Calcipotriol (MC 903), a novel vitamin D₃ analogue stimulates terminal differentiation and inhibits proliferation of cultured human keratinocytes

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Received October 9, 1989

Summary. The hormonally active form of vitamin D₃, 1,25-dihydroxy vitamin D₃ [1,25-(OH)₂-D₃; calcitriol], regulates the differentiation and proliferation of epidermal keratinocytes in vitro. MC 903 (calcipotriol) is a novel vitamin D₃ analogue which is at least 100 times less potent than 1,25-(OH)₂-D₃ in its effects on calcium homeostasis. The present study compared the effects of MC 903 and 1,25-(OH)₂-D₃ on terminal differentiation and proliferation of cultured normal human keratinocytes. Keratinocytes were grown in McCoy's 5A medium supplemented with penicillin (50 IU/ml), streptomycin (50 µg/ml), l-serine (4x10⁻⁴ M), and 10% human type AB serum. MC903, 1,25-(OH)₂-D₃ or 1α-OH-D₃ (10⁻¹² M - 10⁻⁸ M) was added with each feeding when cultures became preconfluent. After incubation for 24 h with D₃ vitamins, cultures were extracted for transglutaminase, and the enzyme activity was indexed against DNA content. The activity of transglutaminase, the enzyme responsible for cross-linking the proteins of the cornified envelope, was maximally stimulated by 388% with MC 903 (10⁻⁸ M), by 328% with 1,25-(OH)₂-D₃ (10⁻⁸ M), and by 27% with 1α-OH-D₃ (10⁻⁸ M) compared with vehicle. After incubation for 2 weeks the number of keratinocytes with cornified envelopes had increased by 288% with MC 903 (10⁻⁸ M), by 360% with 1,25-(OH)₂-D₃ (10⁻⁸ M), and by 149% with 1α-OH-D₃ (10⁻⁸ M) compared with vehicle. After incubation for 2 weeks the number of keratinocytes with cornified envelopes had increased by 288% with MC 903 (10⁻⁸ M), by 360% with 1,25-(OH)₂-D₃ (10⁻⁸ M), and by 149% with 1α-OH-D₃ (10⁻⁸ M) compared with vehicle. Simultaneously the incorporation of (³H)thymidine into DNA was decreased by 64% with MC 903 (10⁻⁸ M), by 71% with 1,25-(OH)₂-D₃ (10⁻⁸ M), and by 10% with 1α-OH-D₃ (10⁻⁸ M). There was a corresponding decrease in cell number. These results demonstrate that both MC 903 and 1,25-(OH)₂-D₃ are potent modulators of keratinocytes differentiation and proliferation in vitro. Because MC 903 is much less active than 1,25-(OH)₂-D₃ in causing hypercalcemia, this compound is a candidate for the treatment of skin diseases characterized by aberrant epidermal differentiation and proliferation.

Key words: Vitamin D₃ analogues - Keratinocyte culture - Differentiation - Proliferation

Recent work indicates that 1,25-dihydroxy vitamin D₃ [1,25-(OH)₂-D₃; calcitriol], the hormonally active form of vitamin D₃, regulates the proliferation and differentiation of epidermal cells. The evidence for a role of 1,25-(OH)₂-D₃ in epidermal growth is based on the presence of receptors specific for 1,25-(OH)₂-D₃ in skin and cultured keratinocytes [14, 15] and on the ability of 1,25-(OH)₂-D₃ to inhibit the proliferation and to stimulate the terminal differentiation of keratinocytes in culture [5, 16].

Administration of 1,25-(OH)₂-D₃ could, therefore, provide a novel approach for the treatment of skin diseases which are characterized by epidermal hyperproliferation and incomplete terminal differentiation such as psoriasis [13, 17]. Due to its potent effects on calcium metabolism the therapeutic use of 1,25-(OH)₂-D₃ may, however, be associated with hypercalcemia and/or hypercalciuria [13, 17]. Therefore, it has been desirable to develop vitamin D₃ analogues with a lower risk of inducing calcium-related side effects.

Calcipotriol (MC 903) is a novel vitamin D₃ analogue [2] (Fig. 1), which is at least 100 times less active than 1,25-(OH)₂-D₃ in causing hypercalcemia and hypercalciuria in rats [1]. In the present study we compared the effects of MC 903 and 1,25-(OH)₂-D₃ on the terminal differentiation and the proliferation of cultured human keratinocytes.

Material and methods

Keratinocyte cultures

All experiments used preconfluent cultures of human epidermal keratinocytes at first passage. Keratinocytes were grown in culture using a modification of the method of Liu and Karasek [10] as
K. Kragballe and I. L. Wildfang: Vitamin D₃ analogue and keratinocyte differentiation and proliferation

1.25 (OH)₂ D₃ (calcitriol) and MC 903 (calcipotriol)

previously described by us [8]. Keratome biopsy specimens incubated with 0.25% trypsin in PBS containing 5 mM glucose (pH 7.0) for 30–40 min at 37°C. After aspiration of trypsin, minimal essential medium with 10% fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin was added. Then epidermis was separated from dermis, and the epidermal cells released into the medium by gently scraping and agitating both the epidermal and the dermal compartment of the biopsy specimen. Epidermal cell suspensions (1.0 × 10⁶ trypan blue-excluding cells per ml) were plated on 16-mm culture dishes precoated with a collagen type I gel. Cells were incubated at 37°C in 100% humidity in a 95% air/5% CO₂ environment. After 24 h the plating medium was replaced with McCoy's 5A medium supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin, 4 × 10⁻⁴M L-serine, and 10% normal human AB serum. The medium was changed three times weekly, and the cultures became confluent after 2–3 weeks. The same batch of human serum was used for all experiments. The complete medium contained 4.1 × 10⁻¹⁵M of 1,25-(OH)₂-D₃ due to the presence of endogenous 1,25-(OH)₂-D₃ in the serum.

**Vitamin D₃ analogues**

Beginning at 1 week in culture, fresh medium containing vehicle alone (control, < 0.1% absolute ethanol), 1,25-(OH)₂-D₃ (10⁻¹²M–10⁻⁸M), or MC 903 (10⁻¹²M–10⁻⁸M) was added to triplicate dishes with each feeding. 1α-OH-D₃ (alfacalcidol; 10⁻¹³M–10⁻⁸M), a prodrug of 1,25-(OH)₂-D₃, was used as a negative control. The D₃ vitamins were kind gifts from Leo Pharmaceutical Products, Copenhagen, Denmark.

**Keratinocyte proliferation**

At the appropriate times, keratinocytes dosed with D₃ vitamins were harvested by trypsinization and counted in a hemocytometer. DNA synthesis via the salvage pathway was determined by (³H)thymidine incorporation into terminally labelled cultures as previously described by Marceto et al. [11]. After exposure for 6 h to 1 µCi (³H)thymidine (60 Ci/mmole) the cultures were rinsed with PBS, harvested by scraping, and extracted for DNA [11]. Aliquots were taken to count (³H)thymidine incorporation into DNA, and the remainder of each sample was assayed for the total DNA content [11].

**Keratinocytes differentiation**

Keratinocyte transglutaminase was assayed as described by Yuspa et al. [18], which measures the enzyme catalyzed formation of ε-amino-γ-glutamyl bonds between (³H)-putrescine and casein. After incubation with D₃ vitamins for 24 h, keratinocyte cultures (6 × 10⁶ cells) were washed with PBS and lysed by freeze-thawing in 300 µl of buffer mixture composed of 50 mM Tris (pH 7.5), 2.5 mM dithiothreitol, 0.13 M NaCl, 0.83 mM EDTA, and 8.3 mM CaCl₂. The reaction mixture consisted of a total of 200 µl as follows: 100 µl cell lysate, 20 µl casein (20 µg/ml), and 30 µl (³H)-putrescine (5 mM final concentration), and the additional 50 µl as buffer or EGTA (100 mM). After 10 min at 37°C, 50 µl of the reaction mixture was spotted on Whatman 3MM filter paper strips (previously washed with 50 µl of 100 mM EGTA and dried) and immediately immersed in ice-cold 10% TCA containing 1.0% putrescine. Filter papers were gently agitated through three TCA washes for 20 min each, rinsed with ice-cold absolute ethanol, and dried. Radioactivity bound to casein which precipitated on filter paper was counted in Instagel in a scintillation counter. Background radioactivity from parallel assays lacking cell lysate was subtracted from all samples.

To count cornified enveloped keratinocyte cultures were trypsinized, centrifuged, and resuspended in 10 mM Tris-HCl (pH 7.4) with 1% sodium dodecyl sulfate and 1% mercaptoethanol [18]. After incubation for 10 min at room temperature, cornified envelopes were counted in a hemacytometer.

**Statistics**

The assessments of statistical significance were based on non-parametric tests only. Statistical significance was tested using either Wilcoxon's one-sample test or two-sample test where appropriate; *P* values above 0.05 were regarded as being not significant.

**Results**

**Keratinocyte differentiation**

After incubation for 2 weeks with 1,25-(OH)₂-D₃ or MC 903 the percentage of keratinocytes with cornified envelopes was significantly and dose-dependently increased (Fig. 2). In comparison with control cultures, the maximal stimulation was 288% for 1,25-(OH)₂-D₃ and 360% for MC 903. The difference between 1,25-(OH)₂-D₃ and MC 903 was not statistically significant. Only at the highest concentration (10⁻⁸M) did 1α-OH-D₃ stimulate (149%) the number of cornified envelopes. The highest concentration of D₃ vitamins used was 10⁻⁸M, because light microscopic signs of degenerative changes were sometimes seen at a concentration of 10⁻⁷M (data not shown).