Pergamon

Biochemical Pharmacology, Vol. 49, No. 5, pp. 621–624, 1995.
Elsevier Science Ltd
Printed in Great Britain.

0006-2952(94)00482-X

STIMULATION OF EPIDERMAL PROLIFERATION IN MICE WITH 1α,25-DIHYDROXYVITAMIN D_3 AND RECEPTOR-ACTIVE 20-EPI ANALOGUES OF 1α,25-DIHYDROXYVITAMIN D_3

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(*Received* 8 *March* 1994; *accepted* 17 *October* 1994)

Abstract—The effects of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) and receptor-active 20-epi vitamin D analogues (MC 1288, 20-epi-1α,25-$(OH)_2D_3$; MC 1301, 20-epi-24*a*-homo-26,27-dimethyl-1α,25-$(OH)_2D_3$, and KH 1060: 20-epi-22-oxa-24a-homo-26,27-dimethyl-1α,25-$(OH)_2D_3$) on epidermal proliferation in mice were studied *in vivo*. Single topical applications of all compounds induced epidermal proliferation in a dose-dependent manner. The relative potencies *in vivo* (KH 1060 > MC1301> MC1288 > 1α,25-$(OH)_2D_3$) correlated well with the known activities of these compounds to inhibit U 937 cell proliferation *in vitro*. Vitamin D_3 and 1β,25-$(OH)_2D_3$, two compounds that do not bind the vitamin D receptor, did not affect epidermal proliferation. Our study shows that vitamin D_3 compounds that bind to the vitamin D receptor stimulate epidermal proliferation in mice.

Key words: calcitriol analogues; epidermis proliferation

It has generally been accepted that the vitamin D hormone (1α,25-$(OH)_2D_3$†), besides its role in calcium homeostasis, is an inhibitor of cellular proliferation [1]. Its inhibitory effects on cell growth may be considerably potentiated by chemical modifications within the side-chain of 1α,25-$(OH)_2D_3$. Studies *in vitro* in a U 937 leukaemic cell culture system revealed that the analogues of 1α,25-$(OH)_2D_3$ with altered stereochemistry of the methyl group at C20 (20-epi) are more active in suppression of cell proliferation than 1α,25-$(OH)_2D_3$ [2, 3] (Table 1).

It has been shown that 1α,25-$(OH)_2D_3$ and the vitamin D_3 analogues inhibit proliferation of cultured keratinocytes [1, 4, 5]. Because the vitamin D receptor (VDR) is expressed in the epidermis, and keratinocytes may synthesise 1α,25-$(OH)_2D_3$ from the precursor 25-$(OH)D_3$ [6], it has been suggested that the vitamin D hormone is a natural inhibitor of growth in the skin [7]. However, the effects of 1α,25-$(OH)_2D_3$ on epidermis have been investigated almost exclusively *in vitro*, where keratinocytes are isolated from their natural environment, and cell–cell and cell–matrix interactions, critical for regulation of growth, are absent. This study shows that in contrast to what is seen in *in vitro* systems, 1α,25-$(OH)_2D_3$ and receptor-active vitamin D_3 analogues (MC 1288, MC 1301, KH 1060) stimulate epidermal growth in mice *in vivo*, with potencies correlated with their growth inhibitory activities *in vitro*.

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†Abbreviations: 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1β,25-$(OH)_2D_3$, 1β,25-dihydroxyvitamin D_3; MC 1288, 20-epi-1α,25-dihydroxyvitamin D_3; MC 1301, 20-epi-24*a*-homo-26,27-dimethyl-1α,25-dihydroxyvitamin D_3; KH 1060, 20-epi-22-oxa-24*a*-homo-26,27-dimethyl-1α,25-dihydroxyvitamin D_3; VDR, receptor for 1α,25-dihydroxyvitamin D_3

MATERIALS AND METHODS

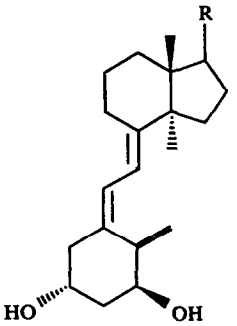
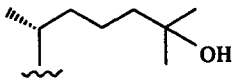
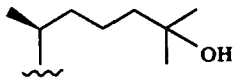
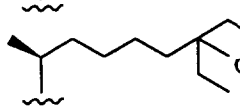
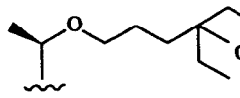
MC 1288, MC 1301, KH 1060, and 1α- and 1β,25-$(OH)_2D_3$ (Table 1) were synthesized in the Chemical Research Department, Leo Pharmaceutical Products (Ballerup, Denmark) [8]. Vitamin D_3 was purchased from Solvay-Duphar (Weesp, The Netherlands). Solutions for topical treatment were prepared in 1:1.2 (w/w) isopropyl alcohol/water vehicle buffered with 1 g/L sodium citrate dihydrate to pH 8.9.

Hairless C3H mice (females, hr/hr C3H/Tif Bom, weight approximately 20 g), three animals to a group, were treated topically on the dorsal skin with 50 μL of the drug solution. The control groups were treated with 1β,25-$(OH)_2D_3$, vitamin D_3, or the vehicle. After 72 hr skin was harvested and haematoxylin and eosin microscopic sections were prepared. Epidermal proliferation was measured with the plastic disc method of Otani *et al.* [9]. Briefly, KH 1060 2.5 pmol/cm^2 was applied on the dorsal skin and mice were killed 2, 10, 24, 48, 72, and 96 hr later. Two hours prior to death, the animals were injected intraperitoneally with 25 μCi of [^{3}H]-thymidine (Amersham International, Amersham, U.K.). Radioactivity of the epidermis was measured in a Minaxi 443 liquid scintillation counter (United Technologies, U.S.A.). Data were compared using one-way or two-way analysis of variance, with the aid of Minitab statistical software (Minitab Inc., U.S.A.), $P < 0.05$ being considered significant.

RESULTS AND DISCUSSION

To study the epidermal effects of 1α,25-$(OH)_2D_3$

Table 1. Vitamin D analogues used in this study

Structure	R	Name	Inhibition of cell proliferation (IC_{50}, M)	Receptor binding 50% displacement (M)
		α,25-$(OH)_2D_3$	1.4×10^{-8}	1.6×10^{-11}
		MC 1288	2.8×10^{-10}	1.3×10^{-11}
		MC 1301	8.2×10^{-11}	1.6×10^{-11}
		KH 1060	1.0×10^{-12}	1.3×10^{-11}

Inhibition of cell proliferation (in U 937 cell culture assay) and receptor binding (data from Ref. 2).

and its 20-epi analogues *in vivo*, 50 μL of 1α,25-$(OH)_2D_3$, MC 1288, MC 1301 and KH 1060 in the dose range 0.06–6.25 pmol/cm^2 were applied topically in hairless mice, and the histological examination of epidermis was performed 72 hr later. 1α,25-$(OH)_2D_3$ and the 20-epi vitamin D analogues caused thickening of the epidermis in a dose-dependent manner (Figs 1 and 2). The increased epidermal thickness was accompanied by the increased number of keratinocyte layers and marked elongation of basal and suprabasal cells, suggesting increased cell proliferation as a mechanism for epidermal thickening.

To confirm that epidermal thickening was caused by increased epidermal mitotic activity, incorporation of [^{3}H]thymidine in the epidermis was measured at different periods after a single application of 2.5 pmol/cm^2 of KH 1060 (Fig. 3). DNA synthesis started to increase after 24 hr, peaked at 48 hr, and declined 72 hr after the application of KH 1060. Thus, increased mitotic activity of the epidermis could explain the epidermal thickening observed after application of 1α,25-$(OH)_2D_3$ and 20-epi analogues.

Our data are confirmed by recent observations of increased epidermal mitotic activity after topical application of 1,25-$(OH)_2D_3$ and vitamin D analogues calcipotriol and KH 1060, both in mice and humans [10–12]. Stimulation of epidermal proliferation by the vitamin D_3 compounds is, however, unexpected, in view of the wealth of data showing that 1α,25-$(OH)_2D_3$ and its analogues inhibit cell proliferation and promote cellular differentiation [1, 2, 4]. In cell culture, 1α,25-$(OH)_2D_3$, MC 1288, MC 1301 and KH 1060 inhibit proliferation of a variety of cancer cell lines and antigen- and mitogen-stimulated T lymphocytes [2, 3]. A decreased DNA synthesis was demonstrated in cultured murine keratinocytes treated with 1α,25-$(OH)_2D_3$ [5] and vitamin D analogues, including 20-epi compounds [13–16].

All 20-epi analogues were more potent inducers of epidermal proliferation than 1α,25-$(OH)_2D_3$ ($P < 0.001$). The order of activity was KH 1060 >($P < 0.001$) MC 1301 >(NS) MC1288 > ($P = 0.002$) 1α,25-$(OH)_2D_3$. This order of activity *in vivo* correlated well with the activity to inhibit the proliferation of U 937 cells *in vitro* (Table 1). However, the difference in activity between analogues is much more pronounced under *in vitro* cell culture conditions than in our *in vivo* system. This may be explained by the different metabolic processing of the vitamin D compounds *in vivo* and/or by differences in sensitivity to the vitamin D analogues between leukaemic U 937 cells and keratinocytes.

The ability of the vitamin D_3 compound to inhibit cell proliferation *in vitro* is believed to be mediated by the VDR [1, 17]. Therefore, it is conceivable that the effects of 1α,25-$(OH)_2D_3$ and its analogues could be attributed to the same specific, receptor-mediated mechanism. This hypothesis is further supported by the fact that structurally related compounds that do not bind to the VDR, vitamin D_3 and 1β,25-$(OH)_2D_3$, did not affect epidermal proliferation, even though the latter compound exhibits some biological activity, being an antagonist of the so-called non-genomic action of 1α,25-$(OH)_2D_3$ [18]. However, in marked contrast to the anti-proliferative effect of the vitamin D compounds which becomes detectable after approximately 4 days of the application of the drug [5, 13, 19], the stimulatory effect in mice *in vivo* is manifested after 24–48 hr. The reason for this discrepancy is unknown, but may rely on participation of secondary mediators, such as transforming growth factor β [20, 21].

1α,25-$(OH)_2D_3$ and vitamin D_3 analogues down-

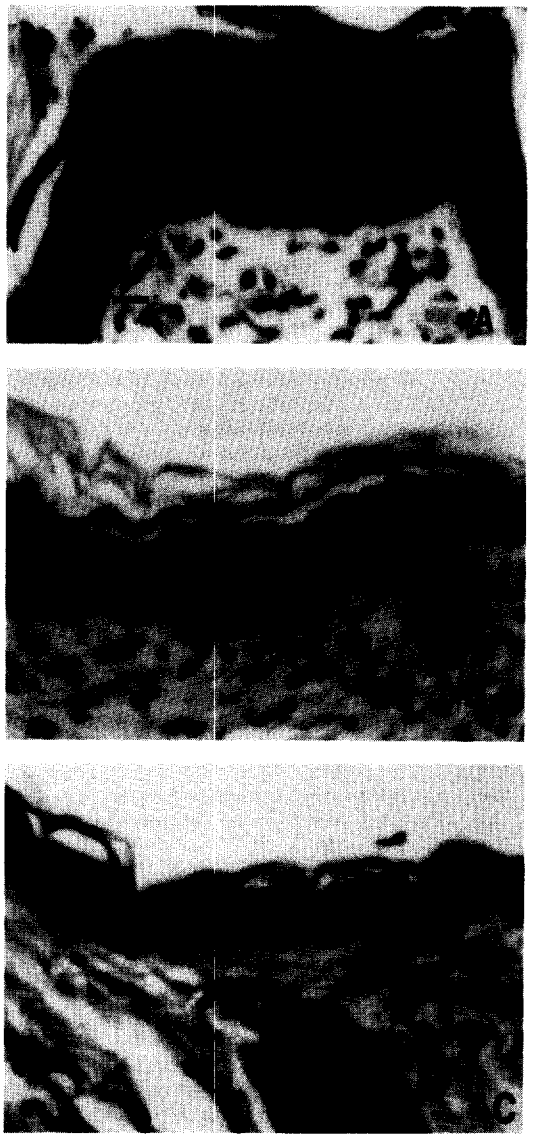


Fig. 1. Epidermal histology 72 hr after a single application of: (A) 6.25 pmol/cm^2 KH 1060; (B) 6.25 pmol/cm^2 1α,25-$(OH)_2D_3$; and (C) vehicle. A, B and C were photographed at the same magnification; bar: 20 μm.

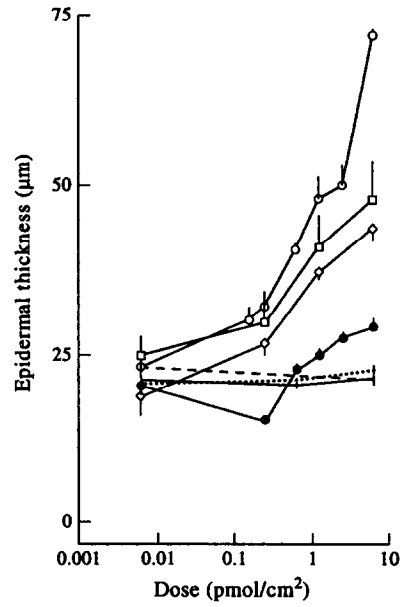


Fig. 2. Dose-dependent stimulation of epidermal thickness 72 hr after a single topical application of 1α,25-$(OH)_2D_3$ (●), MC 1288 (◇), MC 1301 (□), and KH 1060 (○). Control skin treated with 1β,25-$(OH)_2D_3$ (solid line), vitamin D_3 (dashed line), or the vehicle (dotted line). Each point represents mean (N = 3) with SEM.

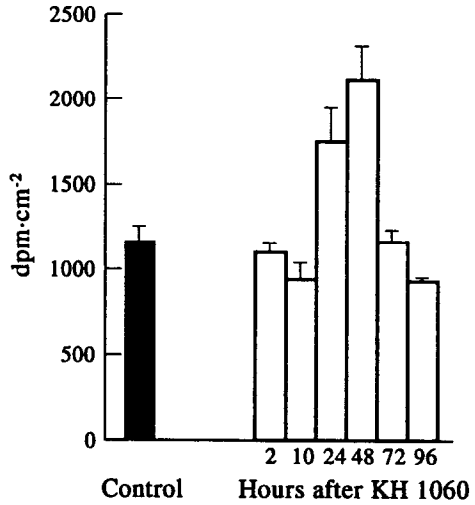


Fig. 3. Effect of a single topical application of 2.5 pmol/cm^2 KH 1060 on epidermal incorporation of [^{3}H]thymidine *in vivo* (open bars). The control value (closed bar) represents the proliferation rate of normal, untreated epidermis. Mean labelling (N = 5) with SEM.

regulate the excessive epidermal proliferation in psoriasis and are used for the topical treatment of this disease [22–24]. The reason why stimulation of VDR causes inhibition of epidermal proliferation in psoriasis and in cultured keratinocytes while triggering proliferation of normal murine epidermis *in vivo* is not clear. Interestingly, other anti-psoriatic drugs, such as dithranol, tar derivatives, and retinoids, as well as ultraviolet irradiation, cause hyperplasia of normal epidermis but a suppression of proliferation of psoriatic keratinocytes. One explanation might be the dependency of the VDR-mediated effects on the functional status of the cell. It has been shown, for example, that all-*trans*-retinoic acid blocks proliferation of mitotically activated keratinocytes, but stimulates the growth of quiescent cells [25]. It is therefore conceivable that stimulation of VDR results in mitotic activation of resting cells, whereas the proliferation of

mitotically-activated keratinocytes becomes down-regulated.

It has been proposed that the vitamin D hormone has a role as a natural inhibitor of epidermal proliferation. The existence of a local miniendocrine system in the epidermis has been postulated, where keratinocytes hydroxylate 25-$(OH)D_3$ to 1α,25-$(OH)_2D_3$ and respond to the latter by ceasing proliferation [7]. Our results suggest that the role of 1α,25-$(OH)_2D_3$ in regulation of cellular proliferation is more complex, since this hormone may also stimulate proliferation of normal epidermis.

Acknowledgements—We are grateful to Drs Ernst Binderup, Frederik Björkling, Martin Calverley and Kai Hansen, for providing vitamin D analogues. We would also like to thank Dr M. Calverley for his valuable suggestions during the preparation of the manuscript.

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