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Effects of 1,25-dihydroxyvitamin D_3 and its 20-epi analogues (MC 1288, MC 1301, KH 1060), on clonal keratinocyte growth: evidence for differentiation of keratinocyte stem cells and analysis of the modulatory effects of cytokines

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1. Keratinocytes are functionally divided into stem cells, transit amplifying cells and terminally differentiated cells. In a hyperproliferative skin disease, psoriasis, increased mitotic activity of the stem cells is chiefly responsible for epidermal hyperplasia. The effects of 1,25dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and potent vitamin D_3 analogues (MC 1288: 20-epi-$1,25(OH)_2D_3$, MC 1301: 20-epi-24*a*-homo-26,27-dimethyl-$1,25(OH)_2D_3$, KH 1060: 20-epi-22-oxa-24*a*-homo-26,27-dimethyl-$1,25(OH)_2D_3$) on the stem cells were investigated.

2 Stem cells were identified retrospectively as those giving rise to large keratinocyte colonies in culture (holoclones). $1,25(OH)_2D_3$ (10^{-8}–10^{-6} M) suppressed formation of holoclones by stimulating the progenitor cell differentiation into the phenotype expressing differentiation markers (keratins K1/K10 and involucrin).

3 20-Epi vitamin D_3 analogues were more potent than $1,25(OH)_2D_3$ in inhibiting the clonal keratinocyte growth. This activity correlated with the ability to induce cell differentiation (KH 1060 > MC 1301 > MC 1288 > $1,25(OH)_2D_3$).

4 Cytokines modulated the effects of $1,25(OH)_2D_3$ on clonal growth. One of the following cytokines (epidermal growth factor, transforming growth factor α, interleukin-1α, interleukin-1β, interleukin-6, interleukin-8) was required for $1,25(OH)_2D_3$ to suppress clonal growth and induce cell differentiation. In contrast, keratinocyte growth factor and insulin-like growth factor I attenuated the effects of $1,25(OH)_2D_3$.

5 In conclusion, $1,25(OH)_2D_3$ and 20-epi vitamin D_3 analogues suppress clonal growth by directly inducing the differentiation of progenitor cells. It is conceivable that stimulation of stem cells differentiation is a major mechanism of action of vitamin D_3 compounds in psoriasis. Balance between different types of cytokines in psoriatic epidermis may be an important factor determining the clinical effect of vitamin D-based therapy.

Keywords: Vitamin D; vitamin D-analogues; keratinocyte; stem cell; differentiation; cytokines

Introduction

Calcitriol ($1,25(OH)_2D_3$) and its synthetic analogues have recently emerged as useful drugs for the treatment of psoriasis (Kragballe *et al*., 1991; Kragballe, 1992). The beneficial effects of vitamin D_3 compounds have been attributed to the antiproliferative and differentiation-promoting activities of $1,25(OH)_2D_3$ on psoriatic keratinocytes (reviewed in Walters, 1992; Bikle & Pillai, 1993; Van de Kerkhof, 1995). In normal keratinocytes in culture, $1,25(OH)_2D_3$ imposes a cell-cycle block in the G1 phase, stimulates formation of cornified envelopes and induces expression of terminal differentiation markers; transglutaminase and involucrin (Hosomi *et al*., 1983; Smith *et al*., 1986; Bikle *et al*., 1993; Sebag *et al*., 1994). In reconstituted epidermis, $1,25(OH)_2D_3$ causes reversible hyperkeratosis and thinning of the suprabasal cell compartment (Regnier & Darmon, 1991). The effects of $1,25(OH)_2D_3$ may be mimicked and potentiated by a wide range of synthetic vitamin D_3 analogues. Very potent analogues have been obtained by chemical modifications at the side chain of $1,25(OH)_2D_3$ (Bouillon *et al*., 1995). Binderup *et al*. (1991) and Gniadecki & Serup (1995) have shown that progressive modification of the side-chain (epimerizing at C20 as in the compound MC 1288, lengthening the side chain by a carbon at C24, C26 and C27 as in MC 1301, and replacing C22 with oxygen as in KH 1060) leads to a substantial increase in efficacy and potency of the compounds, both *in vitro* and *in vivo*. Interestingly, despite the differences in their biological activity, these analogues display virtually identical affinity to the vitamin D receptor (Binderup *et al*., 1991).

It has recently become apparent that human keratinocytes are organized into stem cells, transit amplifying cells and terminally differentiated cells (Barrandon & Green, 1987; Jones & Watt, 1993; Jones *et al*., 1995). The hierarchical organization of keratinocytes is maintained in cell culture conditions (Jones *et al*., 1995). Stem cells have a high proliferative potential, are capable of self-renewing, and express β1 integrin but not K1K10 keratin. The descendants of the stem cells are the transit amplifying cells (TAC) express both β1 integrin and K1/K10 keratin and belong to a partially differentiated pool of cells with a limited proliferative capability. Terminal, mitotically inactive keratinocytes (β1 integrin$^-$, K1K10$^+$) are the end-product of TAC differentiation. Keratinocyte subtypes can be identified retrospectively on the basis of their progeny in the clonogenic assay (Jones & Watt, 1993; Jones *et al*., 1995). Stem cells give rise to large colonies (so-called holoclones; Barrandon & Green, 1987), whereas TAC form colonies of smaller size (meroclones). Terminally differentiated cells give rise to abortive colonies (paraclones). The stem cells play a central pathogenic role in psoriasis. In normal epidermis the

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majority of stem cells are quiescent (arrested in G0), but in psoriasis they shift into cell cycle (Bata Csorgo *et al*., 1993; 1995). Stem cell hyperproliferation is thus considered to be responsible for epidermal hyperplasia seen in psoriasis. The hyperproliferative state of stem cells is probably maintained by a variety of cytokines and lymphokines present in psoriatic epidermis, such as interleukins (IL-1, IL-6, IL-8), peptide growth factors (epidermal growth factor, EGF, transforming growth factor α, TGF-α, insulin-like growth factor I, IGF I), or γ-interferon (Gottlieb, 1990; Krueger & Gottlieb, 1994; Bata-Csorgo *et al*., 1995). Keratinocyte growth in serum-free media resembles the pathological type of proliferation encountered in psoriasis rather than physiological growth pattern in the epidermis. The cells proliferate rapidly in culture and express the keratin pattern characteristic of hyperproliferative skin diseases (Weiss *et al*., 1984; Jiang *et al*., 1993) providing a valid model for investigations in psoriasis.

In this study we investigated whether $1,25(OH)_2D_3$ and the side-chain modified vitamin D_3 analogues (MC 1288, MC 1301, and KH 1060) affect proliferation and differentiation of keratinocyte clonogenic cells and how these effects are modified by inflammatory cytokines involved in the pathogenesis of psoriasis (IL-1, IL-6, IL-8, EGF, TGFα, IGF I, keratinocyte growth factor – KGF). We also aimed to elucidate whether cell differentiation is an active phenomenon induced directly by the vitamin D_3 compounds or rather a passive process triggered by growth arrest.

Methods

Vitamin D_3 compounds

$1,25(OH)_2D_3$ and the following vitamin D_3 analogues: 20-epi-1α,25-dihydroxyvitamin D_3 (MC 1288), 20-epi-24*a*-homo-26,27-dimethyl-1α,25-dihydroxyvitamin D_3 (MC 1301), 20-epi-22-oxa-24*a*-homo-26,27-dimethyl-1α,25-dihydroxyvitamin D_3 (KH 1060), were synthesized in the Chemical Research Department, Leo Pharmaceutical Products (Ballerup, Denmark) (Binderup *et al*., 1991; Hansen *et al*., 1991) (for structural formulae see Table 1). Stock 4 mM solutions were prepared in isopropyl alcohol and kept at 4°C. The purity and concentrations of the compounds have occassionally been checked by high-performance liquid chromatography. Before use, the compounds were further diluted in isopropranol, as required. The final concentration of isopropranol in the culture medium was kept constant at 0.1% (v/v).

Keratinocyte culture

Human neonatal cryopreserved keratinocytes were purchased from PromoCell (Heidelberg, Germany) and cultured in serum-free keratinocyte growth medium (KGM) (Gibco Life Technologies, Gaithersburg, MD) containing 0.09 mM $CaCl_2$, 50 ng ml^{-1} human recombinant EGF and 0.1% bovine pituitary extract (Gniadecki, 1996a). For all cell culture experiments, the media were changed every second day. When appropriate, the concentration of calcium was raised to 1.8 mM by adding $CaCl_2$ from the 100 mM stock solution. To assess the effect of various cytokines on cell growth and differentiation, the medium without EGF was supplemented with the following recombinant proteins: IL-1α (1 ng ml^{-1}), IL-1β (2 ng ml^{-1}), IL-6 (10 ng ml^{-1}), IL-8 (10 ng ml^{-1}), γ-interferon (100 000 u ml^{-1}), IGF I (10 ng ml^{-1}), KGF (10 ng ml^{-1}) (all from Boehringer Mannheim, Mannheim, Germany), TGF-α (10 ng ml^{-1}), or EGF (50 ng ml^{-1}) (Gibco Life Technologies). The concentrations of the cytokines were optimized in the preliminary experiments to give maximal stimulation of clonal growth. No clonal growth was obtained in the presence of γ-interferon and therefore this cytokine was not employed in further studies.

Coating with collagen type IV

Type IV collagen was obtained from Sigma (St. Louis, MO) and dissolved at 100 μg ml^{-1} in phosphate-buffered saline. Culture flasks were coated overnight at 4°C, sterilized with uv light, stored at −20°C, and before use washed twice with phosphate-buffered saline followed by a single wash with an appropriate growth medium.

Colony forming assay

Keratinocyte colonies were divided according to their size into: holoclones (>2 mm), meroclones (size from 32 cells to 2 mm) and abortive colonies, paraclones (<32 cells) (Barrandon & Green, 1987; Jones & Watt, 1993). For the clonogenic assay the keratinocytes were trypsinized at 80% confluence, counted in Coulter cell counter, and plated on a collagen IV-coated 10 cm Petri dish at clonal concentrations (2–6 cells cm^{-2}, i.e. 200–500 cells/dish). After 20 days of culture, the number and size of colonies were determined; smaller colonies were observed with a dissecting microscope. For some experiments the stem cells were enriched in the inoculum by use of the method of Jones & Watt (1993): the keratinocytes were allowed to adhere for 15 min on the collagen type IV-coated culture flasks, adherent cells were harvested with 0.05% trypsin with 0.02% EDTA and used either for clonogenic assays or for immunofluorescent studies. As judged by the frequency of holoclones, stem cells comprised approximately 80% of the fast-adhering cell population.

Bromodeoxyuridine incorporation

Bromodeoxyuridine (BrdU) incorporation assay was performed with the Cell Proliferation Kit (Amersham International, Bucks, U.K.). Keratinocytes were incubated for 6 h with BrdU diluted to 1 : 500. Then the cells were washed with phosphate-buffered saline, trypsinized and counted in a Coulter cell counter. The amount of incorporated BrdU was determined in 10^4 cells with an enzyme-linked immunosorbent assay, according to the procedure described by the producer.

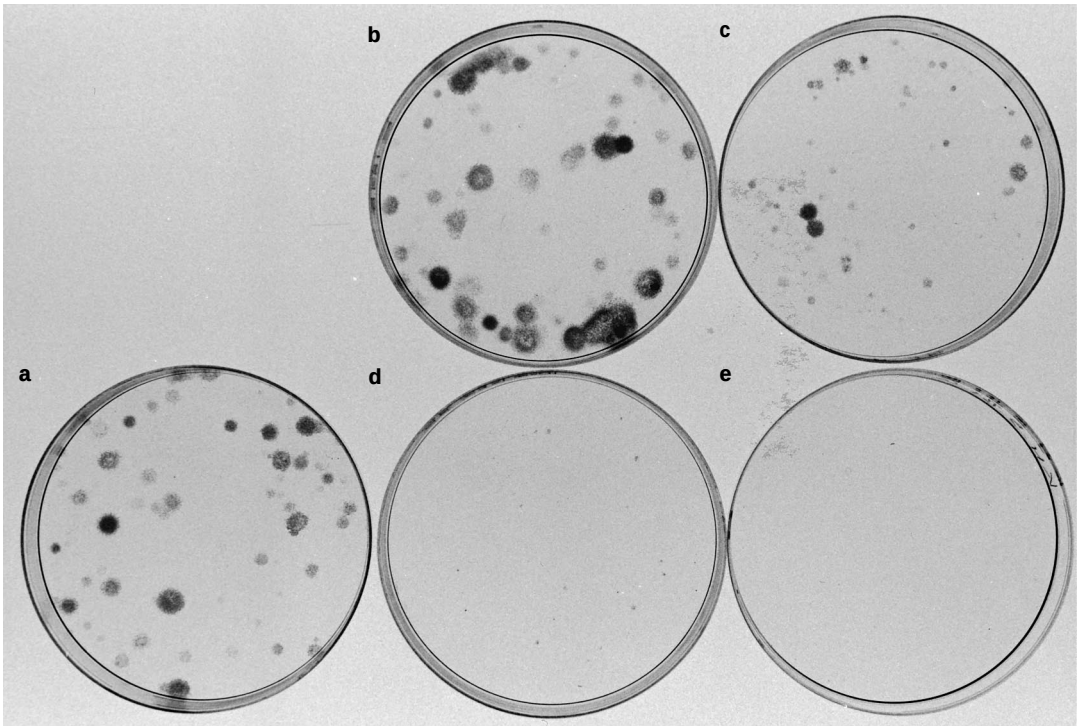
Table 1 Potency of $1,25(OH)_2D_3$ and 20-epi vitamin D analogues in inhibition of the clonogenic keratinocyte growth and stimulation of keratinocyte differentiation

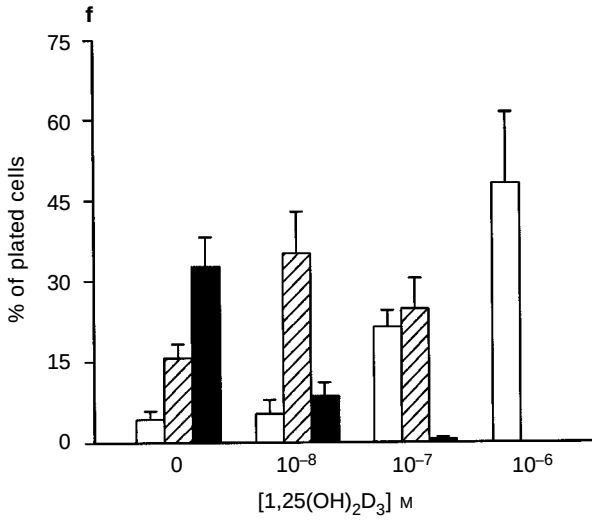
Structure	*R*	*Name*	EC_{50} (M)[a] *Inhibition of proliferation*	*Stimulation of differentiation*
		$1,25(OH)_2D_3$	$14.3\times10^{-9}\pm1.17\times10^{-9}$	$18.0\times10^{-8}\pm1.15\times10^{-8}$
		MC 1288	$2.03\times10^{-11}\pm1.26\times10^{-11}$	$3.05\times10^{-10}\pm0.12\times10^{-10}$
		MC 1301	$4.71\times10^{-12}\pm1.19\times10^{-12}$	$3.72\times10^{-11}\pm0.13\times10^{-11}$
		KH 1060	$2.0\times10^{-12}\pm1.37\times10^{-12}$	$5.63\times10^{-12}\pm0.12\times10^{-12}$

[a]Keratinocytes were cultured with vitamin D compounds as in the legend for Figure 5. At the end of the culture period the number of cells or the proportion of involucrine-positive cells was determined to calculate the EC_{50} for the inhibition of growth and stimulation of differentiation, respectively. Data are means ± s.d. ($n=3$).

Fluorescent microscopy

Immunofluorescence was performed on cells seeded into 16-well glass chambers (Lab-Tek Chamber Slide cat.#178599, Nunc, Naperville, IL). After fixation with methanol/acetone the cells were air-dried, rehydrated in phosphate-buffered saline for 5 min, blocked with 1% BSA in phosphate-buffered saline for 30 min and stained with monoclonal antibodies directed against involucrin (clone SY5, Sigma) and K1K10 keratins (clone LH2, Biogenesis, Franklin, MA) both diluted to 1 : 50. As a second antibody an anti-mouse rabbit isothiocyantate (rodamine) conjugated antiserum was used (1 : 100, Sigma). The dilutions of all antibodies were performed in the blocking solution. Control experiments showed negative staining in samples incubated with the second antibody only. For the detection of apoptotic nuclei the cytochemical detection of internucleosomal DNA fragmentation *in situ* was performed with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method (Gavrieli *et al*., 1992), with an *In Situ* Cell Death Detection Kit (Boehringer Mannheim). Labelling was performed as suggested by the producer. Control experiments showed negative staining of the samples in which the terminal deoxynucleotidyl transferase was omitted. Cells irradiated with ultraviolet light source





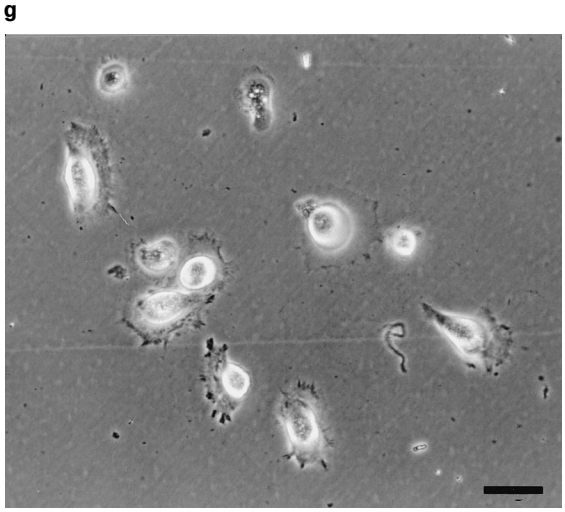


Figure 1 Effect of $1,25(OH)_2D_3$ on the clonal keratinocyte growth. Keratinocytes were seeded at 200 cells/10 cm Petri dish and cultured for 20 days in KGM (a) or the same medium with: (b) 10^{-9} M, (c) 10^{-8} M, (d) 10^{-7} M, (e) 10^{-6} M $1,25(OH)_2D_3$. The concentration of isopropranol (a solvent for $1,25(OH)_2D_3$) was kept constant at 0.1% (v/v) in all cultures. At the end of culture, the cells were fixed with formaldehyde and stained with crystal violet. (f) Quantitative assessment of the influence of $1,25(OH)_2D_3$ on the number of paraclones (open columns), meroclones (hatched columns), and holoclones (solid columns). The number of clones is presented as a proportion of plated cells (ordinates). Abscissa scale: concentration of $1,25(OH)_2D_3$ in the culture medium; the control cells were treated with isopropranolol only (final 0.1%). Columns represent means ($n = 3$) with s.d. $^*P < 0.05$ compared with the vehicle-treated control. (g) Typical morphology of a paraclone (culture in the presence of 10^{-6} M $1,25(OH)_2D_3$) as seen in contrast-phase microscope.

(broad band ultraviolet B, 1500 J m^{-2}) were used as positive controls. The samples were investigated with a confocal laser scanning microscope (TCS4d, Leica Laser Technik GmbH, Heidelberg, Germany). One recording consisted of a series of 5–20 optical sections reaching from top to bottom of the specimen, obtained at a fixed gain.

Statistical analysis

Data are presented as means with s.d. The differences between groups were analysed with a two-sided *t* test for independent samples or the analysis of variance (one way or two-way, as appropriate). Analysis of regression was performed to predict the values of EC_{50} (concentration of a vitamin D compound needed to induce the expression of involucrin of keratins K1/K10 in 50% of the cells or reducing the number of cells by 50%). For all calculations Minitab statistical software was used (Minitab, Inc., State College, U.S.A.). $P<0.05$ was considered significant.

Results

Dynamics of clonal growth inhibition by 1,25(OH)$_2$D$_3$

It has already been shown that $1,25(OH)_2D_3$ inhibits clonal growth of human keratinocytes (Itin *et al*., 1994). These results were confirmed here by showing that culturing of keratinocytes with 10^{-8}–10^{-6} M $1,25(OH)_2D_3$ diminished the size and the number of the holoclones (Figure 1a–f). At 10^{-7} M $1,25(OH)_2D_3$, holoclones were present only occasionally, whereas holoclones did not develop at 10^{-6} M $1,25(OH)_2D_3$. Interestingly, even at high (10^{-6} M) $1,25(OH)_2D_3$ concentrations the keratinocytes could divide the forming abortive colonies (Figure 1g). This suggested that there was a lag period before the onset of the inhibitory action of $1,25(OH)_2D_3$ on clonal growth. This has been further investigated as illustrated in Figure 2. $1,25(OH)_2D_3$ added at the beginning of the culture must be present for at least 4–6 days to exert its inhibitory activity. Addition of $1,25(OH)_2D_3$ for the same period of time at the end of cell culture did not significantly influence clonal growth, which implied that $1,25(OH)_2D_3$ targeted the clonogenic cells rather than the more differentiated cell types emerging in mature colonies.

Stimulation of progenitor cell differentiation is mainly responsible for 1,25(OH)$_2$D$_3$-induced clonal growth arrest

The observed clonal growth arrest could be caused by diverse mechanisms: (i) cell death, (ii) inhibition of cell migration, (iii) induction of cell differentiation, (iv) direct inhibition of growth without inducing of cell differentiation. The direct cytotoxic effect could be excluded since $1,25(OH)_2D_3$ added for 24 h at concentrations of 10^{-8}–10^{-6} M did not impair keratinocyte viability, as assayed by methylene blue staining or counting the detached floating cells (not shown). The possibility that apoptosis might be involved was also investigated. The cells grown at both clonal densities and at approximately 30% confluence were pretreated with 10^{-6} M $1,25(OH)_2D_3$ for 6 days and stained with the TUNEL technique. No staining was seen in adherent keratinocytes and the number of TUNEL positive floating cells was unchanged. These results argue against the involvement of apoptosis in $1,25(OH)_2D_3$-mediated clonal growth arrest. The inhibition of cell migration by $1,25(OH)_2D_3$ was also an unlikely mechanism of clonal growth inhibition because cell densities in the colonies grown in the presence of $1,25(OH)_2D_3$ were similar to the control ones and even in the small abortive colonies the keratinocytes were not closely packed but were laying apart suggesting adequate cell migration (Figure 1g).

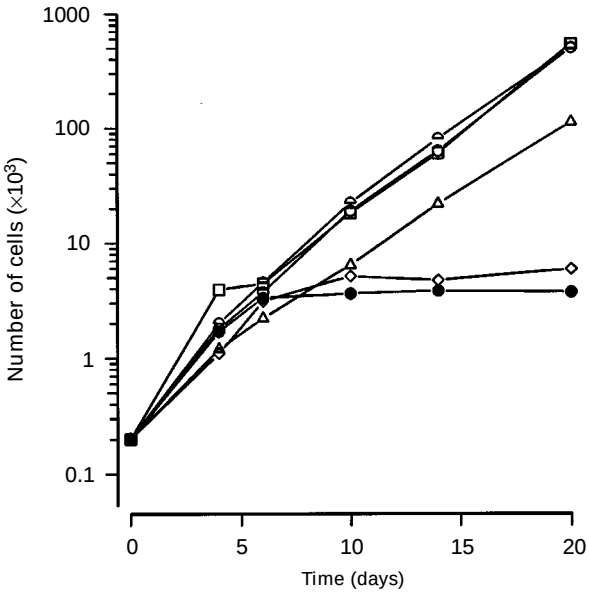


Figure 2 Suppression of clonal keratinocyte growth by $1,25(OH)_2D_3$ added at various times. $1,25(OH)_2D_3$ was added at 10^{-7} M at the beginning of the culture for 2 days (□), 4 days (△), 6 days (◇), or for the last 6 days of culture (⌓). In the control experiments $1,25(OH)_2D_3$ was added for the whole culture period (●) or the cells were cultured without $1,25(OH)_2D_3$ (○). Each symbol represents the mean value of 3 experiments.

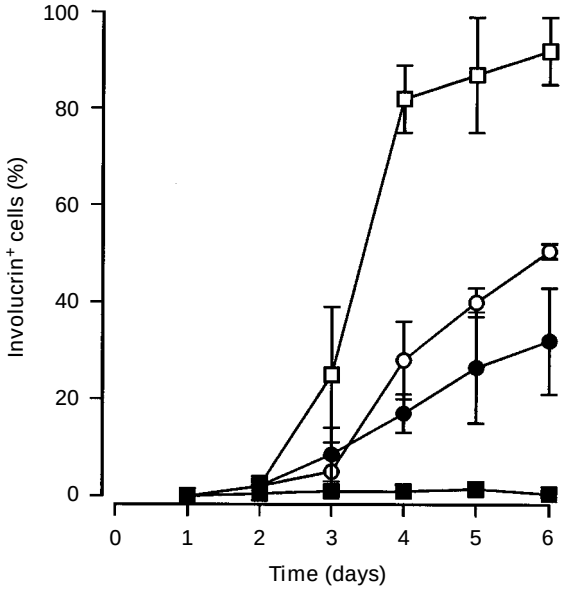


Figure 3 Effects of $1,25(OH)_2D_3$ on the expression of differentiation markers. Keratinocytes were plated on collagen IV-coated culture flasks for 15 min. The adherent cells which represented a population of keratinocytes of a high clonogenic potential (putative stem cells) were trypsinized, replated, and exposed to 10^{-8} M (○, ●) or 10^{-6} M $1,25(OH)_2D_3$ (□, ■) for indicated periods of time. The cells were fixed and stained for involucrin (○, □) or K1K10 keratins (●, ■) as described in Methods section. The proportion of positive cells was determined in a laser-scanning fluorescent microscope. Cells cultured in the presence of the vehicle only did not express any differentiation marker (not shown). Symbols represent the mean values from two independent experiments: vertical lines show range.

We considered the possibility that $1,25(OH)_2D_3$ manifested its inhibitory effects via induction of differentiation of the progenitor pool. As shown in Figure 1, $1,25(OH)_2D_3$ caused an increase in the proportion of meroclones and paraclones at the expense of homoclones. Excess of small colonies was not caused by an increased cell-cycle length since, as shown in

Figure 2 and confirmed by microscopic monitoring of clonal growth, the expansion of meroclones and paraclones was confined to the first week of culture. It was thus conceivable that $1,25(OH)_2D_3$ caused a differentiation of the cells of a high clonogenic potential into those of a more limited proliferative capacity forming meroclones and paraclones. This reasoning was supported by the results showing that markers of differentiation (K1K10 keratin and involucrin) were expressed after a 4 day treatment of clonogenic cells with $10^{-8}-10^{-7}$ M $1,25(OH)_2D_3$. A further increase in the concentration of $1,25(OH)_2D_3$ caused mainly formation of involucrin$^+$ K1K10$^-$ cells, which indicates an accelerated and direct transition of proliferative cells (involucrin$^-$) into terminally differentiated cells (involucrin$^+$) bypassing the TAC-like K1K10$^+$ intermediate (Figure 3).

Data from Figure 2 showed that transient (4–6 days) treatment with $1,25(OH)_2D_3$ caused a permanent suppression of clonal growth. This observation virtually excluded the possibility that a reversible cell-cycle block caused inhibition of colony formation. However, it has been proposed that $1,25(OH)_2D_3$ exerts a rapid, reversible cell-cycle block enabling the keratinocyte to enter the differentiation pathway (Itin *et al*., 1994). Since such rapidly occuring (1–2 days) cell cycle arrest has been demonstrated only for keratinocytes cultured at relatively high densities (Itin *et al*., 1994; Gniadecki, 1996a), we investigated whether a similar phenomenon took place for the cells at clonal densities. As shown in Figure 4, DNA synthesis measured by BrdU labelling was not affected by a short-time exposure to $1,25(OH)_2D_3$. A significant decline in BrdU was apparent only after 4 days when the expression of differentiation markers also took place. These results did not support the concept that $1,25(OH)_2D_3$ exerted a direct, rapid and reversible cell-cycle block in clonogenic cells.

20-Epi vitamin D analogues are potent inhibitors of clonal growth and inducers of cell differentiation

Three 20-epi analogues of $1,25(OH)_2D_3$ were tested for the ability to inhibit clonal growth (Table 1, Figure 5). All the analogues tested were significantly more potent in the clonogenic assay than $1,25(OH)_2D_3$. To investigate whether this increased potency was correlated with the ability to induce clonogenic cell differentiation, the fast-adhering keratinocyte fraction was treated with different concentrations of the analogues for 4 days and the proportion of differentiated involucrin-positive cells was determined. As illustrated in Figure 5 and Table 1 all the analogues tested stimulated cell differentiation at 10^2-10^3 lower concentrations than $1,25(OH)_2D_3$.

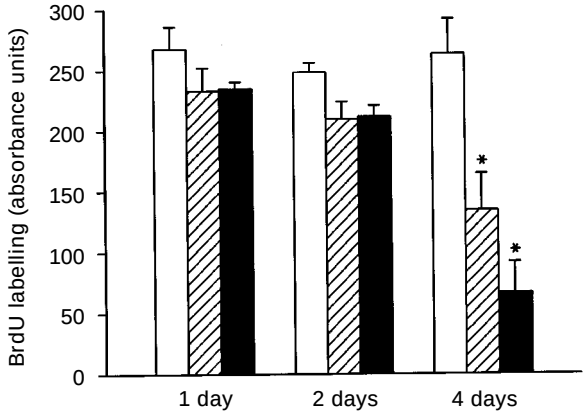


Figure 4 Effects of $1,25(OH)_2D_3$ on incorporation of bromodeoxyuridine (BrdU). Keratinocytes were plated at the density of 3 cells cm^{-2} and cultured in KGM for indicated periods of time with 10^{-8} M (hatched columns) or 10^{-7} M $1,25(OH)_2D_3$ (solid columns). Control cells were not exposed to $1,25(OH)_2D_3$ (open columns). BrdU was added for the last 6 h of culture. Keratinocytes were trypsinized and the amount of BrdU incorporated determined with the enzyme-linked immunosorbent assay. Each column represents the mean with s.d. ($n=3$). *$P<0.001$ as compared with the control.

Effect of extracellular calcium, lymphokines (IL-1, IL-6, IL-8) and growth factors (EGF, TGF-α, KGF, IGF I) on the 1,25(OH)$_2$D$_3$-dependent clonal growth arrest

The results presented above suggested that induction of clonogenic cell differentiation was the major mechanism responsible for the $1,25(OH)_2D_3$-dependent clonal growth arrest. High extracellular calcium concentration triggers keratinocyte differentiation and has been shown to potentiate the effects of $1,25(OH)_2D_3$ (Su *et al*., 1994). However, we showed that the suppressive effects of $1,25(OH)_2D_3$ on colony growth were not dependent on calcium concentration (Figure 6).

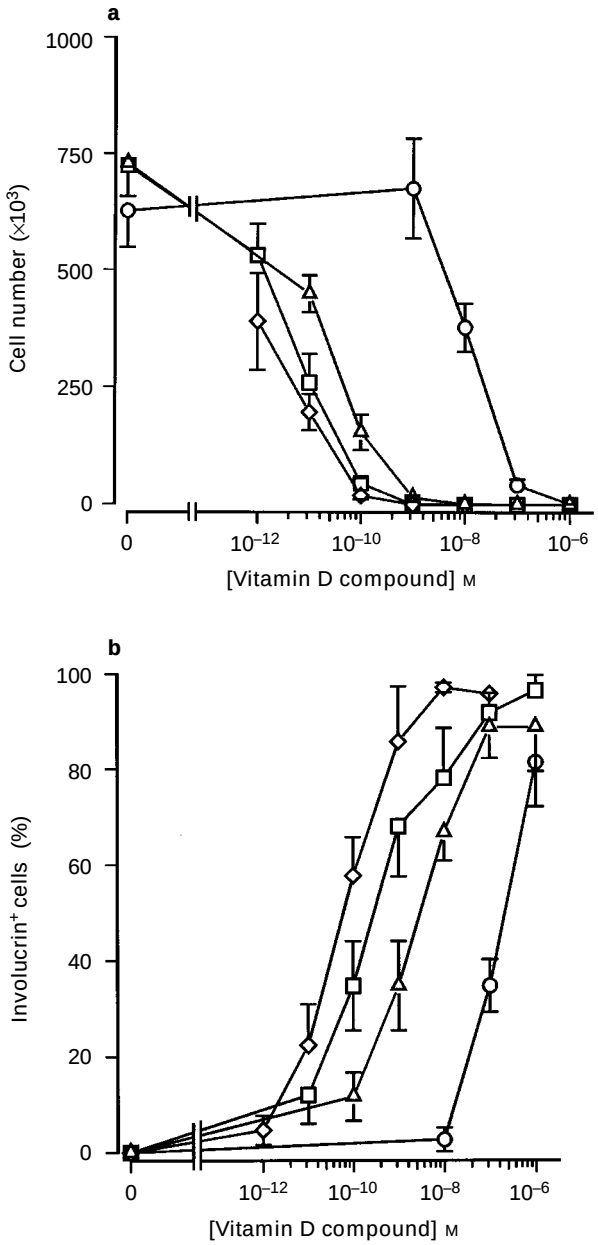


Figure 5 Effects of vitamin D analogues on clonal growth and keratinocyte differentiation. (a) Keratinocytes plated at the density of 3 cells/cm^{-2} and cultured for 20 days with different concentrations of: $1,25(OH)_2D_3$ (○), MC 1288 (△), MC 1301 (□) or KH 1060 (◇). At termination, keratinocytes were trypsinized and the number of cells was determined (in each case viability was >90%, as determined with the trypan blue exclusion test). (b) Keratinocytes of high clonogenic potential were concentrated as described in the legend for Figure 4 and exposed for vitamin D analogues for 6 days. Ordinates: the proportions of involucrin positive cells. Symbols which are identical with those in (a) represent mean values with s.d. ($n=3$).

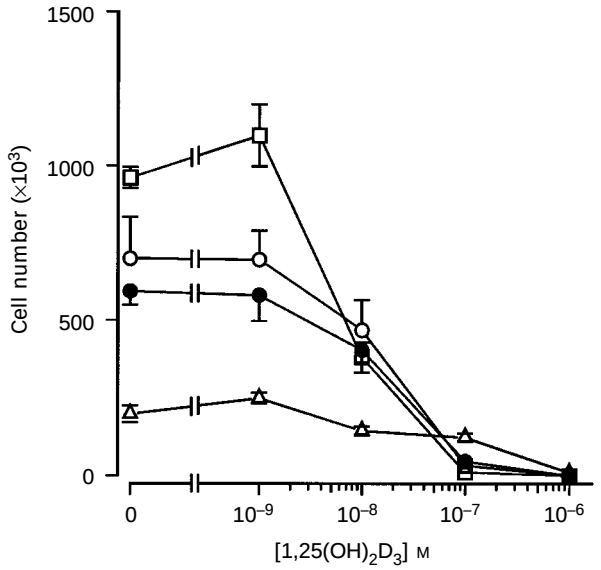


Figure 6 Effects of calcium, EGF and TGFα on the $1,25(OH)_2D_3$-dependent suppression of keratinocyte clonal growth. Cells were plated at 3 cells cm^{-2} and cultured for 18 days in EGF-deficient KGM with: 50 ng ml^{-1} EGF (○), 50 ng ml^{-1} EGF and 1.8 mM Ca^{2+} (●), 0.2 ng ml^{-1} EGF (△), 10 ng ml^{-1} TGFα (□). Each symbol represents the mean with s.d. ($n=3$).

Table 2 Effects of cytokines on clonal keratinocyte growth

	Number of cells at 1,25(OH)₂D₃ concentration (M)[b]		
Cytokine[a]	*0 (control)*[c]	*10^{-7}*	*10^{-6}*
EGF	616±125	33±9	1.5±0.9
KGF[d]	620±126	453±50	117±74
IGF I	667±202	121±40	10±4.9
EGF+KGF[d]	792±64	180±53	40±8
EGF+IGF I	859±85	61±30	7.9±2

[a]EGF (50 ng ml^{-1}), KGF or IGF I (both at 10 ng ng^{-1}) were added to the EGF-deficient KGM. [b]200 cells were plated and cultured for 20 days. At the end the total number of cells was counted. Mean values±s.d. ($n=3$) are shown. [c]No differences between the groups treated with different cytokines. [d]Significant ($P<0.05$) attenuation of the effects of $1,25(OH)_2D_3$ compared to the EGF group (analysis of variance).

At a concentration of 0.2 ng ml^{-1} EGF the keratinocyte colonies were smaller and lower cell yields were obtained. As shown in Figure 6 the addition of $1,25(OH)_2D_3$ caused only a minor inhibition of growth when compared with the cells grown in the presence of 50 ng ml^{-1} EGF ($P<0.001$). The EGF activity could be replaced by TGF-α (Figure 6).

Lymphokines (IL-1α, IL-1β, IL-6, IL-8) and cytokines (IGF I, KGF) promoted clonal cell growth to a degree comparable with EGF and TGF-α (not shown). In the presence of interleukins, the $1,25(OH)_2D_3$-mediated inhibition of clonal growth was similar to that obtained with EGF. In contrast, in the presence of KGF the suppression of colony growth by $1,25(OH)_2D_3$ was attenuated. Culturing of cells with a mixture of EGF and KGF also attenuated the $1,25(OH)_2D_3$-dependent inhibition of colony growth (Table 2). Treatment with IGF I had a similar effect, although the statistical significance was not attained. The $1,25(OH)_2D_3$-dependent expression of involucrin was diminished in the presence of KGF ($P>0.001$) and IGF I ($P=0.045$) indicating that these cytokines blocked stem cell differentiation in response to $1,25(OH)_2D_3$ (Figure 7).

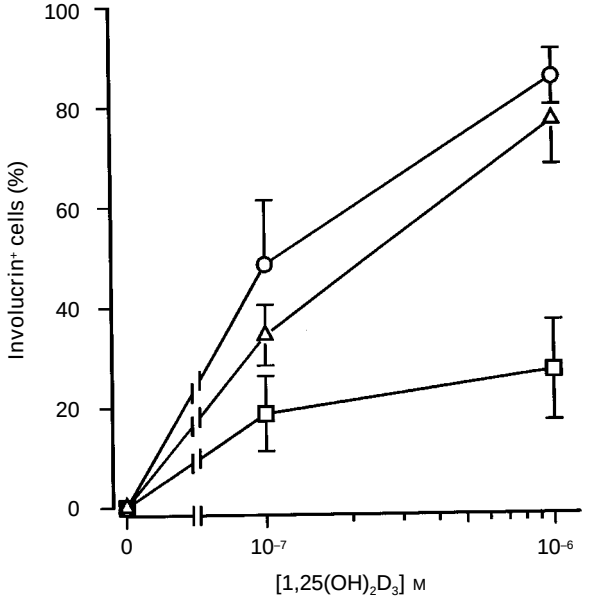


Figure 7 Modulation of $1,25(OH)_2D_3$-induced keratinocyte differentiation by KGF and IGF I. Cells with high clonogenic potential were isolated on type IV collagen, as described in the legend for Figure 4, replated, and incubated overnight in EGF-deficient KGM with: 50 ng ml^{-1} EGF (○), 10 ng ml^{-1} KGF (□), or 10 ng ml^{-1} IGF I (△). $1,25(OH)_2D_3$ was added at a stated concentration for 4 days and the proportions of involucrine-positive cells were counted in a confocal laser-scanning fluorescent microscope. Each symbol represents the mean with s.d. ($n=3$).

Discussion

In this study we employed a clonogenic assay to determine the influence of $1,25(OH)_2D_3$ and vitamin D_3 analogues on keratinocyte stem cells. Keratinocytes plated at clonal densities have different growth characteristics from the cells cultured conventionally at higher densities. In the former instance the keratinocytes require, in addition to the bovine pituitary extract and insulin, a third-party growth factor to develop full-sized colonies (Wille *et al*., 1984; Cook *et al*., 1991; Pittelkow *et al*., 1993). TGF-α and EGF are known to be potent stimulators of clonal growth but we revealed here that they can be replaced by interleukins (IL-1α, IL-1β, IL-6, IL-8) and unrelated growth factors (KGF, IGF I) without any significant loss of clonogenic potential. These cytokines have previously been suggested to be involved in the pathogenesis of psoriasis and are known to enhance keratinocyte growth (Krueger & Gottlieb, 1994) but apart from IL-6 (Elder *et al*., 1992) their clonogenic activities have not been assessed. A primary stimulant activity on stem cells is a likely mechanism of observed clonal growth enhancement, since in the clonogenic assays only 20 cells per ml of culture medium were present, limiting the possible influence of secondarily secreted growth factors.

The ability of $1,25(OH)_2D_3$ to induce terminal differentiation and growth inhibition of cultured keratinocytes has been recognized and extensively investigated. However, it is unclear whether the effects on growth and differentiation are independent phenomena or which of them is relevant for psoriasis treatment. Here we demonstrated that: (i) $1,25(OH)_2D_3$ was unable to block totally cell proliferation even at high concentrations (10^{-6} M) and small abortive colonies were observed, (ii) expression of differentiation markers correlated with the loss of the proliferative potential of the clonogenic cells, (iii) the clonogenic cells were targets for the $1,25(OH)_2D_3$-induced differentiation. These findings strongly indicate that the inhibition of clonal growth by $1,25(OH)_2D_3$ is dependent on the differentiation of clonogenic cells (assumed here to be identical to stem cells) into the more mature phenotype of a limited proliferative potential. These data do not favour the

earlier hypotheses suggesting that $1,25(OH)_2D_3$-induced differentiation is preceded by a period of a reversible growth arrest (Itin *et al*., 1994). Since this has been primarily observed in cultures at higher than clonal cell densities, an involvement of secondary mediators, such as transforming growth β, is possible (Koli & Keski Oja, 1993).

The vitamin D_3 analogues were more potent inducers of stem cell differentiation than $1,25(OH)_2D_3$ ($1,25(OH)_2D_3$ < MC 1288 < MC 1301 < KH 1060) and this activity correlated with the potency of the inhibition of clonal growth. Comparison of EC_{50} values reveals that there is a 4 orders of magnitude difference in potency between KH 1060 and the natural compound $1,25(OH)_2D_3$ in the induction of differentiation markers and inhibition of proliferation. This is in agreement with earlier studies demonstrating the same order of potency for inhibition of the U937 cells (Binderup *et al*., 1991) and induction of epidermal hyperplasia in mice (Gniadecki & Serup, 1995). However, it must be mentioned that in our clonogenic assay effective $1,25(OH)_2D_3$ concentration (approx. 10^{-8} M) exceed those seen normally in the circulation. It implies that endogenous $1,25(OH)_2D_3$ plays only a little role in the regulation of epidermal proliferation and differentiation in man.

The mechanisms of increased potency of the side-chain modified vitamin D_3 analogues has not been fully elucidated. The compounds used in this study have a comparable affinity for the vitamin D receptor (Binderup *et al*., 1991; Bouillon *et al*., 1995). However, a recent study showed that high bioactivity of KH 1060 may be caused by alterations in receptor conformation and increased stability of the receptor-DNA complexes (Van Den Bemd *et al*., 1996). The mechanisms independent of the transcription-factor activity of the vitamin D receptor ('non-genomic' mechanisms) may also be involved. We have recently shown that $1,25(OH)_2D_3$ stimulates the Raf-mitogen-activated protein kinase cascade in keratinocytes which is an essential mechanism of keratinocyte growth regulation by this hormone (Gniadecki, 1996b). Other researchers found that $1,25(OH)_2D_3$ induces different signalling pathways involving the protein kinase C, cyclic nucleotides, or calcium-ion signalling (Merke *et al*., 1989; Barsony & Marx, 1991; Norman *et al*., 1992; Khare *et al*., 1993; 1994; Gniadecki, 1994; Bissonnette *et al*., 1995; Sergeev & Rhoten, 1995; Slater *et al*., 1995). Although the 'non-genomic' signalling has not been extensively investigated for vitamin D_3 analogues, it is conceivable that these mechanisms significantly modulate their effects on cell proliferation and differentiation.

The terminal differentiation of keratinocytes is physiologically coupled with apoptosis (Haake & Polakowska, 1993). Terminally differentiated granular cells in the epidermis and keratinocytes suspended in semi-solid methylcellulose display characteristic DNA fragmentation pattern and other features of apoptosis (Gavrieli *et al*., 1992; Tamada *et al*., 1994; Sachsenmeier *et al*., 1996). However, keratinocyte differentiation induced by $1,25(OH)_2D_3$ was not accompanied by apoptosis. $1,25(OH)_2D_3$ can thus be viewed as an incomplete inducer of terminal differentiation not being able to drive the cell into the final apoptotic pathway.

The vitamin D_3-induced stem cell differentiation was dependent on the presence of certain growth factors. EGF and TGF-α which in some studies prevented cell differentiation (Marchese *et al*., 1990), enhanced the efficacy of $1,25(OH)_2D_3$ in the clonal assay. This effect of EGF has been noticed before and could not be explained by changes in the vitamin D receptor level (Chen *et al*., 1995). It was unlikely that EGF and TGF-α sensitized the cells to $1,25(OH)_2D_3$ via stimulation of their proliferation since KGF and IGF I which stimulated clonal growth equally well had an opposite effect on the $1,25(OH)_2D_3$-dependent clonal growth inhibition. TGF-α is hyperexpressed in psoriasis and is a crucial mediator in the emergence of the psoriatic phenotype (Gottlieb *et al*., 1988; Elder *et al*., 1989; Vassar & Fuchs, 1991). It is thus likely that EGF and TGF-α, and probably other cytokines such as IL-1, IL-6, or IL-8 are not mere stimulators of keratinocyte growth but are able to activate a pathological cell phenotype characterized by high proliferative activity associated with expression of certain markers such as keratins K6 K16 (Jiang *et al*., 1993). Although precise molecular mechanisms have not been elucidated, it is tempting to speculate that such cells are more sensitive to $1,25(OH)_2D_3$-induced differentiation than their normal counterparts.

The attenuation of the cytostatic effect of $1,25(OH)_2D_3$ by IGF I demonstrated in this study has been independently shown by Chen *et al*. (1995). KGF also blocked the $1,25(OH)_2D_3$-induced stem cell differentiation and clonal keratinocyte growth and the effects of this cytokine were more profound than those of IGF I. The mechanism of action of IGF I and KGF is unclear. IGF I and KGF are unrelated and act via different membrane receptors expressed on keratinocytes (Misra *et al*., 1986; Avivi *et al*., 1991). Hyperexpression of IGF I and IGF receptor in psoriasis suggests its role in mediating keratinocyte hyperproliferation (Gottlieb, 1990; Krane *et al*., 1992), although neither IGF I nor KGF alone is able to produce the psoriatic phenotype (Guo *et al*., 1993; Staiano Coico *et al*., 1993). It is not known whether KGF is involved in the pathogenesis, but recent results showing that this cytokine is induced by a range of inflammatory mediators (IL-1, IL-6, TGF-α) (Werner *et al*., 1992; Brauchle *et al*., 1994; Chedid *et al*., 1994) indicate that it may be present in psoriatic epidermis. Interestingly, KGF has recently been shown to suppress keratinocyte differentiation in another experimental system, the methylcellulose suspension-induced keratinocyte differentiation (Hines & Allen-Hoffmann, 1996). KGF may thus represent a cytokine with the ability to block terminal keratinocyte differentiation.

Thus, as far as the $1,25(OH)_2D_3$-mediated cell differentiation is concerned, cytokines may be divided into two groups: those which potentiate the effects of $1,25(OH)_2D_3$ (EGF, TGF-α, IL1-α, IL1-β, IL-6, and IL-8) and those which block its effects on the clonogenic cells (KGF, IGF I). It will be fascinating to see whether the balance between these two groups determines the clinical effect of the vitamin D-based treatment in psoriasis.

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