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Vitamin D Receptor Expression Is Linked to Cell Cycle Control in Normal Human Keratinocytes

Siegfried Segaert,*,† Hugo Degreef,† and Roger Bouillon*,[1]
**Laboratory for Experimental Medicine and Endocrinology and †Department of Dermatology, Katholieke Universiteit Leuven, Campus Gasthuisberg, Onderwijs en Navorsing, Herestraat 49, B-3000 Leuven, Belgium*

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To improve our understanding of the cutaneous vitamin D system, we studied vitamin D receptor (VDR) gene regulation in cultured human keratinocytes. Because VDR and its ligand 1α,25-dihydroxyvitamin D_3 have been implicated in epidermal growth control, we investigated VDR expression as related to cellular proliferation by using different cell cycle synchronization protocols. Keratinocytes, deprived of growth factors, were forced into quiescence and a concomitant loss of VDR expression was observed. Mitogenic stimulation of these G_0 cells however quickly upregulated VDR levels several hours ahead the G_1-S transition point. Growth arrest at the G_1-S border by mimosine treatment or at the metaphase by nocodazole also downregulated VDR levels but a restoration of VDR expression was again quickly achieved after reentering the cell cycle. These findings indicate that VDR expression in keratinocytes is restricted to actively cycling cells, but not limited to one particular phase of the cell cycle.

Key Words: **vitamin D receptor; keratinocytes; cell cycle; growth factors; starvation; mimosine; nocodazole.**

In recent years it has become clear that the action of the vitamin D receptor (VDR) ligand 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] extends far beyond its classical role in bone and mineral metabolism (1). Among the nonclassical vitamin D target tissues the skin occupies a central position as evidenced by the alopecia in humans or mice with a dysfunctional vitamin D receptor (2–4). Moreover, 1,25$(OH)_2D_3$ exerts potent antiproliferative effects on cultured keratinocytes (5–6) and vitamin D analogues have been successfully introduced for the topical treatment of psoriasis (7).

Abbreviations used: 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; UVB, ultraviolet B; VDR, vitamin D receptor.

[1] To whom correspondence should be addressed. Fax: 32-16-345934. E-mail: Roger.Bouillon@med.kuleuven.ac.be.

However, the physiological function of 1,25$(OH)_2D_3$ in the epidermis remains poorly understood. The expression level of VDR is a major determinant of cellular 1,25$(OH)_2D_3$ responsiveness (8); hence, it reflects the cellular need for 1,25$(OH)_2D_3$ and may therefore help to reveal the elusive function of the cutaneous vitamin D endocrine system. We have previously shown that VDR expression in keratinocytes is overtly regulated by a wide variety of environmental factors that influence cell fate such as cell adhesion, cell density, calcium, phorbolesters, interferon γ, ultraviolet B, or flavonoids (9–12). In these experiments, we observed a dramatic reduction in VDR expression under conditions that triggered growth arrest or differentiation. Moreover, VDR levels appeared to fluctuate in concert with the expression of proliferation markers such as *c-myc* and cyclin D1 (9–12). Therefore we further investigated VDR expression as related to cell cycle regulation. Accordingly, we blocked keratinocyte cell cycle progression at different phases of the cell cycle allowing a reentry of the cells in a synchronized way. We show that keratinocytes only express VDR to a normal extent when actively recruited in the cell cycle regardless of the current cell cycle phase.

MATERIALS AND METHODS

Cell culture and reagents. Normal human epidermal keratinocytes were isolated from foreskins as described (6) and propagated in keratinocyte growth medium consisting of keratinocyte serum-free medium containing 0.09 mM calcium and supplemented with bovine pituitary extract and epidermal growth factor (Gibco-BRL, Gaithersburg, MD). For starvation experiments, cells at 25% confluence were rinsed thoroughly with PBS and maintained for 5 days in keratinocyte basal medium (KBM, Clonetics Corp., San Diego, CA). Restimulation of the cells was performed by changing KBM to keratinocyte growth medium supplemented with 50 ng/ml EGF (Sigma, St. Louis, MO). L-mimosine, nocodazole, and cycloheximide were purchased from Sigma (St. Louis, MO); AG1478 and wortmannin were obtained from Calbiochem (La Jolla, CA). We dissolved L-mimosine and cycloheximide in PBS, nocodazole and AG1478 in DMSO, and wortmannin in absolute ethanol.

Northern blot analysis. Northern blot experiments were performed as described (6). The following cDNA probes were used:

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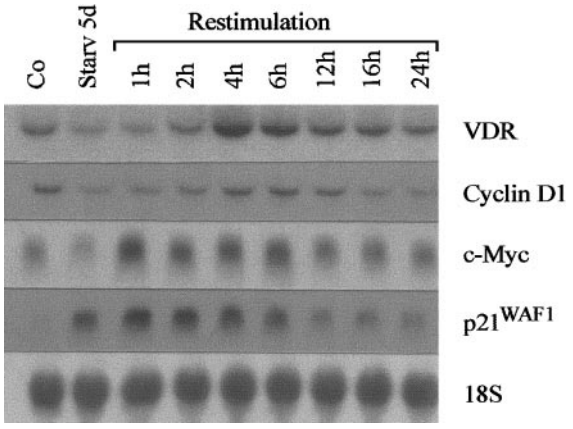

FIG. 1. Exit from quiescence stimulates VDR expression in normal human keratinocytes. Epidermal cells (25% confluence; Co) were maintained for 5 days in keratinocyte basal medium lacking growth factors (Starv) to synchronize them in G_0 and were then restimulated with keratinocyte growth medium supplemented with EGF 50 ng/ml. At different time points total RNA (12 μg) was isolated for Northern blot analysis followed by sequential hybridization with ^{32}P labeled probes for VDR, cyclin D1, *c-myc*, p21^{WAF1}, and 18S RNA.

human VDR cDNA (American Type Culture Collection, Rockville, MD), human cyclin D1 cDNA (13), a 0.479 kb human *c-myc* probe prepared with a RT-PCR amplimer set (Clontech Laboratories Inc., Palo Alto, CA), and human WAF1 cDNA (14). For verification of even loading we used a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S RNA.

Western blot analysis. Total protein samples (100 μg) were subjected to immunoblotting using standard procedures (6). We used a mouse monoclonal antibody to chicken VDR (15), monoclonal anti-retinoblastoma protein (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), rabbit anti-cyclin A (Santa Cruz Biotech., Santa Cruz, CA), and a monoclonal anti-β-actin antibody (Sigma) as a control. Quantification of protein or RNA bands on Western or Northern blots was performed using a laser densitometric scanner (Pharmacia Biotech Inc., Piscataway, NJ).

RESULTS

Regulation of VDR Expression by Synchronization at G_0 (Growth Factor Starvation)

Growth factor starvation was a first approach in order to enrich keratinocyte cultures with quiescent cells of the G_0 phase. We cultured normal keratinocytes for 5 days in the absence of growth factors to bring them into a quiescent state (16). These cells showed a markedly reduced expression of both VDR mRNA (60% reduction; Fig. 1) and protein (85% reduction; Fig. 2). At the same time *c-myc* and cyclin D1 mRNA and cyclin A protein were reduced, whereas p21^{WAF1} mRNA was induced (Figs. 1 and 2). Refeeding of the starved G_0 cells with complete medium supplemented with epidermal growth factor (EGF 50 ng/ml), resulted in a quick upregulation of VDR mRNA (beginning after 2 h and maximal after 4 h), sharing the characteristics of cyclin D1 mRNA induction (Fig. 1), which is expressed as a delayed early gene (17). At the same time p21^{WAF1} mRNA levels returned to normal after a transient increase (Fig. 1). The immediate early gene *c-myc* was induced already 1 h after stimulation. In parallel with its mRNA levels, VDR protein reached normal levels within 4 h of restimulation (Fig. 2). At this point, the cells were still several hours ahead the G_1-S transition point [16 h after stimulation evidenced by the appearance of the hyperphosphorylated form of the retinoblastoma protein and by cyclin A protein elevation (Fig. 2)].

To further investigate the properties of the growth factor-dependent signals that were responsible for the induction of VDR mRNA in quiescent keratinocytes, we performed some additional experiments. VDR mRNA could not be induced in quiescent keratinocytes by the addition of fresh basal medium without growth factors (Fig. 3). The addition of epidermal growth factor to G_0 keratinocytes was sufficient for the induction of VDR expression and did not require protein synthesis (no blocking effect of cycloheximide; Fig. 3). The action of EGF on VDR mRNA could be blocked by inhibitors of EGF receptor tyrosine kinase activity (AG1478) or of phosphatidylinositol 3-kinase activity (wortmannin), suggesting the involvement of these signaling pathways in the regulation of VDR expression by EGF. However, AG1478 and wortmannin did not affect upregulation of VDR mRNA in quiescent keratinocytes following stimulation with keratinocyte

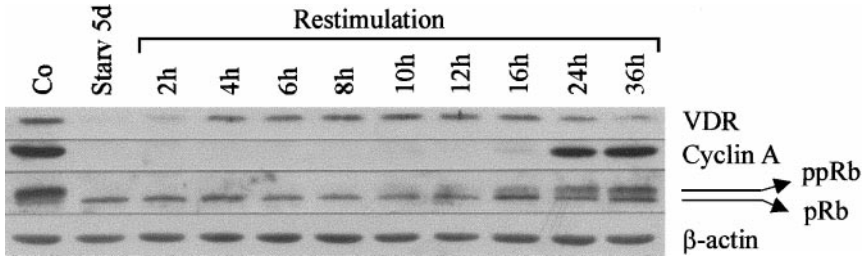

FIG. 2. Upregulation of VDR protein levels in quiescent keratinocytes upon reentry of the cell cycle. Keratinocytes were growth factor starved and restimulated as described for Fig. 1. Total protein samples (100 μg) were used for Western blot analysis to determine VDR and cyclin A protein levels and to assess retinoblastoma protein phosphorylation status (ppRb and pRb designate the hyper- and hypophosphorylated form of retinoblastoma protein, respectively).

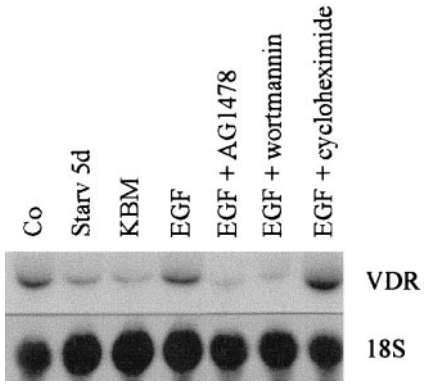

FIG. 3. Effect of chemical inhibitors on growth factor stimulated expression of VDR in G_0 keratinocytes. Keratinocytes were deprived of growth factors for 5 days (Starv) and restimulated for 4 h with keratinocyte basal medium (KBM) lacking growth factors or with KBM supplemented with EGF 50 ng per ml. At the same time several inhibitors were added to the restimulation medium: AG1478 (0.1 μM), wortmannin (0.1 μM), or cycloheximide (10 μg/ml). Northern blot analysis was performed to reveal VDR mRNA levels.

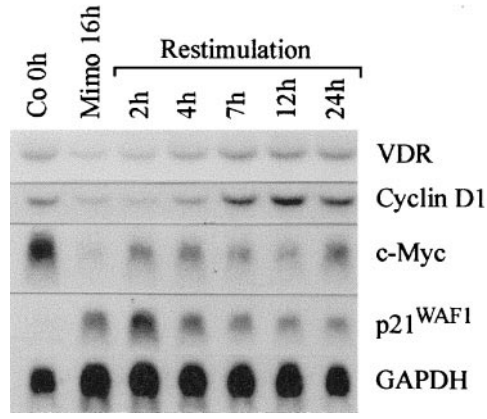

FIG. 5. Regulation of VDR mRNA expression during cell cycle synchronization with mimosine. Normal human keratinocytes were treated for 16 h with mimosine (mimo; 200 mM), resulting in cell cycle arrest at the G_1-S border. The mimosine-containing medium was subsequently removed and replaced by keratinocyte growth medium containing EGF 50 ng/ml. At the indicated time total RNA was isolated to visualize the indicated mRNAs.

growth medium, containing multiple growth factors including EGF (data not shown). This indicates that soluble factors other than EGF are also involved in the regulation of VDR expression. Indeed, various growth factors of different families and using different membrane receptors upregulated VDR levels in quiescent keratinocytes. The keratinocyte mitogenic factors epidermal growth factor (EGF, 50 ng/ml), keratinocyte growth factor (KGF 10 ng/ml), and IGF-I (50 ng/ml) all induced VDR mRNA in keratinocytes to some extent and in a more or less transient way (Fig. 4).

Regulation of VDR Expression by Synchronization at G_1-S (Mimosine)

L-mimosine is a rare plant amino acid which reversibly inhibits cell cycle progression at late G_1 (18) by inhibition of eukaryotic initiation factor 5-A (19) and upregulation of $p21^{WAF1}$ (20). Cell cycle arrest at the G_1-S boundary by 200 μM mimosine (not shown) was accompanied by suppression of c-myc and cyclin D1 mRNA and induction of $p21^{WAF1}$ mRNA (Fig. 5). Concomitantly a reduction of both VDR mRNA (Fig. 5) and protein levels (Fig. 6) took place. After removal of the mimosine and restimulation of the cells with fresh medium supplemented with EGF (50 ng/ml), cells were stimulated to reenter the cell cycle (not shown). Under these conditions, *c-myc*, cyclin D1, and $p21^{WAF1}$ mRNA levels were quickly normalized, and we also observed a rapid reexpression of VDR mRNA and protein within a few hours (Figs. 5 and 6).

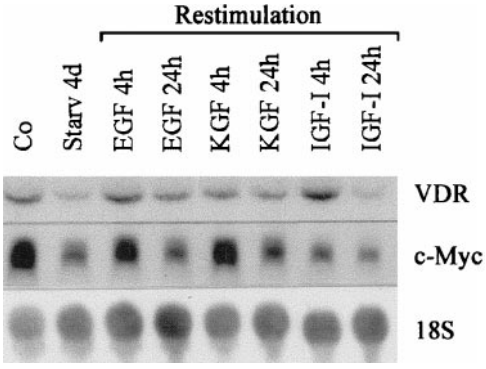

FIG. 4. Stimulation of VDR expression by growth factors in keratinocytes. Keratinocytes, which were maintained for 4 days in medium lacking growth factors to induce quiescence were restimulated with keratinocyte basal medium supplemented with EGF (50 ng/ml), KGF (10 ng/ml), or IGF-I (50 ng/ml) for the indicated time. Total RNA samples were used to detect VDR and *c-myc* RNA by Northern blot.

Regulation of VDR Expression by Synchronization in M Phase (Nocodazole)

We used nocodazole as an inhibitor of tubulin polymerization causing disruption of microtubuli and metaphase arrest (18). As expected this agent caused an enrichment of cells in the G_2 + M phase, as estimated by flow cytometry analysis (data not shown). Concomitantly VDR mRNA, but not *c-myc*, was downregulated (Fig. 7). Restimulation of nocodazole arrested cells led to induction of VDR mRNA levels within 4 h.

DISCUSSION

The presented data suggest an intimate relation between VDR expression and growth control in keratinocytes: VDR levels were markedly decreased by arresting the cells in G_0 (growth factor deprivation), at the G_1-S border (mimosine) or during the mitotic meta-

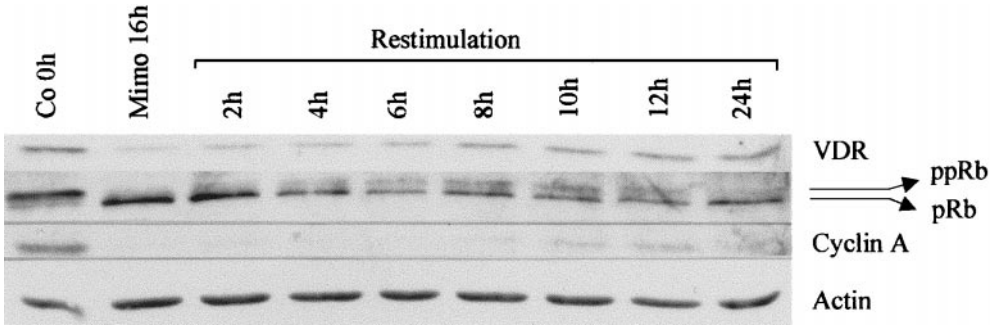

FIG. 6. Upregulation of VDR protein levels after release of synchronization at the G_1-S border by mimosine. Keratinocytes were treated as described in Fig. 5. Subsequently, total protein samples (100 μg) were subjected to immunoblot analysis with specific antibodies against VDR, cyclin A, β-actin, and retinoblastoma protein (ppRb indicates the hyperphosphorylated and pRb the hypophosphorylated form).

phase (nocodazole); in each of these cases VDR was also rapidly induced when the cells were stimulated with different growth factors to reenter the cell cycle. This indicates that VDR expression is restricted to cycling cells but not to a particular cell cycle phase. The interdependence between VDR levels and cell proliferation was also suggested previously by the induction of VDR in quiescent fibroblasts, breast cancer cells, or lymphocytes following mitogenic stimulation with growth factors, serum, or cytokines (21–22) and by the association between the expression of VDR and the proto-oncogene *c-myc* in osteosarcoma cells (23). In the same context, we have previously shown that VDR levels fell dramatically in keratinocytes that were forced to growth arrest and differentiation by suspension culture, density arrest, interferon γ or phorbolesters (9–10). VDR is thus most notably expressed in proliferating keratinocytes which are attached to substrate and are stimulated by growth factors to express growth related genes such as cyclin D1 and *c-myc*. Proliferating cells should therefore be considered as preferential vitamin D target cells in accordance with the identification of basal keratinocytes as the main VDR containing cells in the epidermis (24–25).

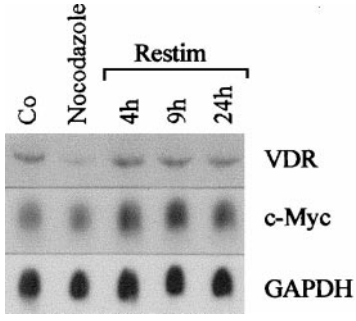

FIG. 7. Regulation of VDR mRNA levels during cell cycle synchronization at the metaphase. Normal human keratinocytes were treated for 16 h with nocodazole (0.5 μg/ml). The drug was subsequently removed and replaced by keratinocyte growth medium containing EGF 50 ng/ml. At the indicated time total RNA samples were used to quantitate the mRNA levels of VDR and *c-myc*.

In our cellular system, VDR was shown to behave as an early gene (17), with a rapid, protein synthesis independent induction in growth factor-stimulated quiescent keratinocytes. The extensive regulation of VDR expression by factors that govern cell proliferation such as growth factors, cell adhesion, and cell shape (9–10) and the short halflife of the transcript (12) and protein (26) are further characteristics of growth related genes. These findings may suggest a physiological function of VDR in the maintenance of proliferation in the epidermis. The alopecia in VDR null mice, resulting from failure of initiation of the proliferative anagen phase (27), may be the expression of such a putative role of VDR in epidermal proliferation. This view is also in line with the preferential localization of VDR in the epidermal cell compartment with the highest growth potential (24–25).

The fluctuation of VDR levels according to the proliferation and differentiation state of the cells is a parameter that may help to explain the paradoxical effects of active vitamin D compounds on keratinocytes *in vitro* and *in vivo*. In this respect, it was shown that the magnitude of the growth inhibitory effect of $1,25(OH)_2D_3$ in cultured keratinocytes correlated with the cellular proliferation rate (16). Vitamin D dependent growth inhibition was even reversed into mitogenic effects in cells that were growth arrested or committed to differentiate (16). Likewise, vitamin D analogues inhibit keratinocyte growth in psoriatic lesions *in vivo* (7) but stimulate epidermal proliferation in normal skin (28), (which is mainly composed of quiescent keratinocytes). The level of VDR expression may be a determining factor in the differential vitamin D response of rapidly growing (antiproliferative effect in psoriasis and cultured keratinocytes) vs quiescent, differentiated keratinocytes (mitogenic in normal *in vivo* skin). Indeed, high VDR levels are known to favor vitamin D antiproliferative actions (29) whereas the mitogenic properties of vitamin D rely on the activation of intracellular signal transduction cascades such as mitogen activated protein kinases (30) with a presum-

ably lower dependency on VDR levels. Hence, it is not surprising that the therapeutic activity of vitamin D analogues in epidermal diseases is largely confined to hyperproliferative disorders such as psoriasis and hyperproliferative ichthyoses (7, 31). Another implication is that culture conditions (e.g., presence of growth factors) that affect cell proliferation and thus VDR expression should be well taken into account when comparing the effects of vitamin D compounds in different experimental systems (32).

The molecular basis of the coupling of the keratinocyte cell cycle with VDR expression remains unclear. Previous studies have shown that regulation of VDR levels in keratinocytes occurs mostly at the transcriptional level (10, 12). Most of the activity of the main VDR promoter depends on multiple Sp1 binding sites in its proximal part (33–34). Recent studies have raised evidence for the physical interaction between Sp1 and cell cycle regulatory proteins such as retinoblastoma and cyclin D1 (35–36) and for growth dependent phosphorylation of Sp1 (37). In this respect, Sp1 may constitute the link between growth regulation and VDR expression. Early growth response 1 (Egr1) is another possible mediator of growth factor induced VDR expression: Egr1 is an immediate early gene encoding a transcription factor, which binds to GC-rich DNA regions that often overlap with Sp1 binding sites and for which a consensus binding sequence was identified in the proximal part of the mouse VDR gene promoter (34). NFκB is a transcription factor that was previously invoked in the suppression of VDR by ultraviolet B or the flavonoid apigenin in keratinocytes (11–12). With the close relationship between proliferation rate and VDR levels in mind, it is of great interest that NFκB was recently implicated in cellular growth arrest in general (38) and in epidermal growth control in particular (39). Inhibition of cylin dependent kinase 2 appears to trigger increased activity of NFκB (38), providing a very attractive model for transcriptional regulation by cell cycle progression. In addition to direct regulation of the VDR promoter by NFκB (33–34), interaction of NFκB with (enhancer binding) Sp1, or Egr1 (40–41) may further contribute to regulation of VDR transcription. The proto-oncogenic transcription factor *c-myc* is a final candidate to explain proliferation dependent expression of VDR. In keratinocytes and other cell types VDR and *c-myc* are often regulated in a coordinate way (9–12, 23). By the presence of a consensus binding sequence for NFκB in their 5′-flanking region, the VDR and *c-myc* genes might be jointly regulated by NFκB (33–34, 42). Direct regulation of the VDR promoter by c-myc (34) may further add to their concordant expression.

In conclusion, our results demonstrate a striking parallelism between growth regulation and VDR expression in cultured human epidermal keratinocytes, thus suggesting a role for VDR in epidermal growth control.

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