Effect of 1α,25-Dihydroxyvitamin D₃ on the Morphologic and Biochemical Differentiation of Cultured Human Epidermal Keratinocytes Grown in Serum-Free Conditions

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The effect of 1α,25-dihydroxyvitamin D₃ [1α,25-(OH)₂-D₃] on the proliferation and morphologic and biochemical differentiation of cultured human epidermal keratinocytes grown under defined, serum-free conditions was studied. 1α,25-(OH)₂-D₃ caused a dose-dependent decrease in proliferation and an increase in the morphologic differentiation of human cultured keratinocytes. The number of attached basal cells decreased when exposed to 1α,25-(OH)₂-D₃, whereas the number of attached squamous cells, terminally differentiated desquamated cells, and cornified cells increased concurrently. In addition, after incubation with 1α,25-(OH)₂-D₃, there was a shift to cells of lighter density. In conjunction with its effect on the basal cells, 1α,25-(OH)₂-D₃ resulted in an inhibition of DNA synthesis. The activity of transglutaminase, the enzyme responsible for cross-linking the proteins of the cornified envelope, was stimulated by 156% with 1α,25-(OH)₂-D₃, but not with 1β,25-(OH)₂-D₃, a biologically inert isomer. Therefore it appears that 1α,25-(OH)₂-D₃ is a potent inhibitor of keratinocyte proliferation as well as a stimulator of epidermal terminal differentiation. J Invest Dermatol 86:709-714, 1986

A known biologically active form of vitamin D₃ in both the small intestine and bone is 1α,25-dihydroxyvitamin D₃ [1α,25-(OH)₂-D₃] [1]. Recently, receptor-like macromolecules specific for 1α,25-(OH)₂-D₃ were characterized in a number of tissues including skin [2-5], suggesting that these tissues also targets for this hormone. In addition, 1α,25-(OH)₂-D₃ was implicated in regulating the growth and differentiation of various tumor cell lines and normal cells, including human skin cells [2,4,6,7]. The effect of 1α,25-(OH)₂-D₃ on the proliferation of skin cells was studied. Our group investigated the growth-inhibition effect of 1α,25-(OH)₂-D₃ on normal human dermal fibroblasts [2], and Hossomi et al [7] investigated the effect of 1α,25-(OH)₂-D₃ on the proliferation and morphologic differentiation of normal murine epidermal keratinocytes. In addition, other groups evaluated the effect of 1α,25-(OH)₂-D₃ on the proliferation of tumor cell lines derived from human melanomas [4,8].

Knowing that receptor-like macromolecules specific for 1α,25-(OH)₂-D₃ exist in human keratinocytes, and knowing the effect of this hormone on growth and differentiation of other cell types, we set out to ascertain whether 1α,25-(OH)₂-D₃ has an effect on the proliferation and morphologic differentiation of cultured human keratinocytes grown under serum-free conditions. The effect of 1α,25-(OH)₂-D₃ on 2 important biochemical markers of epidermal differentiation, transglutaminase activation and keratin content, was examined.

MATERIALS AND METHODS

Keratinocyte Culture Keratinocytes were grown in culture using a modification of the method of Rheinwald and Green [9,10]. 3T3 cells were plated at 0.5 × 10⁵ cells/35-mm tissue-culture dish, and 2 days later were lethally irradiated with a cobalt-60 source (5000 rads). Keratinocytes were obtained from neonatal foreskin after overnight trypsinization at 4°C and treatment with 0.02% EDTA. Keratinocytes were plated in 2 ml of serum-free medium per dish on the lethally irradiated 3T3 cells. Each experiment was performed on primary or secondary keratinocyte cultures obtained from different skin samples. The serum-free medium consisted of Dulbecco’s modified Eagle’s medium (DMEM) with high (1.8 mm) or low (<0.1 mm) concentration of calcium ([M.A. Bioproducts, Walkersville, Maryland) containing 7 growth factors: epidermal growth factor (25 ng/ml); hydrocortisone (203 ng/ml); insulin (5 μg/ml); transferrin (5 μg/ml); prostaglandin E₂ (50 ng/ml); cholela toxin (0.1 μg/ml); Sigma Chemical Co., St. Louis, Missouri); and selenious acid (2 ng/ml; Collaborative Research, Lexington, Massachusetts). Unless otherwise noted, cultures were grown in DMEM with a high calcium concentration. At 1 week in culture, hydrocortisone and cholela toxin were removed from the medium, and the dishes were washed with 0.02% EDTA to remove any remaining 3T3 cells. For the various assays, fresh medium containing vehicle alone (control;...
Quantitation of Morphologic Changes During Keratinocyte Differentiation  Beginning at 1 week in culture, groups of triplicate plates of keratinocytes were incubated with 1α,25-(OH)2-D3 or vehicle alone. After 1 or 2 weeks of dosing, the medium was removed from each culture, spun down, and resuspended for the counting of the desquamated flatter cells. A hemacytometer was used to count the different cell types under a phase-contrast microscope. The attached cells were then trypsinized for 30–40 min with 0.1% EDTA and 0.1% trypsin and then neutralized with medium. The keratinocytes were spun down and resuspended in a known volume of medium. Duplicate aliquots were taken for counting the basal (small, rounded) and squamous (larger, irregular-shaped, flattened) cells. The remaining cells were spun down and treated with 10 mM Tris-HCl (pH 7.4) with 1% β-mercaptoethanol and 1% sodium dodecyl sulfate (SDS) at room temperature for 10 min [11]. Only cells with cornified envelopes were present after this treatment.

Cell Density  Beginning at 1 week in culture, keratinocytes were dosed with 1α,25-(OH)2-D3 or vehicle alone. At the appropriate time, the attached cells were harvested by trypsinization. Both the attached and desquamated flatter cells were spun down by low-speed centrifugation and resuspended in 1.0 ml of Hank's buffered salt solution (M.A. Bioproducts). The cells were layered on 20–60% (1.032–1.090 g/ml) Percoll (Pharmacia Fine Chemicals, Piscataway, New Jersey) linear gradients and were spun for 25 min at 800 g. These experiments used a modification of the method of Simon and Green [12]. The gradients were disrupted in 8 fractions by tube puncture. Gradients with density-marker beads were run simultaneously with the sample gradients to verify density level.

Thymidine Incorporation  Triplicate dishes of keratinocytes were incubated in the presence or absence of 1α,25-(OH)2-D3 at each feeding (3 x/week). At specific times, after 3, 7, 10, or 14 days of incubation with 1α,25-(OH)2-D3, cultures were pulse-labeled with 10 μCi of [methyl-3H]thymidine (20 Ci/mmol, New England Nuclear) for 4 h. The cultures were rinsed with phosphate-buffered saline (PBS), harvested by scraping, and extracted for DNA [13]. Duplicate aliquots were taken to count thymidine incorporation into DNA, and the remainder of each sample was assayed for total DNA content using calf thymus DNA as a standard [14].

Transglutaminase Assay  After 24 h of incubation with 1α,25-(OH)2-D3, 1β,25-(OH)2-D3, or 25-OH-D3, the keratinocyte cultures, grown in DMEM with a low calcium concentration (<0.1 mm), were washed with Hank's buffered salt solution, and the cells were harvested by scraping with a rubber policeman into 10 mM Tris-HCl, 10 mM dithiothreitol (DTT), and 0.5 mM EDTA, pH 7.4. The cells were sonicated disrupted (6 bursts of 15 s each at 20 kHz), extracted, and centrifuged at 12,000 g to obtain a transglutaminase extract, and aliquots of the extracts were removed to measure the protein content [15,16]. Duplicate aliquots of each extract were run in the transglutaminase assay according to the method of Lichte et al [16]. Aliquots of extract were incubated in a Dubnoff metabolic shaking incubator (Precision Scientific Group, GCA Corp., Chicago, Illinois) for 50 min at 25°C in a 50 mM sodium borate buffer (pH 9.5) containing 0.5 mg/ml EDTA, N,N-dimethylated casein (400 μg), 5.0 mM calcium chloride, and 10 μCi of [2,3-3H(N)]putrescine dihydrochloride (37.4 Ci/mmol, New England Nuclear). The enzyme reactions were ended by adding cold trichloroacetic acid and putrescine to each sample. Protein pellets were collected on GF/A glass filter (Whatman, Ann Arbor, Michigan) using a 1225 sampling manifold (Millipore Corp., Bedford, Massachusetts). The protein on the filters was dissolved by incubation in 0.5 ml Protosol (New England Nuclear) at 50°C for 30 min prior to counting the radioactivity on a Tri-Carb 460 C scintillation counter (Packard, Downers Grove, Illinois).

Labeling and Extraction of Keratins  At 1 week in culture, keratinocytes were incubated with either 1α,25-(OH)2-D3, retinyl acetate (Sigma Chemical Co.), or vehicle alone. After 1 week of dosing, 0.5 ml of medium was replaced with DMEM containing a low concentration of methionine (3 μg/ml) to which was added [35S]methionine (1002 Ci/mmol, New England Nuclear) to a final concentration of 10 μCi/ml [17]. After an incubation period of 7.5 h, the plates were washed with Hank’s buffered salt solution, and the cells were harvested by scraping into a Tris-HCl buffer (pH 7.4) containing 1 mM EDTA. Cells were sonically disrupted and centrifuged at 12,000 g. The resulting pellet, containing the keratins, was incubated at 37°C for 20 min in a Tris-HCl buffer (pH 6.8) including 1 mM EDTA, 10 mM DTT, and 2% SDS. The samples were sonically disrupted, heated at 100°C for 2 min, and centrifuged at 12,000 g for 10 min to remove the insoluble residue. Aliquots of cellular extract (20 μg protein/lane) were electrophoresed through a 8.5% SDS-polyacrylamide gel using the Laemmli system [17,18]. Staining was carried out in a solution containing Coomassie blue (0.125%), methanol (50%), and acetic acid (10%), and destaining in a solution of methanol (50%) and acetic acid (10%). Molecular weights of the bands were estimated by comparison with SDS molecular-weight markers (Sigma Chemical Co.), including bovine serum albumin (BSA) (66 kD) and ovalbumin (45 kD), run on the same gel. The gels were then treated with EN'HANCE (New England Nuclear), dried, and exposed to Kodak XAR-5 film at −80°C.

RESULTS
Effect of 1α,25-(OH)2-D3 on the Morphologic Differentiation of Cultured Human Keratinocytes  After 1 week or 2 weeks of incubation with 10–18 M or 10–14 M of 1α,25-(OH)2-D3, there were significant changes in the proportion and the number of keratinocytes at different stages of differentiation (Fig 1). These changes are apparent (Fig 2) when viewing the cells under a phase-contrast microscope (100 x). There was a decrease in total cell number after incubation with 1α,25-(OH)2-D3 at 10–18 M. After 1 week (Fig 1A) and 2 weeks (Fig 1B), the percentage and number of basal cells decreased in a dose- and time-dependent fashion. After 1 week, there were 9.2 x 103 basal cells (93% of total cell number) in control cultures. After incubation with 1α,25-(OH)2-D3 (10–16 M), there were 8.7 x 103 basal cells (89% of total cell number), and after incubation with 10–8 M of 1α,25-(OH)2-D3, there were 4.8 x 103 basal cells (78% of total cell number). In both instances, the difference between the number of basal cells in 1α,25-(OH)2-D3-dosed and control cultures was significant.

Simultaneously with the decrease in basal cells, dosing with 1α,25-(OH)2-D3 resulted in a time- and dose-dependent increase in the percentage and number of squamous cells (Fig 1). After 1 week of dosing, cultures incubated with 10–18 M of 1α,25-(OH)2-D3 had a greater percentage (7.0%) and number (6.6 x 104) of squamous cells than the controls had (5.0%, 5.3 x 104 cells) (Fig 1A). There was a further increase in the percentage (12%) and number (7.5 x 104) of squamous cells in cultures incubated with 1α,25-(OH)2-D3 at 10–8 M.

There was a dose- and time-dependent increase in the percentage of cells that had terminally differentiated and had sloughed off into the medium after incubation with 1α,25-(OH)2-D3 (Fig 1). After 1 week of incubation with 10–8 M of 1α,25-(OH)2-D3, the percentage (10%) and number (6.4 x 104) of desquamated
Figure 1. Effect of 1α,25-(OH)₂-D₃ on the morphologic differentiation of cultured human keratinocytes. The proportion of different keratinocyte cell types after 1 week (A) or 2 weeks (B) of incubation with vehicle alone (open bar); 1α,25-(OH)₂-D₃ at 10⁻¹⁰ M (dotted bar); or 1α,25-(OH)₂-D₃ at 10⁻⁸ M (striped bar). Each bar represents the mean of triplicate determinations ± SEM. Student’s t-test was used to assess level of significance (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

floaters were significantly greater (p < 0.01) than the percentage (2%) and number (1.5 x 10⁴ cells) of floaters in control cultures (Fig 1A). In dishes incubated with 10⁻⁹ M of 1α,25-(OH)₂-D₃, the percentage (4%) and number of floaters (3.4 x 10⁴) were also greater than they were in the control cultures (Fig 1A).

After incubation with 1α,25-(OH)₂-D₃, there was a significant increase in the number of keratinocytes with cornified envelopes, as can be seen in the cultured cells exposed to 1α,25-(OH)₂-D₃ (Fig 2). After 1 week of incubation with 1α,25-(OH)₂-D₃ at 10⁻⁸ M, there was a greater than 4-fold increase in the number of keratinocytes with cornified envelopes (8.9 x 10⁴) in comparison with control cultures (2.0 x 10⁴, p < 0.001). After 2 weeks, there was a further increase in the number of cornified envelopes in both control (4.2 x 10⁴) and 1α,25-(OH)₂-D₃-dosed (9.8 x 10⁴) cultures (p < 0.01).

Effect of 1α,25-(OH)₂-D₃ on Keratinocyte Density
Subsequent to incubation with 1α,25-(OH)₂-D₃ at 10⁻⁸ M, there was a shift to lighter cell density (Fig 3). Fractions collected from linear Percoll density gradients demonstrated that incubation of cells with 1α,25-(OH)₂-D₃ resulted in a dramatic shift in density: from greater than to less than 1.061 g/ml (fraction 4). Greater than 77% of the keratinocytes incubated with 1α,25-(OH)₂-D₃ fell within this density range (fractions 4–8), whereas only 58% of the control cells fell within this density range.

Effect of 1α,25-(OH)₂-D₃ on [³H]Thymidine Incorporation into the DNA of Cultured Human Keratinocytes
After 7 days of incubation with a high concentration of 1α,25-(OH)₂-D₃ (10⁻⁶ M), there was a significant decrease in [³H]thymidine incorporation (776 ± 46 cpm/μg DNA, 10.9% of control value), when compared with control cultures (7060 ± 54 cpm/μg DNA) (Fig 4). After 10 days, there was a significant decrease in the [³H]thymidine incorporation at 10⁻⁶ M and 10⁻⁸ M of 1α,25-(OH)₂-D₃, and, after 14 days, there was a significant decrease in [³H]thymidine incorporation at all dose levels (10⁻⁶, 10⁻⁸, 10⁻⁶ M).

Effect of 1α,25-(OH)₂-D₃ on the Transglutaminase Activity of Cultured Human Keratinocytes
After 1 week in culture, human keratinocytes were incubated with vehicle alone, 1α,25-(OH)₂-D₃ (10⁻⁶, 10⁻⁸, or 10⁻⁶ M); 25-OH-D₃ (10⁻¹⁰, 10⁻⁸, or 10⁻⁴); or 1β,25-(OH)₂-D₃ (10⁻⁶ M). When compared with enzyme extracts of control cultures, extracts of keratinocytes incubated with 1α,25-(OH)₂-D₃ for 24 h had a dose-dependent increase in transglutaminase activity (p < 0.05) (Fig 5). In contrast, the transglutaminase activity of cultures incubated with 10⁻⁶ M

Figure 2. Phase-contrast micrographs of cultured human keratinocytes grown in the absence (a) or presence (b) of 1α,25-(OH)₂-D₃ for 2 weeks (× 1000). Note the formation of enlarged squamous cells in the 1α,25-(OH)₂-D₃-dosed culture (arrow).

Figure 3. Effect of 1α,25-(OH)₂-D₃ on keratinocyte density. At 1 week in culture human keratinocytes were incubated in serum-free medium in the presence (open circle) or absence (closed circle) of 1α,25-(OH)₂-D₃ at 10⁻⁶ M. After 3 days, the cells were harvested and spun on linear Percoll gradients (1.032–1.090 g/ml). Eight fractions were collected from the gradients, and cell number counted in each fraction. Each point represents the mean of triplicate determinations counted in duplicate using a hemacytometer. [Fraction 1, density (d) = 1.090–1.083 g/ml; fraction 2, d = 1.083–1.076; fraction 3, d = 1.076–1.068; fraction 4, d = 1.068–1.061; fraction 5, d = 1.061–1.054; fraction 6, d = 1.054–1.046; fraction 7, d = 1.046–1.039; fraction 8, d = 1.039–1.032]
of 1α,25-(OH)₂-D₃, a biologically inert epimer, was no different from that of control cultures, and incubation with 25-OH-D₃, a precursor of 1α,25-(OH)₂-D₃, resulted in a significant increase in enzyme activity that occurred only at 10⁻⁸ M.

Effect of 1α,25-(OH)₂-D₃ on the Keratin Pattern of Cultured Human Keratinocytes

To determine whether 1α,25-(OH)₂-D₃ has an effect on the keratin pattern of epidermal cells, cultures were incubated for 7 days with vehicle alone, 1α,25-(OH)₂-D₃, or retinyl acetate, then pulse-labeled with [³²P]methionine, and finally extracted for insoluble proteins.

Discussion

Recently, cytosolic and nuclear receptor-like macromolecules specific for 1α,25-(OH)₂-D₃ were characterized in a number of tissues [2–5]. Our laboratory demonstrated the presence of these receptor-like macromolecules in normal cultured human epidermal keratinocytes and dermal fibroblasts [2]. The existence of receptors for this hormone is evidence that normal skin cells may be a target for 1α,25-(OH)₂-D₃. In fact, 1α,25-(OH)₂-D₃ was shown to have an effect on the growth and differentiation of various cell types, including normal human dermal fibroblasts and mouse keratinocytes [2,7]. We, therefore, set out to determine whether 1α,25-(OH)₂-D₃ has an effect on the proliferation and differentiation of normal human keratinocytes.

It has been demonstrated that the effect of 1α,25-(OH)₂-D₃ on cultured cells is lessened when they are incubated in serum-containing medium, possibly due to the presence of vitamin-D-binding protein and vitamin-D metabolites in serum [19,20]. In addition, other factors present in serum, including vitamin A and hydrocortisone, are known to have an effect on epidermal differentiation [17,21,22]. Therefore, we developed a serum-free system for human keratinocytes, and used this system to determine what effect 1α,25-(OH)₂-D₃ had on these cells.

Our findings demonstrated that incubation of cultured human keratinocytes with 1α,25-(OH)₂-D₃ for 1 or 2 weeks resulted in a time- and dose-dependent stimulation of morphologic differentiation. These cells responded to 1α,25-(OH)₂-D₃ at 10⁻¹⁰ M, its normal plasma concentration, which implies that this response may be physiologically significant. The effect of 1α,25-(OH)₂-D₃ on the morphology of keratinocytes was determined by counting the number of different cell types and by measuring cellular density. After incubation with 1α,25-(OH)₂-D₃, there was a decrease in the proliferative basal cells and a concurrent increase in the more differentiated cell types. During in vitro differentiation,
there is a gradual decrease in keratinocyte density [11]. Our study showed that incubation of keratinocytes with 1α,25-(OH)$_2$-D$_3$ results in a shift to lower cellular density, indicative of a more differentiated state. In addition, we demonstrated that incubation with 1α,25-(OH)$_2$-D$_3$ resulted in a time- and dose-dependent inhibition of DNA synthesis. These data correlated well with our morphological data, the latter showing a decrease in the number of proliferative basal cells after incubation with 1α,25-(OH)$_2$-D$_3$.

The results are consistent with the work of Hosomi et al [7] who demonstrated that 1α,25-(OH)$_2$-D$_3$ stimulated morphologic differentiation and inhibited the proliferation of cultured mouse keratinocytes.

In this investigation, we showed that incubation of cultured human keratinocytes with 1α,25-(OH)$_2$-D$_3$ at 10$^{-10}$ to 10$^{-8}$ or 10$^{-10}$ to 10$^{-6}$ M for 24 h resulted in a significant increase in the activity of transglutaminase, the calcium-dependent enzyme responsible for cross-linking the proteins of the corneified envelope. This action was specific for 1α,25-(OH)$_2$-D$_3$, because its precursor, 25-(OH)$_2$D$_3$, stimulated the activity of this enzyme only at a 10,000-fold higher concentration (10$^{-6}$ M) and 1β,25-(OH)$_2$-D$_3$, a biologically inert isomer [23], did not stimulate the activity of this enzyme, even at a 10,000-fold higher concentration. These results suggest that the stimulation of transglutaminase activity by 1α,25-(OH)$_2$-D$_3$ is specific and is a 1α,25-(OH)$_2$-D$_3$ receptor-mediated effect.

When keratinocytes were cultured in medium with a low calcium concentration, the cells proliferated but did not stratify; however, by increasing the calcium concentration of the medium, the keratinocytes began to differentiate [24]. Subsequently, it was shown that transglutaminase activity increases after the induction of differentiation by calcium [25]. Because 1α,25-(OH)$_2$-D$_3$ plays a role in calcium metabolism in other known targets, specifically small intestine and bone, it is possible that this hormone may indirectly stimulate epidermal transglutaminase activity by increasing the availability of calcium to this enzyme.

We were interested in investigating whether 1α,25-(OH)$_2$-D$_3$ had the same effect as other compounds, including vitamin A, phorbol esters, and hydrocortisone, on keratinocyte differentiation [17,21,22,26]. We found that, unlike retinoic acid [16,21], 1α,25-(OH)$_2$-D$_3$ stimulated both corneified-envelope formation and transglutaminase activity in epidermal cells. In addition, other investigators showed that incubation of cultured keratinocytes with vitamin A resulted in an altered keratin pattern [17]. However, in this investigation we demonstrated that incubation of cultured keratinocytes with 1α,25-(OH)$_2$-D$_3$ at 10$^{-8}$ M resulted in a keratin pattern similar to that of control cultures. The absence of an effect by 1α,25-(OH)$_2$-D$_3$ on the keratin pattern of these cells was not surprising. If 1α,25-(OH)$_2$-D$_3$ is exerting its effect on keratinocyte differentiation by altering intracellular calcium concentration, the hormone would not be expected to have an effect on the keratin pattern, inasmuch as other investigators showed that changing the calcium concentration in the medium did not influence the keratin pattern of these cells [24,25].

The physiologic importance of 1α,25-(OH)$_2$-D$_3$ for epidermal differentiation is not known. However, Horiiuchi et al [27] showed that the receptor for 1α,25-(OH)$_2$-D$_3$ appears in the skin of the mouse embryo during the second trimester of gestation and that the number increases throughout gestation and into neonatal development. This evidence suggests that 1α,25-(OH)$_2$-D$_3$ may play a role in the differentiation of skin during development. Once the exact role of 1α,25-(OH)$_2$-D$_3$ in normal epidermal differentiation is elucidated, this information may be of value in investigating the treatment of hyperproliferative diseases and differentiation disorders of the epidermis.

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