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Calcipotriol (MC 903), a novel vitamin D_3 analogue stimulates terminal differentiation and inhibits proliferation of cultured human keratinocytes*

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Summary. The hormonally active form of vitamin D_3, 1,25-dihydroxy vitamin D_3 [1,25-$(OH)_2$-D_3; calcitriol], regulates the differentiation and proliferation of epidermal keratinocytes in vitro. MC 903 (calcipotriol) is a novel vitamin D_3 analogue which is at least 100 times less potent than 1,25-$(OH)_2$-D_3 in its effects on calcium homeostasis. The present study compared the effects of MC 903 and 1,25-$(OH)_2$-D_3 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 (4×10^{-4} *M*), and 10% human type AB serum. MC 903, 1,25-$(OH)_2$-D_3 or 1α-OH-D_3 (10^{-12} *M*–10^{-8} *M*) was added with each feeding when cultures became preconfluent. After incubation for 24 h with D_3 vitamins, cultures were extracted for transglutaminase, and the enzyme activity was indexed against DNA content. The activity of transglutaminase, the enzyme reponsible 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-$(OH)_2$-D_3 (10^{-8} *M*), and by 27% with 1α-OH-D_3 (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-$(OH)_2$-D_3 (10^{-8} *M*), and by 149% with 1α-OH-D_3 (10^{-8} *M*) compared with vehicle. Simultaneously the incorporation of (^{3}H)thymidine into DNA was decreased by 64% with MC 903 (10^{-8} *M*), by 71% with 1,25-$(OH)^2$-D_3 (10^{-8} *M*), and by 10% with 1α-OH-D_3 (10^{-8} *M*). There was a corresponding decrease in cell number. These results demonstrate that both MC 903 and 1,25-$(OH)_2$-D_3 are potent modulators of keratinocytes differentiation and proliferation in vitro. Because MC 903 is much less active than 1,25-$(OH)_2$-D_3 in causing hypercalcemia, this compound is a candidate for the treatment of skin diseases characterized by aberrant epidermal differentiation and proliferation.

Key words: Vitamin D_3 analogues – Keratinocyte culture – Differentiation – Proliferation

Recent work indicates that 1,25-dihydroxy vitamin D_3 [1,25-$(OH)_2$-D_3; calcitriol], the hormonally active form of vitamin D_3, regulates the proliferation and differentiation of epidermal cells. The evidence for a role of 1,25-$(OH)_2$-D_3 in epidermal growth is based on the presence of receptors specific for 1,25-$(OH)_2$-D_3 in skin and cultured keratinocytes [14, 15] and on the ability of 1,25-$(OH)_2$-D_3 to inhibit the proliferation and to stimulate the terminal differentiation of keratinocytes in culture [5, 16].

Administration of 1,25-$(OH)_2$-D_3 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-$(OH)_2$-D_3 may, however, be associated with hypercalcemia and/or hypercalciuria [13, 17]. Therefore, it has been desirable to develop vitamin D_3 analogues with a lower risk of inducing calcium-related side effects.

Calcipotriol (MC 903) is a novel vitamin D_3 analogue [2] (Fig. 1), which is at least 100 times less active than 1,25-$(OH)_2$-D_3 in causing hypercalcemia and hypercalciuria in rats [1]. In the present study we compared the effects of MC 903 and 1,25-$(OH)_2$-D_3 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

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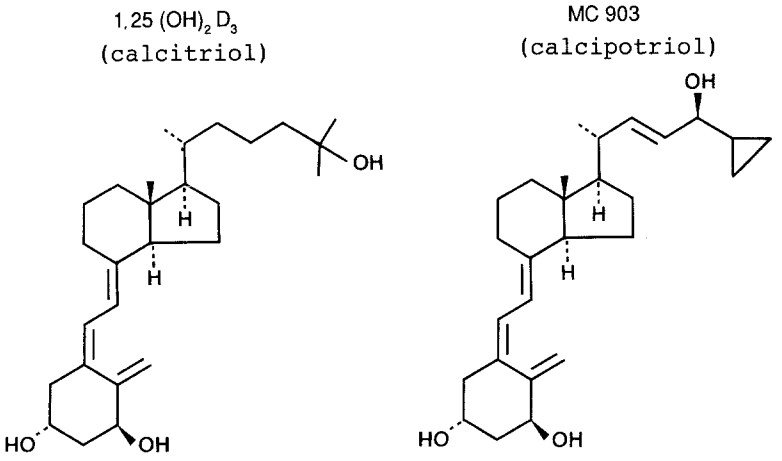

Fig. 1. Structural formulas of 1,25-$(OH)_2$-D_3 (calcitriol) and MC 903 (calcipotriol)

previously described by us [8]. Keratome biopsy specimens incubated with 0.25% trypsin in PBS containing 5 m*M* 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 (1.0×10^6 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% CO_2 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^{-4} *M* 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^{-12} *M* of 1,25-$(OH)_2$-D_3 due to the presence of endogenous 1,25-$(OH)_2$-D_3 in the serum.

Vitamin D_3 analogues

Beginning at 1 week in culture, fresh medium containing vehicle alone (control, < 0.1% absolute ethanol), 1,25-$(OH)_2$-D_3 (10^{-12} *M*–10^{-8} *M*), or MC 903 (10^{-12} *M*–10^{-8} *M*) was added to triplicate dishes with each feeding. 1α-OH-D_3 (alfacalcidol; 10^{-12} *M*–10^{-8} *M*), a prodrug of 1,25-$(OH)_2$-D_3 was used as a negative control. The D_3 vitamins were kind gifts from Leo Pharmaceutical Products, Copenhagen, Denmark.

Keratinocyte proliferation

At the appropriate times, keratinocytes dosed with D_3 vitamins were harvested by trypsinization and counted in a hemocytometer.

DNA synthesis via the salvage pathway was determined by (^{3}H)thymidine incorporation into terminally labelled cultures as previously described by Marcelo et al. [11]. After exposure for 6 h to 1 µCi (^{3}H)thymidine (60 Ci/mmol) the cultures were rinsed with PBS, harvested by scraping, and extracted for DNA [11]. Aliquots were taken to count (^{3}H)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 (^{3}H)-putrescine and casein. After incubation with D_3 vitamins for 24 h, keratinocyte cultures (6×10^6 cells) were washed with PBS and lysed by freeze-thawing in 300 µl of buffer mixture composed of 50 m*M Tris* (pH 7.5), 2.5 m*M* dithiothreitol, 0.13 *M* NaCl, 0.83 m*M* EDTA, and 8.3 m*M* $CaCl_2$. 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 (^{3}H)-putrescine (5 m*M* final concentration), and the additional 50 µl as buffer or EGTA (100 m*M*). 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 m*M* 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 substracted from all samples.

To count cornified enveloped keratinocyte cultures were trypsinized, centrifuged, and resuspended in 10 m*M Tris*-HCl (pH 7.4) with 1% sodium dodecyl sulfate and 1% mercaptoethanol [18]. After incubation for 10 min at room temperature, cornified envelops 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-$(OH)_2$-D_3 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-$(OH)_2$-D_3 and 360% for MC 903. The difference between 1,25-$(OH)_2$-D_3 and MC 903 was not statistically significant. Only at the highest concentration (10^{-8} *M*) did 1α-OH-D_3 stimulate (149%) the number of cornified envelopes. The highest concentration of D_3 vitamins used was 10^{-8} *M*, because light microscopic signs of degenerative changes were sometimes seen at a concentration of 10^{-7} *M* (data not shown).

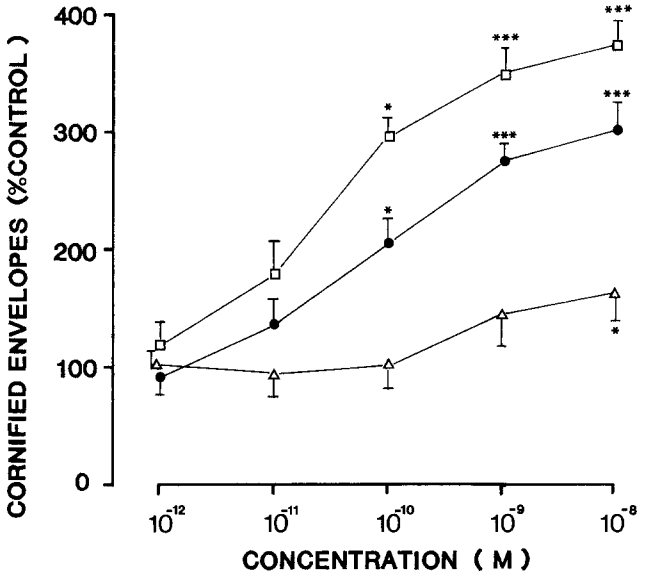

Fig. 2. Effects of 1,25-$(OH)_2$-D_3 (□), MC 903 (●), and 1α-OH-D_3 (△) on the cornified envelope formation of preconfluent cultures of human keratinocytes grown in McCoy's 5A medium supplemented with 10% human serum. After incubation for 2 weeks the number of cornified envelopes was counted. *Bars* represent SEM for eight triplicate experiments. $*p < 0.05$; $**p < 0.02$; $***p < 0.01$

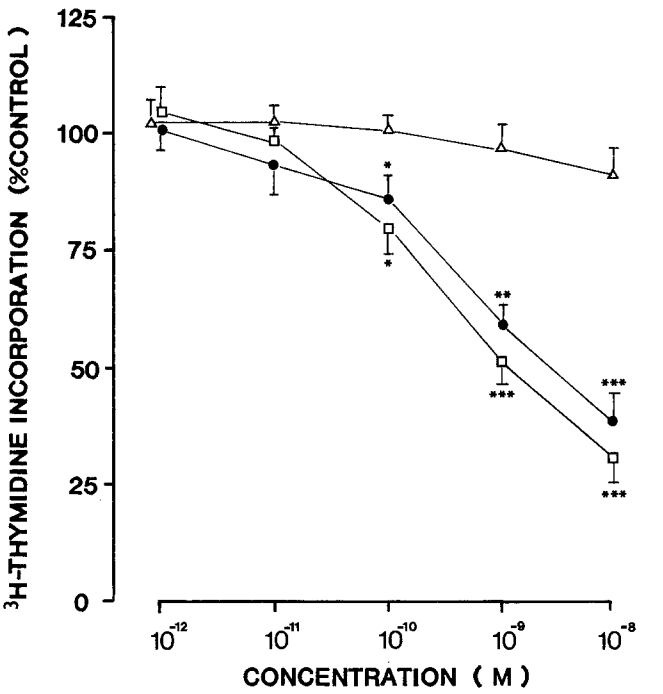

Fig. 3. Effects of 1,25-$(OH)_2$-D_3 (□), MC 903 (●), and 1α-OH-D_3 (△) on the (^{3}H)thymidine incorporation into DNA of preconfluent cultures of human keratinocytes grown in McCoy's 5A medium supplemented with 10% human serum. After incubation for 2 weeks the cultures were pulse-labelled with (^{3}H)thymidine. *Bars* represent SEM for eight triplicate experiments. $*p < 0.05$; $**p < 0.02$; $***p < 0.01$

Table 1. Transglutaminase activity of preconfluent cultures of human keratinocytes incubated with 1,25-$(OH)_2$-D_3, MC 903 and 1α-OH-D_3 for 24 h

Compound	Concentration (M)	n	Transglutaminase (%)	p
Vehicle		6	100	
1,25-$(OH)_2$-D_3	10^{-9}	6	212 ± 25	< 0.01
	10^{-8}	6	328 ± 29	< 0.01
MC 903	10^{-9}	6	205 ± 30	< 0.01
	10^{-8}	6	388 ± 31	< 0.01
1α-OH-D_3	10^{-9}	6	27 ± 12	N.S.

Enzyme activity was indexed against DNA content and expressed in percent of control cultures. Values are mean ± SEM of six triplicate experiments

After incubation for 24 h with the D_3 vitamins, cultures were extracted for transglutaminase, and the enzyme activity indexed against the DNA content (Table 1). When compared with controls, keratinocytes incubated with 1,25-$(OH)_2$-D_3 or MC 903 showed a significant increase of transglutaminase activity. Maximal stimulation was 328% with 1,25-$(OH)_2$-D_3 and 388% with MC 903. At the highest concentration ($10^{-8}M$) 1α-OH-D_3 caused a small, but statistically insignificant increase of transglutaminase activity.

Keratinocyte proliferation

After incubation for 2 weeks 1,25-$(OH)_2$-D_3, MC 903, and to a lesser extent, 1α-OH-D_3 caused a dose-dependent decrease in the incorporation of (^{3}H)thymidine into the DNA of keratinocytes (Fig. 3). There was no difference in the response to 1,25-$(OH)_2$-D_3 and MC 903.

Similar results were obtained when changes in cell number were used as growth assay. Compared with control cultures, the cell number (mean ± SEM, $n = 4$) was decreased by 68% ± 5% with 1,25-$(OH)_2$-D_3 ($10^{-8}M$), by 5% ± 6% with MC 903 ($10^{-8}M$), and by 9% ± 2% with 1α-OH-D_3 ($10^{-8}M$; data not shown).

Discussion

This study demonstrates that MC 903 (calcipotriol) is a potent inducer of terminal differentiation in cultured human keratinocytes. Both the formation of cornified envelope and the activity of transglutaminase, the enzyme responsible for cross-linking the proteins of the cornified envelopes, were stimulated in a dose-dependent way. There was a corresponding inhibition of keratinocyte proliferation. In these effects the potency of MC 903 was comparable to that observed with the naturally occurring, active form of vitamin D_3, 1,25-$(OH)_2$-D_3. In contrast, 1α-OH-D_3 which is a synthetic precursor of 1,25-$(OH)_2$-D_3 had only week effects on keratinocyte differentiation and proliferation. It is unknown whether 1α-OH-D_3 exerts its effects directly on keratinocytes, or whether it becomes active after partial hydroxylation to 1,25-$(OH)_2$-D_3. The liver has a very active 25-hydroxylase [6], but

it remains to be determined whether keratinocytes can metabolize 1α-OH-D_3 into 1,25-$(OH)_2$-D_3.

The effects of 1,25-$(OH)_2$-D_3 and MC 903 on keratinocyte differentiation and proliferation are in accordance with those obtained with 1,25-$(OH)_2D$-$_3$ in cultured human keratinocytes grown in serum-free conditions [16]. It has recently been demonstrated that there may be differences between confluent and preconfluent cultures of human keratinocytes in their responses to 1,25-$(OH)_2$-D_3 [6, 12]. Also, 1,25-$(OH)_2D_3$ may have differential effects on proliferation and differentiation of human keratinocytes grown in different media [12]. It is, therefore, essential to define the experimental conditions when discussing the effects of D_3 vitamins on human keratinocytes in vitro. In the present study the culture medium was supplemented with 10% human serum and contained approximately $4 \times 10^{-12} M$ of endogenous 1,25-$(OH)_2$-D_3. It is, therefore, not surprising that higher concentrations of 1,25-$(OH)_2$-D_3 or MC 903 had to be added to obtain a significant change of keratinocyte growth.

MC 903 has as high an affinity as 1,25-$(OH)_2$-D_3 for the 1,25-$(OH)_2$-D_3 receptor in the human histiocytic lymphoma cell line U 937 [1]. It is likely that MC 903 exert its effects on keratinocyte growth by binding to keratinocyte receptors for 1,25-$(OH)_2$-D_3. There is evidence that receptor binding results in intracellular calcium mobilization [3]. Although it has been established that extracellular calcium has a modulatory effect on keratinocyte differentiation in vitro [4], it remains unknown whether D_3 vitamins act on keratinocyte growth by mobilizing Ca^{2+}. Despite the similar effects of 1,25-$(OH)_2$-D_3 and MC 903 on keratinocyte growth, MC 903 is at least 100 times less active than 1,25-$(OH)_2$-D_3 in causing hypercalciuria and hypercalcemia in rats after oral or intraperitoneal administration [1]. This unique pharmacological profile of MC 903 makes this compound an interesting candidate for the treatment of skin diseases characterized by epidermal hyperproliferation and aberrant epidermal differentiation. Topical MC 903 has shown promising results in the treatment of psoriasis [7, 9] and a large scale multicenter trial is now in progress.

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