrogates for the biologically active 1,25(OH)<sub>2</sub>D compounds. This is because 25(OH)D is much easier to analyze due to its relatively higher concentration in biological samples. The data presented here on relative levels of 25(OH) and 1,25(OH)<sub>2</sub>D<sub>2</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> levels caution that 25(OH) compounds may not be good indicators of the biologically active metabolites.

In conclusion, we have demonstrated that corneal epithelial cells contain VDR and 1 $\alpha$ -hydroxylase mRNA, and that there are significant vitamin D metabolite concentrations in both the aqueous and vitreous humor. In addition, we show that both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> can enhance cornea epithelial barrier function and vitamin D supplementation enhances corneal occludin expression.

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