Vitamin D Activation and Function in Human Corneal Epithelial Cells During TLR-Induced Inflammation

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PURPOSE. Vitamin D is recognized to be an important modulator of the immune system. In the eye, studies have shown that deficiencies and genetic differences in vitamin D-related genes have a significant impact on the development of various ocular diseases. Our current study examines the ability of human corneal epithelial cells (HCEC) to activate vitamin D and the effect of vitamin D treatment on antimicrobial peptide production and cytokine modulation during inflammation, with the ultimate goal of using vitamin D therapeutically for corneal inflammation.

METHODS. Human corneal epithelial cells were treated with 10^{-7} M vitamin D₃ (D₃) or 25-hydroxyvitamin D₃ (25D₃) for 24 hours and 1,25-dihydroxyvitamin D₃ (1,25D₃) detected by immunoassay. Human cathelicidin (LL-37) expression was examined by RT-PCR, immunoblot, and immunostaining following 1,25D₃ treatment and antimicrobial activity of 1,25D₃-treated cells was determined. Cells were stimulated with TLR3 agonist polyinosinic-polycytidylic acid (Poly[I:C]) for 24 hours and cytokine levels measured by RT-PCR, ELISA, and Luminex. Immunostaining determined expression of vitamin D receptor (VDR) and retinoic acid inducible gene-1 receptor (RIG-1) as well as NF-κB nuclear translocation.

RESULTS. When treated with inactive vitamin D metabolites, HCEC produced active 1,25D₃, leading to enhanced expression of the antimicrobial peptide, LL-37, dependent on VDR. 1,25D₃ decreased the expression of proinflammatory cytokines (IL-1β, IL-6, TNFα, and CCL20) and MMP-9 induced by Poly(I:C) as well as pattern recognition receptor expression (TLR3, RIG-1, MDA5). However, early activation of NF-κB was not affected.

CONCLUSIONS. These studies demonstrate the protective ability of vitamin D to attenuate proinflammatory mediators while increasing antimicrobial peptides and antipseudomonas activity in corneal cells, and further our knowledge on the immunomodulatory functions of the hormone.

Keywords: vitamin D, cornea, inflammation, Toll-like receptors, antimicrobial

Vitamin D is a multifunctional hormone that exerts its effects on many tissues and cell types, in addition to its traditional role in calcium homeostasis. Vitamin D is obtained through diet and supplementation, but it also is produced in the skin when exposed to sunlight.^{1,2} Vitamin D must undergo two enzymatic steps to become biologically active.³ The first reaction occurs mainly in the liver, where vitamin D is hydroxylated by the cytochrome p450 enzymes CYP2R1 or CYP27A1, to produce the major circulating form, 25-hydroxyvitamin D₃ (25D₃).⁴ 25-Hydroxyvitamin D₃ then is further modified through 1-α-hydroxylation (CYP27B1) to form the active 1,25-dihydroxyvitamin D₃ (1,25D₃).^{5,6} This activation now is known to occur not only in the kidneys, the site of traditional activation, but also in other tissues, where vitamin D is able to influence a variety of cellular functions.⁷ Active 1,25D₃ binds to its nuclear hormone receptor, the vitamin D receptor (VDR), which then interacts with vitamin D response elements (VDRE) to modulate gene transcription.^{8,9} Activity of 1,25D₃ is regulated by 24-hydroxylation (carried out by CYP24A1), which inactivates the hormone.

In the eye, diverse studies show that vitamin D protects against autoimmune uveitis,¹⁰ restricts retinoblastoma growth^{11,12} and angiogenesis,¹³ stimulates retinal glial cell

phagocytosis,¹⁴ and is associated with a decreased risk of age-related macular degeneration^{15–20} and myopia development.^{21–23} Vitamin D also has been shown to decrease amyloid β plaques in the aging retina²⁴ and lower IOP in nonhuman primates.²⁵ However, relatively little is known about vitamin D function or availability at the ocular surface, including the cornea, which provides protection and a refractive surface. Recent reports demonstrate that a human limbal epithelial cell line can produce vitamin D₃ de novo following ultraviolet B (UVB) exposure and that vitamin D metabolites are present in the aqueous and vitreous humor and tears of rabbits.^{26,27} The widely expressed VDR has been identified in the human corneal epithelium and endothelium, lens, ciliary body, and RPE, as well as the ganglion cell layer and retinal photoreceptors in the eye.²⁸ In addition, several vitamin D hydroxylases are expressed in cultured human corneal epithelial cells and fibroblasts, RPE cells, and nonpigmented ciliary body epithelial cells,^{27,29} suggesting that the cornea and other ocular tissues have the machinery to activate and regulate vitamin D metabolism on their own and that vitamin D is an important biological mediator in the eye.

One of the key roles of vitamin D is immune system regulation. Epidemiologic studies demonstrate that vitamin D

deficiency increases susceptibility to various infections and vitamin D levels are associated with a range of chronic diseases, including several autoimmune conditions.^{30,31} Vitamin D modulates innate and adaptive immune responses through the production of antimicrobial peptides,³² regulation of antigen presenting cell differentiation and maturation,³³ and direct action on T-cell function, enhancing development of a Th2 response.³⁴ In addition, vitamin D influences the expression and activity of various pattern recognition receptors (PRR), including Toll-like receptors (TLR).^{1,35–37} Toll-like receptors are a family of highly conserved PRRs that are activated in response to microbial and endogenous ligands.^{38,39} Vitamin D also increases CD14 expression, a coreceptor that mediates the response to bacterial antigens in coordination with several other proteins, including TLR2 and TLR4.^{40–42}

An important mechanism for innate immune influence came with the discovery that vitamin D directly induces human cathelicidin antimicrobial peptide (hCAP) expression via a VDRE located in the promoter region of the cathelicidin gene.³² While first identified as an antimicrobial peptide, human cathelicidin (LL-37), the functional cleavage product of hCAP, is now recognized to be a multifunctional peptide, with important immunomodulatory properties, in addition to killing microbes.^{43–45} Human cathelicidin LL-37 neutralizes lipopolysaccharide (LPS) signaling directly^{46,47} and indirectly,⁴⁸ dampening inflammation caused by infection. This peptide also influences the response to other TLR ligands^{48–50} and cytokines,^{51,52} demonstrating its capacity to modulate innate immunity. Human cathelicidin LL-37 is expressed by human corneal epithelial cells and is upregulated during inflammation, infection, and injury to the ocular surface.^{45,53,54}

The cornea is the transparent tissue covering the eye that functions to protect the rest of the eye from damage and to refract light for vision. Inflammation during infection and injury must be regulated carefully in this tissue to prevent damage and disruption of corneal transparency, vitally important for vision. Because of vitamin D's known role in immunoregulation, we investigated the functional activity of vitamin D in human corneal epithelial cells (HCEC). We demonstrated that HCEC are able to activate vitamin D, inducing target gene expression through the VDR. We also showed that vitamin D is able to enhance antimicrobial peptide production and interact with TLR stimulation to decrease inflammatory mediators, important aspects in protecting the cornea from infection and damage.

MATERIALS AND METHODS

Human Corneal Cells and VDR Small Interfering RNA (siRNA) Treatment

Human cadaveric donor corneas were obtained from Heartland Lions Eye Bank (St. Louis, MO, USA) within 1 week of death and primary HCEC were isolated by enzymatic digestion (Dispase II; Roche Diagnostics, Indianapolis, IN, USA) as described previously.⁵⁵ Cells were maintained in EpiLife medium with defined growth supplement (Invitrogen, Grand Island, NY, USA), 60 μ M calcium chloride, and penicillin-streptomycin (5000 U/mL; 5000 μ g/mL). In addition, scraped epithelial cells and freshly isolated keratocytes were collected from donor corneas and lysed for RNA collection.⁵⁶ Human telomerase-immortalized corneal epithelial cells (hTCEpi) were cultured in KGM-2 medium (Lonza, Allendale, NJ, USA)⁵⁷ and SV40-immortalized HCECs were maintained in supplemental hormonal epithelium medium (SHEM) as described previously.⁵⁸ All cells were grown to approximately 80% confluency before use in

experiments. Human corneal epithelial cells were stimulated with 10^{-7} M vitamin D compounds (D_3 and $1,25D_3$; Sigma-Aldrich Corp., St. Louis, MO, USA; $25D_3$, EMD Millipore, San Diego, CA, USA), or TLR agonists (1 μ g/mL Pam3CSK4 [TLR1/2], FSL1 [TLR6/2], Poly[I:C] [TLR3], Flagellin *Salmonella typhimurium* [TLR5], or 10^8 cells/mL HKLM [TLR2], or 50 μ g/mL zymosan [TLR2: Invivogen, Inc., San Diego, CA, USA]) for 24 hours, unless otherwise specified.

To examine the effect of VDR silencing, negative control siRNA and VDR-specific siRNA (s14777 and s14779) were purchased from Life Technologies (Grand Island, NY, USA). 10 nM siRNAs were transfected into hTCEpi in 12-well plates using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Vitamin D receptor knock-down was confirmed by RT-PCR and Western analysis at 24, 48, and 72 hours after transfection. Cells were stimulated with $1,25D_3$ 24 hours after transfection.

Quantitative Real-Time PCR

Relative expression of vitamin D hydroxylases, VDR, LL-37, and CD14 in cultured cells was determined by real-time RT-PCR analysis. Total RNA was extracted from cell samples using RNeasy kits (Qiagen, Valencia, CA, USA) and reverse transcribed using the Optimax First Strand cDNA Synthesis Kit (BioChain, Newark, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed using intron-spanning primers (see Table) and Brilliant II SYBR Green QPCR master mix (Agilent Technologies, Santa Clara, CA, USA). Products were amplified with a Mx3005P QPCR System (Agilent Technologies) with the following program: 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 1 minute at 58°C, and 1 minute at 72°C. Dissociation curve analysis confirmed primer specificity and no template/no RT samples were used for controls. Samples were analyzed using the $\Delta\Delta C_t$ method. Briefly, C_t values for the housekeeping gene, *GAPDH*, were subtracted from C_t values of the gene of interest (ΔC_t). The ΔC_t values for untreated controls were subtracted from the ΔC_t for the treated samples ($\Delta\Delta C_t$). Messenger RNA (mRNA) fold change was determined using $2^{\Delta\Delta C_t}$.

Immunoblot Analysis for LL-37, CYP24A1, and VDR Expression

The LL-37 protein was detected in hTCEpi culture supernatants from control cells and cells stimulated with $25D_3$ or $1,25D_3$ (10^{-7} M) for 24 hours by dot blot as reported previously,⁵⁹ using a C-terminal rabbit anti-LL-37 antibody (1:5000, donated by Fu Shin Yu, PhD, Wayne State University, sourced from Panatecs, Heilbronn, Germany). Expression of CYP24A1 and VDR was detected in whole cell lysates by SDS-PAGE followed by Western blot analysis. Briefly, cells were collected in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Roche, Nutley, NJ, USA), lysed on ice for 15 minutes, vortexed, and centrifuged for 10 minutes at 20,000g. Protein concentration was determined in supernatants using the BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL, USA) and equal amounts of reduced protein were loaded onto 10% SDS-polyacrylamide gels and separated by electrophoresis. After transfer, the nitrocellulose membranes were incubated overnight with primary antibodies (rat anti-VDR 1:1000 or rabbit anti-CYP24A1 1:1500, EMD Millipore) diluted in 5% milk/TTBS (Tris-buffered saline/Tween-20), followed by horseradish peroxidase-conjugated goat secondary antibodies (1:5000; R&D Systems, Minneapolis, MN, USA). Chemiluminescence was detected using a Pierce ECL Plus Western Blotting Substrate (Thermo Scientific).

TABLE. Primer Sequences for Real-Time PCR

Gene Name	Forward Primer	Reverse Primer	NCBI Reference Sequence
<i>GAPDH</i>	5'-GACCACAGTCCATGCCATCA-3'	5'-CATCACGCCACAGTTTCCC-3'	NM_002046
<i>CYP27A1</i>	5'-CCATCGGGTTAATGTTCCAG-3'	5'-AGTGCAGGTAGCCAGACACC-3'	NM_000784
<i>CYP2R1</i>	5'-AGTGGGTGAACTCATCATTCG-3'	5'-AAGAAGGCTTCCCATTAGGG-3'	NM_024514
<i>CYP24A1</i>	5'-TTCTCTGGAAAGGGGTCTC-3'	5'-GTGCACCGACTCAAAGGAAC-3'	NM_000782
<i>CYP27B1</i>	5'-AACCCTGAACAACGTAGTCTGCCA-3'	5'-ATGGTCAACAGCGTGGACACAAA-3'	NM_000785
<i>VDR</i>	5'-ACGCCCACCATAAGACCTAC-3'	5'-GCTGGGAGTGTGTCTGGAG-3'	NM_000376
<i>CAMP</i> (LL-37)	5'-GGACAGTGACCCCTCAACCAG-3'	5'-AGAAGCCTGAGCCAGGGTAG-3'	NM_004345.4
<i>CD14</i>	5'-GAGCTCAGAGGTTCCGAAGAC-3'	5'-GCTGAGGTTCCGAGAAGTTG-3'	NM_000591
<i>CXCL8</i> (IL-8)	5'-GACCACACTGCCCAACAC-3'	5'-CTTCTCCACAACCTCTGCAC-3'	NM_000584
<i>TNF</i>	5'-TGGAGAAGGGTGACCGACTC-3'	5'-TCCTCACAGGGCAATGATCC-3'	NM_000594.3
<i>IL1B</i>	5'-GCTGAGGAAGATGCTGGTTC-3'	5'-GCTGAGGAAGATGCTGGTTC-3'	NM_000576.2
<i>IL6</i>	5'-TACCCCCAGGAGAAGATTCC-3'	5'-AGTGCCTCTTTGCTGCTTTC-3'	NM_000600.3
<i>CCL20</i> (MIP3)	5'-CTGGCTGCTTTTGATGTCAGTG-3'	5'-GCAGTCAAAGTTGCTTGCTGC-3'	NM_001130046.1
<i>MMP9</i>	5'-CCAACTACGACACCGACGAC-3'	5'-TTGGCCTTGAAGATGAATG-3'	NM_004994.2
<i>TLR3</i>	5'-GCTGCAGTCAGCAACTTCAT-3'	5'-AGGAAAGGCTAGCAGTCATCC-3'	NM_003265
<i>DDX58</i> (RIG-1)	5'-GACTGGACGTGGCAAAACAA-3'	5'-TTGAATGCATCCAATATACACTTCTG-3'	NM_014314
<i>IFIH1</i> (MDA-5)	5'-ACCAAATACAGGAGCCATGC-3'	5'-CGTTCTTTGCGATTTCCTTC-3'	NM_022168.3

Immunostaining for VDR, RIG-1, NFκB, and LL-37

Human telomerase-immortalized corneal epithelial cells were grown on Lab-Tek 8-well chamber slides (Sigma-Aldrich Corp.) and were stimulated with 1,25D₃ or left untreated. After 24 hours, cells were fixed in 4% paraformaldehyde (retinoic acid inducible gene-1 receptor [RIG-1]) or methanol (VDR), blocked (1% BSA/10% goat serum/0.3 M glycine/0.1% Tween-20), and incubated with 5 µg/mL primary antibodies overnight (rabbit anti-DDX58 RIG-1; Abcam, Cambridge, MA, USA; rat anti-VDR, Millipore clone 9A7). This was followed by incubation with Alexa Fluor 488-conjugated goat anti-rat (VDR) or Alexa Fluor 546-goat anti-rabbit (RIG-1) with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) nuclear stain added along with the secondary antibodies. To quantify nuclear VDR staining, mean fluorescence intensity values of nuclear regions (6.5-µm fixed diameter circle) were determined using Image J analysis software, with vehicle compared to 1,25D₃-treated cells.

For NF-κB p65 nuclear translocation, hTCEpi were stimulated for 2 hours with Poly[I:C] and/or 1,25D₃. After treatment, cells were fixed in 4% paraformaldehyde, permeabilized in methanol, and then blocked with 3% BSA. Cells were incubated with rabbit anti-p65 NF-κB (1:200; Abcam), followed by Alexa Fluor 488-conjugated donkey anti-rabbit with 1 µg/mL DAPI nuclear stain.

For human corneal staining, human donor corneas were incubated, epithelial side up, in Optisol with or without 1,25D₃ (10⁻⁷M) for 24 hours. Following incubation, corneas were fixed in 4% paraformaldehyde for 2 hours, equilibrated in 30% sucrose/PBS overnight, and snap frozen in optimum cutting temperature media (OCT) with liquid nitrogen. Cryosections (10 µm) were blocked for 2 hours at room temperature (2% BSA/0.5% Tween-20/1 µg/mL Fc-block/PBS) followed by overnight incubation with 2.5 µg/mL primary antibody to LL-37 or rabbit IgG control (Santa Cruz Biotechnology, Dallas, TX, USA) in PBS containing 2% BSA/0.5% Tween-20. Donkey anti-rabbit Alexa Fluor 488 conjugated secondary antibody (1:500; Invitrogen) was applied for 1 hour and coverslips mounted with DAPI counterstain. Fluorescent images of the cells and tissue sections were obtained with a DeltaVision Core inverted microscope (Applied Precision, Issaquah, WA, USA).

Antimicrobial Assay

To determine if vitamin D was able to influence cell antimicrobial activity, SV40-HCEC were incubated in serum and antibiotic-free media with and without 1,25D₃ (10⁻⁷M) for 24 hours. Supernatants then were collected and tested for antimicrobial activity against *Pseudomonas aeruginosa* ATCC 19660 and *Staphylococcus aureus* ATCC 29213 as previously described.⁶⁰ Media that was not incubated with cells was used as a control in this assay.

ELISA and Luminex Assay

Human telomerase-immortalized corneal epithelial cells were treated with D₃ or 25D₃ for 24 hours and 1,25D₃ was quantitated in cell supernatants with a 1,25D₃ enzyme immunoassay (EIA) kit according to the manufacturer's instructions (Immunodiagnostic Systems, Scottsdale, AZ, USA). Following stimulation with 1,25D₃ and Poly(I:C) for 24 hours, IL-8 and MMP-9 were detected in cell supernatants by ELISA, as per the manufacturer's instructions (Human IL-8 ELISA MAX; BioLegend, San Diego, CA, USA; MMP-9 Human ELISA kit; Abcam). Interleukin-1β, IL-6, TNFα and MIP3α levels in culture supernatants were determined with the MILLIPLEX MAP Human Magnetic Bead Panel (EMD Millipore) using the MAGPIX system.

Flow Cytometry

Intracellular TLR3 expression was determined by flow cytometry, as previously described.⁵⁹ Briefly, hTCEpi were permeabilized in 0.1% Triton X-100/3% BSA for 15 minutes on ice. After blocking in 3% BSA, cells were incubated with 10 µg/mL mouse anti-TLR3 (Imgenex, San Diego, CA, USA) for 30 minutes followed by Alexa Fluor 488-conjugated goat anti-mouse (Life Technologies). Samples were run on a FACS Canto II (BD Biosciences, San Jose, CA, USA) and data were analyzed with FACS Diva software (BD Biosciences).

Statistical Analyses

Statistical analyses were performed using unpaired, 2-tailed, Student's *t*-tests in experiments comparing two samples. When more than two samples were analyzed, 1-way ANOVA was

FIGURE 1. Human corneal epithelial cells express the enzymes for vitamin D metabolism, a functional VDR, and are able to convert inactive vitamin D compounds to the biologically active metabolite, 1,25D₃. **(A)** Reverse transcription-PCR analysis of hydroxylase and VDR expression in primary HCEC and hTCEpi. Cytochrome p450 (CYP) hydroxylases: 27A1 and 2R1 (25-hydroxylases—catalyze D₃ to 25D₃); 27B1 (1 α -hydroxylase—catalyzes 25D₃ to 1,25D₃). **(B)** Human telomerase-immortalized corneal epithelial cells were stimulated with 1,25D₃ (10⁻⁷M) or vehicle for 24 hours, then fixed and stained for VDR (FITC/green) and DAPI nuclear stain (blue). Rat IgG served as a negative control. Images are representative of three independent experiments. Vitamin D receptor nuclear localization is significantly increased with 1,25D₃ treatment compared to control, as determined by nuclear staining intensity of the FITC signal; $P < 0.0001$, Student's *t*-test. Scale bars: 40 μ m. Human telomerase-immortalized corneal epithelial cells were treated with **(C)** 25D₃ (10⁻⁶M–10⁻⁹M) or **(D)** D₃ (10⁻⁷M) for 24 hours and 1,25D₃ (pmol/L) was quantitated in cell supernatants by immunoassay. Data represent mean $\pm$ SEM of four independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons **(C)** and Student's *t*-test **(D)**; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

used, with Bonferroni's test for multiple comparisons. All data are representative of a minimum of three independent experiments with $P \leq 0.05$ considered statistically significant. These tests were performed with GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Corneal Cells Express the Machinery for Vitamin D Metabolism and Are Able to Convert Inactive Vitamin D Compounds to the Fully Functional 1,25D₃

As the cornea is directly exposed to UVB irradiation, we hypothesized that corneal cells also express the machinery to fully activate vitamin D and to produce the functional 1,25D₃ metabolite. Cultured primary HCEC and the HCEC immortalized cell line, hTCEpi, expressed the activating 25-hydroxylases (CYP27A1 and CYP2R1) and 1 α -hydroxylase CYP27B1, and the deactivating enzyme 24-hydroxylase CYP24A1 (Fig. 1A). In

addition, freshly isolated corneal epithelium, endothelium, and stromal cells from human donor corneas and SV40-HCEC also expressed these genes (data not shown). We confirmed prior reports that HCEC express the VDR^{27–29} and also showed that stimulating these cells with 1,25D₃ resulted in a significant increase in the nuclear localization of the VDR compared to vehicle control treatment (vehicle, 38.4 $\pm$ 1.4 nuclear pixel intensity value; 1,25D₃, 73 $\pm$ 1.9 nuclear pixel intensity value; $P < 0.0001$, Student's *t*-test; Fig. 1B).

Importantly, when stimulated with 25D₃, hTCEpi were able to produce functional 1,25D₃ that was detectable in culture supernatants (Fig. 1C). There was a statistically significant increase ($P < 0.05$) in 1,25D₃ levels with increasing concentrations of 25D₃ up to a peak of 300 pmol/L with 10⁻⁷M 25D₃ treatment ($n = 4$). Additionally, when cells were treated with 10⁻⁷M of the unhydroxylated D₃, there also was a statistically significant ($P < 0.05$) increase in 1,25D₃ above unstimulated control supernatants, although at very low levels (Fig. 1D, $n = 4$). This demonstrates that HCEC can directly activate vitamin D metabolites through hydroxylation to produce 1,25D₃.

HCEC Respond to Vitamin D by Inducing the Expression of CYP24A1, Regulated by the VDR

To determine if HCEC respond functionally to locally activated vitamin D, primary cultured HCEC were treated with inactive D₃ and 25D₃, or 1,25D₃ for 24 hours. Human corneal epithelial cells showed an increase in *CYP24A1* gene expression with all vitamin D compounds tested (Fig. 2A, $n = 3$). The unhydroxylated D₃ increased CYP24A1 expression 18-fold while 25D₃ stimulation yielded a 655-fold increase relative to untreated control cells. 25-Vitamin D₃ was actually slightly more effective in stimulating CYP24A1 mRNA production than 1,25D₃ (400-fold increase), although this difference was not significant. However, CYP24A1 protein expression was increased more by 1,25D₃. These results demonstrated that HCEC can activate then use vitamin D to influence gene transcription.

While most effects of 1,25D₃ are mediated through the classical activation of its nuclear receptor, the VDR, 1,25D₃ also has been shown to influence cytoplasmic signaling pathways and interact with G-protein coupled receptors at the cell membrane.⁶¹ To assess if the upregulation of CYP24A1 was dependent on the VDR, we effectively knocked down its expression using siRNA, with decreases in mRNA expression of 80% ($\pm 5\%$) after 24 hours of transfection and 85% ($\pm 3\%$) after 72 hours (Fig. 2B). Vitamin D receptor protein also was knocked down to almost undetectable levels as determined by Western blot by 24 hours. When hTCEpi were treated with 1,25D₃, CYP24A1 mRNA expression increased 3000-fold (± 279) after 24 hours and VDR silencing completely abolished this response ($P < 0.0001$, $n = 3$; Fig. 2C), demonstrating not only that vitamin D can act through the VDR in these cells but also that the VDR is functional in corneal epithelial cells.

Vitamin D Increases the Expression of Vitamin D-Regulated Genes Involved in Innate Immune Defense

We next sought to determine if vitamin D was able to regulate the expression of several genes important in innate immune defense. When hTCEpi were treated with vitamin D metabolites for 24 hours, expression of LL-37, as well as CD14, increased more than 4-fold (Fig. 3A, left, $n = 3$). The increase in LL-37 also was detected in supernatants from cells treated with either 25D₃, or 1,25D₃, as determined by dot blot analysis (Fig. 3A, right). This upregulation was dependent on signaling through the VDR, as knockdown of the receptor significantly inhibited LL-37 ($P < 0.0001$) and CD14 ($P < 0.01$) expression following vitamin D treatment (Fig. 3B). In addition, VDR silencing significantly lowered the basal transcript levels of LL-37 by 75% ($P < 0.05$).

To confirm the upregulation of innate immune response genes in primary tissue, whole human donor corneas were treated with 1,25D₃ and examined for antimicrobial peptide expression by immunohistochemistry. There was an increase throughout the epithelial layer in LL-37 expression in the vitamin D-treated corneas compared to matched controls (Fig. 3C). In addition, human β defensin-2, hBD-2, another antimicrobial peptide that is expressed at the ocular surface, also was increased in the 1,25D₃ treated corneas (data not shown).

Vitamin D Enhances Antimicrobial Activity of HCEC and Increases TLR-Induced Antimicrobial Peptide Production

To investigate a possible functional result of the upregulation of innate immunity-related genes, SV40-HCEC were treated

with 1,25D₃ and the supernatants were incubated with a clinical isolate of *P. aeruginosa* (ATCC 19660), a common ocular pathogen, to determine antimicrobial activity. The SV40-HCEC cells had some natural antimicrobial activity on their own, as cell conditioned media (Cells) decreased *P. aeruginosa* growth by 50% ($P < 0.01$) compared to media that had not been exposed to cells (Media, Fig. 4A). Supernatants treated with 1,25D₃ (Cells+1,25D) were even more effective at killing *P. aeruginosa* than cells alone, with a further decrease in bacterial colonies after incubation (40%, $P < 0.01$). This suggests that 1,25D₃ initiated the production of an antimicrobial agent by the SV40-HCEC, leading to a decrease in colony growth. However, the same effect was not seen against the gram-positive organism *S. aureus* (data not shown).

As TLRs are important sensors of the innate immune system and can interact with vitamin D's activation and antimicrobial response, we investigated the ability of TLRs to regulate vitamin D hydroxylase expression in HCEC. Human telomerase-immortalized corneal epithelial cells were stimulated with TLR agonists specific for TLR2 (Pam3CSK4, FSL1, Zymosan), TLR3 (Poly[I:C]), or TLR5 (Flagellin) for 24 hours. Both CYP27B1 and CYP24A1 expression were significantly increased by TLR activation (Fig. 4B; $P < 0.05$, $n = 4-5$). Specifically, TLR2 and TLR3 agonists induced the expression of the activating enzyme, CYP27B1, with the largest increase (21-fold ± 5) initiated through Poly(I:C), a double-stranded (ds) RNA analog similar to that of viruses.

We next sought to determine if LL-37 expression could be augmented by combined TLR agonist-vitamin D treatment. Focusing on TLR2 and TLR3 agonists, when 1,25D₃ was added to cells along with FSL1, Pam3CSK4, or HKLM (TLR2), or with Poly(I:C) (TLR3), there was an increase in LL-37 expression (Fig. 4C). Poly(I:C) was the most effective in augmenting LL-37 expression in combination with 1,25D₃, with a 9-fold increase above control ($n = 4$). These results suggested that vitamin D can cooperate with TLRs to enhance innate immunity in HCEC.

Vitamin D Modulates the HCEC Response to Poly(I:C) Through Decreased Production of Proinflammatory Cytokines and MMP-9

Because vitamin D is known to have anti-inflammatory activities in other tissues, we hypothesized that vitamin D treatment could affect cytokine production in HCEC. As shown in Figure 5A, when hTCEpi were treated with Poly(I:C) for 24 hours, there was a large increase in IL-8 at mRNA and protein levels (~1000- and ~230-fold increases, respectively). When 1,25D₃ was added to cells with Poly(I:C), there was a significant decrease in IL-8 production (50% decrease in mRNA and 86% decrease in protein, $P < 0.001$, $n = 4$). Similarly, IL-1 β , IL-6, TNF α , and CCL20 (MIP3 α) levels were attenuated by 1,25D₃ treatment (relative fold changes with Poly[I:C] treatment versus Poly[I:C] + 1,25D₃: IL-1 β [22 $\pm$ 3 vs. 9 $\pm$ 0.8]; IL-6 [973 $\pm$ 123 vs. 301 $\pm$ 44]; TNF α [84 $\pm$ 10 vs. 44 $\pm$ 3]; CCL20 [243 $\pm$ 49 vs. 62 $\pm$ 16]). When 1,25D₃ was added to the cells four hours after Poly(I:C) stimulation, IL-8 levels also were decreased (38%, $n = 4$, data not shown). The suppression of these inflammatory mediators appears to be dependent on signaling through the VDR, as treatment with VDR siRNA blocked the downregulation of IL-8 by 1,25D₃ (data not shown). Interestingly, although 1,25D₃ was able to decrease proinflammatory cytokines, the anti-inflammatory mediators, IL-10 and IL-1 receptor antagonist (IL-1RA) were not increased (data not shown).

In addition to cytokines and chemokines, matrix metalloproteinases (MMPs) also are upregulated by inflammatory stimuli at the ocular surface.^{62,63} Matrix metalloproteinase-9

FIGURE 2. Human corneal epithelial cells respond to vitamin D by inducing the expression of CYP24A1, regulated by the VDR. **(A)** Human telomerase-immortalized corneal epithelial cells were treated with D₃, 25D₃, or 1,25D₃ (10⁻⁷M) for 24 hours and CYP24A1 expression was determined by real-time PCR (*left*) and Western blotting (*right*). **(B)** Human telomerase-immortalized corneal epithelial cells were left untreated (control), transfected with a nonspecific control siRNA (Neg. siRNA), or VDR siRNA for 24, 48, or 72 hours and analyzed for VDR expression by real-time PCR (*left*) and Western blot (*right*). **(C)** Human telomerase-immortalized corneal epithelial cells were left untreated (control and 1,25D₃) or transfected with VDR siRNA for 24 hours and then treated with 1,25D₃ for 24 hours to determine if VDR expression is required for 1,25D₃-mediated gene induction. Expression of CYP24A1 was analyzed by real-time PCR. Data represent mean $\pm$ SEM of three independent experiments. Statistical analysis was by Student's *t*-test (**A**, **B**) and ANOVA with Bonferroni's test for multiple comparisons (**C**), **P* < 0.05, ***P* < 0.01, *****P* < 0.0001.

FIGURE 3. Vitamin D increases the expression of vitamin D-regulated genes involved in innate immune defense. (A) Human telomerase-immortalized corneal epithelial cells were treated with 10^{-7} M D₃, 25D₃, or 1,25D₃ for 24 hours and expression of LL-37 and CD14 was determined by real-time PCR (*left*) and LL-37 production was determined by dot blot (*right*). (B) Human telomerase-immortalized corneal epithelial cells were left untreated (control and 1,25D₃) or transfected with VDR siRNA for 24 hours and then treated with 1,25D₃ for 24 hours to determine if VDR expression is required for 1,25D₃-mediated gene induction. LL-37 (*left*) and CD14 (*right*) expression was analyzed by real-time PCR. Graphs represent mean $\pm$ SEM of three independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons, * $P < 0.05$, ** $P < 0.01$ compared to control; **** $P < 0.0001$ compared to 1,25D₃. (C) Human corneas were treated with 1,25D₃ for 24 hours, fixed in paraformaldehyde, and frozen sections stained for LL-37 or a control rabbit IgG (*green*) and DAPI nuclear stain (*blue*).

FIGURE 4. Vitamin D increases HCEC antimicrobial activity against *P. aeruginosa* and enhances TLR-mediated LL-37 production. (A) SV40-HCEC were left untreated (Cells) or treated with 1,25D₃ (10⁻⁷M) for 24 hours (Cells+1,25D₃) and supernatants used in an antimicrobial assay. For controls, media only (Media) or 1,25D₃ in media (Media+1,25D₃) were used without cells. Data represent mean $\pm$ SEM of three independent experiments. Statistical analysis was by Student's *t*-test, ***P* < 0.01. (B) Human telomerase-immortalized corneal epithelial cells were treated with 1 μ g/mL TLR agonists for 24 hours (Pam3CSK4 [TLR2/1] [Pam], FSL1 [TLR2/6], Poly[I:C] [TLR3], Flagellin [TLR5] [Flag] or 50 μ g/mL zymosan (TLR2) [Zym] for 6 hours. Expression of CYP27B1 and CYP24A1 was determined by real-time PCR. nd, not determined. (C) Human telomerase-immortalized corneal epithelial cells were treated with TLR agonists (Flag, FSL1, HKLM, Pam, or Poly[I:C]), in combination with 1,25D₃ (10⁻⁷M) for 24 hours and LL-37 expression analyzed by real-time PCR. Data represent mean $\pm$ SEM of four to six independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

levels were significantly decreased by 20% when hTCEpi were treated with 1,25D₃ and Poly(I:C) together (*P* < 0.05, *n* = 4; Fig. 5B). 1,25D₃ also reduced basal levels of MMP-9 transcript (42%, *P* < 0.0001) and suppressed its expression induced by other TLR agonists, FSL1 (TLR2; *P* < 0.0001), and flagellin (TLR5, *P* < 0.05; Fig. 5C).

Vitamin D Decreases the Expression of PRRs That Are Activated in Response to Poly(I:C) But Activation of Downstream NF- κ B Is Not Affected

To understand this anti-inflammatory response, specifically to Poly(I:C)-induced inflammation, we investigated if vitamin D can influence PRR expression in HCEC. Following 24 hours of treatment, 1,25D₃ lowered the RNA expression of TLR3 that was induced by Poly(I:C) 36% (*P* < 0.05, *n* = 5; Fig. 6A, left). Using flow cytometry, we observed that 1,25D₃ also decreased the intracellular expression of TLR3 (Fig. 6A, right). Poly(I:C) is a ligand for TLR3 but also can activate the RIG-I-like receptors RIG-I and MDA5, cytoplasmic dsRNA helicases that recognize viral RNA. Human telomerase-immortalized corneal epithelial

cells not only expressed these receptors, but the addition of 1,25D₃ decreased RIG-I and MDA5 to approximately 50% of control levels (*P* < 0.01, 0.001, *n* = 5; Figs. 6B, 6C). Similar to TLR3 expression, Poly(I:C) stimulation increased levels of RIG-I and MDA5, which were lowered by combined treatment with 1,25D₃ (data not shown).

After determining that vitamin D decreased PRR expression, we next wanted to investigate if the hormone had an effect on Poly(I:C) activation of NF- κ B. When hTCEpi were stimulated with Poly(I:C) for 2 hours, nuclear staining of the NF- κ B p65 subunit (green) increased and there was a corresponding decrease in fluorescent staining in the cytoplasm (Fig. 6D). However, concurrent treatment with 1,25D₃ did not affect p65 translocation significantly.

DISCUSSION

The cornea is the avascular tissue that forms the anterior surface of the eye, covering the pupil and iris. One of its major functions is to refract light, focusing the entry of light back

FIGURE 5. Vitamin D modulates HCEC response to Poly(I:C) through decreased production of proinflammatory cytokines and MMP-9. Human telomerase-immortalized corneal epithelial cells were treated with 1,25D₃ (10⁻⁷M) and/or TLR agonists (1 μg/mL) for 24 hours. (A) Cell lysates were collected for RNA isolation followed by real-time PCR analysis (*top row*) and supernatants were used to quantify protein levels (*bottom row*) of IL-8 (ELISA), and IL-1β, IL-6, TNFα or CCL20 (Luminex assay). (B) For MMP-9 expression, cell lysates were collected for RNA isolation followed by real-time PCR analysis (*top*) and supernatants were used to quantify protein levels by ELISA (*bottom*). (C) Human telomerase-immortalized corneal epithelial cells were treated with 1,25D₃ (10⁻⁷M) for 24 hours (*top*) or 1,25D₃ in combination with FSL1 or Flagellin (Flag, *bottom*). Relative quantity of MMP-9 was assessed by real-time PCR. Graphs represent mean ± SEM of four to five independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons or Student's *t*-test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

onto the retina, for photodetection. The cornea is responsible for two-thirds of the eye's total focusing power and tissue transparency is essential for this function. The cornea also functions to protect the rest of the eye from damage, providing a barrier from physical, microbial, UV, and other insults. Given vitamin D's known role in modulating inflammation and antimicrobial peptide production, we hypothesized that vitamin D could aid in protecting the cornea, both from inflammation and infection, and, thus, help maintain the function of this tissue. In this study, we examined the ability of HCEC to produce active vitamin D, 1,25D₃, and to respond to

vitamin D treatment through a functional VDR, resulting in altered gene expression of target genes. In addition, we explored the ability of vitamin D to dampen the expression of inflammatory mediators induced by TLR stimulation, while enhancing antimicrobial peptide production.

To ascertain the capacity of the cornea to use vitamin D and its metabolites and determine the source of vitamin D in the cornea, a better understanding is needed of the local production of active vitamin D. Corneal epithelial cells, like keratinocytes of the skin, are directly exposed to UVB from sunlight. Upon exposure, keratinocytes are able to convert 7-

FIGURE 6. Vitamin D lowers the expression of PRRs TLR3 and RIG-1/MDA-5 but does not block NF- κ B p65 nuclear translocation following Poly(I:C) stimulation. (A) Human telomerase-immortalized corneal epithelial cells were treated with 1,25D₃ (10⁻⁷M) and/or Poly(I:C) (1 μ g/mL) for 24 hours. TLR3 expression was determined by real-time PCR (left) and flow cytometry analyses (middle and right). (B) Cytoplasmic pattern recognition receptors RIG-1 (left) and MDA-5 (right) expression was determined by real-time PCR. Data represent mean $\pm$ SEM of five independent experiments. Statistical analysis was by ANOVA with Bonferroni's test for multiple comparisons (A) or Student's *t*-test (B), **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. (C) Human telomerase-immortalized corneal epithelial cells were stimulated with 1,25D₃ (10⁻⁷M) or vehicle for 24 hours, then fixed and stained for RIG-1 (red) and DAPI nuclear stain (blue). (D) Human telomerase-immortalized corneal epithelial cells were stimulated with 1,25D₃ and/or Poly(I:C) for 2 hours and stained with NF- κ B subunit p65 (green) and DAPI nuclear stain (blue). Images are representative of two independent experiments. Scale bars: 40 μ m.

dehydrocholesterol into previtamin D, D₃, and then produce the major circulating form of the hormone, 25D₃.⁶⁴ The epidermis then is able to fully activate 25D₃ through 1- α -hydroxylation (CYP27B1) to 1,25D₃, and use it locally, where vitamin D exerts a range of cellular effects. It has been shown that human limbal epithelial cells also are able to produce D₃ and 25D₃ following stimulation with UVB.²⁶ However, this study did not detect the active 1,25D₃ in cell supernatants. Our current study directly demonstrated for the first time to our knowledge that HCECs are able to produce the biologically active form of vitamin D, 1,25D₃, from precursors D₃ and 25D₃. In addition, these cells express the hydroxylases necessary for full vitamin D activation and inactivation and express a functional receptor. Therefore, it is reasonable to hypothesize that the cornea is able to perform *de novo* synthesis of vitamin D when exposed to sunlight, providing this tissue with a local source of the hormone.

Upon activation and binding to the VDR in other tissues, 1,25D₃ exerts transcriptional influence through receptor interactions on the promoter regions of genes containing

specific response elements, VDREs. Vitamin D regulates its own activity through the production of the *CYP24A1* gene, which is strongly induced by 1,25D₃ binding to the VDR.^{5,65,66} The resulting enzyme is able to hydroxylate 25D₃ and 1,25D₃, inducing their catabolism, and maintaining vitamin D homeostasis.^{6,67} We have shown that HCEC respond to stimulation with vitamin D metabolites by inducing the expression of *CYP24A1*. This induction is mediated through a functional VDR, as silencing of the receptor blocks this response. Therefore, HCEC are able to regulate the activity of vitamin D when it is present.

The cornea is responsible for protecting the rest of eye not only from physical damage, but from infection as well. Therefore, it is critically important for this tissue to be able to respond quickly and effectively when exposed to pathogens. Vitamin D has been shown to directly induce the production of the antimicrobial peptide, LL-37,³² which has broad spectrum activity against not only bacteria, but viruses and fungi as well.^{43,44} Like LL-37, hBD-2 is a potent antimicrobial peptide that is expressed at the ocular surface and is known to be

increased by inflammatory signals.^{53,55} Vitamin D indirectly regulates, positively and negatively, hBD-2 expression in some cell types, including keratinocytes.^{68–70} In this study, vitamin D treatment induced an increase in LL-37 in HCEC at the RNA and protein levels. Additionally, 1,25D₃ increased LL-37 and hBD-2 in the epithelium of human donor corneas. However, no induction of hBD-2 mRNA was seen in cultured cells. It would be interesting to examine further the regulation of antimicrobial peptide production, including β -defensins, by vitamin D during infection or pathogenic challenge. Notably, vitamin D increased the bactericidal activity of HCEC against *P. aeruginosa*, as supernatants from vitamin D-treated cells killed more bacteria than control. Although not directly tested, we speculated that increased antimicrobial peptide expression likely contributes to this effect. This functional consequence of vitamin D exposure would be expected to provide a significant benefit to the ocular surface, helping to protect it from infection.

Also important for innate defense in the cornea, TLRs recognize microbial ligands and initiate recruitment of immune cells to the site of infection.³⁹ The TLRs are expressed at the ocular surface and contribute to the inflammatory process during infection and wound healing.⁵⁹ Engagement of these innate immune receptors leads to the robust production of cytokines and chemokines that facilitate the recruitment and activation of an adaptive immune response. Previous studies have shown that TLR engagement can lead to increased vitamin D activation and enhanced antimicrobial peptide production. For example, TLR2/1 ligands upregulate the VDR and CYP27B1 in human macrophages, increasing responsiveness to 1,25D₃, which in turn augments LL-37 production, leading to *Mycobacterium tuberculosis* killing.⁷¹ Skin injury also results in increased vitamin D activation, causing induction of LL-37, CD14, and TLR2 expression.⁷² In a positive feedback loop, TLR2 activation further upregulates LL-37 in the presence of 1,25D₃. In respiratory epithelial cells, double-stranded RNA, a TLR3 agonist, also stimulates CYP27B1 expression, LL37, and CD14.⁷³ Similarly, here we showed that TLR2 and TLR3 agonists increased the expression of regulatory hydroxylases CYP24A1 and CYP27B1 in HCEC, demonstrating the potential of the cornea to regulate vitamin D activity. These TLR agonists also acted in concert with 1,25D₃ to further enhance antimicrobial peptide production, increasing the protective ability of HCEC during inflammation.

Engagement of TLRs in the cornea also results in activation of an inflammatory response. While important for defense against infection, this response must be regulated to protect against tissue damage. dsRNA (Poly[I:C] agonist) in particular induces a robust response in human corneal epithelial cells, producing potentially harmful levels of proinflammatory mediators. In an immunosuppressive role, vitamin D is known to decrease inflammatory mediators in a variety of tissues during disease, infection, and TLR activation.^{1,36,37,74} Several studies demonstrate that vitamin D also can have this effect at the ocular surface. Vitamin D decreased inflammatory cytokines and infiltrates in animal models of corneal transplantation and injury^{75,76} and suppressed the expression of IL-1 α ,⁷⁷ IL-1 β , IL-6, and IL-8⁷⁸ in cultured human corneal epithelial cells. In our studies, 1,25D₃ acted through the VDR to downregulate the expression of IL-1 β , IL-6, TNF α , IL-8, and CCL20 (MIP3 α) induced by TLR3 agonist Poly(I:C). In addition, 1,25D₃ lowered MMP-9 expression. Mechanistically, these results could be explained in part by the decrease in PRR expression seen with vitamin D treatment. Interestingly, this anti-inflammatory response was not an immediate response, as vitamin D did not decrease IL-8 levels before 6 hours of

cotreatment with Poly(I:C) (data not shown) and did not prevent the NF- κ B nuclear translocation that occurs quickly after Poly(I:C) treatment.

This pattern of response to vitamin D, allowing the initiation of inflammatory signals, could be helpful, particularly during infection and wound healing, where limited amounts of cytokine and chemokine production are beneficial for the initial inflammatory event. Vitamin D then is able to attenuate levels of proinflammatory mediators later in the response, potentially protecting the local tissue environment from damage or enhancing corneal immune privilege. Indeed, vitamin D was able to suppress corneal graft rejection in a rat model of penetrating keratoplasty, partly through inhibition of IL-1 α , TNF α , and VEGF expression.⁷⁵ Vitamin D has been shown to suppress effector T cell function and promote regulatory T cells in other tissues; therefore, vitamin D's ability to promote immune privilege in the cornea would be very interesting to pursue further. In the cornea it is critically important to minimize inflammation that could disrupt transparency. Therefore, vitamin D's role in immunomodulation and antimicrobial peptide production, as we have shown, will be important to study in the context of ocular surface inflammation, such as that occurs during infection or dry eye syndrome.

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