SPANDIDOS PUBLICATIONS

# Vitamin $D_3$ inhibits expression of pemphigus vulgaris antigen desmoglein 3: Implication of a partial mechanism in the pharmacological effect of vitamin $D_3$ on skin diseases

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**Abstract.** Desmoglein-3 (DSG3) is a critical molecule for adhesion between keratinocytes. Its expression can affect interactions between keratinocytes as well as keratinocyte morphology. Vitamin $D_3$ (VD3) has been known for its role in mineral homeostasis, but has now been shown to be involved in cell differentiation, cell proliferation, immune responses and inflammation as well. Topical application of VD3 is used for the treatment of various skin diseases in present clinical practice. This study examined the effect of VD3 on DSG3 gene expression in keratinocytes. Incubation of the cultured keratinocytes with VD3 resulted in a significant decrease in Dsg3 mRNA expression. Cycloheximide treatment did not alter the inhibition rate of Dsg3 gene expression by VD3. We additionally measured the effect of VD3 on the promoter activity of the DSG3 gene. VD3 failed to inhibit promoter activities. These results suggest that down-regulation of Dsg3 expression by VD3 is independent of active protein synthesis, and may also result from keratinocyte regulatory mechanisms operating at the post-transcriptional level. Thus, the pharmacological effect of VD3 was partially mediated by the modulation of DSG3 expression through a simple pathway, without either new transcription or protein synthesis being necessary.

## Introduction

Desmosomes are intercellular junctional apparatuses that connect intracellular intermediate filaments to the cell surface and mediate strong cell-cell adhesion. They are composed of two major transmembrane proteins, desmoglein (Dsg) and desmocollin (1-3). Until now, four desmogleins expressed in tissue- and differentiation-specific manners have been characterized (4-6). Desmoglein-3 is located in the basal and suprabasal layers of stratifying epithelia and has been shown to be the antigen recognized by the autoantibodies of patients with pemphigus vulgaris (PV), an autoimmune blistering disease characterized by suprabasal acantholysis (the loss of cell-cell adhesion between keratinocytes) in the epidermis (7,8). Currently, the activity of the disease can be evaluated in clinical practice by ELISA for the DSG3 antibody (9,10).

Vitamin $D_3$ (VD3) was initially known for its role in mineral (calcium and phosphorus) homeostasis and the maintenance of normal skeletal structures. It is now clear that VD3 is also involved in cell differentiation, cell proliferation, immune responses and inflammation (11-13). In the area of clinical dermatology, topical application of VD3 is widely used for the treatment of psoriasis (14). Recently, patients with vitiligo vulgaris and other keratinizing diseases have also been successfully treated using topical VD3, and systemic VD3 treatment has been administered to numerous patients with osteoporosis, cancers and autoimmune diseases (14).

As mentioned above, since DSG3 is critical for adhesion between keratinocytes, its expression can affect interactions between keratinocytes and also keratinocyte morphology. The modulation of DSG3 expression through VD3 treatment may therefore facilitate the treatment of skin diseases. This study examined the effect of VD3 on DSG3 gene expression. In addition to investigating the molecular mechanisms behind this effect, we measured the effect of VD3 on changes in the promoter activity of the DSG3 gene.

## Materials and methods

*Culture of epidermal keratinocytes.* Normal human epidermal keratinocytes were cultured in keratinocyte growth medium (KGM), containing epidermal growth factor (0.1 $\mu$g/ml), bovine pituitary extract (0.1 $\mu$g/l), insulin (0.5 mg/ml) and hydrocortisone (0.5 mg/ml) (Clonetics Corp., San Diego, CA) at 37˚C in a 5% $CO_2$-air atmosphere. The calcium concentration of standard medium was adjusted to 0.15 mmol/ml. Cultures were passaged by trypsinization and passages 3-4 were examined.

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*Abbreviations:* DSG3, desmoglein-3; VD3, vitamin $D_3$; PV, pemphigus vulgaris

*Key words:* keratinocytes, vitamin $D_3$, desmoglein-3, differentiation, transcription, protein synthesis

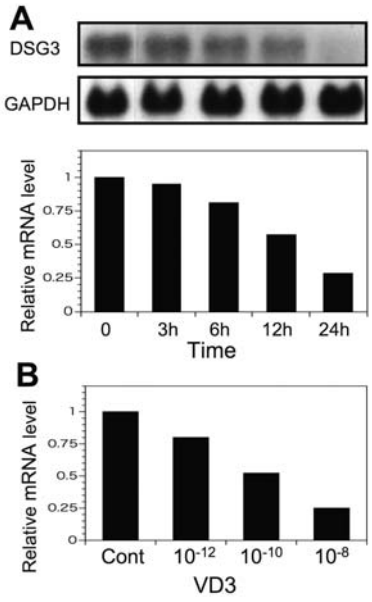


Figure 1. Effects of VD3 on DSG3 mRNA expression. Normal human epidermal keratinocytes were cultured routinely using keratinocyte serum-free medium. (A) Keratinocytes were grown in VD3, $1\alpha,25(OH)_2D3$ at $10^{-8}$ M. After cultured keratinocytes were treated with VD3, total RNA was fractionated on 1.0% agarose gels and transferred to nitrocellulose membranes, which were hybridized with 2.3-kb DSG3 gene cDNA and with 1.3-kb human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA labeled with $^{32}P$. The intensity of the autoradiographic band was quantified by scanning densitometry. (B) Keratinocytes were treated with different concentrations of VD3 for 24 h, and the DSG3 mRNA level was determined by Northern blot analysis. Data are the means of two samples.

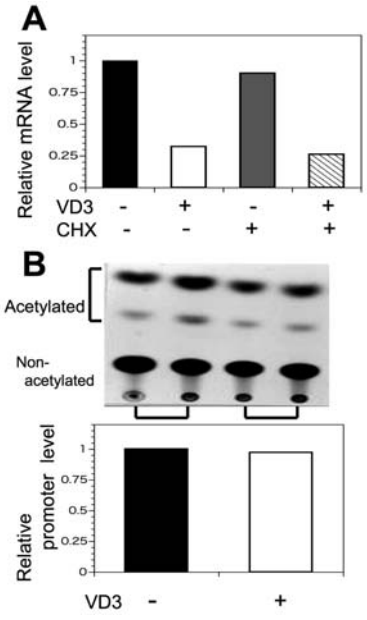


Figure 2. Mechanisms of the inhibitory effects of VD3. (A) Keratinocyte cultures were incubated in the presence (+) or absence (-) of VD3 ($10^{-8}$ M) and/or cycloheximide (CHX; 10 mg/ml) for 24 h. Relative DSG3 mRNA levels were quantified by Northern blot analysis. (B) The human DSG3 promoter-DNA/chloramphenicol acetyl transferase (CAT) reporter gene plasmid, containing the BPAG1 promoter region extending from -2300 to -1, was used for transient transfection of cultured human keratinocytes. Transfection was performed with a commercial kit, and VD3 ($10^{-8}$ M) was added to the cultures. CAT activity in the cell cultures was determined by the incubation of cell extracts with [$^{14}C$]chloramphenicol, followed by the separation of the acetylated and non-acetylated forms by thin-layer chromatography. Promoter activity was determined by computing the radioactivity in the acetylated forms of chloramphenicol as a percentage of total radioactivity. Data are means of two samples.

*Total RNA preparation and Northern blot analyses*. Total RNA was isolated from cultured keratinocytes treated with $1\alpha,25(OH)_2D3$ (Teijin, Japan). For Northern hybridizations, total RNA (20 mg/lane) was fractionated on 1.0% agarose gels and transferred to nitrocellulose membranes. The products were prehybridized and then hybridized with 2.3-kb human Dsg3 cDNA (15) and with 1.3-kb human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, both of which were radioactively labeled with [a-32P] dCTP (NEN Life Science Products Inc., Boston, MA) using a random primer DNA labeling kit (Stratagene, La Jolla, CA). The filters were then exposed to X-ray film (Fuji Film, Tokyo, Japan). Blots were scanned, and the intensity of each band was measured by the computer software program NIH image.

*Transient transfection of keratinocytes with the Dsg3 promoter/reporter gene plasmid constructs*. The human Dsg3 promoter-DNA/chloramphenicol acetyl transferase (CAT) reporter gene plasmid pPV2.3CAT, containing the human Dsg3 promoter region extending from positions -2300 to -1 (in relation to the transcription initiation site), was used for the transient transfection of cultured human keratinocytes (15). The transfections were performed by Lipofectamine (Invitrogen, San Diego, CA), and VD3 was added to the cultures 3 h after transfection. CAT activity in the cell cultures was determined by incubation of the cell extracts with [$^{14}C$]chloramphenicol, followed by separation of the acetylated and non-acetylated forms by thin-layer chromatography. Promoter activity was determined by computing the radioactivity in acetylated forms of chloramphenicol as a percentage of total radioactivity.

## Results and Discussion

First, we examined the effects of VD3 on Dsg3 mRNA expression. Incubation of the cultured cells with $10^{-8}$ M VD3 resulted in a significant time-dependent decrease in Dsg3 mRNA levels, whereas GAPDH mRNA expression, used as the control, was constant (Fig. 1A). After 24 h of incubation with VD3, Dsg3 gene expression was inhibited by ~75% after the mRNA steady-state levels had been corrected against their corresponding GAPDH levels (Fig. 1A). Inhibition of Dsg3 gene expression was moreover found to be dose-dependent; in the presence of 10-12 and 10-10 M VD3, expression was inhibited by 20 and 45%, respectively (Fig. 1B).

Next, to ascertain whether the VD3-elicited down-regulation of Dsg3 was dependent on ongoing protein synthesis, a similar inhibition study was performed in the presence and absence of cycloheximide (10 mg/ml). A 24-h incubation of cultured keratinocytes with VD3 ($10^{-8}$ M) in the absence of cycloheximide resulted in a reduction in Dsg3 mRNA levels (Fig. 2A). Incubation of parallel cultures with cycloheximide (10 mg/ml) alone resulted in very mild inhibition of Dsg3 mRNA levels after correction against GAPDH mRNA levels (Fig. 2A). However, cycloheximide treatment did not alter the inhibition rate of Dsg3 gene expression by VD3, suggesting that the down-regulation of Dsg3 expression is independent of active protein synthesis.

In addition to examining the potential mechanisms responsible for VD3-elicited down-regulation of Dsg3 gene expression detected at the mRNA level, transient transfection with Dsg3 promoter-CAT reporter gene constructs was carried out in cultured keratinocytes. The construct consisted of 2.3 kb of the 5'-flanking DNA of the Dsg3 gene. These plasmid constructs have previously been shown to possess active cis-elements conferring keratinocyte-specific expression of the gene (15). Transfection of control keratinocytes with the plasmid constructs led to the exhibition of strong promoter activities, detected by CAT assay (Fig. 2B). However, the addition of $10^{-8}$ M VD3 to parallel duplicate cultures failed to inhibit the promoter activities of the Dsg3 gene measured at 24 h after incubation (Fig. 2B). These results suggest that the 5'-flanking DNA of the Dsg3 gene does not contain VD3-dependent down-regulatory elements, and that the VD3-elicited inhibition of these genes might result from keratinocyte regulatory mechanisms operating at the post-transcriptional level.

In this study, we revealed the inhibitory effect of VD3 on the expression of the DSG3 gene in cultured human keratinocytes. However, VD3 is known to increase expression of involucrin in keratinocytes, and a recent detailed study revealed the partial mechanism of this effect (16). It is possible that VD3 has opposite effects on these two molecules, as DSG3 and involucrin are differentially-expressed in the lower and upper epidermis, respectively. VD3 increased expression of the transcription factor peroxisome proliferation-activated receptor (PPAR)γ, which regulates involucrin expression by controlling the AP-1 signal and p38 activation (16). This indicates that the up-regulation of involucrin expression may require at least the process of active protein synthesis. On the other hand, we clearly showed that down-regulation of Dsg3 expression was independent of active protein synthesis. Consequently, VD3 may have differential molecular mechanisms to modulate the expression of gene encoding protein in keratinocytes.

DSG3 has been found to be overexpressed in head and neck cancer, with the degree of overexpression associated with the clinicopathologic features of the tumor (17). Inhibition of DSG3 significantly suppresses carcinogenic potential in cellular and *in vivo* animal studies. Furthermore, a transgenic mouse study demonstrated that overexpression of DSG3 in the suprabasal area of the epidermis of transgenic mice resulted in hyperproliferation and abnormal differentiation (18). The significance of DSG3 in skin conditions suggests that the inhibitory effect had on it by VD3 plays a role in the pharmacological effect of VD3 in skin diseases. Furthermore, VD3 has been shown to reduce DSG3 expression though a simple pathway without either new transcription or protein synthesis being necessary. Thus, it might provide potential therapy for other skin diseases which require effective treatments.

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