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Regulation of late cornified envelope genes relevant to psoriasis risk by plant-derived cyanidin

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ABSTRACT

The PSORS4 genetic risk factor for psoriasis is a deletion of two late cornified envelope (LCE) genes (LCE3C_LCE3Bdel) in a cluster of five LCE3 genes with a proposed role in skin repair. We previously showed that 1,25-dihydroxyvitamin D_3 (1,25D) modestly upregulates transcripts from all five LCE3 genes as monitored by real time PCR in primary human keratinocytes. Herein we report that cyanidin, a plant-derived compound with anti-inflammatory/anti-oxidant properties, upregulates expression of all five LCE3 genes in cultures of differentiating primary human keratinocytes to a greater extent that does 1,25D. This action of cyanidin is dependent on the differentiation state of the keratinocytes, with a stronger effect after the cells have been incubated with 1.2 mM calcium for 24 h. Competition displacement assays using radiolabeled 1,25D revealed that cyanidin directly competes as a ligand for vitamin D receptor (VDR) binding with an estimated IC_{50} of 500 μM. However, 20 μM cyanidin is sufficient to upregulate LCE3 genes. The 25-fold discrepancy between the cyanidin concentration required for upregulating LCE3 genes in intact keratinocytes vs. that required for direct binding to VDR in vitro suggests that cyanidin may be: (a) metabolized to a more active VDR ligand in keratinocytes and/or (b) functioning via a non-VDR mediated mechanism. The fact that cyanidin is the most potent upregulator of global LCE3 gene expression reported to date suggests that this or related compounds may have potential in psoriasis therapy.

1. Introduction

Psoriasis is a skin disorder of unknown etiology that affects over 7 million individuals in the USA [1]. Analogs of the vitamin D hormone, 1,25-dihydroxyvitamin D_3 (1,25D), are routinely used topically to treat psoriasis. These actions are presumably mediated by the nuclear vitamin D receptor (VDR) which is expressed in skin [2]. However, specific genes that are regulated by 1,25D to improve psoriasis symptoms remain uncharacterized.

Linkage studies identify over 30 genetic loci associated with increased psoriasis susceptibility, with 12 of these loci denoted as PSORS1–12 [3]. The PSORS4 locus lies within an assembly of skin differentiation genes on human chromosome 1 [4] designated the epidermal differentiation complex (EDC) [5]. Studies of PSORS4 have implicated a common deletion (LCE3C_LCE3Bdel, see Fig. 1) of two genes encoding late cornified envelope 3B and 3C (LCE3B and LCE3C) proteins as being a risk allele [6–8], with a frequency of 21–75% depending on ethnicity [9]. Typically, LCE gene products are expressed late in keratinocyte differentiation and are cross-linked to help form the cornified envelope [10]. LCE3B and LCE3C belong to a cluster of five LCE3 genes with minimal expression in healthy skin. However, transcripts of LCE3A, -3C, -3D and -3E are upregulated in psoriasis [6,11]. Tape stripping to induce superficial skin injury also induces LCE3A/3C/3D/3E [11]. In contrast, other nearby LCE genes, such as LCE2B, are expressed at moderate levels in normal skin and downregulated in psoriasis or superficial injury [11]. These observations suggest that LCE3 genes play a role in skin repair in lesional psoriatic epidermis or after injury. The current study examines the expression of all five LCE3 genes, along with LCE2B, which was shown to be upregulated by 1,25D in a human keratinocyte (CCD-1105 KERTr) line in a previous study [12].

If LCE3 proteins play a role in skin repair, then the deletion of two of these genes (as in LCE3C_LCE3Bdel) might lead to a slower or less complete repair response. The fact that a substantial portion of the population (9–40% of persons of European descent [9]), is homozygous for LCE3C_LCE3Bdel, yet the prevalence of psoriasis

Abbreviations: 1,25D, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; LCE, late cornified envelope.

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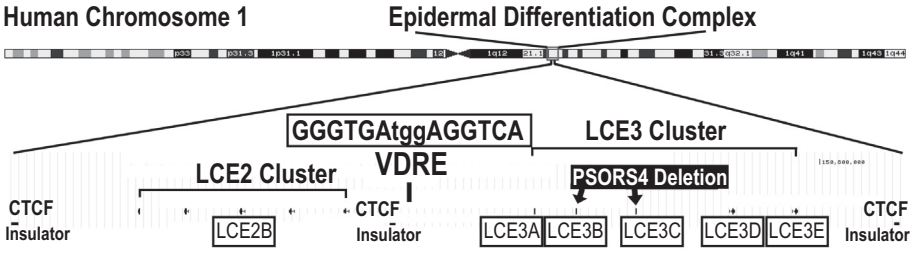

Fig. 1. Location of PSORS4 deletion (LCE3C_LCE3Bdel) in the context of the LCE2 and LCE3 gene clusters. One of the genetic loci conferring risk of developing psoriasis has been localized to the epidermal differentiation complex (EDC) on human chromosome 1 which consists of numerous genes expressed in differentiated keratinocytes. Specifically, the PSORS4 risk allele contains a deletion that removes the LCE3B and LCE3C genes from the LCE3 cluster, but retains other LCE genes intact. Both the LCE3 and the adjacent LCE2 gene clusters are bracketed by CTCF binding sites, which may serve as regulatory insulators to allow for coordinated control of each cluster [31]. The location of the candidate vitamin D responsive element (VDRE) used in this study is also shown.

is only about 1% [1], clearly indicates that this deletion cannot cause psoriasis by itself. However, if it occurs in combination with other susceptibility alleles, the risk for developing psoriasis may become substantially greater [9,13,14].

Plant-derived anthocyanidins such as delphinidin and cyanidin have been shown to possess protective effects in skin [15,16]. In the current study, we examine the ability of cyanidin to upregulate LCE genes in differentiating primary human keratinocytes, and also to activate VDR-mediated transcription from a reporter gene linked to a vitamin D responsive element corresponding to a sequence within the LCE3 locus.

2. Materials and methods

2.1. Source of ligands

Crystalline 1,25D was a kind gift from Milan Uskokovic of Hoffmann-LaRoche. *cis*-4,7,10,13,16,19-Docosahexaenoic acid was obtained from Sigma Aldrich Corp. (St. Louis, MO) and cyanidin chloride was from Santa Cruz Biotechnology (Dallas, TX).

2.2. Cell lines and real time PCR

The human keratinocyte line CCD-1106 KERTr (KERTr) was obtained from ATCC (Manassas, VA) and cultured in SFM keratinocyte medium with recommended supplements along with 5 μg/ml Genticin; media ingredients were obtained from Gibco (Invitrogen Corp., Carlsbad, CA). Human primary neonatal keratinocytes (HEKn) were purchased from Invitrogen Corp. (Carlsbad, CA) and cultured in serum free EpiLife medium containing recommended HKGS supplement kit reagents. Human embryonic kidney cells (HEK-293) were obtained from ATCC and maintained in DMEM high glucose plus 10% fetal bovine serum and penicillin/streptomycin (Gibco).

2.3. Real time PCR analysis of LCE gene expression

RNA was isolated using an Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA) from HEKn cells (550,000 cells per 60 mm plate grown to approximately 70% confluence). First strand cDNA was synthesized using an iScript kit (Bio-Rad). Quantitative real time PCR (qrtPCR) was performed with Fast Start Universal SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN) in a System 7500 Fast thermal cycler using 2 μl of first strand DNA and 1 μl of 18 μM primer mixture in 20 μl total volume. For detection of human LCE transcripts, primers were as follows: LCE2B: forward primer 5′-GCCAGCCCCCTCCCAAGTGT and reverse primer 5′-GGG CACTGGGGCAGGCATTT; LCE3A forward primer 5′-GAGTCACCA CAGATGCC and reverse primer 5′-CTTGCTGACCACTTCCC (3A primers from [11]); LCE3B, forward primer 5′- CCCAAAGAGCTCAGCA CAGT and reverse primer 5′-TGCCTCTGTCACAGGAGTTG; LCE3C, forward primer 5′-AGTTGTCCCTCACCCAAGTG and reverse primer 5′-ATTGATGGGACCTGAAGTGC; LCE3D forward primer 5′-CTCT GCACCTGGACAACTCA and reverse primer 5′-CACTTGGGTGAGGG ACACTT; LCE 3E forward primer 5′-CTGATGCTGAGACAAGCGATCTT and reverse primer 5′-GATCCCCCACAGGAAAACCT (3E primers also from [11]). Human CYP24A1 was detected using forward primer 5′-CAGCGAACTGAACAAATGGTCG and reverse primer 5′-TCTCTTC TCATACAACACGAGGCAG, and human GAPDH was amplified using forward primer 5′-TGACAACTTTGGTATCGTGGAAGG and reverse primer 5′-AGGGATGATGTTCTGGAGAGCC. PCR products were sequenced to confirm their specificity. Real time PCR data were analyzed using the comparative Ct method, normalized to GAPDH. Fold effects were calculated relative to vehicle-treated control samples and expressed as $2^{-\Delta\Delta Ct}$.

2.4. Competition assay to analyze VDR binding

The protocol for competition binding was described previously [17]. Briefly, VDR-deficient COS-7 cells (2.5×10^6 cells per 150-mm plate) were transfected with expression plasmids for human VDR (pSG5-hVDR) and human RXRα (pSG5-hRXRα) using Express-In reagent (Open Biosystems, Lafayette, CO). Cells were harvested by trypsinization and a lysate was prepared as described [18]. For the competition assay, 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol ([^{3}H] 1,25D, 155 Ci/mmol, Perkin Elmer, Waltham, MA) was diluted to 54 Ci/mmol. Each assay tube received [^{3}H]1,25D (final concentration 0.4 nM), 2–4 μL cell lysate, 196 μL of buffer containing 0.3 mM $ZnCl_2$ and 4 μL of diluted ligands. After overnight incubation on ice, unbound 1,25D was removed with 80 μL of dextran-coated charcoal (Sigma–Aldrich, St. Louis, MO) for 15 min, followed by a 2 min microcentrifugation. Supernatants (200 μL) were combined with 4 ml of ScintiSafe 30% (Fisher Scientific, Pittsburgh, PA) and counted in a Beckman LS 5801 scintillation counter. Data were analyzed with Prism 4 software (GraphPad Software, San Diego, CA).

2.5. Genotyping at the LCE3B_LCE3C locus

Genomic DNA was isolated from 4–5 million cells using a DNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. A triple primer set was utilized to detect the LCE3B_LCE3C deletion as described by de Cid et al. [6]: LCE3CF 5′-TCAC CCTGGAACTAGACCTCA; LCE3CR 5′CTCCAACCACTTGTTCTTCTCA; and LCE3CR2D 5′-CATCCCAGGGATGCTGCATG. PCR reactions included 50–80 ng of DNA, 0.5 μL of an 18 μM primer stock and 5 μL of Fast Start Universal SYBR Green Master Mix (Roche Applied Science, Indianapolis, IN) in 10 μL total volume. An ABI 2400

machine was programmed for 35 cycles (94 C for 30 s, 60 C for 30 s, 72 C for 1 min), followed by a 72 C step for 10 min. PCR products were resolved on 3% agarose gels. A single band at 199 bp indicated a homozygous deletion, a single band at 240 bp indicated a homozygous intact locus and the presence of both bands indicated a heterozygote.

2.6. LCE3 VDRE reporter plasmid, transfections and luciferase assays

A VDRE sequence was identified ~29 kb upstream of the LCE3A gene. Two copies of this sequence with four bases on either side (5′-TTCAGGGTGAtggAGGTCAAATG; the two VDRE half sites are underlined) were synthesized with an additional four base overhang for cloning into the HindIII/BglII sites of the pLUC-MCS reporter plasmid (Invitrogen) to create pLUC-LCE3. HEK-293 cells were transfected in 24-well plates at 60,000 cells/well as described [17]. Briefly, each well received 2.0 µl ExpressIn Reagent, 250 ng of either empty pLUC-MCS plasmid or pLUC-LCE3, 25 ng of pSG5-hVDR, 20 ng of pRL-null (*Renilla* luciferase reporter) and 1 µl of 100X sodium pyruvate. KERTr cells were similarly transfected utilizing 2.3 µL of TransIT keratinocyte reagent (Mirius Bio, Madison, WI) and the same amounts of each plasmid DNA. After transfection, wells were treated with ligands or ethanol control for 20 h. Whole cell lysates were harvested and analyzed for Firefly luciferase and Renilla luciferase activity using a Dual Luciferase assay kit (Promega) and a Sirius Luminometer (Zylux Corp.) according to the manufacturers' protocols.

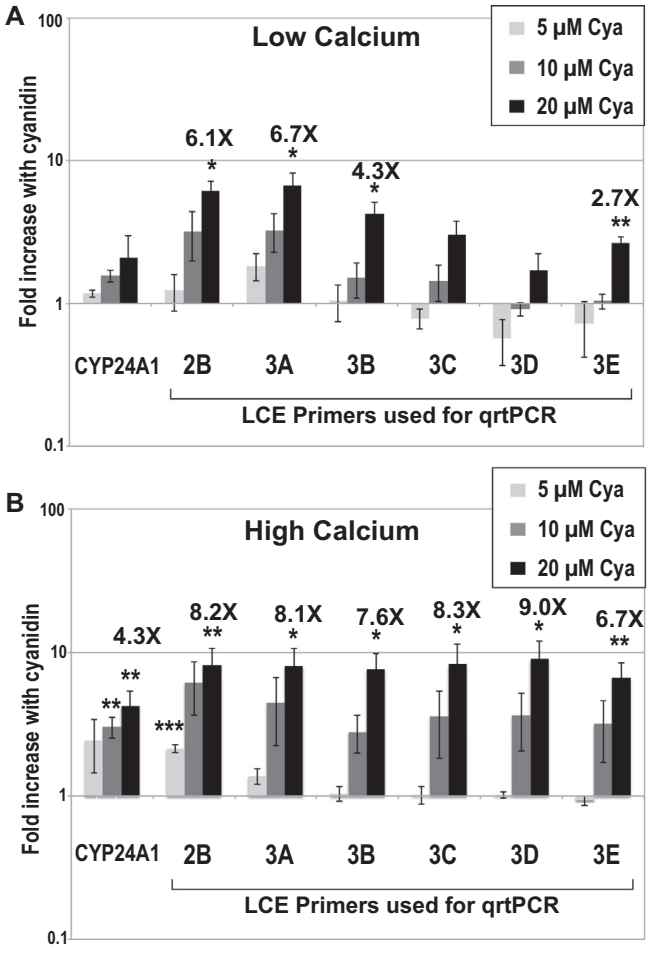

Fig. 2. Regulation of LCE genes by cyanidin. HEKn cells were plated as described in Methods, incubated overnight, then treated as follows: A, 24 h with cyanidin or ethanol vehicle without high calcium preincubation; B, preincubation for 15 h with 1.2 mM calcium, then with cyanidin or ethanol vehicle for 24 h. RNA isolation, synthesis of first strand DNA and real time PCR are described in Methods. Results are from three independent experiments, each in triplicate, ± SEM. A single asterisk (*) denotes averages that are statistically significant from ethanol control, $p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3. Results

3.1. Regulation of LCE3 genes in primary human keratinocyte culture

Primary neonatal human keratinocytes (HEKn) were cultured under two conditions: 60 µM calcium (low calcium) and 1.2 mM calcium (high calcium). Cells grown at low calcium maintain an undifferentiated state, while high calcium causes a marked change in cell morphology associated with keratinocyte differentiation [19]. The ability of cyanidin (Cya) to modulate the expression of LCE3 genes was assessed under both conditions (Fig. 2, panel A vs. B). Genotyping indicated that this keratinocyte donor is heterozygous for LCE3C_LCE3Bdel. Expression of mRNAs for LCE2B and CYP24A1 was also assessed, since these are known targets of 1,25D upregulation [12,20,21]. As shown in Fig. 2A and B, cyanidin dose-dependently upregulated CYP24A1 in both states, reaching 4.3-fold over ethanol control at 20 µM cyanidin and high calcium (Fig. 2B, left). These results suggested that cyanidin is a low affinity VDR ligand, since ligand-occupied VDR is a major regulator of CYP24A1 transcription [20]. Cyanidin also regulated LCE genes, both in low and high calcium. At low calcium, incubation with 20 µM cyanidin yielded a statistically significant upregulation of LCE2B, 3A, 3B and 3E, with fold inductions over ethanol control as indicated in Fig. 2A. After 24 h preincubation with high calcium, cyanidin induced all LCE3 genes, as well as LCE2B and CYP24A1, with fold-induction values of 4.3- to 9.0-fold (Fig. 2B). The fold effects of cyanidin on the mRNA levels of LCE genes are consistently greater than those observed with 10^{-8} M 1,25D in an earlier study [17].

To test whether cyanidin is a VDR ligand, radioinert cyanidin was incubated along with radiolabeled 1,25D in a competition binding assay using lysates containing overexpressed human VDR and RXRα. As a positive control, unlabeled 1,25D competes

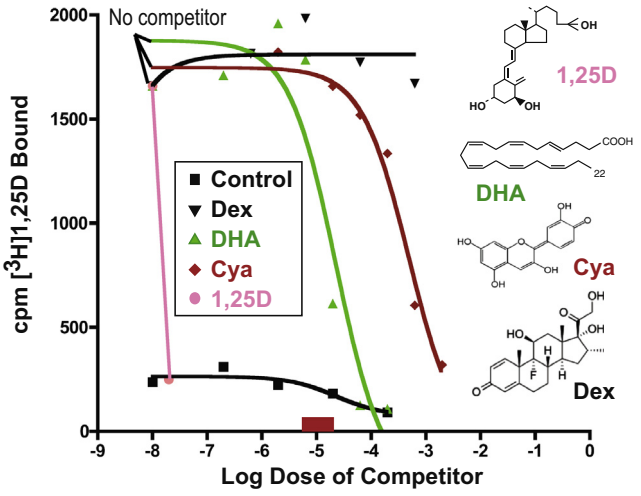

Fig. 3. Competition curves displaying the concentration range in which each ligand is able to compete for binding to VDR with approximately 0.4 nM [^{3}H]1,25D. Dexamethasone (Dex) is a ligand for the glucocorticoid receptor with no appreciable binding to VDR and thus serves as a negative control. Radioinert 1,25D (pink line) serves as a high affinity ligand to show the full range of competition possible in this assay. Docosahexaenoic acid (DHA, green line) is a low affinity VDR ligand [22]. Cyanidin (Cya, red line) is able to compete with [^{3}H]1,25D with an estimated IC_{50} of 500 µM. The competition results shown here are representative of two independent experiments.

effectively for binding to VDR (Fig. 3, pink line). DHA is capable of displacing [^{3}H]1,25D (IC_{50} at log −5.5, or approximately 3 μM) [22]. In contrast, dexamethasone (Dex) does not compete for binding to VDR [18]. Cyanidin (Fig. 3, red line) competes with an estimated IC_{50} of approx. 500 μM (log_{10} -3.3). However, as shown in Fig. 2, cyanidin upregulates transcripts of LCE genes in the range of 5–20 μM (log_{10} −4.7 to −5.3; see red box on *X*-axis of Fig. 3), which is approximately 25-fold lower than the concentration required to compete for VDR binding in this cell-free assay.

For a final test of cyanidin as a potential VDR ligand, human cell lines were transfected with a reporter plasmid containing a candidate VDRE corresponding to a sequence located near the human LCE3 gene cluster (Fig. 1). Two recipient human cell lines were chosen: HEK-293 embryonic kidney and CCD-1106 KERTr keratinocytes. As expected, 1,25D upregulated transcription from the VDRE-containing reporter plasmid, but not from the vector lacking the VDRE, in both cell lines (Fig. 4A and B, left pair of bars); an ethanol control, not shown, was used to calculate the fold effects. The activity of cyanidin was, however, markedly different in the two cell lines. Cyanidin showed essentially no activity in HEK-293 cells either alone or in combination with 1,25D (Fig. 4A). However, in the KERTr cells, 20 μM cyanidin upregulated the VDRE-containing reporter equivalently to 1,25D and significantly above either the ethanol control or the reporter containing no VDRE (Fig. 4B), thus demonstrating an ability to drive VDR/VDRE-dependent transcription in this cell line.

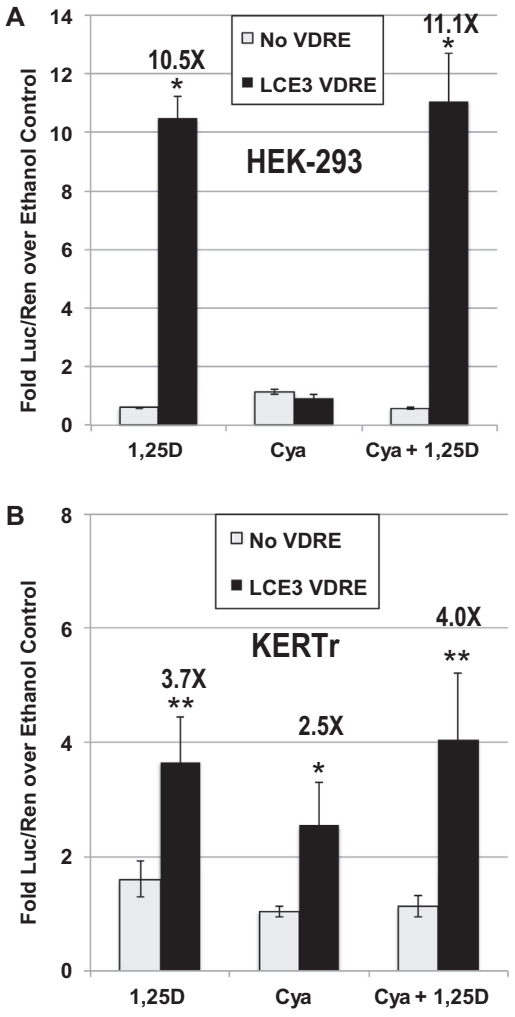

Fig. 4. Ability of 1,25D and cyanidin to modulate VDR-mediated transcription of a luciferase reporter gene under the control of a VDRE from the LCE3 gene cluster. HEK293 cells (panel A) or KERTr cells (panel B) were cotransfected with a VDR expression plasmid and either a reporter plasmid lacking a VDRE (No VDRE) or a plasmid containing two copies of the LCE3 VDRE element upstream of the firefly luciferase reporter gene (LCE3 VDRE). Cells also received a *Renilla* luciferase plasmid to control for transfection efficiency. Treatments included ethanol control, 10^{-8} M 1,25D, 20 μM cyanidin (Cya), or a combination of both. The firefly/Renilla ratios were calculated for each group, and the results are shown as fold inductions over the ethanol vehicle (ethanol results not shown). Results of Student's *t*-test are indicated by asterisk: $^*p < 0.05$; $^{**}p < 0.01$.

4. Discussion

Psoriasis therapy with 1,25D or its analogs, even when combined with an anti-inflammatory drug (e.g., betamethasone), is not effective in approximately 40% of patients with mild to moderate psoriasis [23]. Severe cases can be treated with biological response modifiers [24], but these injectable drugs are not available to patients with mild/moderate psoriasis [25]. The current study therefore sought to test a natural, dietary compound to determine if it could regulate psoriasis-relevant genes even more potently than 1,25D.

Anthocyanidins, notably delphinidin, have been shown to exert protective effects in skin, as well as antiproliferative and prodifferentiative actions [15]. These properties, plus their steroid-like structure, suggested that these compounds could act as VDR ligands. In the current study, cyanidin was tested in three stages: (i) regulation of LCE3 transcripts in primary human keratinocytes as monitored by qrtPCR; (ii) direct binding to VDR in a competition binding assay and (iii) ability to stimulate a luciferase reporter gene in transfected cells under control of a VDRE corresponding to a sequence near the LCE3 locus.

Experiments to probe cyanidin regulation of LCE3 transcripts in HEKn cells included two calcium concentrations: low calcium (60 μM), which is the maintenance medium for basal keratinocytes, and high calcium (1.2 mM) to induce differentiation to a more mature keratinocyte phenotype [19,26–28]. The results in Fig. 2A and B demonstrate that cyanidin upregulates the expression of all LCE genes tested in a dose-dependent fashion and that this effect is more pronounced in the cells pretreated for 24 h with high calcium. Presumably this difference reflects the differentiation state of the keratinocytes in accordance with the notion that LCE genes are expressed primarily in the more differentiated epidermal layers, as has been demonstrated for LCE2 proteins [11].

The effect of 1,25D to upregulate LCE3 mRNAs was tested previously in a format identical to that used in the current studies at doses up to 100 nM, a supraphysiological dose that is capable of promoting skin differentiation [29,30]. The fold increases above the ethanol control elicited by 1,25D were only in the range of 1.4- to 3.1-fold [17], whereas the current results with 20 μM cyanidin are between 6.7- and 9-fold (Fig. 2B). Although it is possible that higher doses of either 1,25D (or cyanidin) could elicit greater effects, the present data suggest that the effects of cyanidin on LCE3 gene expression may be substantially greater than those of 1,25D.

Competition binding assays indicate that although cyanidin is indeed capable of binding to VDR in this cell-free assay, the concentrations required to displace radiolabeled 1,25D (Fig. 3) are 25-fold higher than those required to induce LCE3 genes (Fig. 2). All LCE transcripts tested are induced by cyanidin concentrations of 20 μM (Fig. 2), whereas concentrations of 500 μM are required for 50% displacement of [^{3}H] 1,25D from VDR (Fig. 3). One explanation for this discrepancy could be that cyanidin is converted to a more active metabolite in HEKn cells or, alternatively, that cyanidin is acting via a novel, non-VDR-mediated mechanism to control LCE gene expression.

The results in Fig. 4 support the former hypothesis. This experiment, in which a luciferase reporter gene linked to a VDRE

sequence corresponding to a sequence near the LCE3 locus was transfected into recipient human cells, reveals that KERTr keratinocytes, but not HEK-293 embryonic kidney cells, support cyanidin induction of the reporter construct in a VDRE-dependent manner. One straightforward explanation for this dramatic difference is that the keratinocyte line, but not HEK-293, metabolizes cyanidin to a more active compound that binds VDR with higher affinity to activate VDRE-controlled genes. Future experiments will be directed toward testing this hypothesis.

The present investigation reveals novel attributes of gene regulation by VDR ligands in skin, focusing on genes implicated in psoriasis risk. Studies relating to the mechanism(s) of gene regulation by novel VDR ligands such as cyanidin could ultimately lead to a molecular basis for developing and testing candidate psoriasis therapies.

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