ABBREVIATIONS AND SYNONYMS (*alphabetical*):

1,25-$(OH)_2D_3$: 1α,25-dihydroxy-vitamin D_3
AJ: adherens junctions
DR: direct repeat
EC_{50}: 50% effective concentration
EGF: epidermal growth factor
IGF-I: insulin-like growth factor I
IL: interleukin
IP: inverted palindrome
KGF: keratinocyte growth factor
KH 1060: 20-*epi*-22-oxa-24a-homo-26,27-dimethyl-1α,25-dihydroxy-vitamin D_3
MAPK: mitogen-activated protein kinase
MC 1301: 20-*epi* -24a-homo-26,27-dimethyl-1α,25-dihydroxy-vitamin D_3
MC 1288: 20-*epi*-1α,25-dihydroxy-vitamin D_3
PKC: protein kinase C
RAR: retinoic acid receptor
Ro 24 – 2090: 16-ene-23-yne-25-hydroxy-vitamin D_3
RXR: retinoid X receptor
SH: Src homology domain
SD: standard deviation
T_3R: triiodothyronine receptor
TAC: transit amplifying cells
TGFα: transforming growth factor a
VD: vitamin D compounds (1α,25-$(OH)_2D_3$ and receptor-active vitamin D analogues)
VDR: vitamin D receptor
VDRE: vitamin D responsive element

DEFINITIONS

Genomic signaling: the mode of steroid hormone signal transduction dependent on the transcription factor-like activity of the hormone receptor(s).

Non-genomic signaling: a term proposed by Nemere and Norman (ref. 88) to describe various signaling pathways activated by 1,25-$(OH)_2D_3$ and other steroid hormones which are independent of gene transcription and protein synthesis.

Keratinocyte stem cell: a keratinocyte of a large proliferative and self-renewal potential giving rise to a large colony (holoclone) during culturing *in vitro* (4). Alternative term: prokeratinocyte (124).

Transit amplifying cell: a keratinocyte giving rise to an intermediate-size colony (meroclone) during culturing *in vitro*. Transit amplifying cells are committed to differentiation and have a limited proliferative potential.

Denne afhandling er af Det Sundhedsvidenskabelige Fakultet ved Københavns Universitet antaget til offentligt at forsvares for den medicinske doktorgrad.

København, den 30. September 1998
Hans Hultborn
dekan

This thesis is based on the following original publications:

I Gniadecki, R., Serup, J. (1995). Stimulation of epidermal proliferation in mice with 1α,25-dihydroxyvitamin D_3 and receptor-active 20-epi analogues of 1α,25-dihydroxyvitamin D_3. Biochem. Pharmacol. *49*, 621 – 624.

II Gniadecki, R., Gniadecka, M., Serup, J. (1995). The effects of KH 1060, a potent 20-epi analogue of the vitamin D_3 hormone, on hairless mouse skin *in vivo*. Br. J. Dermatol. *132*, 841 – 852.

III Gniadecki, R. (1994). A vitamin D analogue KH 1060 activates the protein kinase C-c-*fos* signalling pathway to stimulate epidermal proliferation in murine skin. J. Endocrinol. *143*, 521 – 525.

IV Gniadecki, R. (1996). Stimulation *versus* inhibition of keratinocyte growth by 1,25-dihydroxyvitamin D_3: dependence on cell culture conditions. J. Invest. Dermatol. *106*, 510 – 516.

V Gniadecki, R. (1997). 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. Br. J. Pharmacol. *120*, 1119 – 1127.

VI Gniadecki, R., Gajkowska, B., Hansen, M. (1997). 1,25-$(OH)_2D_3$ stimulates the assembly of adherens junctions in keratinocytes: Involvement of protein kinase C. Endocrinology *138*, 2241 – 2248.

VII Gniadecki, R. (1996). Activation of Raf-mitogen-activated protein kinase signaling pathway by 1,25-dihydroxyvitamin D_3 in normal human keratinocytes. J. Invest. Dermatol. *106*, 1212 – 1217.

VIII Gniadecki, R. (1997). Involvement of Src in the vitamin D signaling in human keratinocytes. Biochem. Pharmacol. *55*, 499 – 503.

Forsvaret finder sted den 26. Februar 1999 kl. 14.30 i auditoriet, Dermatologisk afdeling D, bygning 9, 1. sal, Bispebjerg Hospital, Bispebjerg bakke 23, 2400 København NV

CONTENTS

PREFACE

The present thesis is the culmination of investigations which have occupied me for the last seven years. These studies concern the mechanisms of action of 1,25-dihydroxy-vitamin D_3 and various vitamin D analogs in keratinocytes, in relation to the regulation of cell growth and differentiation. The most consequential ideas of this project were developed during my employment in the Department of Dermatological Research at Leo Pharmaceutical Products in Ballerup (1991 – 1994). This was possible due to innumerable discussions with Dr. Jørgen Serup, the head of this department. I was immensely helped not only by his wise scientific judgment and criticism, but also by his friendly and positive attitude and willingness to aid me with all kinds of practical issues. He was an ideal mentor and a source of scientific inspiration.

The completion of this work would not be possible without engagement and support of the head of research and development in Leo, Dr. Poul Rasmussen and the head of the Department of Biology, Dr. Lise Binderup. Dr. Martin Calverley from the Chemical Research Department provided many vitamin D analogues (the MC series) and shared his extensive chemical expertise with me. He also sacrificed a lot of his time correcting my manuscripts. Drs. Kai Hansen, Claus Bretting and Ernst Binderup from the same department, and Dr. Milan Uskokovic from Roche, New Jersey, have kindly provided their vitamin D compounds for research. This study could not have been finished on time without the technical help of Mrs. Pavla Setina and Mrs. Helle Flaga from the Department of Dermatological Research, Leo Pharmaceutical Products.

In 1994 I moved from Leo to the Department of Dermatology in Rigshospitalet (from 1996 Department of Dermatology, Bispebjerg Hospital) where I met with instant support from the head of the department, Professor Hans Christian Wulf. The research-oriented atmosphere created by him was ideal to continue this project. His vast research experience and sound advice which he generously shared with me were of great value. I will also mention that I am indebted to Dr. Michael Hansen from the Section of Microbiology at the Royal Veterinary and Agricultural University for his expert help with confocal laser scanning microscopy and Prof. Barbara Gajkowska, head of the Electron Microscopy Laboratory, Polish Academy of Sciences in Warsaw for performing the electron microscopy photographs. Research performed during this period was partially financed from the grant donated by the Haensch Foundation.

I dedicate this thesis to my wife Monika who has been a treasure to me throughout these years. Our love has been a continual source of energy which enabled me to complete this work.

Charlottenlund, August 1997
Robert Gniadecki

1. INTRODUCTION

In 1981 Abe et al. reported that treatment of M1 myeloid leukemia cells with 1,25-dihydroxy-vitamin D_3 (1,25-$(OH)_2D_3$) induces differentiation of this line to a more mature phenotype (1). This finding opened a new era in vitamin D research. 1,25-$(OH)_2D_3$, previously considered exclusively to be responsible for calcium homeostasis, appeared to be capable of regulation of cell growth and differentiation (121). 1,25-$(OH)_2D_3$ has been shown to block proliferation of a variety of cells *in vitro* and this cytostatic effect is often associated with a stimulation of cell differentiation (Table I). In very well investigated models of promyelocytic leukemia, the HL-60 or U 937 cell lines, treatment with 1,25-$(OH)_2D_3$ causes cell-cycle block in the G_1 phase and differentiation into a mature phenotype producing superoxide and expressing macrophage-specific α-naphtyl acetate- and non-specific esterase species. Other cancer cells are also susceptible to the antiproliferative effects of 1,25-$(OH)_2D_3$ (Table I). These results generated considerable interest in the clinical potential of 1,25-$(OH)_2D_3$ as an anti-cancer agent and stimulated search for the vitamin D analogs for the use in oncology.

The interest in 1,25-$(OH)_2D_3$ in dermatology arose after Hosomi et al. had revealed that 1,25-$(OH)_2D_3$ inhibited murine keratinocyte growth and stimulated their terminal differentiation (53). These findings were subsequently confirmed using human keratinocytes in culture by showing that 1,25-$(OH)_2D_3$ caused inhibition of DNA synthesis, an increase in proportion of terminally differentiated, cornified cells, and an increase in transglutaminase activity (105). This led to the concept that 1,25-$(OH)_2D_3$ might be used for treatment of skin diseases with keratinocyte hyperplasia. Psoriasis was the first hyperproliferative skin disease to be successfully treated with 1,25-$(OH)_2D_3$ (82, 83, 106).

Table I. *Examples of cell types responding to the antiproliferative activity of 1,25-$(OH)_2D_3$ or vitamin D analogues*

Normal cells	keratinocytes*
	fibroblasts
	chondrocytes*
	adipocytes*
	osteoblasts*
	colonocytes
	endothelial cells
	mesangial glomerular cells
	parathyroid cells
	prostatic epithelial cells
	thyroid follicular cells
	vascular smooth muscle cells
Cancer cells	squamous cell carcinoma
	colon cancer
	pancreatic cancer
	prostate cancer
	ovarian carcinoma
	leukemia and lymphoma*
	glioma
	glioblastoma
	neuroblastoma
	breast cancer
	sarcoma

*stimulation of cell differentiation.

Vitamin D analogs

To avoid the calcemic side effects of the natural compound, various research groups and pharmaceutical companies synthesized vitamin D analogs which were equally or more potent than 1,25-$(OH)_2D_3$ with respect to the suppression of proliferation but less capable of inducing hypercalcemia. Reports of several hundred analogs appeared in the literature, however the biological effects of only few have been investigated extensively (reviewed in refs. 19, 56). Calcipotriol was the first selective analog shown to be clinically effective (62–64, 85) and is now extensively used for the treatment of plaque psoriasis. The analysis of the psoriatic epidermis treated with calcipotriol or 1,25-$(OH)_2D_3$ confirmed the inhibition of extensive cell proliferation and stimulation of differentiation of psoriatic keratinocytes (30, 39, 114). Due to its modulation of keratinocyte differentiation, calcipotriol may also be efficacious in the treatment of skin diseases associated with disordered keratinization (66, 74, 115). *In vitro*, calcipotriol is able to suppress the proliferation of lymphoma and leukemia cells (51, 96, 107), MCF-7 breast cancer cell line (27), squamous cell carcinoma (24), and prostate cancer (103) and these results support its use for the treatment of malignant tumors (20, 94).

Calcipotriol is equipotent to 1,25-$(OH)_2D_3$ with respect to inhibition of cell proliferation, its advantage being a significantly reduced calcemic effect. In an attempt to obtain analogs of higher potency, the compounds with altered stereochemistry of carbon 20 in the side chain (so-called 20-*epi* analogs) were synthesized (Fig. 1).

The 20-*epi* analog of 1,25-$(OH)_2D_3$ (MC 1288) inhibits U 937 cell proliferation at concentrations 100 times lower than the natural hormone, and the most potent compound from this family, 20-*epi*-22-oxa-24a-homo-26,27-dimethyl-1,25-$(OH)_2D_3$ (KH 1060) is up to 10 000 times more potent in the same assays (Table II) (13, 32, 33, 80). Because of its very high potency, this drug is a promising candidate for the treatment of certain hematologic neoplasms (34, 35). Topical KH 1060 is also beneficial in psoriasis, however contrary to what could be expected, its clinical efficacy does not seem to be higher than that of calcipotriol (65).

The mechanism of action: Genomic and non-genomic signaling pathways

The major cellular 1,25-$(OH)_2D_3$-binding protein is the 48-60 kD vitamin D receptor (VDR). VDR is widely expressed in a variety of cells, including keratinocytes (78, 109). This receptor is a member of a larger family of receptors for steroid hormones which also contains receptors for all-*trans*-retinoic acid (RAR), 9-*cis*-retinoic acid (RXR), the thyroid hormone (T_3R) and other biologically active steroids (androgens, estrogens, progesterone, mineralocorticoids, and glucocorticoids). These receptors are active as transcription factors and they regulate gene expression upon binding to the appropriate responsive elements in gene promoters (reviewed in refs. 22, 25). The vitamin D responsive elements (VDRE) host VDR homo- and heterodimers that bind to 2 hexameric core motifs, arranged either as direct repeats (DRs) or inverted palindromes (IPs). The most often encountered is the heterodimer VDR-RXR, but other combinations are at least theoretically possible (VDR-VDR, VDR + T_3R, VDR + RAR)

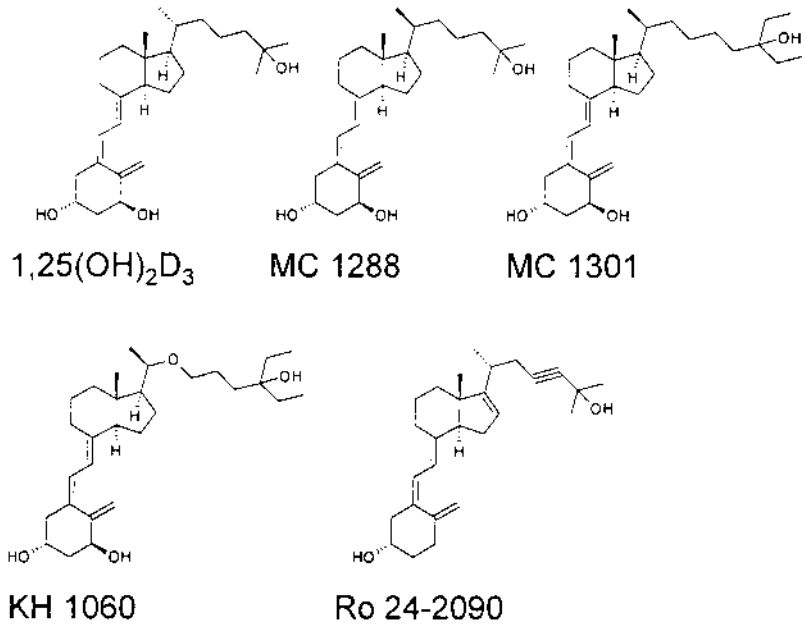

Fig. 1. Structural formulae of selected vitamin D compounds used in this study.

substantially increasing signaling flexibility and plasticity (23, 59, 99, 100).

Expression of VDR has been considered necessary for 1,25-$(OH)_2D_3$ and the receptor-active vitamin D analogs (collectively referred to as the vitamin D compounds, VD) to manifest their growth-regulatory properties (12, 26, 48). However, the mechanistic basis of growth regulation and of higher potencies of certain vitamin D analogs have not been satisfactorily elucidated on the basis of known VDR-mediated mechanisms. It has been impossible to identify a common pattern of chemical modifications leading to increased biological activity. There is no correlation between biological activity and receptor-binding properties of the analogs (Table II). Some analogs may though increase VDR stability or cause subtle changes in the conformation of this receptor, as demonstrated recently for KH 1060 (73, 116). Recently, cyclin-dependent kinase inhibitors, such as $p21^{Cip1/WAF1}$ or $p27^{Kip1}$, have been implicated in mediating of the antiproliferative activity of VD (72, 84, 122). Cyclin-dependent kinase inhibitors suppress cell cycle by binding in quaternary complexes with cyclin-dependent kinases, their associated cyclins, and the proliferating cell nuclear antigen (reviewed in refs. 42, 101) and may directly inhibit DNA replication by blocking the ability of the proliferating cell nuclear antigen to activate DNA polymerase δ (119). Treatment with 1,25-$(OH)_2D_3$ induces p21 expression via a DR3-type VDRE (DRs spaced by 3 base pairs) and causes accumulation of the p21 protein leading to cell cycle arrest and differentiation of examined myeloid leukemia cell lines (72). However, recently obtained data indicate that the IP9-type VDRE, rather than DR3, is mainly involved in growth regulation (86, 87). It is therefore unresolved whether the antiproliferative potency correlates with the ability to activate a specific type of VDRE.

1,25-$(OH)_2D_3$, in addition to the VDR-mediated genomic signaling, may activate certain transcription-independent signaling pathways similar to those used by peptide hormones and cytokines. The latter phenomena termed "non-genomic", comprise stimulation of calcium fluxes, activation of protein kinase C (PKC), accumulation of cyclic nucleotides cAMP and cGMP, or sphingomyelin hydrolysis (reviewed in refs. 89, 91, 108). These non-genomic events have been particularly well investigated in a model known as transcaltachia, where

Table II. *20 – epi vitamin D analogues: receptor binding, inhibition of proliferation and stimulation of cell differentiation*

Compound	Receptor binding	EC_{50} (*M*)		
		Inhibition of U 937 cell proliferation[a]	Inhibition of keratinocyte proliferation[b]	Stimulation keratinocyte differentiation[b]
1,25-$(OH)_2$ D_3	$1.6 \cdot 10^{-11}$	$1.4 \cdot 10^{-8}$	$14.3 \cdot 10^{-9}$	$18.0 \cdot 10^{-8}$
MC 1288	$1.3 \cdot 10^{-11}$	$2.8 \cdot 10^{-10}$	$2.03 \cdot 10^{-11}$	$3.05 \cdot 10^{-10}$
MC 1301	$1.6 \cdot 10^{-11}$	$8.2 \cdot 10^{-11}$	$4.71 \cdot 10^{-12}$	$3.72 \cdot 10^{-11}$
KH 1060	$1.3 \cdot 10^{-11}$	$1.0 \cdot 10^{-12}$	$2.0 \cdot 10^{-12}$	$5.63 \cdot 10^{-12}$

Mean values are shown.
[a] Data from Binderup et al. (ref. 13).
[b] Data from publication **V**.

$1,25\text{-}(OH)_2D_3$ induces a transcription-independent calcium flux in the intestine (88, 90). Non-genomic events have also been demonstrated in other cell types, but their functional importance and molecular mechanisms remain obscure.

The aim of the study

To date, the investigations on the growth-regulatory properties of VD have been focused on their antiproliferative activity. However, my own preliminary investigations revealed that in certain situations treatment with $1,25\text{-}(OH)_2D_3$ and vitamin D analogs may lead to an *increase* rather than suppression of cell proliferation (41). In a hairless mouse model, topical application of $1,25\text{-}(OH)_2D_3$ or KH 1060 caused a dose-dependent epidermal hyperplasia. This was a new finding suggesting that the pattern of cell growth regulation by $1,25\text{-}(OH)_2D_3$ is very complex and cannot be reduced to a simple inhibition of growth as postulated earlier. It has therefore been my intention to further investigate the effects of $1,25\text{-}(OH)_2D_3$ and different vitamin D analogs on murine epidermis and to develop appropriate *in vitro* models to examine the factors modulating keratinocyte response to VD. The goal of this study was also to deepen the understanding of the mechanisms of regulation of keratinocyte growth and differentiation by VD with reference to novel signaling pathways not directly linked to the transcription factor-like activity of VDR. Sections 2 – 8 will review the results of those investigations. In sections 2 and 3 the results dealing with the effects of VD on murine epidermis will be presented. Sections 4 – 6 will describe the pattern of keratinocyte growth regulation by VD in two different culture models. Part 7 and 8 deal with the description of signaling pathways activated by VD in cultured keratinocytes. Finally, the relevance of the findings for the understanding of the effects of VD in dermatological pharmacology will be outlined in section 9.

2. INFLUENCE OF VD ON THE EPIDERMIS: EPIDERMAL HYPERPLASIA AFTER TOPICAL APPLICATION OF $1,25\text{-}(OH)_2D_3$ AND THE 20-*EPI* VITAMIN D ANALOGS IN HAIRLESS MICE

The effects of VD *in vivo* were described in detail in papers **I** and **II**. In these studies hairless mice were topically treated with solutions containing different concentrations of $1,25\text{-}(OH)_2D_3$ and selected 20-*epi* analogs of different potencies **(I)**. Seventy-two hours after a single treatment with $1,25\text{-}(OH)_2D_3$ or the analogs MC 1288, MC 1301, and KH 1060, an epidermal thickening accompanied by an increased number of keratinocyte layers was present (Fig. 2). The observed epidermal hyperplasia was most likely caused by increased epidermal mitotic activity, since epidermal DNA synthesis measured by [^{3}H]thymidine incorporation was transiently increased 24 – 48 h after application of the compounds **(I)**. Analysis of the dose-response curves revealed that all 20-*epi* analogs were more potent and more active in inducing epidermal proliferation than $1,25\text{-}(OH)_2D_3$. The order of activity was KH 1060 > MC 1301 > MC 1288 > $1,25\text{-}(OH)_2D_3$, thus being in a striking correlation with their known ability to inhibit the proliferation of U 937 cells *in vitro* described by Binderup et al. (13) and shown in Table II. The ability of $1,25\text{-}(OH)_2D_3$ and KH 1060 to induce epidermal hyperplasia could also be observed during the course of a prolonged, repeated topical treatment for 10 – 28 days **(II)**. In

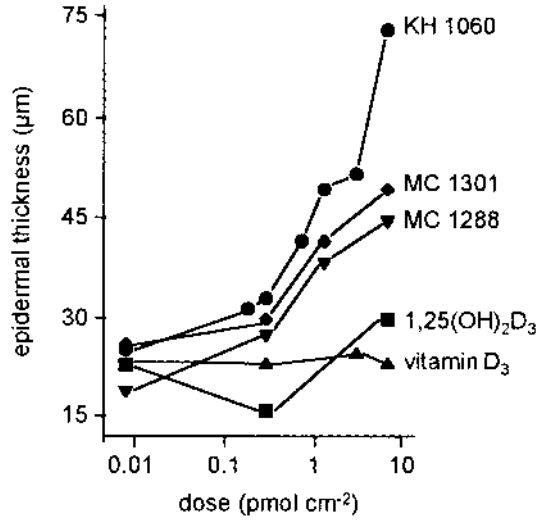

Fig. 2. Stimulation of epidermal hyperplasia in mice by $1,25\text{-}(OH)_2D_3$ and vitamin D analogs: the dose-dependent increase in epidermal thickness 72 h after topical application of $1,25\text{-}(OH)_2D_3$, MC 1288, MC 1301, KH 1060. Vitamin D_3 or $1\beta,25\text{-}(OH)_2D_3$ (not shown) which are receptor-inactive, did not trigger epidermal hyperplasia. Modified from publication **I**.

accordance with the short-term studies, KH 1060 was more potent and active than $1,25\text{-}(OH)_2D_3$.

The observation of epidermal hyperplasia after topical application of $1,25\text{-}(OH)_2D_3$ and 20-*epi* analogs was independently made by Lützow-Holm et al. (75) who demonstrated that $1,25\text{-}(OH)_2D_3$ and KH 1060 increased epidermal mitotic rate. In an attempt to explain their findings, these authors raised a hypothesis that epidermal effects of $1,25\text{-}(OH)_2D_3$ and 20-*epi* analogs were caused by a non-specific skin irritation. I approached this problem in papers **I** and **II** by treating the skin with $1\beta,25\text{-}(OH)_2D_3$ or vitamin D_3, compounds of a very similar structure to the active 1α-hydroxylated hormone but unable to bind to VDR. Neither $1\beta,25\text{-}(OH)_2D_3$ nor vitamin D_3 affected epidermis when applied for periods 3 – 28 days, even if used at concentrations exceeding those of KH 1060 by a factor of 100. This makes non-specific irritation very unlikely and suggested that epidermal hyperplasia was dependent on the activation of VDR. Possible involvement of irritation was also argued against by assessment of the transepidermal water loss in KH 1060-treated mice: When compared to the vehicle-treated mice, KH 1060 did not cause any increase in this parameter (2.3 ± 0.8 $gm^{-2}\cdot h$ versus control value of 2.4 ± 0.9 $gm^{-2}\cdot h$, means with SD) (43).

The alternative possibility that non-genomic signaling mechanisms mediated the observed hyperplastic effect of VD was also considered. In an established model of the non-genomic process, transcaltachia, the response to VD may be blocked by the analog $1\beta,25\text{-}(OH)_2D_3$ and stimulated by the analog Ro 24-2090 (16-ene-23-yne-25-hydroxy-vitamin D_3) which does not bind to VDR either (91, 92). To probe for the potential involvement of the non-genomic signaling, mice were treated with $1\beta,25\text{-}(OH)_2D_3$ and KH 1060 at molar ratio of 10 : 1 or 100 : 1, or with a concentration range of Ro 24-2090. In the first case, no modulation of the KH 1060 response was observed and in the second case no effect was seen. Therefore, VDR-binding activity seems to be required for VD to stimulate epidermal hyperplasia in mice.

3. INVOLVEMENT OF PROTEIN KINASE C IN KH 1060-INDUCED EPIDERMAL HYPERPLASIA

The morphological features of 1,25-$(OH)_2D_3$-induced hyperplastic response of the epidermis resemble those seen during epidermal regeneration or following the topical application of retinoic acid or phorbol esters. The resemblance to the phorbol ester-induced hyperplasia was especially striking: Both 1,25-$(OH)_2D_3$ (or KH 1060) **(I, II)** and phorbol-12-myristate-13-acetate (47) cause epidermal hyperplasia with enlargement and elongation of basal cells, papillomatosis with elongation of rete-ridges, granulomatosis, and hyperkeratosis. Since phorbol esters are potent activators of PKC, I hypothesized that PKC activation was involved in the mechanism of action of 1,25-$(OH)_2D_3$ and vitamin D analogs *in vivo*. This reasoning was supported by available evidence that in some types of cells, including keratinocytes, 1,25-$(OH)_2D_3$ is able to activate PKC (10, 29, 81, 117, 120, 125).

The role of PKC as a mediator of KH 1060-induced epidermal hyperplasia was investigated in study **III**. This paper documented that KH 1060 induced a translocation of PKC activity from the cytoplasmic to the membrane fraction of homogenized epidermis as soon as 15 min after the topical application of the analog. This was regarded as an evidence that KH 1060 causes PKC activation in murine epidermis *in vivo*. Moreover, when the skin was topically treated with KH 1060 and the PKC blocker, sphingosine, a reduction in the hyperplastic response to this vitamin D analog was observed. Together, these results strongly indicate that KH 1060 induce epidermal hyperplasia in mice *via* PKC.

In response to PKC activation, an upregulation of c-*fos* gene transcription takes place (16, 40, 110). Using the polymerase chain reaction to amplify c-*fos*-specific mRNA it was possible to demonstrate the up-regulation in the epidermal expression of this gene after treatment with KH 1060 **(III)**. Expression of c-*fos* was rapid, transient, and was most clearly observed 2 h after KH 1060 treatment. The fact that the transient c-*fos* expression was abrogated by sphingosine suggested dependence on PKC activation.

The conclusion from studies **I** and **II** was that topically applied 1,25-$(OH)_2D_3$ and 20-*epi* vitamin D analogs stimulated keratinocyte proliferation to the degree proportional to their *antiproliferative* potencies *in vitro*. The data showing activation of PKC and its participation in the development of epidermal hyperplasia suggested that VD may act indirectly via secondary mediators such as protein kinases. The mechanistic basis of PKC activation could not be determined, but the rapid dynamics of this phenomenon suggested that a non-genomic pathway of activation was involved. This putative non-genomic pathway was probably different from the previously described because it was insensitive to the modulation by a known inhibitor (1β,25-$(OH)_2D_3$) and activator (Ro 24-2090). For a better understanding of the nature of growth modulation by VD several issues needed further elucidation: *i)* The data supporting the involvement of PKC were based on the blocking experiment with sphingosine. However, this compound is not a pure PKC antagonist, but is also able to suppress activity of other protein kinases. Therefore, use of more selective PKC blockers would be desirable, an approach which has not been feasible using the *in vivo* model; *ii)* Demonstration of a PKC translocation to membranes was only an indirect proof of PKC activation. A measurement of intracellular PKC activity towards a specific substrate was needed to confirm the findings, but this methodology could not be applied in the *in vivo* model; *iii)* Epidermis is a tissue containing several cell types and thus heterogeneous intercellular interactions, either via direct cell-cell contacts or secreted cytokines, could dramatically influence the proliferation ratio of keratinocytes.

Therefore, to further investigate the mechanistic basis of VD-dependent regulation of keratinocyte growth it became necessary to develop relevant *in vitro* models.

4. DEVELOPMENT OF A CELL CULTURE MODEL FOR STUDYING THE EFFECTS OF VD ON KERATINOCYTE GROWTH

Keratinocytes can be cultured in the MCDB 153 basal nutrient medium supplemented with epidermal growth factor (EGF), ethanolamine, phosphoethanolamine, hydrocortisone, insulin, and bovine pituitary extract as a source of growth factors, mainly the fibroblast growth factor (21).

For the purpose of these investigations, EGF was completely removed from the culture medium or its concentration was reduced from standard 50 – 100 ng/ml to 0.1 ng/ml. As documented in study **IV**, subconfluent keratinocytes still proliferated in such modified media although at a rate lower than that in the medium containing standard EGF concentration. Intermediate rate of cell growth allowed investigation of both growth-stimulatory and growth-inhibitory effects of 1,25-$(OH)_2D_3$.

A biphasic, concentration-dependent effect of 1,25-$(OH)_2D_3$

Results of study **IV** showed that the effects of 1,25-$(OH)_2D_3$ on keratinocyte proliferation depended on two factors: the concentration of 1,25-$(OH)_2D_3$ and the concentration of the extracellular calcium. At high (1.8 mM) calcium concentration, a biphasic, concentration-dependent response to 1,25-$(OH)_2D_3$ was observed (Fig. 3). At low concentrations ($10^{-11} – 10^{-9}$ M) 1,25-$(OH)_2D_3$ stimulated cell proliferation by increasing the proportion of cells entering S phase whereas a proliferation block was observed at the concentrations $\geq 10^{-8}$ M. In the low-calcium conditions (0.15 mM) the stimulatory effect of 1,25-$(OH)_2D_3$ was not apparent and a cell cycle block in the late G_1 phase was observed at hormone concentrations $10^{-11} – 10^{-6}$ M (Fig. 3).

The biphasic response of keratinocytes to 1,25-$(OH)_2D_3$ seen in study **IV** resembles growth responses to VD in other cell types. In hepatic Ito cells and quiescent vascular smooth muscle cells, 1,25-$(OH)_2D_3$ has been shown to stimulate cell growth in the concentration range $10^{-11} – 10^{-9}$ M and $10^{-11} – 10^{-8}$ M, respectively (71, 79), values comparable with those obtained in this study. Moreover, the main results of study **IV** were independently confirmed by other investigators who using different culture conditions also observed a biphasic, concentration-dependent response of murine and human keratinocytes to VD (17, 111).

Role of cell differentiation

It is well documented that in the presence of elevated calcium concentrations cell stratification and differentiation takes place, keratinocyte shape changes and cell-cell contacts are being formed (49). It was thus conceivable that cell differ-

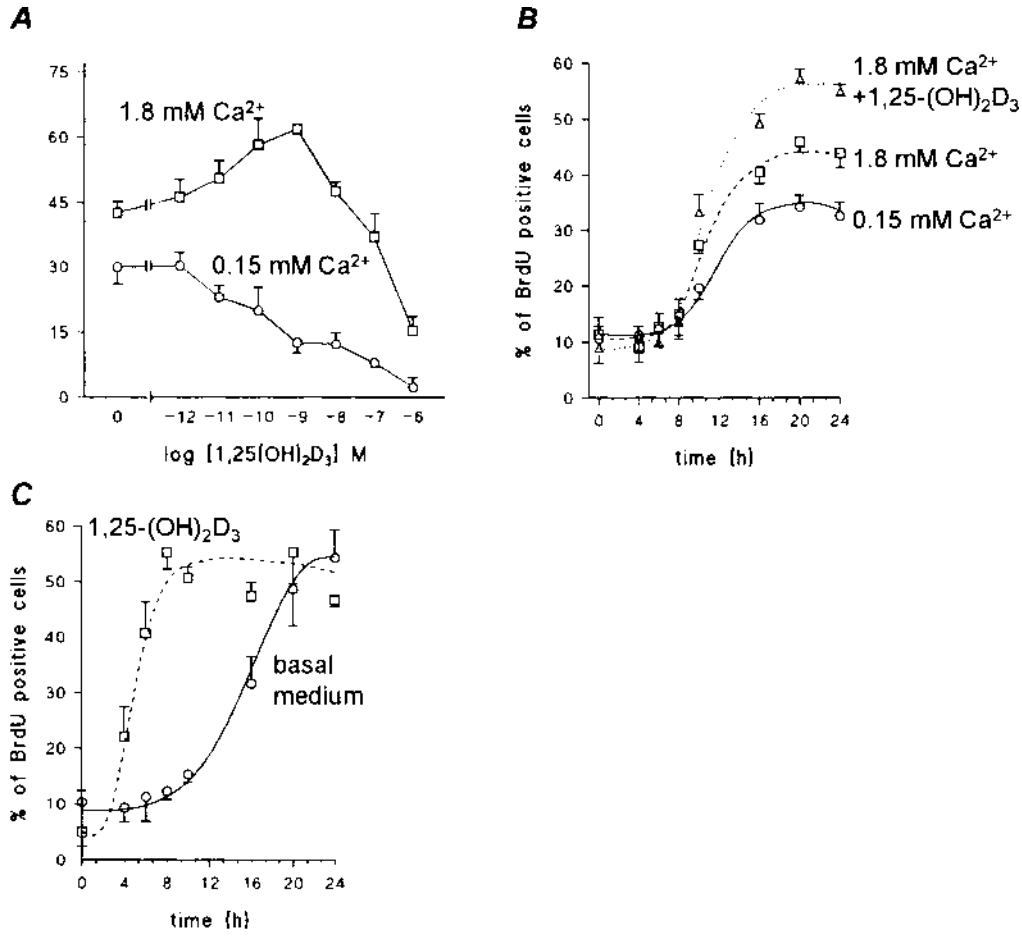

Fig. 3. Effects of 1,25-$(OH)_2D_3$ on keratinocyte cell proliferation *in vitro*. *A* – Subconfluent keratinocyte cultures were incubated with different concentrations of 1,25-$(OH)_2D_3$ in the medium containing suboptimal concentration of EGF (0.1 ng/ml) and 0.15 mM calcium (circles) or 1.8 mM calcium (squares). A stimulation of keratinocyte proliferation was seen (assessed by BrdU labeling index determination) when the cells were cultured at low concentrations of 1,25-$(OH)_2D_3$ in media containing 1.8 mM calcium. High concentrations of 1,25-$(OH)_2D_3$ caused inhibition of cell proliferation at any calcium concentration. *B-C* – Cell-cycle analysis of 1,25-$(OH)_2D_3$-treated cells. In *B*, keratinocytes which were partially synchronized in G_0/G_1 by a preincubation in the medium without any growth factors (keratinocyte basal medium) were stimulated with EGF-deficient medium with 0.15 mM calcium (circles), 1.8 mM calcium (squares) or 1.8 mM calcium with 10^{-9} M 1,25-$(OH)_2D_3$ (triangles). Significantly higher proportion of cells entering the S phase was seen in the 1,25-$(OH)_2D_3$-treated group. In *C*, the cells were growth-arrested by incubating with 10^{-9} M 1,25-$(OH)_2D_3$ (squares) or the growth factor-depleted medium (circles). After restimulation with the full growth medium, the cells treated with 1,25-$(OH)_2D_3$ demonstrated a shorter G_1 phase suggesting that 1,25-$(OH)_2D_3$ arrested the cells in the late G_1. Shown means with SD. Modified from publication **IV** .

entiation status and/or cell-cell interactions, rather than calcium concentration regulated the proliferative response to 1,25-$(OH)_2D_3$. The experiments attempting to distinguish between these possibilities were described in study **IV**. Cells were pre-treated with EGF deficient medium with 1.8 mM calcium for different periods of time (15 min – 24 h) and then restimulated with the same medium containing 0.15 mM calcium and 10^{-9} M 1,25-$(OH)_2D_3$ for 24 h. Stimulation of growth was observed when 1,25-$(OH)_2D_3$ was applied on cells pretreated for 5, 8 and 24 h with 1.8 mM calcium, but not on those pretreated for 15 min or 1 h. This clearly indicated that the presence of calcium during exposure to 1,25-$(OH)_2D_3$ was not absolutely required for the proliferative response to occur and suggested that the keratinocytes differentiating in response to calcium ions were induced to proliferate by 1,25-$(OH)_2D_3$. This was supported by further experiments showing that induction of partial differentiation by suspension in the semi-solid medium had the same effect as calcium pretreatment and that suppression of calcium-induced differentiation by antibodies against E- and P-cadherin abrogated the hyperproliferative response to 1,25-$(OH)_2D_3$ (**IV**). It was therefore concluded that the direction of the proliferative response to 1,25-$(OH)_2$ D_3 in keratinocytes depended on the degree of cell differentiation.

The process of keratinocyte differentiation is complex and temporal separation of proliferation and differentiation seems not to be obligatory. Actively proliferating cells have been found among differentiated involucrin expressing keratinocytes and mitoses may take place in the presence of intact intercellular junctions (113). Recent studies revealed that two types of proliferating cells exist in the epidermis and cultured keratinocytes: stem cells with high proliferative potential and transit amplifying cells (TAC) (57, 58). The latter population, despite being committed to terminal differentiation, retains the proliferative capacity. During the process of differentiation, daughters of stem cells move continuously through the transit

amplifying cell stage to the committed, terminally differentiated cells. It could thus be speculated that the proliferative response to 1,25-$(OH)_2D_3$ differs between different subpopulations of keratinocytes. 1,25-$(OH)_2D_3$ would block proliferation of actively growing, undifferentiated stem cells, whereas more differentiated transit amplifying cells would react by increasing their mitotic activity. To address this possibility, the influence of 1,25-$(OH)_2D_3$ on clonal keratinocyte growth was investigated.

5. EFFECTS OF VD ON CLONAL KERATINOCYTE GROWTH

Keratinocytes belonging to different subpopulations may be distinguished either by their phenotypic markers or functionally, on the basis of their progeny in the clonogenic assay (4, 57, 58, 98). The most commonly used phenotypic markers are β1 integrin and keratin K1K10. Stem cells express β1 integrin but not K1K10, TAC express both β1 integrin and K1K10, and terminal, mitotically inactive keratinocytes are β1 integrin negative but express K1K10. The high level of expression of β1 integrin allows for stem cell purification based on their high affinity to matrix proteins, e.g. collagen type IV. In the clonogenic assays, stem cells give rise to large colonies (so-called holoclones) whereas TAC form colonies of smaller size (meroclones). Terminally differentiated cells give rise to abortive colonies (paraclones).

In study **V**, I examined the effect of VD on formation of keratinocyte colonies of different sizes. It has already been reported that 1,25-$(OH)_2D_3$ inhibits clonal growth of human keratinocytes (54). I confirmed these results 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 (Fig. 4). 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-$(OH)_2D_3$.

A new finding in study **V** was that a decrease in the number of holoclones was accompanied by an increase in proportion of smaller colonies: the meroclones and the paraclones (Fig. 4). Moreover, the effect of 1,25-$(OH)_2D_3$ depended on the timing of the addition to culture media and duration of the exposure to the hormone. 1,25-$(OH)_2D_3$ must have been present for at least 4–6 days to exert its inhibitory activity. Growth inhibition was seen only when the hormone was added in the beginning of cell culture whereas addition of 1,25-$(OH)_2D_3$ for the same amount of time at the end of culturing had only a marginal effect on colony size. This implied that the clonogenic cells rather than the more differentiated cell types were targeted.

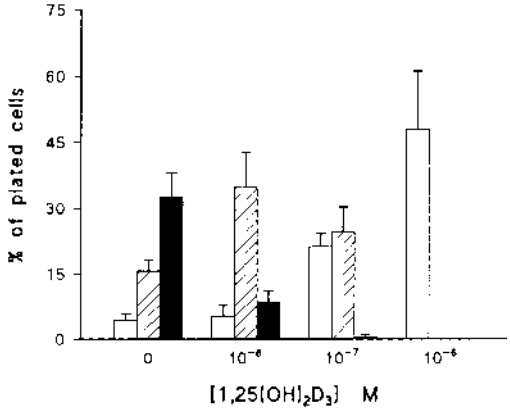

Fig. 4. Effects of 1,25-$(OH)_2D_3$ on clonal keratinocyte growth. The cells were plated and cultured for 20 days in the media containing $10^{-8}-10^{-6}$ M 1,25-$(OH)_2D_3$, the solvent used for 1,25-$(OH)_2D_3$ (isopropanol) was added in the control experiment (0 M). The number of holoclones (solid columns), meroclones (hatched columns), and paraclones (open columns) expressed as a proportion of plated cells was determined for different 1,25-$(OH)_2D_3$ concentrations. Modified from publication **V**.

The long time required for 1,25-$(OH)_2D_3$ to exert its effects contrasted with the dynamics of growth inhibition seen in the subconfluent keratinocyte cultures **(IV)**. It was therefore conceivable that the mechanisms of growth inhibition differed in those two culture assays. Indeed, rather than a reversible G_1 cell cycle block seen in the subconfluent cultures 24 h after 1,25-$(OH)_2D_3$ treatment, clonogenic cells required a minimum of 4-day-treatment with 1,25-$(OH)_2D_3$ for the growth inhibition to occur. Because 1,25-$(OH)_2D_3$ caused an increase in the proportion of meroclones and paraclones at the expense of holoclones (Fig. 4), it was conceivable that 1,25-$(OH)_2D_3$ manifested its inhibitory effects via induction of differentiation of the progenitor pool rather than a direct interference with the cell cycle. This was supported by the results showing that treatment with 1,25-$(OH)_2D_3$ caused a permanent suppression of clonal growth. Moreover, 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-$(OH)_2D_3$. Further increase in 1,25-$(OH)_2D_3$ concentration caused mainly a formation of involucrin-positive K1K10-negative cells, which indicated an accelerated and direct transition of proliferative cells (involucrin-negative) into terminally differentiated cells (involucrin-positive) bypassing the TAC-like, K1K10-positive intermediate.

The effects of 20-*epi* analogs on clonal growth was also tested. All tested compounds (MC 1288, MC 1301, and KH 1060) were significantly more potent 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 keratinocytes (a fraction enriched in stem cells) were treated with different concentrations of the analogs for 4 days and the proportion of differentiated involucrin-positive cells was determined. As illustrated in Table II, all tested analogs stimulated cell differentiation at 10^2-10^3 lower concentrations than 1,25-$(OH)_2D_3$ and the potency for inhibition of clonal growth correlated with the ability of the compounds to stimulate progenitor cell differentiation. These observations further supported the conclusion that the stimulation of progenitor cell differentiation was mainly responsible for 1,25-$(OH)_2D_3$-induced clonal growth arrest.

6. MODULATION OF THE 1,25-$(OH)_2D_3$-DEPENDENT CLONAL GROWTH ARREST BY LYMPHOKINES AND CYTOKINES

The reviewed results of study **V** indicated that the progenitor, stem cell-like cells, were the main target for 1,25-$(OH)_2D_3$ during clonal growth. Stem cells play a central pathogenic role in psoriasis. In normal epidermis the majority of stem cells are quiescent, but in psoriasis they shift into cell cycle (7, 8). 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 (EGF, transforming growth factor α, TGFα, insulin-like growth factor I, IGF-I), or γ-interferon (reviewed in refs. 42, 44). Because of supraphysiological concentrations of EGF and other growth factors in culture media, clonal keratinocyte growth resembles the pathological type of proliferation encountered in psoriasis rather than physiological growth pattern in the epidermis, providing a valid model for investigations in psoriasis (55). In view of the previously demonstrated modulatory effects of EGF on 1,25-$(OH)_2D_3$-dependent growth regulation **(IV)** and the use of VD in the treatment of psoriasis, it was of interest to determine the modulatory role of other cytokines and lymphokines utilizing the clonogenic assay.

Investigations pertaining to this question were described in study **V**. Lowering EGF concentration from standard 50 ng/ml to the suboptimal 0.2 ng/ml resulted in the formation of smaller keratinocyte colonies. However, 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 EGF. The EGF activity could be mimicked by TGFα which is understandable in view of the fact that those cytokines share a common membrane receptor. When EGF in the media was replaced by another lymphokine or cytokine (IL-1α, IL-1β, IL-6, IL-8, IGF-I, KGF), clonal growth comparable to that in the presence of EGF or TGFα was observed. 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 III). Treatment with IGF-I had a similar effect, although statistical significance was not attained. The 1,25-$(OH)_2D_3$-dependent expression of involucrin was diminished in the presence of KGF and IGF-I indicating that these cytokines blocked stem cell differentation in response to 1,25-$(OH)_2D_3$. These results underscore the important role of cytokines in modulating the 1,25-$(OH)_2D_3$ activity and show that certain cytokines may attenuate the effects of VD. The biological effects of VD should therefore be analyzed in the context of other biological response modifiers (cytokines, lymphokines) present in the culture medium.

Table III. *Effects of cytokines on clonal keratinocyte growth*

Cytokine	Number of cells at 1,25-$(OH)_2D_3$ concentration (*M*):		
	0 (control)	10^{-7}	10^{-6}
EGF	616 ± 125	33 ± 9	1.5 ± 0.9
KGF[a]	620 ± 126	453 ± 50	117 ± 74
IGF-I	667 ± 202	121 ± 40	10 ± 4.9
EGF + KGF[b]	792 ± 64	180 ± 53	40 ± 8
EGF + IGF-I	859 ± 85	61 ± 30	7.9 ± 2

EGF (50 ng/ml), KGF or IGF-I (both at 10 ng/ml) were added to the EGF-deficient culture media. 200 cells were plated and cultured for 20 days. At termination the total number of cells was counted. Mean values with SD (data from publication **V**).

[a] no differences between the groups treated with different cytokines.

[b] significant ($p < 0.05$) attenuation of the effects of 1,25-$(OH)_2$ D_3 compared to the EGF group.

7. INVOLVEMENT OF PKC IN 1,25-$(OH)_2D_3$-DEPENDENT FORMATION OF ADHERENS JUNCTIONS

The experiments *in vivo* described in publication **III** suggested that PKC activation may be responsible for certain effects of VD on keratinocytes, most notably for the stimulation of keratinocyte proliferation and keratinocyte hyperplasia. It is however difficult to model the effects of PKC *in vitro*. In contrast to the clear-cut hyperproliferative effects of phorbol esters after topical application, treatment of cultured cells with those compounds leads to a rapid cell-cycle block, induction of keratinocyte terminal differentiation with formation of cornified envelopes, and formation of cell-cell adherens junctions (69, 95, 126, 127). Treatment with phorbol esters causes a short-term up-regulation of PKC activity followed by a prolonged period of enzyme down-regulation associated with the shedding of the differentiated keratinocytes and emergence of hyperproliferating cell population. Therefore, to investigate the possible involvement of PKC in 1,25-$(OH)_2D_3$ signaling *in vitro*, I chose to study the effects of this hormone on keratinocyte differentiation rather than on cell growth.

One of the aspects of epidermal cell differentiation is formation of cell-cell junctions which enable intercellular communication and are essential for regulation of epithelial morphogenesis, growth and differentiation (61). During epidermal differentiation caused e.g. by elevating of calcium concentration in culture medium, two types of junctional structures are induced: the classic desmosomes and the adherens junctions (AJ) (3, 46). Ultrastructurally, desmosomes consist of two submembranous plaques separated by an electron-lucent 20 – 30 nm wide desmoglea with (a) distinct electron-dense midline(s) (52). The assembly of the desmosome is mediated by a homophilic interaction between the transmembrane proteins of cadherin superfamily, desmoglein and desmocolin, which cytoplasmic tails bind to desmosome plaque proteins, placoglobin and desmoplakin. AJ are ultrastructurally similar to the desmosome but are biochemically and functionally different from the latter. AJ are stabilized due to the homophilic binding between N-terminal domains of the "classic" cadherins, E- and P-cadherin. The cytoplasmic tails of the cadherins interact with the proteins of the catenin family, α-, β-, and γ-catenin, and with a number of other accessory proteins, e.g. placoglobin or vinculin. Catenin α is required for cadherin-mediated cell adhesion and has an actin-binding activity (97). Thus, AJ are associated with actin cytoskeleton rather than with the keratin intermediate filaments like the desmosomes. AJ have been implicated in the regulation of morphogenesis, tissue remodeling, cell migration and stratification, cell spreading, epithelial compactness, and apoptosis (46, 50, 68, 70, 123). Thus, rather than mainly strengthen the epidermis, AJ are dynamic structures capable of signal transduction (45, 61, 93).

Induction of adherens junction formation by 1,25-(OH)$_2$D$_3$

The ability of 1,25-$(OH)_2D_3$ to promote the assembly of intercellular junctions was investigated in study **VI**. Treatment of confluent keratinocyte cultures with 10^{-7} M 1,25-$(OH)_2D_3$ for 4 days resulted in cell stratification. Stratification was focal,

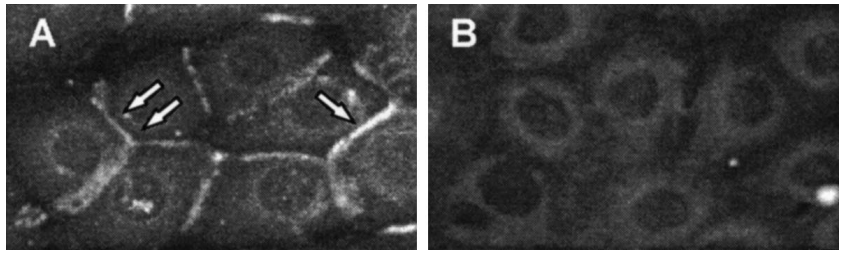

Fig. 5. Assembly of adherens junctions in keratinocytes treated with 1,25-$(OH)_2D_3$. Keratinocyte monolayers were treated with 10^{-7} M 1,25-$(OH)_2$ D_3 for 4 days (*A*) or the vehicle (*B*) for the same period of time and stained with antibodies against E-cadherin. 1,25-$(OH)_2D_3$-treated cells show clear junctional E-cadherin staining (arrows).

with emergence of two cell layers: the upper one containing involucrin-positive cells, and the lower one containing undifferentiated involucrin-negative cells. Stratification was accompanied by formation of the junctional structures consisting of two thin subplasmalemmal discs separated by a slit of a moderate electron density. The absence of a electron dense midline and no visible connection between the junctions and intermediate cytoskeletal elements indicated that observed structures were AJ. This was further confirmed by immunofluorescent staining for the AJ components. Treatment with 1,25-$(OH)_2D_3$ induced translocation of these proteins (E-cadherin, P-cadherin, α-catenin and vinculin) to the cell membrane (Fig. 5). In contrast, staining for desmosome components, desmoplakin and desmoglein did not reveal the junctional pattern of staining. This indicated that AJ, but not desmosomes, were induced by the treatment with 1,25-$(OH)_2D_3$. AJ were considered functional because blocking of the vital AJ component with the anti-E-cadherin antibody impaired the 1,25-$(OH)_2D_3$-induced keratinocyte stratification. 1,25-$(OH)_2D_3$-induced AJ were stable only in the constant presence of the hormone and disappeared 24 h after removal of 1,25-$(OH)_2D_3$ from the culture medium.

Involvement of PKC in 1,25-$(OH)_2D_3$-mediated adherens junction formation

1,25-$(OH)_2D_3$ stimulated the intracellular activity of PKC in keratinocyte monolayers, which was assayed *in situ* using a PKC-specific peptide substrate **(VI)**. The increase in PKC activity was rapid and resembled that seen after a topical application of KH 1060 in mice **(III)** in achieving the peak activity 15 min after 1,25-$(OH)_2D_3$ treatment (Fig. 6). Furthermore, 1,25-$(OH)_2D_3$ caused translocation of PKC isosymes from the cytoplasm to the membranes. To determine whether PKC activation was involved in the formation of AJ, the effects of selective inhibitors, H-7 [1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine] and BIM (bisindolylmaleimide) on the formation of AJ was assessed. A single treatment with PKC inhibitors for 1 day or repeated application for 4 days prevented

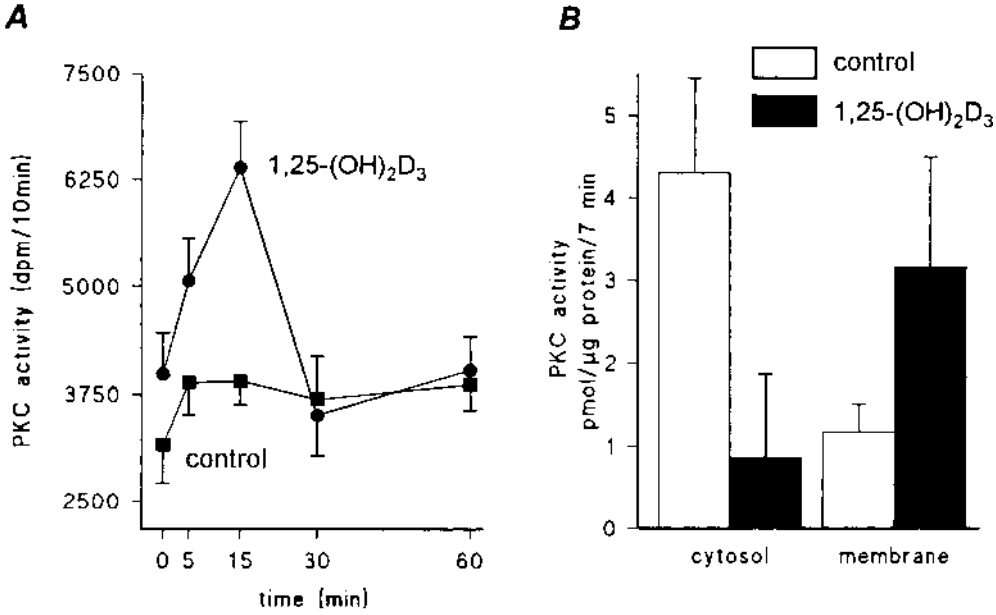

Fig. 6. Activation of PKC by 1,25-$(OH)_2D_3$ in vitro. *A* – Keratinocytes were treated with 10^{-7} M 1,25-$(OH)_2D_3$ or 0.1% isopropanol solvent (control) for indicated periods of time. The PKC activity was determined *in situ* by measuring phosphorylation of the added specific PKC substrate. *B* – Keratinocytes were incubated with 10^{-7} M 1,25-$(OH)_2D_3$ for 15 min (closed columns); control keratinocytes were incubated for the same period of time with 0.1 % isopropanol (open columns). PKC activity was determined in the cytoplasmic and membrane fractions. Note a shift of PKC activity from the cytosol to the membranes. Means with SD. Data from publication **VI**.

1,25-$(OH)_2D_3$-induced translocation of E-cadherin to cell-cell borders and formation of AJs. Together, these results indicated that PKC was activated by 1,25-$(OH)_2D_3$ and that PKC isosymes were involved in formation of AJ by this hormone. However, in view of the discussed effects of phorbol esters on cell proliferation it was unlikely that activation of PKC explained the observed stimulation of keratinocyte growth *in vitro* induced by low concentrations of 1,25-$(OH)_2D_3$. Therefore, studies were undertaken to further elucidate a possibility that other signaling pathways are involved in VD action.

8. INVOLVEMENT OF SRC AND MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY IN SIGNAL TRANSDUCTION BY 1,25-$(OH)_2D_3$

Activation of mitogen-activated protein kinase (MAPK) takes place upon autophosphorylation of receptor tyrosine kinase or an activation of non-receptor tyrosine kinases, such as Src. The receptor proteins form complexes with Sos (the product of mammalian homologue of the *Drosophila Son of sevenless* gene) which activates Ras by catalyzing GDP/GTP exchange. The GTP-bound Ras activates MAPK kinase kinase (identified as a product of the c-*raf* protooncogene, Raf), which stimulates sequentially MAPK kinase and MAPK isoforms by activatory phosphorylations. Activated MAPK is a potent regulator of cell growth able to activate a wide range of kinases and other mediators (15, 28).

The mode by which the receptor protein forms complexes with Sos has been partially elucidated recently. The tyrosine phosphorylated receptors bind directly to the adapter molecule Grb2 via its SH2 (Src-homology 2) binding domains and Grb2 binds to the C-terminal portion of Sos via the SH3 domains (31). However, in some cases the adapter molecules of Shc family (46, 52 and 66 kD) are involved. These proteins are tyrosine phosphorylated at position 317 within their central proline/glycine-rich domain by the activated receptor or non-receptor tyrosine kinases and dock the receptor proteins to Grb2 employing the SH2 domains and the phosphotyrosine, respectively (18, 31).

Activation of the enzymatic activity of Raf, MAPK and Src by 1,25-$(OH)_2D_3$

Keratinocyte growth and differentiation seem to depend on tyrosine phosphorylation events (36) and probably involve the activation of MAPK signaling cascade. Therefore, I asked whether activation of MAPK occurred in keratinocytes after application of 1,25-$(OH)_2D_3$. This issue was investigated in study **VII**. The cells were treated with the hormone and the enzymes were precipitated from cell lysates with specific antibodies. The kinase activities of MAPK and Raf in the immunoprecipitates were determined by measuring the radiolabeling of the specific substrates with [γ-^{32} P]ATP.

In the conditions where growth of keratinocytes was stimulated by 1,25-$(OH)_2D_3$, incubation with this hormone caused a rapid activation of MAPK (Fig. 7). The enzymatic activity of Raf was also rapidly stimulated by 1,25-$(OH)_2D_3$. The activation of MAPK and the growth-stimulatory effects of 1,25-$(OH)_2D_3$ depended on Raf because the down-regulation of Raf with the antisense oligonucleotides reduced the 1,25-$(OH)_2D_3$-dependent hyperproliferation and MAPK activity (Fig. 7). Importantly, activation of neither MAPK nor Raf could be blocked by the inhibitors of transcription and protein synthesis which indicated that those processes could represent a non-genomic pathway.

The activity of Src, a non-receptor tyrosine kinase regulating cell growth and differentiation, was also stimulated by 1,25-$(OH)_2D_3$. Similarly to the response pattern of Raf and MAPK, preincubation of the cells in media containing high calcium concentration was required for Src activation **(VIII)**. In an attempt to elucidate the mechanism of Src activation, the tyrosine phosphorylation status of the molecule was assessed by immunoprecipitation and Western blotting. It is known that the tyrosine kinase activity of Src is regulated by the phosphorylation state of the molecule. Commonly, activation of Src occurs via dephosphorylation of the tyrosine residue in position 527. In accordance with this mechanism, 1,25-$(OH)_2D_3$ caused tyrosine dephosphorylation of Src protein, a process that was time-correlated with the observed increase in Src activity **(VIII)**. However, 1,25-$(OH)_2D_3$-induced dephosphorylation of Src was also observed in the cells cultured under low calcium conditions when no activation of Src activity was present. Thus, tyrosine dephosphorylation alone is insufficient for Src activation by 1,25-$(OH)_2D_3$ in keratinocytes.

Formation of Shc-Grb2-Sos complexes

To examine whether formation of typical Shc-Grb-Sos complexes took place due to the treatment with 1,25-$(OH)_2D_3$, an immunoprecipitation analysis was performed. Phosphotyrosine-containing proteins were immunoprecipitated with a monoclonal antibody, separated by polyacrylamide gel electrophoresis and immunoblotted with an anti-Shc antibody. 1,25-$(OH)_2D_3$ (10^{-9} M) provoked tyrosine phosphorylation of $p66^{Shc}$, and to a lower extend $p52^{Shc}$. Phosphorylation $p66^{Shc}$ was detected in both calcium-pretreated and non-pretreated cells. Grb2 and Sos were found to associate with Shc selectively in the lysates from calcium-pretreated cells **(VII)**. These results suggested that upon 1,25-$(OH)_2D_3$ treatment the p66 isoform of Shc was tyrosine phosphorylated allowing for the formation of a complex Shc-Grb2-Sos.

Signal transmission from activated Src occurs via complex formation between Shc and Src (31). As described in publiation **VIII** and shown in Fig. 8, 15–30 min incubation of calcium pretreated keratinocytes with 10^{-8} M 1,25-$(OH)_2D_3$ induced complex formation between Src and Shc. Src-Shc complexes were also detected in the cells cultured with low calcium concentration 15 min after 1,25-$(OH)_2D_3$ treatment. However, these complexes were unstable, because they could not be detected 30 min after 1,25-$(OH)_2D_3$ treatment, and less prominent than in the cells exposed to high calcium concentration.

Association of Src with Shc was likely to lead to signal propagation since Grb2 signaling molecule was present in Src-Shc complexes (Fig. 8). However, it was unlikely that observed activation of Raf was caused by the series of events initiated by stimulation of Src, because the concentration required to activate Src were 10-fold higher than that needed for Raf-MAPK activation. Thus, if Shc-Grb2-Sos complexes played a role in Raf-MAPK activation, other kinases would have to be implicated in the activatory Shc phosphorylation.

It was impossible in this study to get a detailed mechanistic insight into the process of activation of Shc. However, some preliminary evidence was obtained indicating involvement of

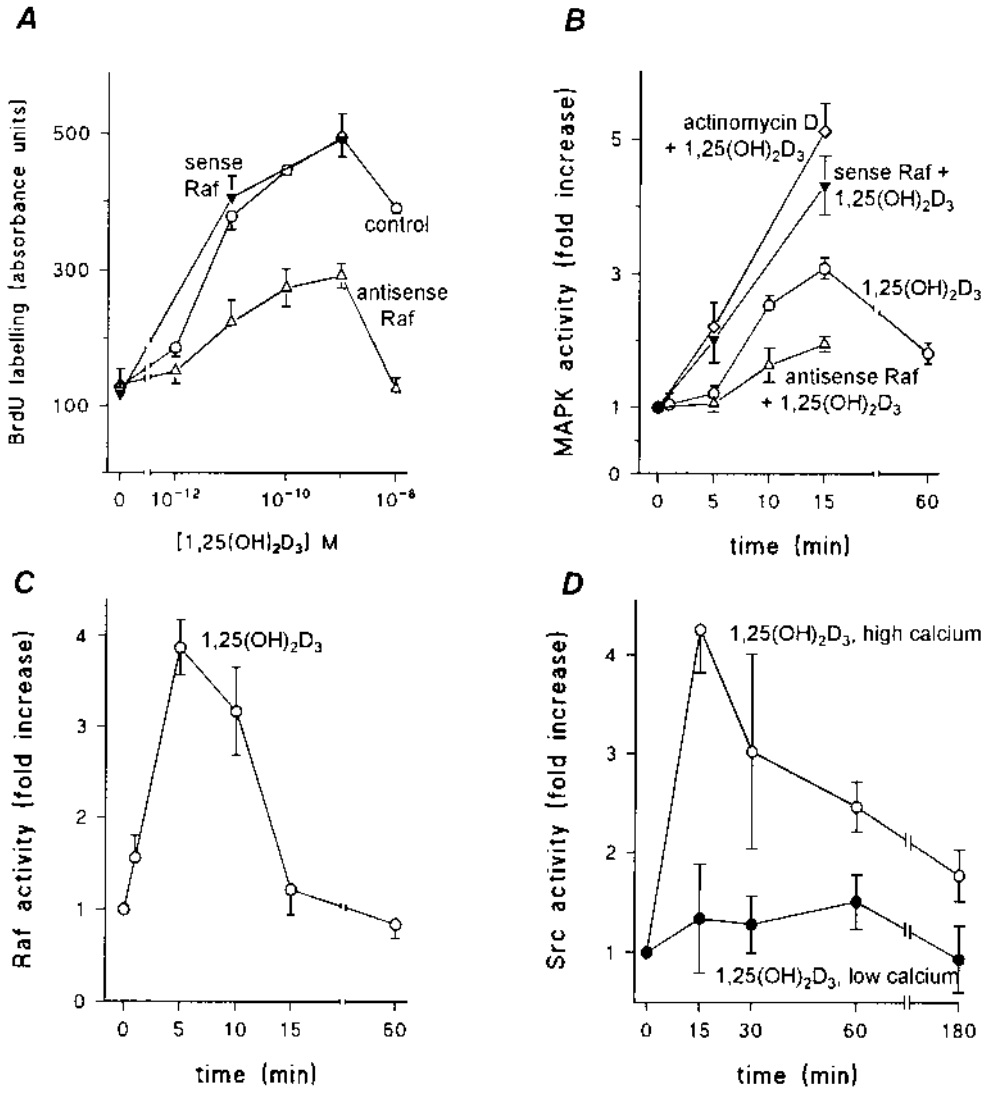

Fig. 7. Evidence for involvement of MAPK, Raf, and Src in signaling by 1,25-$(OH)_2D_3$. *A* – Keratinocytes were cultured in the media containing 1.8 mM calcium and treated with anti-sense Raf oligonucleotides (open triangles), sense oligonucleotides (closed inverted triangles) or were not treated with any oligonucleotides (circles). Anti-sense Raf oligomers caused a specific inhibition of 1,25-$(OH)_2D_3$-dependent growth stimulation. *B* – Keratinocytes cultured at 1.8 mM calcium were treated with 1,25-$(OH)_2D_3$, lysed, MAPK was immunoprecipitated with a monoclonal antibody, and MAPK activity was measured in the immunocomplexes. 1,25-$(OH)_2D_3$ (circles) stimulated MAPK activity. Antisense Raf oligomers (triangles) but not sense oligonucleotides (inverted solid triangles) inhibited this effects indicating that MAPK activation was dependent of Raf. Activation of MAPK was not blocked with actinomycin D (diamonds), an inhibitor of mRNA synthesis suggesting that the effects of 1,25-$(OH)_2D_3$ were transcription-independent. *C* – 1,25-$(OH)_2D_3$-dependent activation of Raf in keratinocytes cultured with 1.8 mM calcium and 10^{-9} M 1,25-$(OH)_2D_3$. *D* – activation of Src by 1,25-$(OH)_2D_3$ in the cells precultured in media containing high (1.8 mM) calcium concentration (open circles), but not in keratinocytes cultured with low calcium (0.15 mM)-closed circles. Shown means with SD. Modified from publications **VII** and **VIII**.

VDR in this process. In the 1,25-$(OH)_2D_3$-treated cells, VDR co-precipitated with Shc **(VII)**. This finding may suggest that VDR is a part of an active complex Shc-Grb2-Sos.

9. CONCLUSIONS AND PERSPECTIVES

The following main conclusions may be drawn on the basis of the presented results:

1. 1,25-$(OH)_2D_3$ and receptor-active vitamin D analogs may both stimulate and inhibit keratinocyte proliferation.
2. The direction and magnitude of the proliferative response of keratinocytes to 1,25-$(OH)_2D_3$ depend on the concentration of the hormone, degree of cell differentiation and availability of certain growth factors.
3. The MAPK cascade, Src activity, and PKC activity are stimulated by VD. Those signaling pathways are likely to mediate some of the effects of 1,25-$(OH)_2D_3$ on keratinocyte proliferation and differentiation. There is evidence that MAPK and PKC activations occur via non-genomic signaling.

In contrast to earlier views, 1,25-$(OH)_2D_3$ can no longer be considered as a mere inhibitor of cellular growth. Under favorable conditions (topical application, suboptimal concentrations of growth factors, conditions promoting keratinocyte

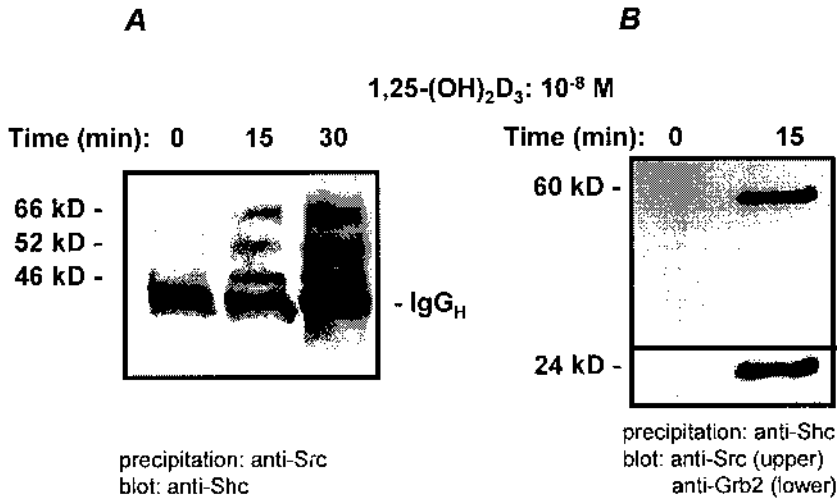

Fig. 8. Formation of complexes between Src, Shc, and Grb2. Cells were pretreated in media containing 1.8 mM calcium and stimulated for the indicated periods of time with 1,25-$(OH)_2D_3$. Lysates were immunoprecipitated and the blots were probed with appropriate antibodies, as indicated. Positions of three Shc species (46 kD, 52 kD, and 66 kD) are indicated in *A* , and positions of the Src band (60 kD) or the Grb2 band (24 kD) are marked in *B*. Modified from publication **VIII**.

differentiation) this hormone will stimulate, rather than inhibit keratinocyte growth. These results may be of importance for understanding the pharmacological effects of VD and the physiological effects of endogenously produced 1,25-$(OH)_2D_3$. The physiological levels of 1,25-$(OH)_2D_3$ in humans are in picomolar range (approx. 10 pM), which may suggest that endogenous 1,25-$(OH)_2D_3$ stimulates rather than inhibits cell growth. However, some cells (e.g. keratinocytes) are able to synthesize 1,25-$(OH)_2D_3$ from the precursor 25-$(OH)D_3$ which is available at 1000 × higher concentrations. It is thus conceivable that the concentrations of the endogenously produced 1,25-$(OH)_2D_3$ reach the nanomolar range inside the cells, concentrations that lead to a suppression in cell cycle progression (76). The intracellular production of 1,25-$(OH)_2D_3$ is an important but often overlooked mechanism modulating the effects of this hormone on proliferation in physiological conditions.

From the clinical point of view, the two main targets for VD application are treatment of hyperplastic disorders such as psoriasis and cancer treatment. Since the main mechanism of therapeutic action seems to be a direct inhibition of cell growth and induction of differentiation, it is likely that the discovered enhancing effect on cell growth could present an undesirable factor limiting the clinical efficacy of the drugs. The ideal vitamin D analog should therefore, in addition to a high potency in cell growth inhibition and a low calcemic activity, be devoid of the ability to stimulate cell proliferation. Inclusion of a screening of vitamin D analogs for their ability to stimulate cell growth may be a useful test enabling selection of promising candidates for clinical evaluation.

The finding that the 1,25-$(OH)_2D_3$-dependent suppression of clonal keratinocyte growth is modulated by certain cytokines and lymphokines **(V)** may aid in the understanding of the mechanism of action of VD in psoriasis. It appears that cytokines may be divided into two groups: those which potentiate the suppressive effects of 1,25-$(OH)_2D_3$ (EGF, TGFα, interleukins) and those which block its effects on the clonogenic cells (KGF, IGF-I). The pattern of cytokine expression in psoriasis plaques varies widely and expression of cytokine receptors may be differentially regulated, as shown for the receptors for IGF and EGF/TGFα (67). It is thus conceivable that the balance between these two groups determines the clinical effect of the vitamin D-based treatment in psoriasis. It will be interesting to see whether patients with high levels of KGF and IGF-I in psoriatic plaques are less susceptible to treatment with VD.

Elucidation of the mechanisms of the regulation of cell growth and differentiation by 1,25-$(OH)_2D_3$ is one of the most important aspects of vitamin D research and has received considerable interest in recent years. I detected at least four qualitatively different phenomena related to the regulation of growth and differentiation in keratinocytes: a rapid cell-cycle block in G_1, a protracted and irreversible suppressive effect, stimulation of proliferation in the G_1 cell cycle phase, and formation of the cell-cell contacts of the adherens junction type **(IV – VI)**. These effects may be mediated by different effector signaling pathways, as shown for the growth stimulation *in vitro* (MAPK pathway) and AJ assembly (PKC pathway). The effects on cell proliferation may, at least partially, depend on the VD-induced differentiation processes. Induction of terminal differentiation is likely to be chiefly responsible for the observed irreversible growth arrest during clonal growth. AJ formation is an example of another phenomenon, which may have a powerful influence on keratinocyte growth and terminal differentiation. It is known that disruption of cell-cell contacts is associated with increased proliferation, dedifferentiation and acquisition of the capacity to invade (2, 9, 37, 50, 118). Tumors of keratinocyte origin, basal cell carcinoma and squamous cell carcinoma, demonstrate low E-cadherin and b-catenin expression (38, 102, 112) and up-regulation of AJ has been considered a promising therapeutic approach to inhibit growth and invasion of malignant tumors (77). It remains to be seen whether induction of AJ constitutes a novel mechanism of the antiproliferative effects of VD.

1,25-$(OH)_2D_3$ seems to activate both genomic and nongenomic signaling pathways, however the mode of growth regulation by either pathway has been unclear. The results presented here indicate that 1,25-$(OH)_2D_3$ is able to produce very rapid biological responses in keratinocytes independent

of gene transcription and protein synthesis. PKC activation in keratinocytes **(III, VI)** and other cell types (14, 11, 104) is probably an example of a non-genomic phenomenon, an issue that requires further studies. An example of another non-genomic mechanism is activation of MAPK cascade that was shown here to be independent of transcription and translation. MAPK and Raf activation turned out to be an important mechanism mediating the stimulatory effects of 1,25-$(OH)_2D_3$ on keratinocyte growth, a finding showing the physiological importance of non-genomic signaling. Besides the MAPK cascade, 1,25-$(OH)_2D_3$ activates also the enzymatic activity of Src in keratinocytes **(VIII)**. Based on the study of Zhao et al. (128) who showed that activation of Src is important for keratinocyte differentiation, it may be speculated that Src also plays a role in the process of 1,25-$(OH)_2D_3$-dependent differentiation. A very recent study of Khare et al. (60) revealed that Src is a component of VD signaling in another cell type, the colonocyte. Thus, Src and possibly other non-receptor tyrosine kinases may be commonly involved in VD signal transduction. This possibility deserves future studies employing e.g. Src knockout cells or mice.

The major source of contention when discussing the non-genomic events, has been the involvement of VDR. "Non-genomic" has incorrectly been considered to be a synonym for "VDR-independent". There is accumulating evidence that VDR mediates other events than binding to DNA. It has been conclusively shown that VDR resides not only in the nucleus but also in the cytoplasm, especially in endoplasmic reticulum, the Golgi complex, and microtubules (6). 1,25-$(OH)_2D_3$ treatment causes subcellular VDR redistribution and a very rapid accumulation of cGMP in the vicinity of cytoplasmic VDR (5). Interestingly, these processes are disrupted in patients with VDR mutations (5, 6). Own immunoprecipitation studies suggested that VDR has the ability to form complexes with cytoplasmic signaling molecules, Shc, Grb2, and Sos **(VII)**. All together, these findings support a fascinating hypothesis that VDR (and possibly other steroid hormone receptors) utilize signaling pathways similar to those of peptide hormone and cytokine receptors. Mechanistic studies on VDR signaling are difficult in keratinocytes because of the low level of signaling protein expression and the relative instability of the complexes. Overexpression of the protein of the interest and use of dedicated models, such as the yeast double-hybrid assay, will be necessary to reveal the nature of interactions between VDR and cytoplasmic proteins involved in signal transduction, and to understand the functional importance of those complexes. It must therefore be stressed that the presented data suggesting an involvement of VDR in non-genomic signaling are preliminary. Further work is needed to elucidate the nature and significance of VDR involvement in non-genomic signaling and in the interactions between genomic and non-genomic pathways.

10. SUMMARY

1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is a hormone able to regulate proliferation and differentiation of a variety of cells. In this thesis, the results of own investigations pertaining to the regulation of growth and differentiation of keratinocytes by 1,25-$(OH)_2D_3$ and selected vitamin D analogs are reviewed.

The effects of 1,25-$(OH)_2D_3$ and its analogs, collectively referred to as vitamin D compounds (VD) were initially studied *in vivo* in the hairless mouse model. In contrast to what could be expected from earlier studies in cell culture, 1,25-$(OH)_2D_3$ and a potent analog KH 1060 (20-*epi*-22-oxa-24a-homo-26,27-dimethyl-1α,25-dihydroxy-vitamin D_3) stimulated, rather than inhibited, epidermal proliferation. Other active vitamin D analogs, MC 1288 (20-*epi*-1α,25-dihydroxy-vitaminD_3) and MC 1301 (20-*epi*-24a-homo-26,27-dimethyl-1α,25-dihydroxy-vitamin D_3), of potencies intermediate between KH 1060 1,25-$(OH)_2D_3$, also produced epidermal hyperplasia. The vitamin D compounds that do not bind to the vitamin D receptor (1β,25-$(OH)_2D_3$, vitamin D_3, or Ro 24-2090) were inactive in this assay. Treatment with KH 1060 caused PKC activation in the epidermis (a translocation of PKC isosymes from the cytosol to the membranes) and a PKC-dependent stimulation of the c-*fos* gene transcription. The observed activation of PKC was considered to be of biological significance because its inhibition with sphingosine attenuated the KH 1060-induced epidermal hyperplasia.

In the next step, a cell culture model was developed to further characterize signaling pathways involved in the regulation of keratinocyte growth and differentiation by VD. It was found that when subconfluent keratinocytes were cultured in media containing low concentration of epidermal growth factor (EGF) and elevated calcium concentration, 1,25-$(OH)_2D_3$ stimulated cell proliferation. 1,25-$(OH)_2D_3$-dependent growth stimulation was absent when calcium-dependent differentiation was abrogated with anti-E-cadherin antibodies. The effects of calcium could be mimicked by other stimuli inducing keratinocyte differentiation, such as short-term suspension in the semi-solid methylcellulose medium. At low calcium concentration and high EGF concentration the inhibition of growth in the G_1 phase of cell cycle was observed. The effects of 1,25-$(OH)_2D_3$ on keratinocyte growth depended on the concentration of the hormone. Growth stimulation was observed only below 10^{-9} M, and at higher concentrations a cell cycle block was imposed regardless the composition of the media.

Another factor governing the proliferative response of keratinocytes to 1,25-$(OH)_2D_3$ was the density of the cells in culture. When cells were cultured at very low densities in the clonogenic assay, 1,25-$(OH)_2D_3$ and vitamin D analogs suppressed keratinocyte colony growth regardless the concentration of calcium in the media. Suppression of growth was associated with an increased rate of differentiation of clonogenic cells (putative epidermal stem cells) into more mature phenotypes expressing involucrin. By analyzing the effects of VD on colony size and the dynamics of clonogenic growth it was possible to determine that 1,25-$(OH)_2D_3$ suppressed growth by targeting the stem cell rather than the more differentiated progeny of clonogenic cells. The ability of VD to inhibit clonal growth was modulated by cytokines and lymphokines; the suppression of growth was attenuated in media containing suboptimal concentrations of EGF or supplemented with keratinocyte growth factor or insulin-like growth factor I.

It was observed that when keratinocytes were stimulated to differentiate by 1,25-$(OH)_2D_3$, stratification and formation of adherens junctions took place. The assembly of adherens junctions was dependent on the activation of PKC, since 1,25-$(OH)_2D_3$ stimulated PKC activity in the cells and inhibition of this enzyme with specific blockers abrogated formation of the junctions. In subconfluent keratinocytes cultured in the absence of EGF and at high calcium ion concentrations, 1,25-$(OH)_2D_3$ stimulated also the activity of the mitogen-activated protein kinase (MAPK), Raf kinase, and Src kinase. These events are probably of importance in the regulation of growth, since down-regulation of Raf expression by antisense oligonucleotides attenuated the growth-stimulatory properties of 1,25-$(OH)_2D_3$ normally seen under those conditions. Activation of neither MAPK nor Raf could be inhibited by transcription and translation blockers suggesting that the effects of 1,25-$(OH)_2D_3$ are independent of the transcription factor activity of the vitamin D receptor. Moreover, it was found that treatment of keratinocytes with 1,25-$(OH)_2D_3$ triggered formation of complexes involving the vitamin D receptor, Src, and other cellular signaling proteins such as Shc, Grb2 and Sos.

In conclusion, this study shows that 1,25-$(OH)_2D_3$ and receptor-active vitamin D analogues may both stimulate and inhibit keratinocyte proliferation. The direction and the magnitude of the proliferative response depends on cell culture conditions, especially the presence or absence of certain growth factors, and the degree of cell differentiation. Some biological effects of 1,25-$(OH)_2D_3$ may be mediated by PKC and MAPK signaling cascades, and there is evidence that at least some of these signaling pathways are not dependent on the transcription factor-like activity of the vitamin D receptor. These results are relevant for the understanding of the mechanism of action of vitamin D compounds in hyperplastic and neoplastic skin diseases.

11. DANSK RESUMÉ

Det er veldokumenteret, at den hormonale form af vitamin D_3 (1,25-dihydroxy-vitamin-D_3, 1,25-$(OH)_2D_3$) er en vigtig regulator af celleproliferation og differentiering. Hudceller, inklusiv epidermale keratinocytter, indeholder D vitamin receptorer, og der er omfattende dokumentation for, at 1,25-$(OH)_2D_3$ hæmmer keratinocytproliferation både i cellekultur og ved topikal anvendelse hos patienter med psoriasis. D vitamin analoger med nedsat kalcæmisk virkning har været anvendt til behandling af psoriasis. Egne præliminære undersøgelser af effekter af topikalt anvendt D vitamin analoger viste, at disse substanser fremkaldte epidermal hyperplasi i hårløse mus. Denne observation var interessant, idet den viste, at D vitaminer også kan stimulere cellevækst i særlige situationer.

Formålet med denne afhandling var at undersøge denne stimulatoriske effekt og at karakterisere de faktorer, som modulerer D vitamins effekt på keratinocytvækst. Cellulære signaler, som formidler D vitamin effekter i epidermale celler, blev også undersøgt.

Initialt blev effekten af 1,25-$(OH)_2D_3$ og en række af potente, lavkalcemiske D vitamin analoger (MC 1288, MC 1301, KH 1060) undersøgt i hårløse mus. Topikal påføring af disse substanser stimulerede proliferation af epidermale celler og fremkaldte epidermal hyperplasi. KH 1060 var den mest effektive analog, efterfulgt af MC 1301, MC 1288, og 1,25-$(OH)_2D_3$. Samme rækkefølge af analogernes potens er tidligere blevet fundet i model baseret på *hæmning* af leukæmiske cellers vækst. Effekten af D vitaminer var afhængig af D vitamin receptor aktivering, idet analoger, som ikke binder til denne receptor (1β,25-$(OH)_2D_3$ og Ro 24-2090) ikke var i stand til at påvirke epidermal vækst. Endvidere aktiverede KH 1060 protein kinase C i epidermis i hårløse mus. Protein kinase C var en vigtig mediator af D vitamin aktivitet, idet blokering af kinase aktivitet med sfingosin hindrede KH 1060 induceret epidermal hyperplasi.

Mekanismerne bag D vitaminernes vækstregulering blev også undersøgt i keratinocyt cellekultur. Cellerne blev dyrket i modificerede substrater, enten i høj densitet ("subkonfluent" kultur) eller i lav densitet, hvor keratinocytter danner kolonier af forskellige størrelser. Høje 1,25-$(OH)_2D_3$ koncentrationer ($10^{-7}-10^{-6}$ M) fremkaldte væksthæmning, hvorimod lave koncentrationer af dette hormon stimulerede vækst af subkonfluente differentierede celler. I tilfælde af klonal keratinocytvækst sås kun væksthæmning i form af dannelse af kolonier af mindre størrelse end normalt. Mekanismen bag væksthæmning i subkonfluent kultur og klonal kultur var forskellig, idet 1,25-$(OH)_2D_3$ i subkonfluent kultur fremkaldte en hurtig og reversibel blokade af cellecyklus i G_1, hvorimod hæmning af den klonale vækst var langtrukken, ikke reversibel, og forårsaget af differentiering af umodne keratinocytter.

Klonal keratinocytvækst minder om den patologiske keratinocytproliferation i psoriasis. Idet cytokiner og lymfokiner spiller en rolle i psoriasis patogenese, blev effekten af forskellige vækstfaktorer og lymfokiner på væksthæmning med 1,25-$(OH)_2D_3$ undersøgt. Epidermal vækstfaktor (EGF), transforming growth factor a (TGFα), samt interleukinerne 1, 6, og 8 stimulerede keratinocyt kolonidannelse og fremmede væksthæmning forårsaget af D vitaminer. To andre vækstfaktorer (keratinocyt vækstfaktor, KGF, og insulin-lignende vækstfaktor I, IGF I), som normalt også stimulerer kolonidannelse, svækkede D vitamins effekt.

Mekanismerne bag 1,25-$(OH)_2D_3$ effekt på cellevækst og differentiering blev undersøgt i keratinocytter dyrket i høj densitet. 1,25-$(OH)_2D_3$ stimulerede dannelse af intercellulære forbindelser af adherens type via protein kinase C. Src kinase blev aktiveret af 1,25-$(OH)_2D_3$ i koncentrationer $10^{-8}-10^{-7}$ M. Anvendelse af lave koncentrationer af 1,25-$(OH)_2D_3$ ($<10^{-8}$ M) stimulerede aktiviteten af såkaldt mitogen-activated protein kinase (MAPK), forudgået af aktivering af protein kinase Raf og dannelse af komplekser mellem signal proteiner Shc, Grb2, og Sos. Aktivering af denne MAPK kaskade synes at være relevant, idet hæmning af Raf med antisense oligonukleotider ophævede den stimulerende virkning af 1,25-$(OH)_2D_3$ på keratinocytvækst. MAPK aktivering kunne ikke blokeres med RNA- og protein syntese hæmmer. Interessant nok, kunne både Src og D vitamin receptor protein findes i komplekser med Shc. Disse fund tyder på, at D vitamin, udover den velkendte direkte modulation af gentransskription via D vitamin receptoren, også kan aktivere de signalveje som er karakteristiske for peptide hormoner og cytokiner, fx. MAPK eller protein kinase C.

Sammenfattende fandtes det, at D vitaminer (1,25-$(OH)_2D_3$ og nogle D vitamin analoger) både kan hæmme og stimulere keratinocytvækst. Endelig effekt afhænger af flere faktorer. Vigtigst var koncentration af D vitamin, tilstedeværelse af bestemte cytokiner og lymfokiner, og differentieringsstatus af keratinocytterne. I de hurtige voksende, umodne celler udøver D vitamin hæmning af cellevækst, hvorimod vækst af delvis differentierede keratinocytter kan stimuleres med små koncentrationer af 1,25-$(OH)_2D_3$. Nogle effekter af 1,25-$(OH)_2D_3$ på cellevækst og differentiering afhænger af MAPK, Src, eller protein kinase C aktivering. Resultaterne af denne afhandling kan bidrage til bedre forståelsen af de farmakologiske effekter af D vitamin præparater til bl.a. behandling af hudsygdomme som psoriasis og hudkræft. Afhandlingen åbner nye muligheder for rationel udvikling af dermatologiske lægemidler.

REFERENCES

1. Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamazaki T, Yoshiki S, Suda T. Differentiation of mouse myeloid leukemia cells induced by 1α,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA 1981; 78: 4990 – 4994.
2. Allen E, Yu Q.-C, Fuchs E. Mice expressing a mutant desmosomal cadherin exhibit abnormalities in desmosomes, proliferation, and epidermal differentiation. J Cell Biol 1996; 13: 1367 – 1382.
3. Amagai M. Adhesion molecules. I: Keratinocyte-keratinocyte interactions; cadherins and pemphigus. J Invest Dermatol 1995; 10: 146 – 152.
4. Barrandon Y, Green H. Three clonal types of keratinocyte with different capacities for multiplication. Proc Natl Acad Sci USA 1987; 84: 2302 – 2306.
5. Barsony J, Marx SJ. Rapid accumulation of cyclic GMP near activated vitamin D receptors. Proc Natl Acad Sci USA 1991; 88: 1436 – 1440.
6. Barsony J, Renyi I, McKoy W. Subcellular distribution of normal and mutant vitamin D Receptors in living cells. Studies with a novel fluorescent ligand. J Biol Chem 1997; 27: 5774 – 5782.
7. Bata-Csorgo Z, Hammerberg C, Voorhees JJ, Cooper KD. Flow cytometric identification of proliferative subpopulations within normal human epidermis and the localization of the primary hyperproliferative population in psoriasis. J Exp Med 1993; 17: 1271 – 1281.
8. Bata-Csorgo Z, Hammerberg C, Voorhees JJ, Cooper KD. Kinetics and regulation of human keratinocyte stem cell growth in short-term primary ex vivo culture. Cooperative growth factors from psoriatic lesional T lymphocytes stimulate proliferation among psoriatic uninvolved, but not normal, stem keratinocytes. J Clin Invest 1995; 95: 317 – 327.
9. Behrens J, Vakaet L, Friis R, Winterhager E, Roy FV, Mareel MM, Birchmerier W. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/β-catenin complex in cells transformed with a temperature-sensitive v-*src* gene. J Cell Biol 1993; 12: 757 – 766.
10. Bellido T, Morelli S, Fernandez LM, Boland R. Evidence for the participation of protein kinase C and 3′,5′-cyclic AMP-dependent protein kinase in the stimulation of muscle cell proliferation by 1,25-dihydroxy-vitamin D_3. Mol Cell Endocrinol 1993; 90: 231 – 238.
11. Berry DM, Antochi R, Bhatia M, Meckling-Gill KA. 1,25-dihydroxyvitamin D_3 stimulates expression and translocation of protein kinase Cα and Cδ via a nongenomic mechanism and rapidly induces phosphorylation of a 33-kDa protein in acute promyelocytic NB4 cells. J Biol Chem 1996; 27: 16090 – 16096.
12. Bhatia M, Kirkland JB, Meckling Gill KA. Monocytic differentiation of acute promyelocytic leukemia cells in response to 1,25-dihydroxyvitamin D_3 is independent of nuclear receptor binding. J Biol Chem 1995; 27: 15962 – 15965.
13. Binderup L, Latini S, Binderup E, Bretting C, Calverley M, Hansen K. 20-epi-vitamin D_3 analogues: a novel class of potent regulators of cell growth and immune responses. Biochem Pharmacol 1991; 42: 1569 – 1575.
14. Bissonnette M, Wali RK, Hartmann SC, Niedziela SM, Roy HK, Tien X.-Y, Sitrin MD, Brasitus TA. 1,25-dihydroxyvitamin D_3 and 12-*O*-tetradecanoyl phorbol 13-acetate cause differential activation of Ca^{2+}-dependent and Ca^{2+}-indpendent isoforms of protein kinase C in rat colonocytes. J Clin Invest 1995; 95: 2215 – 2221.
15. Blenis J. Signal transduction via the MAP kinases: Proceed at your own RSK. Proc Natl Acad Sci USA 1993; 90: 5889 – 5892.
16. Bollag WB, Xiong Y, Ducote J, Harmon CS. Regulation of fos-lacZ fusion gene expression in primary mouse epidermal keratinocytes isolated from transgenic mice. Biochem J 1994; 30: 263 – 270.
17. Bollag WB, Ducote J, Harmon CS. Biphasic effect of 1,25-dihydroxyvitamin D_3 on primary mouse epidermal keratinocyte proliferation. J Cell Physiol 1995; 16: 248 – 256.
18. Bonfini L, Migliaccio E, Pelicci G, Lanfrancone L, Pelicci P. Not all Shc's roads lead to Ras. Trends Biochem Sci 1996; 21: 257 – 261.
19. Bouillon R, Okamura WH, Norman AW. Structure-function relationships in the vitamin D endocrine system. Endocr. Rev 1995; 16: 200 – 257.
20. Bower M, Colston KW, Stein RC, Hedley A, Gazet JC, Ford HT, Combes RC. Topical calcipotriol treatment in advanced breast cancer. Lancet 1991; 33: 701 – 702.
21. Boyce ST, Ham RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal cultures and serum-free serial cultures. J Invest Dermatol (Suppl.) 1983; 81: 33 – 40.
22. Carlberg C. Mechanisms of nuclear signalling by vitamin D_3. Interplay with retinoid and thyroid hormone signalling. Eur J Biochem 1995; 23: 517 – 527.
23. Carlberg C, Bendik I, Wyss A, Meier E, Sturzenbecker LJ, Grippo JF, Hunziker W. Two nuclear signalling pathways for vitamin D. Nature 1993; 36: 657 – 660.
24. Cho KH, Son YS, Lee DY, Chung EK, Hur KC, Hong SI, Fuchs E. Calcipotriol (MC 903), a synthetic derivative of vitamin D_3 stimulates differentiation of squamous carcinoma cell line in the raft culture. Anticancer Res 1996; 16: 337 – 347.
25. Christakos S, Raval Pandya M, Wernyj RP, Yang W. Genomic mechanisms involved in the pleiotropic actions of 1,25-dihydroxyvitamin D_3. Biochem J 1996; 31: 361 – 371.
26. Clemens TC, Adams JS, Horiguchi N, Gilchrest BA, Cho H, Tsuchiya TY, Matsuo N, Suda T, Holick MC. Interaction of 1,25-dihydroxyvitamin D_3 with keratinocytes and fibroblasts from skin of normal subjects and a subject with vitamin-D-dependent rickets, type II: a model for study the mode of action of 1,25-dihydroxyvitamin D_3. J Clin Endocrinol Metab 1983; 56: 824 – 830.
27. Colston KW, Chander SK, Mackay AG, Coombes RC. Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro. Biochem. Pharmacol 1992; 44: 693 – 702.
28. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 1993; 26: 14553 – 14556.
29. de Boland AR, Norman A. Evidence for involvement of protein kinase C and cyclic adenosine 3′,5′ monophosphate-dependent protein kinase in the 1,25-dihydroxy-vitamin D_3-mediated rapid stimulation of intestinal calcium transport (transcaltachia). Endocrinology 1990; 12: 39 – 45.
30. de Jong EM, van de Kerkhof PC. Simultaneous assessment of inflammation and epidermal proliferation in psoriatic plaques during long-term treatment with the vitamin D_3 analogue MC903: modulations and interrelations. Br J Dermatol 1991; 12: 221 – 229.
31. Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature 1993; 36: 45 – 51.
32. Elstner E, Lee YY, Hashiya M, Pakkala S, Binderup L, Norman AW, Okamura WH, Koeffler HP. 1α,25-dihydroxy-20-epi-vitamin D_3: an extraordinarily potent inhibitor of leukemic cell growth in vitro. Blood 1994; 84: 1960 – 1967.
33. Elstner E, Linker Israeli M, Said J, Umiel T, de Vos S, Shintaku IP, Heber D, Binderup L, Uskokovic M, Koeffler HP. 20-epi-vitamin D_3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. Cancer Res 1995; 55: 2822 – 2830.
34. Elstner E, Linker Israeli M, Umiel T, Le J, Grillier I, Said J, Shintaku IP, Krajewski S, Reed JC, Binderup L, Koeffler HP. Combination of a potent 20-epi-vitamin D_3 analogue (KH 1060) with 9-cis-retinoic acid irreversibly inhibits clonal growth,

decreases bcl-2 expression and induces apoptosis in HL-60 leukemic cells. Cancer Res 1996; 56: 3570 – 3576.
35. Elstner E, Linker-Israeli M, Le J, Umiel T, Michl P, Said JW, Binderup L, Reed JC, Koeffler HP. Synergistic decrease of clonal proliferation, induction of differentiation, and apoptosis of acute promyelocytic leukemia cells after combined treatment with novel 20-epi vitamin D_3 analogs and 9-*cis* retinoic acid. J Clin Invest 1997; 99: 349 – 360.
36. Filvaroff E, Stern DF, Dotto GP. Tyrosine phosphorylation is an early and specific event involved in primary keratinocyte differentiation. Mol Cell Biol 1990; 10: 1164 – 1173.
37. Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, Lochner D, Birchmeir W. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J. Cell Biol 1991; 11: 173 – 185.
38. Fuller LC, Allen MH, Montesu M, Barker JNWN, MacDonald DM. Expression of E-cadherin in human epidermal non-melanoma cutaneous tumours. Br J Dermatol 1996; 13: 28 – 32.
39. Gerritsen MJ, Rulo HF, Van Vlijmen Willems I, van Erp PE, van de Kerkhof PC. Topical treatment of psoriatic plaques with 1,25-dihydroxyvitamin D_3: a cell biological study. Br J Dermatol 1993; 12: 666 – 673.
40. Gilman MZ. The c-fos serum responsive element responds to protein kinase C-dependent and-independent signals but not to cyclic AMP. Genes Dev 1988; 2: 394 – 402.
41. Gniadecki R. Cutaneous effects of KH 1060, a potent analogue of vitamin D_3. Ph.D. Thesis University of Copenhagen 1995.
42. Gniadecki R. Regulation of keratinocyte proliferation. Gen. Pharmacol 1998; 30: 619 – 622.
43. Gniadecki R. Disruption of the vertical calcium gradient in murine epidermis treated with a vitamin D analog KH 1060. Acta Derm Venereol (Stockh) 1998; 78: 164 – 168.
44. Gottlieb AB. Immunologic mechanisms in psoriasis. J Invest Dermatol (Suppl.) 1990; 95: 18S – 19S.
45. Gumbiner BM. Signal transduction of β-catenin. Curr Opin Cell Biol 1995; 7: 634 – 640.
46. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 1996; 84: 345 – 357.
47. Gupta AK, Fisher GJ, Elder JT, Nickoloff BJ, Voorhees JJ. Sphingosine inhibits phorbol-ester-induced inflammation, ornithine decarboxylase activity, and activation of protein kinase C in mouse skin. J Invest Dermatol 1988; 91: 486 – 491.
48. Hedlund TE, Moffatt KA, Miller GJ. Stable expression of the nuclear vitamin D receptor in the human prostatic carcinoma cell line JCA-1: evidence that the antiproliferative effects of 1α, 25-dihydroxyvitamin D_3 are mediated exclusively through the genomic signaling pathway. Endocrinology 1996; 13: 1554 – 1561.
49. Hennings H, Holbrook K. Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. Exp. Cell Res. 1983; 14: 127 – 142.
50. Hermiston ML, Gordon JI. In vivo analysis of cadherin function in the mouse intestinal epithelium: essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death. J Cell Biol 1995; 12: 489 – 506.
51. Hickish T, Cunningham D, Colston K, Millar BC, Sandle J, Mackay AG, Soukop M, Sloane J. The effect of 1,25-dihydroxyvitamin D_3 on lymphoma cell lines and expression of vitamin D receptor in lymphoma. Br J Cancer 1993; 68: 668 – 672.
52. Hino H, Kobayasi T, Asboe-Hansen G. Desmosome formation in normal human epidermal cell culture. Acta Derm. Venereol 1982; 62: 185 – 191.
53. Hosomi J, Hosoi J, Abe E, Suda T, Kuroki T. Regulation of terminal differentiation of cultured mouse epidermal cells by 1,25-dihydroxyvitamin D_3. Endocrinology 1983; 113: 1950 – 1957.
54. Itin PH, Pittelkow MR, Kumar R. Effects of vitamin D metabolites on proliferation and differentiation of cultured human epidermal keratinocytes grown in serum-free or defined culture medium. Endocrinology 1994; 13: 1793 – 1798.
55. Jiang CK, Magnaldo T, Ohtsuki M, Freedberg IM, Bernerd F, Blumenberg M. (1993). Epidermal growth factor and transforming growth factor α specifically induce the activation- and hyperproliferation-associated keratins 6 and 16. Proc Natl Acad Sci USA 1993; 90: 6786 – 6790.
56. Jones G, Calverley MJ. A dialogue on analogues. Trends Endocrinol Metab 1993; 4: 297 – 303.
57. Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 1993; 73: 713 – 724.
58. Jones PH, Harper S, Watt FM. Stem cell patterning and fate in human epidermis. Cell 1995; 80: 83 – 93.
59. Kahlen JP, Carlberg C. Identification of a vitamin D receptor homodimer-type response element in the rat calcitriol 24-hydroxylase gene promoter. Biochem. Biophys Res Commun 1994; 20: 1366 – 1372.
60. Khare S, Bolt MJG, Wali RK, Skarosi SF, Roy HK, Niedziela S, Scaglione-Sewell B, Aquino B, Abraham C, Sitrin MD, Brasitius TA, Bissonnette M. 1,25 dihydroxyvitamin D_3 stimulates phopsholipase C-γ in rat colonocytes: role of c-Src in PLC-γ activation. J Clin Invest 1997; 99: 1831 – 1841.
61. Klymkowsky MW, Parr B. The body language of cells: the intimate connection between cell adhesion and behavior. Cell 1995; 83: 5 – 8.
62. Kragballe K. Treatment of psoriasis with calcipotriol and other vitamin D analogues. J Am Acad Dermatol 1992; 27: 1001 – 1008.
63. Kragballe K, Wildfang IL. Calcipotriol (MC 903), a novel vitamin D_3 analogue stimulates terminal differentiation and inhibits proliferation of cultured human keratinocytes. Arch Dermatol Res 1990; 28: 164 – 167.
64. Kragballe K, Gjertsen BT, De Hoop D, Karlsmark T, van de Kerkhof PC, Larko O, Nieboer C, Roed Petersen J, Strand A, Tikjøb G. Double-blind, right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris. Lancet 1991; 33: 193 – 196.
65. Kragballe K, Bam TN, Hansen ER, Baadsgaard O, Grønhøj Larsen F, Søndergaard J, Axelsen MB. Efficacy and safety of the 20-epi-vitamin D_3 analogue KH 1060 in the topical therapy of psoriasis: Results of a dose-ranging study. Acta Derm Venereol 1994; 74: 398 – 402.
66. Kragballe K, Steijlen PM, Ibsen HH, van de Kerkhof PC, Esmann J, Sørensen LH, Axelsen MB. Efficacy, tolerability, and safety of calcipotriol ointment in disorders of keratinization. Results of a randomized, double-blind, vehicle-controlled, right/left comparative study. Arch Dermatol 1995; 13: 556 – 560.
67. Krane JF, Gottlieb AB, Carter DM, Krueger JG. The insulin-like growth factor I receptor is overexpressed in psoriatic epidermis, but is differentially regulated from the epidermal growth factor receptor. J Exp Med 1992; 17: 1081 – 1090.
68. Larue L, Ohsugi M, Hirchenhaim J, Kemler R. E-cadherin null mutant embryos fail to form a trophoderm epithelium. Proc Natl Acad Sci USA 1994; 91: 8263 – 8267.
69. Lewis JE, Jensen PJ, Johnson KR, Wheelock MJ. E-cadherin mediates adherens junction organization through protein kinase C. J Cell Sci 1994; 10: 3615 – 3621.
70. Lewis JE, Jensen PJ, Wheelock MJ. Cadherin function is required for human keratinocytes to assemble desmosomes and stratify in response to calcium. J Invest Dermatol 1994; 10: 870 – 877.
71. Lissoos TW, Beno DW, Davis BH. 1,25-Dihydroxyvitamin D_3 activates Raf kinase and Raf perinuclear translocation via a protein kinase C-dependent pathway. J Biol Chem 1993; 26: 25132 – 25138.
72. Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP. Transcriptional activation of the Cdk inhibitor p21 by vitamin D_3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev 1996; 10: 142 – 153.
73. Liu Y.-Y, Collins ED, Norman AW, Peleg S. Differential interaction of 1α-dihydroxyvitamin D_3 analogues and their 20-*epi*

homologues with the vitamin D receptor. J Biol Chem 1997; 27: 3336 – 3345.
74. Lucker GP, Steijen PM, Suykerbuyk EJ, Kragballe K, Brandrup F, van de Kerkhof PC. Flow-cytometric investigation of epidermal cell characteristics in monogenic disorders of keratinization and their modulation by topical calcipotriol treatment. Acta Derm Venereol 1996; 76: 97 – 101.
75. Lützow Holm C, De Angelis P, Grosvik H, Clausen OP. 1,25-Dihydroxyvitamin D_3 and the vitamin D analogue KH1060 induce hyperproliferation in normal mouse epidermis. A BrdUrd/DNA flow cytometric study. Exp Dermatol 1993; 2: 113 – 120.
76. Matsumoto K, Azuma Y, Kiyoki M, Okumura H, Hashimoto K, Yoshikawa K. (1991). Involvement of endogenously produced 1,25-dihydroxyvitamin D_3 in the growth and differentiation of human keratinocytes. Biochim Biophys Acta 1991; 1092: 311 – 318.
77. Mbalaviele G, Dunstan CR, Sasaki A, Williams PJ, Mundy GR, Yoneda T. E-Cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in an experimental metastasis model. Cancer Res 1996; 56: 4063 – 4070.
78. Milde P, Hauser U, Simon T, Mall G, Ernst V, Haussler MR, Frosch P, Rauterberg EW. Expression of 1,25-dihydroxyvitamin D_3 receptors in normal and psoriatic skin. J Invest Dermatol 1991; 97: 230 – 239.
79. Mitsuhashi T, Morris RC, Jr Ives HE. 1,25-dihydroxyvitamin D_3 modulates growth of vascular smooth muscle cells. J Clin Invest 1991; 87: 1889 – 1895.
80. Moore TB, Koeffler HP, Yamashiro JM, Wada RK. Vitamin D_3 analogs inhibit growth and induce differentiation in LA-N-5 human neuroblastoma cells. Clin Exp Metastasis 1996; 14: 239 – 245.
81. Morelli S, de Boland AR, Boland RL. Generation of inositol phosphates, diacylglycerol and calcium fluxes in myoblasts treated with 1,25-dihydroxyvitamin D_3. Biochem J 1993; 28: 675 – 679.
82. Morimoto S, Onishi T, Imanaka S. Topical administration of 1,25-dihydroxyvitamin D_3 for psoriasis: report of five cases. Calcif Tissue Int. 1986a; 38: 119 – 122.
83. Morimoto S, Yoshikawa K, Kozuka T. Treatment of psoriasis vulgaris by oral administration of 1α,25-dihydroxyvitamin D_3-open-design study. Calcif. Tissue Int 1986b; 39: 209 – 212.
84. Munker R, Kobayashi T, Elstner E, Norman AW, Uskokovic M, Zhang W, Andreeff M, Koeffler HP. A new series of vitamin D analogs is highly active for clonal inhibition, differentiation, and induction of WAF1 in myeloid leukemia. Blood 1996; 88: 2201 – 2209.
85. Murdoch D, Clissold SP. Calcipotriol. A review of its pharmacological properties and therapeutic use in psoriasis vulgaris. Drugs 1992; 43: 415 – 429.
86. Mørk Hansen C, Danielsson C, Carlberg C. The potent anti-proliferative effect of 20-epi analogues of 1,25 dihydroxyvitamin D_3 in human breast-cancer MCF-7 cells is related to promoter selectivity. Int J Cancer 1996; 67: 739 – 742.
87. Nayeri S, Danielsson C, Kahlen JP, Schrader M, Mathiasen IS, Binderup L, Carlberg C. The anti-proliferative effect of vitamin D_3 analogues is not mediated by inhibition of the AP-1 pathway, but may be related to promoter selectivity. Oncogene 1995; 11: 1853 – 1858.
88. Nemere I, Norman AW. Rapid action of 1,25-dihydroxyvitamin D_3 on calcium transport in perfused chick duodenum: effects of inhibitors. J Bone Miner Res 1987; 2: 99 – 107.
89. Nemere I, Zhou L.-X, Norman A. Nontranscriptional effects of steroid hormones. Receptor 1993; 3: 277 – 291.
90. Nemere I, Dormanen MC, Hammond MW, Okamura WH, Norman AW. Identification of a specific binding protein for 1α,25-dihydroxyvitamin D_3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. J Biol Chem 1994; 26: 23750 – 23756.
91. Norman AW, Nemere I, Bishop JE, Lowe KE, Maiyar AC, Collins ED, Taoka T, Sergeev I, Farach-Carson MC. 1,25-$(OH)_2$-vitamin D_3: a steroid hormone that produces biologic effects via both genomic and nongenomic pathways. J Steroid Biochem Molec Biol 1992; 41: 231 – 240.
92. Norman AW, Bouillon R, Farach Carson MC, Bishop JE, Zhou LX, Nemere I, Zhao J, Muralidharan KR, Okamura WH. Demonstration that 1β,25-dihydroxyvitamin D_3 is an antagonist of the nongenomic but not genomic biological responses and biological profile of the three A-ring diastereomers of 1α,25-dihydroxyvitamin D_3. J Biol Chem 1993; 26: 20022 – 20030.
93. Nusse R. A versatile transcriptional effector of Wingless signaling. Cell 1997; 89: 321 – 323.
94. Palmieri Sevier A, Palmieri GM, Baumgartner CJ, Britt LG. Case report: long-term remission of parathyroid cancer: possible relation to vitamin D and calcitriol therapy. Am J Med Sci 1993; 30: 309 – 312.
95. Parkinson EK, Emmerson A. The effects of tumour promoters on the multiplication and morphology of cultured human epidermal keratinocytes. Carcinogenesis 1982; 3: 525 – 531.
96. Rebel VI, Ossenkoppele GJ, van de Loosdrecht AA, Wijermans PW, Beelen RH, Langenhuijsen MM. Monocytic differentiation induction of HL-60 cells by MC 903, a novel vitamin D analogue. Leuk Res 1992; 16: 443 – 451.
97. Rimm DL, Koslov ER, Kebriaei P, Cianci CD, Morrow JS. α1(E)-Catenin is an actin-binding and-bundling protein mediating the attachment of F-actin to the membrane adhesion complex. Proc Natl Acad Sci USA 1995; 92: 8813 – 8817.
98. Rochat A, Kobayashi K, Barrandon Y. Location of stem cells of human hair follicles by clonal analysis. Cell 1994; 76: 1063 – 1073.
99. Schrader M, Muller KM, Carlberg C. Specificity and flexibility of vitamin D signaling. Modulation of the activation of natural vitamin D response elements by thyroid hormone. J Biol Chem 1994; 26: 5501 – 5504.
100. Schrader M, Muller KM, Nayeri S, Kahlen JP, Carlberg C. Vitamin D_3-thyroid hormone receptor heterodimer polarity directs ligand sensitivity of transactivation. Nature 1994; 37: 382 – 386.
101. Sherr CJ. Mammalian G1 cyclins. Cell 1993; 73: 1059 – 1065.
102. Shiozaki H, Tahara H, Oka H, Miyata M, Kobayashi T, Tamura S, Iihara K, Doki Y, Hirano S, Takeichi M, Mori T. Expression of immunoreactive E-cadherin adhesion molecules in human cancer. Am J Pathol 1991; 13: 17 – 23.
103. Skowronski RJ, Peehl DM, Feldman D. Actions of vitamin D_3: analogs on human prostate cancer cell lines: comparison with 1,25-dihydroxyvitamin D_3. Endocrinology 1995; 13: 20 – 26.
104. Slater SJ, Kelly MB, Taddeo FJ, Larkin JD, Yeager MD, McLane JA, Ho C, Stubbs CD. Direct activation of protein kinase C by 1α,25-dihydroxyvitamin D_3. J Biol Chem 1995; 27: 6639 – 6643.
105. Smith EL, Walworth NC, Holick MF. Effects of 1α,25-dihydroxyvitamin D_3 on the morphologic and biochemical differentiation of cultured human keratinocytes grown in serum-free conditions. J Invest Dermatol 1986; 86: 709 – 714.
106. Smith EL, Pinkus SH, Donovan L, Holick MF. A novel approach for the evaluation and treatment of psoriasis. Oral or topical use of 1,25-dihydroxyvitamin D_3 can be a safe and effective therapy for psoriasis. J Am Acad Sci 1988; 19: 516 – 528.
107. Song LN, Cheng T. Effects of 1,25-dihydroxyvitamin D_3 analogue 1,24$(OH)_2$-22-ene-24-cyclopropyl D_3 on proliferation and differentiation of a human megakaryoblastic leukemia cell line. Biochem Pharmacol 1992; 43: 2292 – 2295.
108. Studzinski GP, McLane JA, Uskokovic MR. Signaling pathways for vitamin D-induced differentiation: implications for therapy of proliferative and neoplastic diseases. Crit Rev Eukaryot Gene Expr 1993; 3: 279 – 312.
109. Stumpf WE, Sar M, Reid FA, Tanaka Y, Deluca HF. Target cells

for 1,25-dihydroxyvitamin D_3 in intestinal tract, stomach, kidney, skin, pituitary, and parathyroid. Science 1979; 20: 1188 – 1190.
110. Stumpo DJ, Stewart TN, Gilman MZ, Blackshear PJ. Identification of c-fos sequence involved in induction by insulin and phorbol esters. J Biol Chem 1988; 26: 1611 – 1614.
111. Svendsen M, Daneels G, Geysen J, Binderup L, Kragballe K. Proliferation and differentiation of cultured human keratinocytes is modulated by $1,25(OH)_2D_3$ and synthetic vitamin D_3 analogues in a cell density-, calcium- and serum-dependent manner. Pharmacol. Toxicol. 1997; 80: 49 – 56.
112. Takayama T, Shiozaki H, Shibamoto S, Oka H, Kimura Y, Tamura S, Inoue M, Monden T, Ito F, Monden M. β-Catenin expression in human cancers. Am J Pathol 1996; 14: 39 – 46.
113. Tseng H, Green H. Association of basonuclin with ability of keratinocytes to multiply and with absence of terminal differentiation. J Cell Biol 1994; 12: 495 – 506.
114. van de Kerkhof PCM. Biological activity of vitamin D analogues in the skin, with special reference to antipsoriatic mechanisms. Br J Dermatol 1995; 13: 675 – 682.
115. van de Kerkhof PC, Steijlen PM. Topical treatment of pityriasis rubra pilaris with calcipotriol. Br J Dermatol 1994; 13: 675 – 678.
116. van den Bemd GC, Pols HA, Birkenhager JC, van Leeuwen JP. Conformational change and enhanced stabilization of the vitamin D receptor by the 1,25-dihydroxyvitamin D_3 analog KH1060. Proc Natl Acad Sci USA 1996; 93: 10685 – 10690.
117. van Leeuwen JPTM, Birkenhäger JC, van den Bemd G-JCM, Buurman CJ, Staal A, Bos MP, Pols HAP. Evidence for the functional involvement of protein kinase C in the action of 1,25-dihydroxyvitamin D_3 in bone. J Biol Chem 1992; 26: 12562 – 12569.
118. Vleminckx K, Vakaet L, Jr Mareel M, Fiers W, Van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell 1991; 66: 107 – 119.
119. Waga S, Hannon GJ, Beach D, Stillman B. Differential effects by the p25 CDK inhibitor on PCNA-dependent DNA replication and repair. Nature 1994; 36: 574 – 578.
120. Wali RK, Baum CL, Sitrin MD, Brasitus TA. $1,25(OH)_2$ vitamin D_3 stimulates membrane phosphoinositide turnover, activates protein kinase C and increases cytosolic calcium in rat colonic epithelium. J Clin Invest 1990; 85: 1296 – 1303.
121. Walters MR. Newly identified actions of the vitamin D endocrine system. Endocr Rev 1992; 13: 719 – 764.
122. Wang QM, Luo X, Studzinski GP. Cyclin-dependent kinase 6 is the principal target of p27/Kip1 regulation of the G_1-phase traverse in 1,25-dihydroxyvitamin D_3-treated HL-60 cells. Cancer Res 1997; 57: 2851 – 2855.
123. Wheelock MJ, Jensen PJ. Regulation of keratinocyte intercellular junction organization and epidermal morphogenesis by E-cadherin. J Cell Biol 1992; 11: 415 – 425.
124. Wille JJ, Pittelkow MR, Shipley GD, Scott RE. Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: clonal analyses, growth kinetics, and cell-cycle studies. J Cell Physiol 1984; 12: 31 – 44.
125. Yada Y, Ozeki T, Meguro S, Mori S, Nozawa Y. Signal transduction in the onset of terminal keratinocyte differentiation induced by 1α,25-dihydroxyvitamin D_3: role of protein kinase C translocation. Biochem Biophys Res Commun 1989; 16: 1517 – 1522.
126. Yuspa SH, Ben TB, Hennings H, Lichti U. Divergent responses in epidermal basal cells exposed to the tumor promotor 12-*O*-tetradeconoyl phorbol-13-acetate. Cancer Res 1982; 42: 2344 – 2349.
127. Yuspa SH, Ben TB, Hennings H. The induction of epidermal transglutaminase and terminal differentiation by tumour promoters in cultured human epidermal cells. Carcinogenesis 1983; 4: 1413 – 1418.
128. Zhao Y, Sudol M, Hanafusa H, Krueger JG. Increased tyrosine kinase activity of c-Src during calcium-induced keratinocyte differentiation. Proc Natl Acad Sci USA 1992; 89: 8298 – 8302.