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An evaluation of the biologic activity and vitamin D receptor binding affinity of the photoisomers of vitamin D_3 and previtamin D_3

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Skin is the site of previtamin D_3 and vitamin D_3 synthesis and their isomerization in response to ultraviolet irradiation. At present, little is known about the function of the photoisomers of previtamin D_3 and vitamin D_3 in skin cells. In this study we investigated the antiproliferative activity of the major photoisomers and their metabolites in cultured human keratinocytes by determining their influence on ^{3}H-thymidine incorporation into DNA. Our results demonstrated that vitamin D_3 and 25-hydroxytachysterol$_3$ were effective in inhibiting ^{3}H-thymidine incorporation at both 10^{-8} and 10^{-6} M in a dose-dependent manner. Lumisterol, tachysterol$_3$, 5,6-trans-vitamin D_3, and 25-hydroxy-5,6-transvitamin D_3 only induced significant inhibition at 10^{-6} M. 25-Hydroxytachysterol$_3$ was approximately 10- to 100-fold more active than tachysterol$_3$. 7-Dehydrocholesterol was not active even at 10^{-6} M. The dissociation constants of vitamin D receptor (VDR) for 25-hydroxytachysterol$_3$, 25-hydroxy-5,6-trans-vitamin D_3, and 5,6-trans-vitamin D_3 were 22, 58, and 560 nM, respectively. The dissociation constants for 7-dehydrocholesterol, tachysterol, and lumisterol were greater than 20 μM. In conclusion, vitamin D_3, its photoisomers, and the photoisomers of previtamin D_3 have antiproliferative activity in cultured human keratinocytes. However, the antiproliferative activity did not correlate with their binding affinity for VDR. The results suggest that some of the photoproducts may be metabolized to their 25-hydroxylated and 1α,25-dihydroxylated counterparts before acting on VDR. Alternatively, a different receptor may recognize these photoproducts or another mechanism may be involved in modulating the antiproliferative activity of the photoisomers examined. (J. Nutr. Biochem. 11:267–272, 2000)

Keywords: skin; keratinocytes; thymidine incorporation; antiproliferation

Introduction

Vitamin D is absolutely essential for the maintenance of calcium homeostasis and for the development and maintenance of healthy bones in animals.[1] There are two major sources of vitamin D; one is diet and the other is the cutaneous synthesis. However, because vitamin D is rare in foods, it is casual exposure to sunlight that is responsible for providing humans with most of their vitamin D requirement. During exposure to sunlight, the ultraviolet B (UVB) portion of the solar spectrum (295–315 nm) causes the photolysis of epidermal stores of 7-dehydrocholesterol (7-DHC) to previtamin D_3. Previtamin D_3 then thermoisomerizes to vitamin D_3, a process that is facilitated by the lipid-membrane environment.[2] After vitamin D_3 is made in the skin or ingested in the diet, it must be hydroxylated at carbon positions 25 and 1 in the liver and kidney, respectively, to form 1α,25-dihydroxyvitamin D_3 [1α,25 $(OH)_2D_3$], the active form of vitamin D_3. 1α,25$(OH)_2D_3$ is responsible for promoting intestinal calcium absorption and the mobilization of calcium from bone for the purpose of

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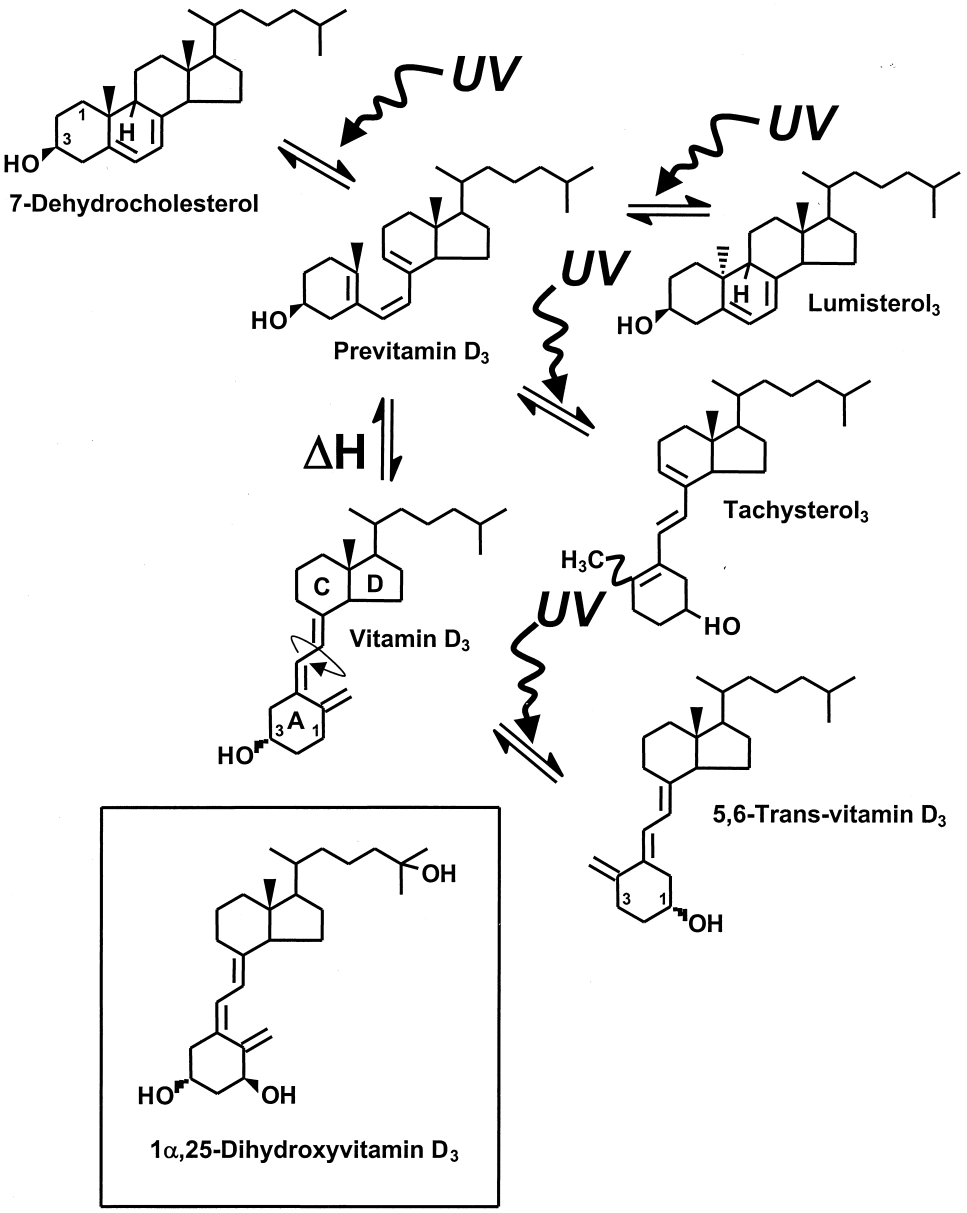

Figure 1 Structures of vitamin D_3 and its related compounds

maintaining a normal level of serum calcium. During the past 20 years, it has been recognized that $1\alpha,25(OH)_2D_3$ also can influence a variety of other biologic processes through its vitamin D receptor (VDR) in tissues other than those that regulate calcium metabolism, including skin. In cultured human keratinocytes, $1\alpha,25(OH)_2D_3$ inhibited basal cell proliferation and stimulated differentiation to form the cornified envelope.[3–5]

During chronic exposure to sunlight, both previtamin D_3 and vitamin D_3 in the skin can be photoisomerized to a variety of photoproducts, including 5,6-trans-vitamin D_3, tachysterol, and lumisterol.[6,7] Because the concentrations of these photoproducts in skin cells are likely to be very high, it is possible that some of these photoproducts may have direct biologic properties on skin cells. Because tachysterol and 5,6-trans-vitamin D_3, which are photoproducts of previtamin D_3 and vitamin D_3, respectively, have a pseudo-1-α-hydroxyl structure due to the 180-degree rotation of the 3-hydroxyl group during isomerization (*Figure 1*), it is possible that these analogs may act like $1\alpha,25(OH)_2D$ in the epidermis. It has been previously reported that 5,6-trans-vitamin D_3 and tachysterol can mimic the intestinal calcium transport activity of $1\alpha,25(OH)_2D$ in anephric rats and that their 25-hydroxy derivatives were more active in the same assay system.[8] Therefore, we evaluated the potential antiproliferative activity of the major photoproducts of previtamin D_3 and vitamin D_3 by determining their effects on ^{3}H-thymidine incorporation into cultured normal human keratinocytes and compared the antiproliferative activity with their binding to VDR using a calf thymus cytosol receptor preparation.

Methods and materials

Cell cultures

Normal human keratinocytes were obtained from neonatal foreskins as described previously.[4,9] Keratinocytes were plated and grown on lethally irradiated 3T3 fibroblast feeder layer in a serum-free basal medium supplemented with amino acids and growth factors as previously described.[10]

^{3}H-Thymidine incorporation

When the second-passage cells grown on 24-well plates reached approximately 40% confluency, they were fed with basal MCDB-153 medium supplemented with amino acids without growth factors.[10] Two days later, cells were incubated with epidermal growth factor (EGF; 25 ng/mL) and with or without 1α,25(OH)$_2$D$_3$, vitamin D$_3$, 7-DHC, and photoisomers of vitamin D$_3$ and previtamin D$_3$ (10^{-10}, 10^{-8}, and 10^{-6} M), as indicated in the figure legends. Eighteen hours later, the medium was replaced with 0.5 mL of fresh basal medium containing 1 μCi of [methyl-^{3}H]thymidine and incubated at 37°C for 3 hr. ^{3}H-Thymidine incorporation into DNA was stopped by placing the 24-well plates on ice; unincorporated ^{3}H-thymidine was removed and the cells were washed three times with ice-cold phosphate-buffered saline. DNA labeled with ^{3}H-thymidine was extracted by the perchloric acid method, as described previously.[4]

Receptor binding assay

The binding of vitamin D$_3$ and the photoisomers of previtamin D$_3$ and vitamin D$_3$ to VDR was performed using a nonequilibrium binding assay in which calf thymus 1α,25(OH)$_2$D receptor was used.[11] The procedures for the determination of binding affinity for calf thymus 1α,25(OH)$_2$D receptor have been described in detail elsewhere.[12] The K_d was defined as the concentration at which it caused a 50% reduction in the binding of ^{3}H-labeled 1α,25(OH)$_2$D$_3$ to thymus receptor.

Synthesis of photoisomers

Tachysterol$_3$ was prepared by irradiating 7-DHC solution in methanol (50 μg/mL) in a quartz tube placed on ice at 254 nm using a monochromatic light source (Oriel Corp., Stamford, CT USA) for 9 min. The irradiated solution was dried under a stream of nitrogen gas and redissolved in 200 μL of 1.2% 2-propanol in n-hexane. The solution was then applied to a high performance liquid chromatography (HPLC; Waters Associates, Milford, MA USA) using a preparative Econosphere silica column (10 μ particle size, 250 × 10 mm; Alltech Associates, Deerfield, IL USA) with a mobile phase of 1.2% 2-propanol in n-hexane and a flow rate of 2 mL/min. The fraction containing tachysterol$_3$ was dried under a stream of nitrogen gas, redissolved in 0.1% 2-propanol in n-hexane, and applied to HPLC using SB-CN column (250 × 4.6 mm; MAC MOD Analytical Inc., Chaddsford, PA USA) with a mobile phase of 0.1% 2-propanol in n-hexane and a flow rate of 1 mL/min. The fraction containing the purified tachysterol$_3$ was dried under a stream of nitrogen gas and redissolved in 100% ethanol, and its quantity was determined. The ultraviolet (UV) absorption spectrum showed λ_{max} of 279 nm. The yield was approximately 50%. 25-Hydroxytachysterol$_3$ [25(OH)tachysterol$_3$] was synthesized from 25-hydroxy-7-DHC by a procedure similar to that used for tachysterol$_3$. Lumisterol$_3$ was prepared by exposing 7-DHC solution in methanol (400 μg/mL) in a quartz tube to a UVB light box (National Biological Corp., Cleveland, OH USA) containing FS-40 fluorescent tubes with a spectral output of 280 to 450 nm for 30 min at room temperature. The exposed solution was dried under a stream of argon gas. The residue was redissolved in an aliquot of 0.5% 2-propanol in n-hexane and applied to HPLC using an Econosphere Sil silica column (5 μ, 250 × 4.6 mm; Alltech Associates) with a flow rate of 1.6 mL/min. The fraction containing lumisterol$_3$ was dried and redissolved in 100% ethanol. The UV absorption spectrum and the concentration were determined using a Perkin-Elmer 552A spectrophotometer (Norwalk, CT USA). The yield was approximately 40%. 5,6-Trans-vitamin D$_3$ and 25-hydroxy-5,6-transvitamin D$_3$ were prepared from vitamin D$_3$ and 25-hydroxyvitamin D$_3$ [25(OH)D$_3$], respectively, as previously described.[8]

Materials

Crystalline 7-DHC was obtained from Salsburg Laboratories Inc. (Charies City, IA USA) and vitamin D$_3$ was purchased from Sigma Chemical Co. (St. Louis, MO USA). 25-Hydroxy-7-DHC and 1α,25(OH)$_2$D$_3$ were gifts from Dr. R. Gray of Amoco BioProducts Corporation (Naperville, IL USA) and Dr. M. Uskokovic of Hoffmann-La Roche Inc. (Nutley, NJ USA), respectively. Tissue culture media, insulin, hydrocortisone, and prostaglandin E$_1$ were obtained from Sigma Chemical Co. Human recombinant EGF was purchased from PeproTech (Rock Hill, NJ USA). ^{3}H-Thymidine (specific activity, 72.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA USA). ^{3}H-1α,25(OH)$_2$D$_3$ [1α,25-dihydroxy(26,27-methyl-^{3}H)cholecalciferol] with a specific activity of 180 Ci/mmol (TRK 656) was obtained from Amersham (Arlington Heights, IL USA).

Statistics

Results are reported as mean ± SEM, and the unpaired Student's *t*-test was used to calculate *P*-values.

Results

Figure 2 demonstrates that 1α,25(OH)$_2$D$_3$ was highly effective in inhibiting ^{3}H-thymidine incorporation in a dose-dependent manner as reported previously.[10] At concentrations of 10^{-10}, 10^{-8}, and 10^{-6} M, 1α,25(OH)$_2$D$_3$ caused a 48 ± 2, 65 ± 3, and 85 ± 1% inhibition, respectively, in ^{3}H-thymidine incorporation compared with the control. Lumisterol$_3$, tachysterol$_3$, 5,6-trans-vitamin D$_3$, and 25(OH)5,6-trans-vitamin D$_3$ at either 10^{-10} or 10^{-8} M did not cause any significant inhibition in ^{3}H-thymidine incorporation. Only at 10^{-6} M did lumisterol$_3$, tachysterol$_3$, 5,6-trans-vitamin D$_3$, and 25(OH)5,6-trans-vitamin D$_3$ induce a 27 ± 3 ($P < 0.01$), 58 ± 3 ($P < 0.001$), 64 ± 2 ($P < 0.001$), and 67 ± 3% ($P < 0.001$) inhibition, respectively. On the other hand, vitamin D$_3$ and 25(OH)tachysterol$_3$ were active in inhibiting ^{3}H-thymidine incorporation at both 10^{-8} and 10^{-6} M in a dose-dependent manner, with no significant inhibitory effect found at 10^{-10} M. Vitamin D$_3$ caused a 25 ± 8 ($P < 0.01$) and 55 ± 3% ($P < 0.001$) inhibition at 10^{-8} and 10^{-6} M, respectively; 25(OH)tachysterol$_3$ induced a 33 ± 6 ($P < 0.01$) and 81 ± 2% ($P < 0.001$) inhibition at 10^{-8} and 10^{-6} M, respectively. 25(OH)Tachysterol was approximately 10- to 100-fold more active than tachysterol and vitamin D$_3$ in this respect. There was no inhibition in ^{3}H-thymidine incorporation induced by 7-DHC at doses ranging from 10^{-10} to 10^{-6} M.

Because the biologic activity of 1α,25(OH)$_2$D$_3$ is believed to be mediated by VDR, we next determined the binding affinity of 1α,25(OH)$_2$D$_3$, vitamin D$_3$, and selected

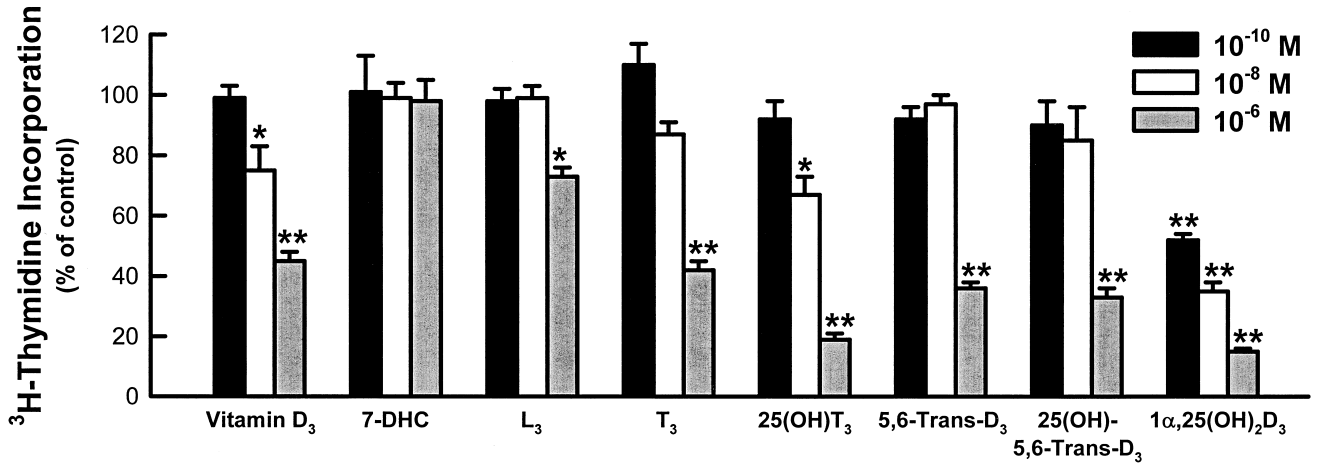

Figure 2 Dose-dependent inhibition of ^{3}H-thymidine incorporation into keratinocytes in the presence of vitamin D_3 and its related compounds. After keratinocytes were fed with basal medium without growth factors for 2 days, cells were incubated with fresh basal medium containing 25 ng/mL of epiderman growth factor (EGF) and 0, 10^{-10}, 10^{-8}, or 10^{-6} M of vitamin D_3 or its related compounds for 18 hr at 37°C. At the end of incubation period, ^{3}H-thymidine incorporation was performed. The results are presented as the percent of control in the absence of test compounds. Each value is the mean ± SEM of six to eight determinations. *$P < 0.01$, **$P < 0.001$ compared with respective controls. 7-DHC, 7-dehydrocholesterol; L_3, lumisterol$_3$; T_3, tachysterol$_3$; 25(OH)T_3, 25-hydroxytachysterol$_3$; 5,6-trans-D_3, 5,6-trans-vitamin D_3; 25(OH)5,6-trans-D_3, 25-hydroxy-5,6-trans-vitamin D_3; 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D_3.

photoisomers of vitamin D_3 and previtamin D_3 to VDR using a calf thymus cytosol preparation (*Figure 3*). The K_ds for VDR were 3.8 pM, 22 nM, 58 nM, and 560 nM for 1α,25(OH)$_2$D$_3$, 25(OH)tachysterol$_3$, 25(OH)trans-vitamin D_3, and 5,6-trans-vitamin D_3, respectively. The K_ds for 7-DHC, vitamin D_3, tachysterol$_3$, and lumisterol$_3$ were all greater than 20 μM with the following order of decreasing binding affinity: 7-DHC > vitamin D_3 > tachysterol$_3$ > lumisterol$_3$.

Discussion

Our results demonstrate that vitamin D_3 itself and some of the photoisomers of vitamin D_3 and previtamin D_3 including tachysterol$_3$, 5,6-trans-vitamin D_3, and their 25-hydroxylated metabolites are active in inhibiting ^{3}H-thymidine incorporation into cultured normal human keratinocytes (*Figure 2*). The activity found for vitamin D_3 could be attributed to its metabolism to 1α,25(OH)$_2$D$_3$ in cultured keratinocytes, which have been shown to possess both vitamin D-25-hydroxylase and 25(OH)D-1α-hydroxylase activities.[13–15] The recent demonstraton of vitamin D-25-hydroxylase activity in keratinocytes suggests that the photoisomers of previtamin D_3, such as tachysterol$_3$, may be substrates for this enzyme and be hydroxylated to 25(OH)tachysterol$_3$, which is a pseudo-1α,25(OH)$_2$D$_3$ analog. Thus, 25(OH)tachysterol$_3$ could be recognized by the VDR in keratinocytes and cause a direct biologic response.

The antiproliferative activity of the photoisomers did not correlate with their binding affinity for VDR (*Figure 3*). For

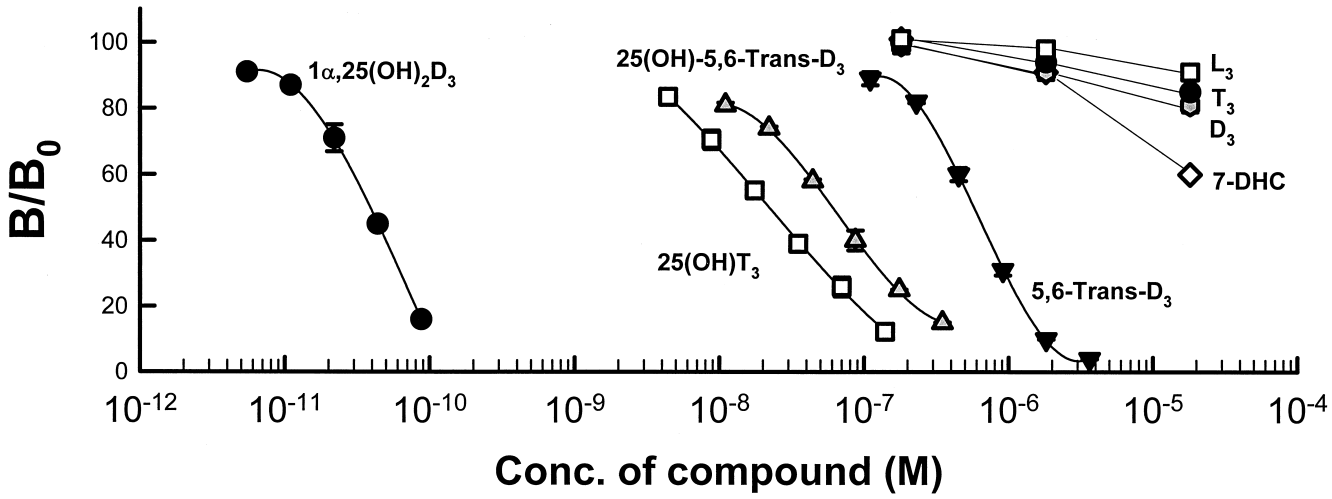

Figure 3. Competition for ^{3}H-1α,25(OH)$_2$D$_3$ binding to the thymus 1α,25(OH)$_2$D receptor by nonradioactive 1α,25(OH)$_2$D$_3$, vitamin D_3, and vitamin D_3-related compounds. 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D_3; 25(OH)5,6-trans-D_3, 25-hydroxy-5,6-trans-vitamin D_3; 25(OH)T_3, 25-hydroxytachysterol$_3$; 5,6-trans-D_3, 5,6-trans-vitamin D_3; 7-DHC, 7-dehydrocholesterol; L_3, lumisterol$_3$; T_3, tachysterol$_3$.

example, the binding affinity of 25(OH)tachysterol$_3$ to calf thymus VDR was at least 1,000-fold higher than that between the receptor and tachysterol$_3$ and yet only a 10-fold difference in their antiproliferative activity was found. Likewise, although 25(OH)5,6-trans-vitamin D_3 and 5,6-trans-vitamin D_3 bound to thymus VDR with a 10-fold difference in binding affinity, both compounds were only active in inhibiting keratinocyte proliferation at 10^{-6} M. Furthermore, 7-DHC bound to thymus VDR better than tachysterol$_3$, yet tachysterol$_3$ had much higher antiproliferative activity than did 7-DHC.

There are several possible explanations for the discrepancy between the antiproliferative activity and VDR binding affinity. First, it is possible that VDR might be different among different species or different tissues. Thus, the binding affinities of various photoproducts to calf thymus may not reflect the biologic activity in human skin. At the present time, there is no evidence to support this.

Second, the effect of vitamin D_3, 1α,25$(OH)_2D_3$, and the photoisomers of vitamin D_3 and previtamin D_3 on inhibiting keratinocyte proliferation may be mediated by a receptor other than the classic VDR.[15] Recent evidence suggests that different 1α,25$(OH)_2D_3$ receptor forms may be responsible for the signal transduction processes associated with genomic and nongenomic actions of 1α,25$(OH)_2D_3$. For example, 1α,25$(OH)_2D_3$ rapidly increases intracellular Ca^{2+} in osteoblast-like cells (ROS 17/2.8) that lack VDR. The effect can be inhibited by its inactive epimer 1β,25$(OH)_2D_3$, which does not displace 1α,25$(OH)_2D_3$ from VDR.[16] The rapid action of 1α,25$(OH)_2D_3$ on calcium transport was also observed in perfused duodena of normal chicks.[17] This non-nuclear action has been shown to be mediated by a plasma membrane receptor and not VDR.[18] Norman et al.[18] synthesized 1α,25$(OH)_2$-9,14,19,19,19-pentadeuterio-pre-D_3 to investigate whether it had any biological activity. The presence of the deuterium atoms in the molecules of 1α,25$(OH)_2$-9,14,19,19,19-pentadeuterio-pre-D_3 suppressed its isomerization from the previtamin to the vitamin form and thus functioned primarily as an analog of the 6-s-cis form of 1α,25$(OH)_2D_3$. They demonstrated that both nongenomic actions were equally responsive to 1α,25$(OH)_2D_3$ and 1α,25$(OH)_2$-9,14,19,19,19-pentadeuterio-pre-D_3, whereas 1α,25$(OH)_2$-9,14,19,19,19-pentadeuterio-pre-D_3 was significantly less active than 1α,25$(OH)_2D_3$ in the genomic induction of osteocalcin gene.

Third, the biologic activity may be determined by the availability of receptors induced by different ligands. This possibility is supported by a recent report indicating that the antiproliferative and the differentiation effect of vitamin D analogues in skin is determined by the ability of the compounds to induce VDR transcription rather than the binding affinity for VDR only.[19]

Fourth, during the 18-hr incubation at 37°C, the putative 25-hydroxylase activity present in keratinocytes may metabolize 5,6-trans-vitamin D_3 and tachysterol$_3$ to 25(OH)5,6-trans-vitamin D_3 and 25(OH)tachysterol$_3$, respectively. Therefore, the activity seen with the addition of 5,6-trans-vitamin D_3 or tachysterol$_3$ may be due in part to their 25-hydroxylated counterparts. It is also likely that 25-hydroxylated photoisomers, which were either added to the incubation media or derived from the 25-hydroxylation of photoisomers during the incubation, may be further metabolized to their respective 1α,25-dihydroxylated compounds by the 1α-hydroxylase present in keratinocytes,[13] which in turn acted on VDR to exert their biological activity. This possibility is strengthened by the observation that dihydrotachysterol, which has an A-ring orientation similar to that of tachysterol and 5,6-trans-vitamin D_3, can be hydroxylated at the C-1 position to form 1α,25-dihydroxy-dihydrotachysterol and 1β,25-dihydroxy-dihydrotachysterol in vivo.[20]

Thus, the present study suggests that sunlight is not only the major provider of vitamin D_3 for humans for maintaining calcium and phosphorus homeostasis, but also provides vitamin D_3 and photoproducts that may act directly to regulate epidermal proliferation. The amounts of vitamin D_3 and tachysterol$_3$ that can be made in the epidermal cell during solar irradiation are much higher than the picogram quantities of 1α,25$(OH)_2D_3$ that are exposed to the epidermis from the circulation. Thus, the results suggest the possibility that vitamin D_3 and some of the photoproducts of previtamin D_3, such as tachysterol$_3$, can regulate epidermal proliferation and differentiation under physiologic conditions.

UVB phototherapy has been used to treat psoriasis since 1925 when Goeckerman[21] observed the benefit of combined treatment using tar and UV light. New developments in phototechnology during the past decade have made UVB phototherapy alone emerge as a reasonably effective and fairly practical treatment for psoriasis.[22] The mechanisms of the therapeutic effect of UV radiation on psoriasis are not well understood.[22] Most hypotheses have focused on a direct effect of UV radiation on inhibiting DNA synthesis in the hyperproliferating cells. Alternatively, it has long been suspected that during UV radiation therapy some photoproducts that are produced were having therapeutic benefits.[7] Our present results strongly suggest that the formation of vitamin D_3 and previtamin D_3 photoisomers could be a major factor in the therapeutic benefit that UVB phototherapy provides for psoriasis.

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