Journal of Steroid Biochemistry & Molecular Biology 113 (2009) 9–16

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Characterization of the vitamin D endocrine system in human sebocytes *in vitro*

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ARTICLE INFO

Article history:
Received 5 October 2007
Received in revised form 6 October 2008
Accepted 21 October 2008

Keywords:
Vitamin D
Vitamin D receptor
Vitamin D-25-hydroxylase
25-Hydroxyvitamin D-1α-hydroxylase
1,25-Dihydroxyvitamin D-24-hydroxylase
Sebaceous gland
Cells cultured
Cell proliferation
Neutral and polar lipids
Interleukin-6
Interleukin-8

ABSTRACT

Sebocytes are sebum-producing cells that form the sebaceous glands. We investigated the role of sebocytes as target cells for vitamin D metabolites and the existence of an enzymatic machinery for the local synthesis and metabolism of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$, calcitriol], the biologically active vitamin D metabolite, in these cell types. Expression of vitamin D receptor (VDR), vitamin D-25-hydroxylase (25OHase), 25-hydroxyvitamin D-1α-hydroxylase (1αOHase), and 1,25-dihydroxyvitamin D-24-hydroxylase (24OHase) was detected in SZ95 sebocytes *in vitro* using real time quantitative polymerase chain reaction. Splice variants of 1αOHase were identified by nested touchdown polymerase chain reaction. We demonstrated that incubation of SZ95 sebocytes with 1,25$(OH)_2D_3$ resulted in a cell culture condition-, time-, and dose-dependent modulation of cell proliferation, cell cycle regulation, lipid content and interleukin-6/interleukin-8 secretion *in vitro*. RNA expression of VDR and 24OHase was upregulated along with vitamin D analogue treatment. Although several other splice variants of 1αOHase were detected, our findings indicate that the full length product represents the major 1αOHase gene product in SZ95 cells. In conclusion, SZ95 sebocytes express VDR and the enzymatic machinery to synthesize and metabolize biologically active vitamin D analogues. Sebocytes represent target cells for biologically active metabolites. Our findings indicate that the vitamin D endocrine system is of high importance for sebocyte function and physiology. We conclude that sebaceous glands represent potential targets for therapy with vitamin D analogues or for pharmacological modulation of 1,25$(OH)_2D_3$ synthesis/metabolism.

1. Introduction

Sebaceous lipogenesis leads to accumulation of lipid droplets and finally to cell content excretion in a holocrine manner. Excessive sebum production is supposed to be of crucial importance in the pathogenesis of acne [1]. Over the past decade, considerable progress has been made in our understanding of the molecular events regulating adipocyte differentiation. Several transcription factors, including CCAAT/enhancer binding proteins and peroxisome proliferator-activated receptors, which act cooperatively and sequentially to trigger the terminal differentiation program in cultured pre-adipocytes and sebocytes as well as in sebaceous gland cells *in vivo* have been identified [2–5]. However, the key molecular mechanisms that regulate the differentiation process in sebaceous gland cells still remain to be identified. In this study, we tested the hypothesis whether key components of the vitamin D endocrine system are present in human sebocytes and whether vitamin D analogues may regulate the activity of human sebocytes *in vitro*.

1,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$, calcitriol), the biologically active metabolite of vitamin D, has been shown to regulate the growth and multiple other biological functions in various cell types, including human keratinocytes [6,7]. This potent seco-steroid hormone acts via binding to a corresponding intranuclear receptor [vitamin D receptor (VDR)], present in target tissues. VDR belongs to the superfamily of trans-acting transcriptional regulatory factors, which includes the steroid and thyroid hormone receptors as well as the retinoid-X receptors and retinoic acid receptors [8]. It

Abbreviations: 1αOHase, 25-hydroxyvitamin D-1α-hydroxylase; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24OHase, 1,25-dihydroxyvitamin D-24-hydroxylase; 25OHase, vitamin D-25-hydroxylase; 25$(OH)D_3$, 25-hydroxyvitamin D_3; AFU, absolute fluorescence units; CV, crystal violet; FACS, Fluorescence-activated cell sorting; FCS, fetal calf serum; FDA, fluorescein diacetate; IL-6, interleukin-6; IL-8, interleukin-8; LDH, lactate dehydrogenase; MUH, 4-methylumbelliferyl heptanoate; PBS, phosphate-buffered saline without Ca^{2+} and Mg^{2+}; PCR, polymerase chain reaction; RTqPCR, real time quantitative polymerase chain reaction; VDR, vitamin D receptor.

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doi:10.1016/j.jsbmb.2008.10.010

has been demonstrated that VDR require heterodimerization with auxiliary proteins for effective DNA interaction. These auxiliary proteins have been identified as the retinoid-X receptors-α, -β, and -γ.

There are two principal enzymes involved in the formation of circulating $1,25(OH)_2D_3$ from vitamin D, the hepatic microsomal or mitochondrial vitamin D 25-hydroxylase (CYP27A1; 25OHase) and the renal mitochondrial enzyme 1α-hydroxylase (CYP27B1; 1αOHase) for vitamin D and 25-hydroxyvitamin D_3 [$25(OH)D_3$], respectively [7]. Metabolism of $1,25(OH)_2D_3$ is mediated via its principal catabolizing enzyme, 1,25-dihydroxyvitamin D 24-hydroxylase (CYP24A1, 24OHase). These hydroxylases belong to a class of proteins known as cytochrome P450 mixed function monooxidases. During recent years, extrarenal activity of 1αOHase has been reported in various cell types including macrophages, keratinocytes, prostate and colon cancer cells [9–11].

The aim of this study was to gain first insight into the function of the vitamin D endocrine system in sebaceous glands. We have analyzed effects of vitamin D analogues on SZ95 sebaceous gland cell proliferation, cell cycle regulation, and lipid content under different culture conditions *in vitro*. Additionally, we asked the question whether SZ95 sebocytes express VDR and/or the main enzymes involved in the synthesis/metabolism of $1,25(OH)_2D_3$, thereby identifying sebaceous glands as potential targets for therapy with vitamin D analogues or for pharmacological modulation of $1,25(OH)_2D_3$ synthesis/metabolism.

2. Materials and methods

2.1. Cell culture

Immortalized human facial SZ95 sebocytes, which have been shown to conserve the major characteristics of normal sebocytes [12], were maintained in Sebomed® Basal Medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS (Biochrom), 5 ng/ml human epidermal growth factor (Sigma, Deisenhofen, Germany), 50 μg/ml gentamicin (Gibco/BRL, Karlsruhe, Germany), and 1 mM Ca^{2+} in a humidified atmosphere of 5% CO_2 at 37 °C. Cell culture medium was changed every 2 days. SZ95 sebocytes were allowed to grow to about 70% confluence. For experiments under serum-free conditions, cells were cultured in Sebomed® medium containing 5 ng/ml human epidermal growth factor, 50 μg/ml gentamycin (Gibco/BRL), 10^{-6} M linoleic acid, 10 μg/ml insulin and 1 mg/ml bovine serum albumin (all from Sigma). The content of Ca^{2+} in Sebomed® medium amounts to 0.05 mM and basal medium supplemented with FCS amount to ~0.4 mM Ca^{2+}. SZ95 sebocytes were cultivated using 75 cm^2 cell culture flasks or 96 well plates (Nunc, Naperville IL). Semi-confluent cells were incubated for 120 h with or without $25(OH)D_3$, $1,25(OH)_2D_3$, or seocalcitol (EB 1089) at different doses (10^{-12} to 10^{-6} M). Cells were re-treated with or without vitamin D analogs every 24 h. Vitamin D analogues were kindly provided by Lise Binderup (Leo Pharmaceuticals, Ballerup, Denmark). Following incubation, the supernatants were collected for detection of protein/cytokine expression and the attached cells were used for lipid analysis, RNA extraction and proliferation.

2.2. Detection of cytotoxicity

SZ95 sebocytes were cultured in 96-well tissue culture plates at a density of 10,000 cells per well for 24 h. Fresh medium without or with active compounds was then given to the cells. The supernatants were collected 24 h later and were centrifuged to remove cell detritus, and 100 μl was proceeded to measurement of LDH release with a LDH assay kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. Experiments were performed in triplicate, with 5 wells evaluated for each data point in each experiment.

2.3. Analysis of cell proliferation

Cell proliferation was quantified by the MUH fluorescence assay [13] or by CV dye staining [14], as previously described. For MUH assay, cells were cultured in 96-well tissue culture plates at a density of 2000 cells per well for 2 days. The wells were then washed with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) and medium with or without active compounds was added. On the day of evaluation the medium was removed, the cells were washed twice with PBS, and 100 μl of a 100 μg/ml MUH solution in PBS was added to each well. The plates were then incubated at 37 °C for 30 min, and the released fluorescence, which is representative for cell numbers, were read on a Molecular Devices SPECTRAmax Gemini spectrofluorometer using 355 nm excitation and 460 nm emission filters. Experiments were performed in triplicate, with 10 wells evaluated for each data point in each experiment. For CV assay, cells were washed once with PBS and fixed with ethanol (70%) for 30 min at room temperature. Cells were then incubated with a CV solution (1%, w/v, in 20% ethanol) for 30 min at room temperature and rinsed with water thoroughly. After drying, the dye was extracted with 70% ethanol and its absorbance determined at 550 nm using a microplate reader.

2.4. Detection of lipid content

The cells were cultured in 96-well tissue culture plates at a density of 8000 cells per well for 2 days. The wells were then washed with PBS, and fresh medium was added. After 1 and 3 days, the supernatants were harvested. The wells were washed twice with PBS, and 100 μl of a 10 μg/ml Nile red solution in PBS was added to each well. The plates were then incubated at 37 °C for 20 min, and the released fluorescence was read on a Molecular Devices SPECTRAmax Gemini spectrofluorometer. The results are presented as percentages of the absolute fluorescence units (AFU) in comparison with the controls, using 485 nm excitation and 565 nm emission filters for neutral lipids and 540 nm excitation and 620 nm emission filters for polar lipids. Experiments were performed in triplicate, with 5 wells evaluated for each data point in each experiment. Cell numbers were measured indirectly by the fluorescein diacetate (FDA) assay (Sigma) as previously described [15]. Briefly, cells were stained with FDA, whereas fluorescence is dependent on cellular hydrolysis of the non-fluorescent substrate into its fluorescent product. Fluorescence signals from the two dyes were compared to give the amount of intact and viable cells in the culture. Untreated cultures served as controls. Results were considered biologically relevant at a statistical significance of $p < 0.05$ and at a mean difference of at least 10%.

2.5. Determination of IL-6 and IL-8

IL-6 and IL-8 were measured in the supernatants of challenged SZ95 sebocytes using Quantikine ELISA kits (R&D System, Wiesbaden, Germany) according to the manufacturer's protocols. Interleukin release was measured in triplicate wells for all individual treatments. The results are presented as relative fold increase compared with the results of the untreated controls at the same time, whereas the ratio represents the interleukin release of the controls.

2.6. RNA expression analysis

RNA isolation was carried out with RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturers manual. Expression of VDR, 25OHase, 1αOHase, and 24OHase was analyzed in SZ95 sebocytes using RTqPCR (LightCycler, 50 cycles) and gene-specific primers as published previously [16] (TIB Mol Biol, Berlin, Germany; Table 1). In order to calculate the normalized ratio, the relative amount of target gene (VDR, 25OHase, 1αOHase, or 24OHase) and a reference gene (β2-microglobulin) was determined for each sample and one calibrator, integrated in each Lightcycler run. The relative ratio of target to reference for each sample and for the calibrator was first calculated to correct sample to sample variations caused by differences in the initial quality and quantity of the nucleic acid (Roche, technical note LC 13/2001) The target/reference ratio of each sample was then divided by the target/reference ratio of the calibrator using relative quantification software (Relquant, Roche Molecular Biochemicals, Mannheim, Germany). This second step normalized different detection sensitivities of target and reference amplicons. Thus the normalization to a calibrator provided a constant calibrator point between PCR runs. Experiments were done in duplicates and final results, expressed as median of *N*-fold differences in target gene expression in treated samples relative to the control ones.

2.7. Analysis of 1αOHase splice variants by nested touchdown PCR

Prior to utilization, RNA was treated with DNaseI (Promega). The absence of genomic DNA was confirmed by Alu-PCR. First-strand cDNA was synthesized with Omniscript reverse transcriptase (Qiagen) using oligo-d$(T)_{15}$ primers. The existence of genomic DNA contaminants was excluded via RT-minus reaction. After the first PCR using primers Sp1aFor (5′-GGA GAA GCG CTT TCT TTC G-3′) and Sp1aRev3 (5′-AAA CCA GGC TAG GGC AGA TT-3′) with 10 cycles (10 s at 94 °C, 20 s at 62 °C, 4 s at 72 °C) the PCR reaction was purified (PCR purification kit; Qiagen) and 2 μM were used as template for the second PCR using primers Sp1aRev3 (5′-TGG GGC AAA CCC ACT TAA TA-3′) and HE1 (5′-CAG ACC CTC AAG TAC GCC-3′). This PCR consisted of first 12 cycles with a touchdown from 68 to 62 °C (10 s at 94 °C, 20 s at 68 °C with touchdown in 0.5 °C intervals to 62 °C, 4 s at 68 °C) followed by 18 cycles (10 s at 94 °C, 20 s at 62 °C, 4 s at 8 °C). Both PCR reactions were performed with RedAccuTaq (Sigma). The PCR products were separated on a 1% agarose gel.

2.8. Statistical analysis

All data are presented as mean ± S.E.M. for at least 3 experiments. Statistical significance was calculated by the Student's *t*-test. Mean differences were considered to be significant when $p < 0.05$. One-way analysis of variance (ANOVA) was used to assess statistical significance ($p < 0.05$) between means in vitamin D analogue-treated groups versus controls.

2.9. Immunohistochemical detection of VDR in human skin in situ

Freshly excised biopsies of normal skin ($n = 5$, healthy volunteers, no history of skin disease) were immediately embedded in OCT Tissue-Tek II (Miles Laboratories, Naperville, IL, USA) snap-frozen in liquid nitrogen, and stored at −80 °C. Serial sections (5 μm) were cut on a cryostat (Frigocut 2800, Reichert-Jung, Heidelberg, Germany) and mounted on pretreated glass slides. Pretreatment of slides with 2% aminopropylmethoxysilane (Sigma, München, Germany) in acetone for 5 min was performed to enhance sticking of sections during the staining procedure. Frozen sections to be stained for VDR were fixed in 3.7% paraformaldehyde (Merck 4005, Darmstadt, Germany) in phosphate buffered saline (PBS) for 10 min at room temperature (RT), incubated in methanol (Merck 6009, 3 min, −20 °C) and acetone (Merck 22, 1 min, −20 °C), and transferred into PBS. The rat monoclonal antibody 9A7γ (IgG_{2b}; MU 193-UC, BioGenex, CA, USA) was used as primary antibody. This mAb is directed against partially purified vitamin D receptor from chicken intestine and cross-reacts with human, mouse, and rat VDRs, but does not bind to glucocorticoid or estrogen receptors.

Incubation steps were performed in a moist chamber at RT. The slides were incubated with the mAb 9A7γ (16 h, 4 °C) at a dilution of 1:1000. After intermediate washing steps (PBS/TBS, 2× 5 min), the sections were incubated with biotin-labeled rabbit anti-rat IgG (DAKO) at a dilution of 1:400 (30 min, RT), and then with streptavidin–peroxidase complexes (DAKO) at a dilution of 1:400 (30 min, RT). After rinsing, the sections were incubated with 3-amino-9-ethylcarbazole (AEC, Sigma A 5754, München, Germany) as a substrate for the peroxidase reaction, transferred into tap water, and mounted with AquaTex (Merck). In control sections, primary antibody was replaced with polyclonal rat IgG (DAKO). No immunoreactivity was observed in these control sections.

2.10. Western analysis of VDR and 1αOHase in SZ 95 sebocytes

Western analysis was performed as published previously [17–19]. In short, cell lysates were subjected to 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Filters were blocked and incubated with primary antibody (mAb 9A7γ for VDR and pAb 462 for 1αOHase, 17-19) at a final dilution of 1:1000 (mAb 9A7γ) or 1:500 (pAb 462). Monoclonal anti-actin or anti-GAPDH antibodies (Sigma) were used in a 1:500 dilution as loading control.

3. Results

3.1. High pharmacologic doses of 1,25(OH)$_2$D$_3$ inhibit the growth of rapidly proliferating SZ95 sebocytes

We demonstrate that human sebocytes represent target cells for biologically active vitamin D metabolites. Incubation of rapidly proliferating SZ95 sebocytes [semi-confluent cells in the logarithmic phase of cell growth cultured with medium that contained fetal calf serum (FCS)] with 1,25(OH)$_2$D$_3$ (10^{-10} to 10^{-6} M) resulted in a dose-dependent and significant suppression of cell proliferation [up to approximately 30% after treatment with 10^{-7} M 1,25(OH)$_2$D$_3$ as detected using crystal violet (CV) dye staining], that was most pronounced after 120 h (Fig. 1). In additional experiments using a lactate dehydrogenease (LDH) assay, we had shown that no cytotoxicity occurs under these experimental conditions (data not shown).

3.2. The less calcemic vitamin D analogue seocalcitol (EB 1089) exerts antiproliferative effects on rapidly proliferating SZ95 sebocytes

Incubation of rapidly proliferating SZ95 sebocytes with EB 1089 (10^{-7} to 10^{-6} M) resulted in a dose-dependent and significant inhibition of cell proliferation (CV dye staining) that was most pronounced after 120 h (Fig. 1). At lower concentration (10^{-10} M), antiproliferative effects of EB 1089 were more pronounced as compared to 1,25(OH)$_2$D$_3$, while effects of both compounds were comparable at higher concentrations (10^{-7} to 10^{-6} M).

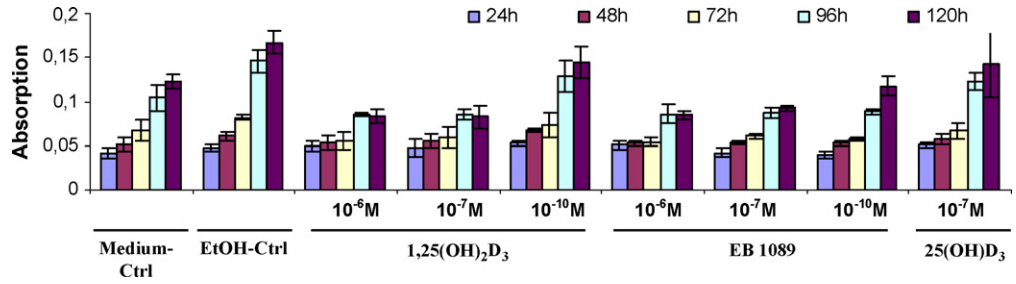

Fig. 1. Effect of vitamin D metabolites on the proliferation of rapidly proliferating SZ95 sebocytes. Proliferation analysis in SZ95 sebocytes treated with $1,25(OH)_2D_3$ (10^{-10} to 10^{-6} M), EB 1089 (10^{-10} to 10^{-6} M), or $25(OH)D_3$ (10^{-6} M). The strong inhibition of cell proliferation after treatment of sebocytes with $1,25(OH)_2D_3$ or EB 1089 in high pharmacological concentrations has to be noted.

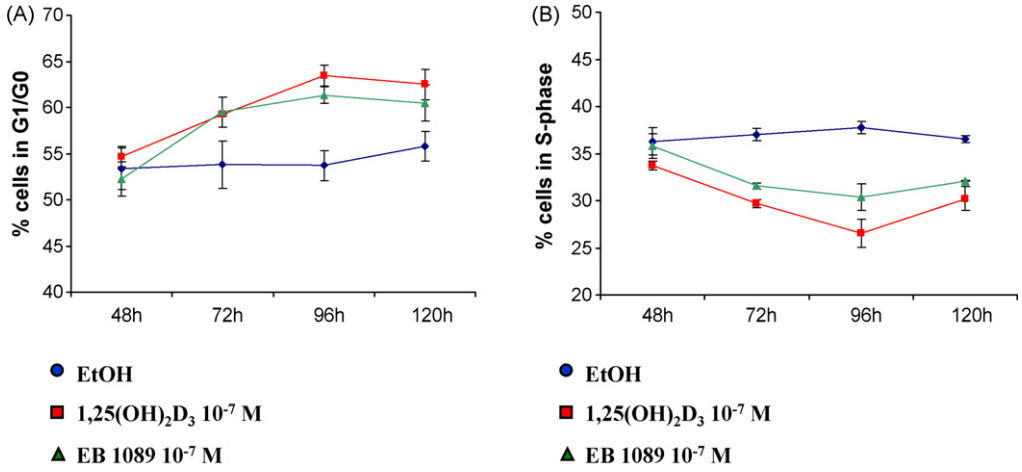

Fig. 2. Vitamin D analogues induce a G1 cycle arrest on SZ95 sebocytes. Flow cytometric cell cycle analysis in SZ95 sebocytes treated with $1,25(OH)_2D_3$ (10^{-10} to 10^{-6} M), EB 1089 (10^{-10} to 10^{-6} M), as compared to control (ethanol). Note increased percentage of cells in the G1-phase and reduced percentage of cells in the S-phase after treatment of SZ95 sebocytes with vitamin D analogues in high pharmacological concentrations.

3.3. 25(OH)D_3, the precursor of biologically active 1,25(OH)$_2D_3$, exerts at high dose (10^{-7} M) moderate antiproliferative effects on rapidly proliferating SZ95 sebocytes

Incubation of rapidly proliferating SZ95 sebocytes with $25(OH)D_3$ (10^{-7} M) resulted in a moderate suppression of cell proliferation (up to approximately 10% as detected using CV dye staining), that was most pronounced after 120 h (Fig. 1). This antiproliferative effect points at a stimulation of endogenous production of $1,25(OH)_2D_3$.

3.4. High pharmacologic doses of 1,25(OH)$_2D_3$ or its analogs induce an arrest of rapidly proliferating SZ95 sebocytes in the G1 phase

Flow cytometric cell cycle analysis of rapidly proliferating SZ95 sebocytes treated with $1,25(OH)_2D_3$ (10^{-10} to 10^{-6} M), EB 1089 (10^{-10} to 10^{-6} M), or $25(OH)D_3$ (10^{-7} M) revealed increased percentage of cells in the G1-phase after treatment of sebocytes with vitamin D analogues in high pharmacological concentrations (Fig. 2A and B).

3.5. 1,25(OH)$_2D_3$ stimulates the proliferation of slowly proliferating SZ95 sebocytes

Under serum free conditions (0.4 mM Ca^{2+}), incubation of slowly proliferating SZ95 sebocytes for 3 days with $1,25(OH)_2D_3$ (10^{-9} to 10^{-7} M) resulted in a dose-dependent and significant stimulation of cell proliferation, as measured by MUH assay. Note that incubation with 10^{-7} M calcitriol resulted in an approximately 100% increase in cell proliferation, as measured by 4-methylumbelliferyl heptanoate (MUH) assay (Fig. 3).

3.6. 1,25(OH)$_2D_3$ modulates the content of neutral and polar lipids in SZ95 sebocytes

Under serum free conditions (0.4 mM Ca^{2+}), incubation of slowly proliferating SZ95 sebocytes for 3 days with $1,25(OH)_2D_3$ (10^{-9} to 10^{-7} M) resulted in a time- and dose-dependent, statistically significant reduction of neutral and polar lipids in SZ95 sebocytes (Fig. 4).

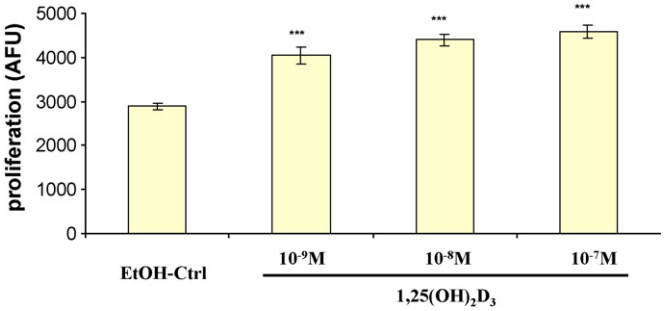

Fig. 3. Effect of vitamin D metabolites on the proliferation of slowly proliferating SZ95 sebocytes. Incubation with $1,25(OH)_2D_3$ (10^{-9} to 10^{-7} M; 3 days) stimulated the proliferation of slowly proliferating SZ95 sebocytes under serum free conditions (0.4 mM Ca^{2+}). Cell treatment with 10^{-7} M $1,25(OH)_2D_3$ resulted in an approximately 100% increase in cell proliferation, as measured by MUH assay.

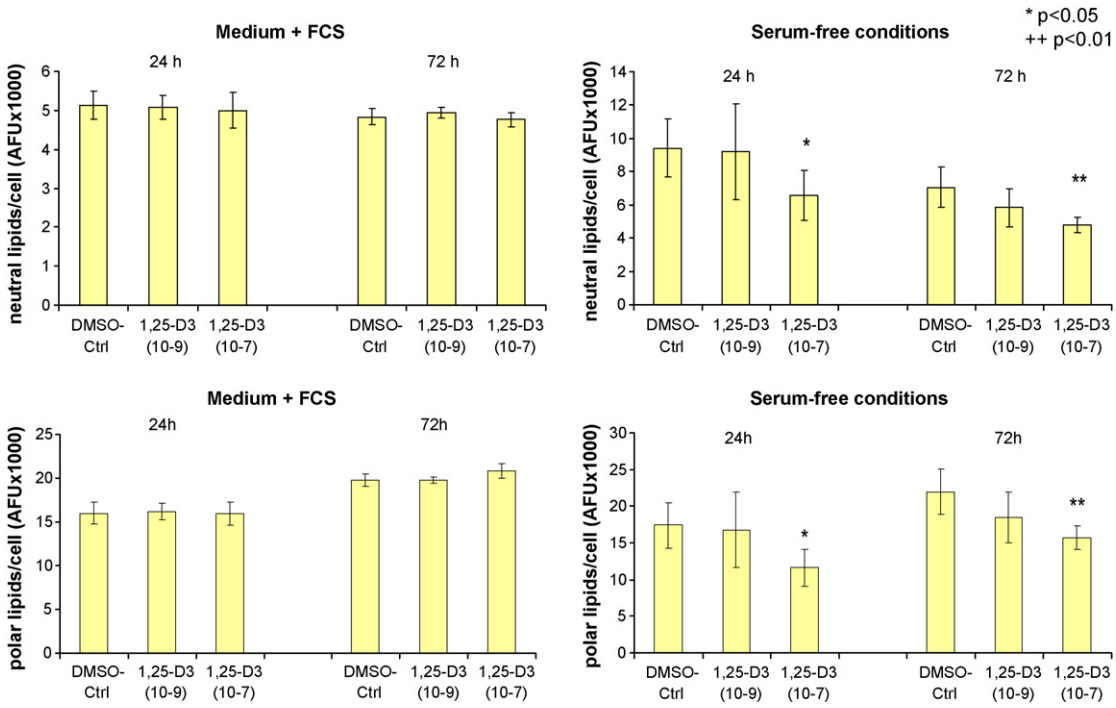

Fig. 4. $1,25(OH)_2D_3$ modulates content of neutral and polar lipids in SZ95 sebocytes. No effect on content of polar (A) and neutral (C) lipids was seen when cells were incubated with $1,25(OH)_2D_3$ and cultured with medium containing serum. In contrast, under serum free conditions (0.4 mM Ca^{2+}), incubation of slowly proliferating SZ95 sebocytes for 3 days with $1,25(OH)_2D_3$ (10^{-9} to 10^{-7} M) resulted in a time- and dose-dependent, significant reduction of polar (B) and neutral (D) lipids in SZ95 sebocytes.

In contrast, no effect on content of neutral and polar lipids was seen when cells were cultured with medium containing serum (Fig. 4).

3.7. $1,25(OH)_2D_3$ reduces secretion of interleukin-6 (IL-6) and interleukin-8 (IL-8) in supernatant of SZ95 sebocytes

Both under serum containing and under serum free (0.4 mM Ca^{2+}) conditions, incubation of SZ95 sebocytes for 24 h with $1,25(OH)_2D_3$ (10^{-7} M) resulted in a significant reduction of the cytokines IL-6 and IL-8 in supernatants of SZ95 sebocytes (Fig. 5).

3.8. Key components of the vitamin D system are expressed in human sebocytes and are regulated by $1,25(OH)_2D_3$ and its analogues

As shown by real-time quantitative polymerase chain reaction (RTqPCR), RNA for VDR, 25OHase, 1αOHase, and 24OHase is present in SZ95 sebocytes. Levels of 24OHase RNA are higher and of 25OHase RNA are lower as compared to various melanoma cell lines (Fig. 6). After treatment with $1,25(OH)_2D_3$ the RNA expression of VDR and 24OHase was increased, while expression of 1αOHase was not suppressed (Fig. 7).

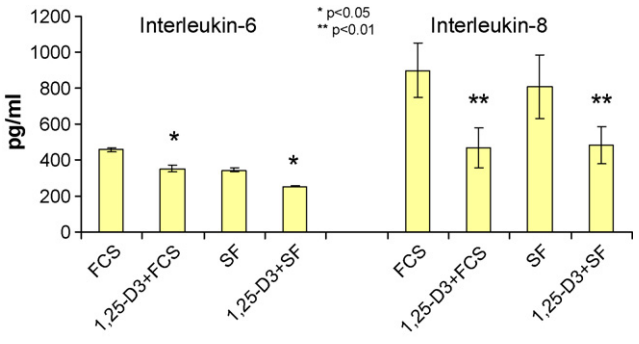

Fig. 5. $1,25(OH)_2D_3$ reduces content of IL-6 and IL-8 in supernatant of SZ95 sebocytes. Both under serum containing and under serum free (0.4 mM Ca^{2+}) conditions, incubation of SZ95 sebocytes for 24 h with $1,25(OH)_2D_3$ (10^{-7} M) resulted in a significant reduction of the cytokines IL-6 and IL-8 in supernatants of SZ95 sebocytes.

3.9. Different alternate splice variants of the 1αOHase gene in human sebocytes

We have previously reported partial PCR-amplification of the 1αOHase cDNA including variants that lack exons 4 and 5 in various tissues and cell lines [17–19]. This approach was, however, restricted to identification of truncated 1αOHase splice variants. To adequately describe the full length expression of 1αOHase gene, we now developed a highly specific approach that combined nested and touchdown polymerase chain reaction. Using this method, we here demonstrate the expression of the normal enzyme (2.15 kb) as the major 1αOHase gene product in human SZ95 sebocytes (Fig. 8). In addition, we here describe the presence of several other splice variants than the previously reported variants at 1.78 kb (Hyd-V2), 1.97 kb (Hyd-V3), and 2.23 kb (Hyd-V4). Using specific primers from exons 1 and 9, we detected additional PCR products (1–4 kb).

3.10. Detection of VDR in human skin immunohistochemically in situ and western analysis of VDR and 1αOHase in cultured human sebocytes in situ

To confirm our RNA in vitro data on the protein level *in situ*, VDR was detected immunohistochemically in normal human skin. Strong nuclear immunoreactivity was detected in a majority of sebaceous gland cells and in epidermal keratinocytes in all cell layers of the viable epidermis (Fig. 9). This staining pattern was comparable to results that we and others have reported previously ([6], rev. in [7]). Additionally, we detected VDR protein expression that was induced by treatment with $1,25(OH)_2D_3$ and several

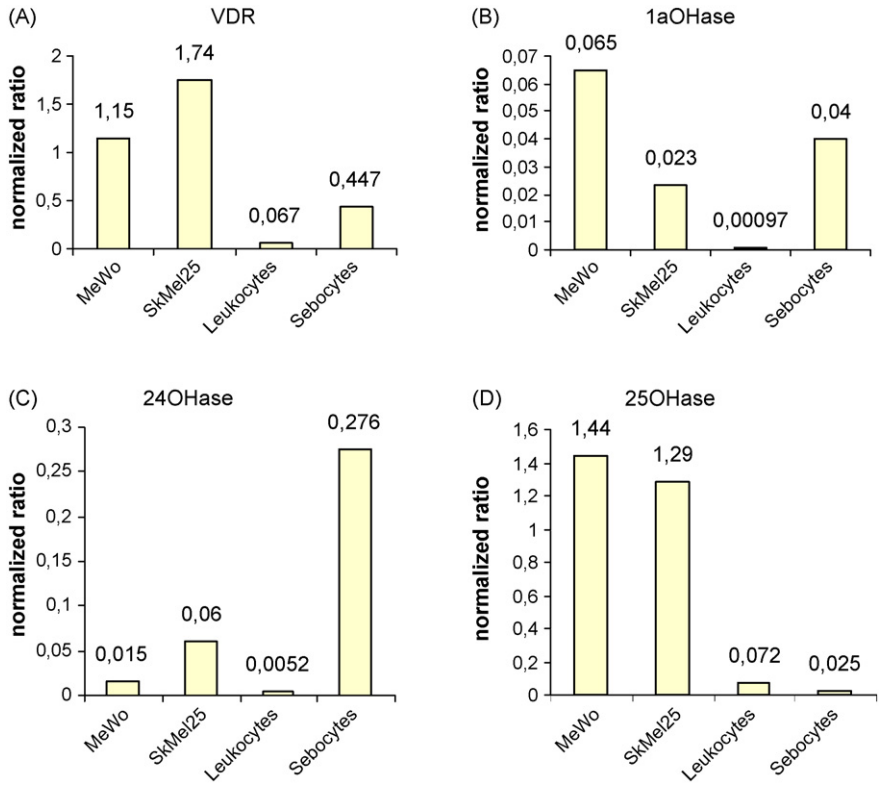

Fig. 6. Basal RNA levels of VDR and vitamin D metabolizing enzymes in SZ95 sebocytes. (A) VDR, (B) 1αOHase, (C) 24OHase and (D) 25OHase in cultured human SZ95 sebocytes, melanoma cells (MeWo, SkMel25) and leukocytes.

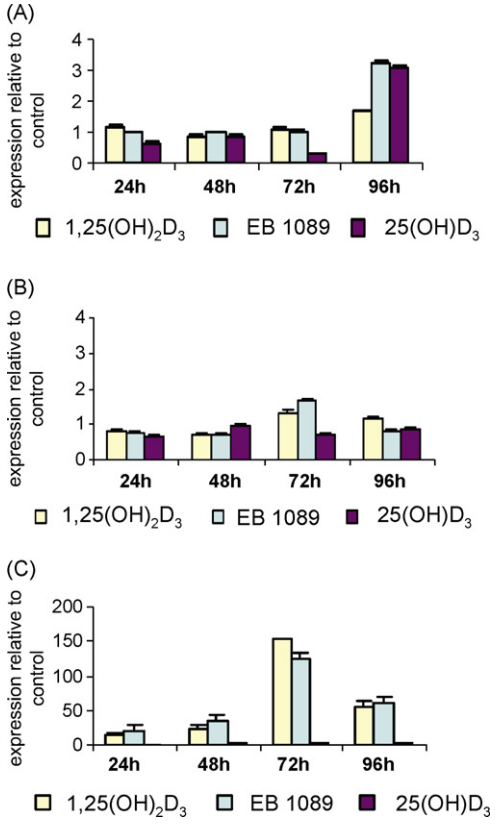

Fig. 7. RNA levels of VDR and vitamin D metabolizing enzymes in SZ95 sebocytes under treatment with vitamin D analogues. (A) VDR, (B) 1αOHase and (C) 24OHase after treatment with $1,25(OH)_2D_3$, EB1089 or $25(OH)D_3$ (10^{-7} M).

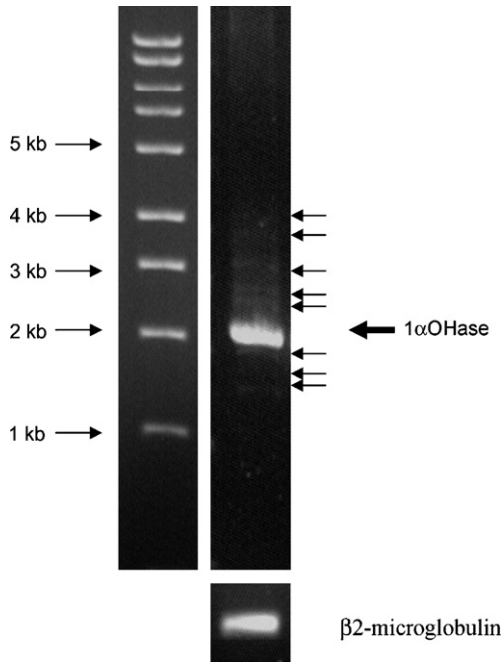

Fig. 8. Expression analysis of the 1αOHase gene in SZ95 sebocytes by nested-touchdown PCR. The full length product represents the predominant variant of the 1αOHase gene in SZ95 sebocytes. Note that weak expression of some additional splicing variants can be detected.

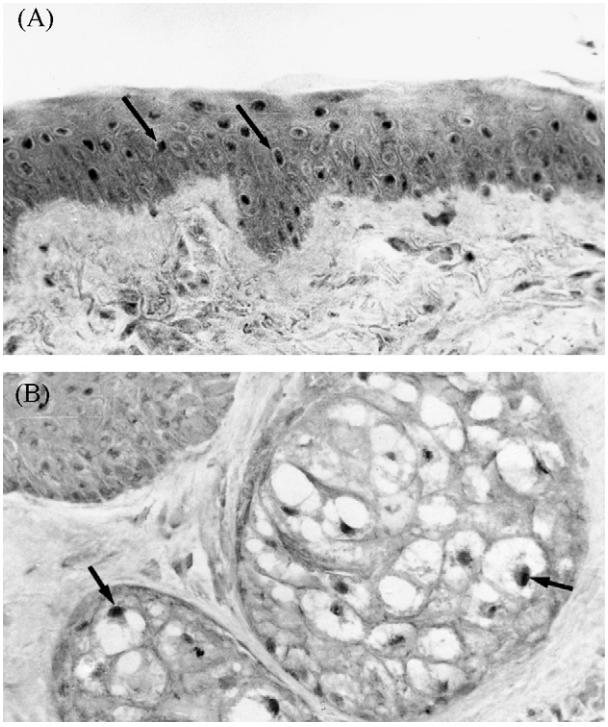

Fig. 9. Immunohistochemical detection of VDR in human skin. Strong nuclear immunoreactivity can be seen in a majority of sebaceous gland cells (A, arrow) and in epidermal keratinocytes in all cell layers of the viable epidermis (B, arrow).

protein variants of 1αOHase in cultured SZ95 sebocytes *in vitro* (Fig. 10).

4. Discussion

In conclusion, our results identify the sebaceous gland cells as a new target for vitamin D. The findings indicate that vitamin D analogues modify the activity of human sebaceous gland cells and may, therefore, represent promising compounds for the treatment of sebaceous gland disorders, especially for acne. We detected RNA and protein expression of VDR in cultured human SZ95 sebocytes and demonstrated that $1,25(OH)_2D_3$ and a biologically active vitamin D analogue at high pharmacologic concentrations inhibit the growth of rapidly proliferating SZ95 sebocytes *in vitro*. In addition, we demonstrated that the inhibition of cell proliferation is, at least in part, mediated via an arrest of sebocytes in the G1-phase, which is most likely induced by VDR-mediated genomic effects. Interestingly, stimulation with $1,25(OH)_2D_3$ exerted a biphasic effect on SZ95 sebocyte proliferation that was dependent on the presence of serum (and Ca^{2+} levels). In contrast to rapidly proliferating ones, the proliferation of slowly proliferating SZ95 sebocytes cultured under serum-free conditions was stimulated by $1,25(OH)_2D_3$. Differential effects of $1,25(OH)_2D_3$ on cell proliferation, that may depend on dose or cell culture conditions, have been shown for keratinocytes and other cell types previously [20,21]. Interestingly, such a biphasic effect on human sebocytes has also been demonstrated for vitamin A and retinoids [22].

On the other hand, induction of differentiation of human sebocytes leads to an increase in the content of polar and neutral lipids [23]. Interestingly, incubation of slowly proliferating SZ95 sebocytes with $1,25(OH)_2D_3$ under serum-free conditions (0.4 mM Ca^{2+}) resulted in a time- and dose-dependent reduction of neutral and polar lipids. These findings indicate that $1,25(OH)_2D_3$-induced stimulation of SZ95 sebocyte proliferation is associated with inhibition of sebocyte differentiation. In contrast, no effect on cellular lipid content was detected when cells were cultured with medium containing serum indicating that $1,25(OH)_2D_3$-induced inhibition of SZ95 sebocyte proliferation is not associated with induction of sebocyte differentiation. In contrast, retinoic acid-induced inhibition of cell proliferation has been shown to be associated with induction of sebocyte differentiation [22,24].

Both under serum containing and serum-free conditions, incubation of SZ95 sebocytes with $1,25(OH)_2D_3$ resulted in a significant reduction of cytokine IL-6 and IL-8 secretion. These findings indicate that $1,25(OH)_2D_3$ exerts a potent anti-inflammatory effect on human sebocytes and suggest that vitamin D analogues may be effective in the treatment of acne, which has currently been established as an inflammatory disease [25,26].

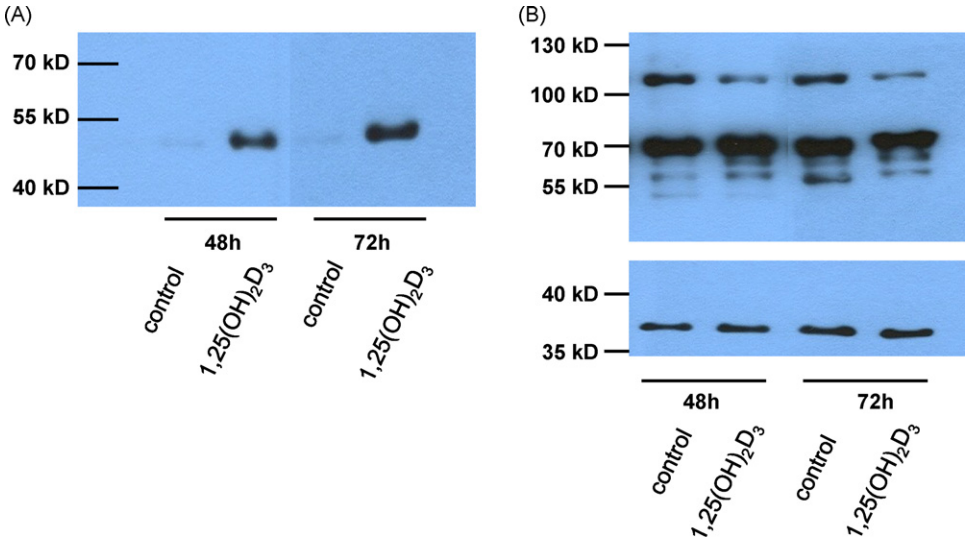

Fig. 10. Detection of VDR and 1αOHase in cultured human sebocytes by western analysis in situ. (A) VDR protein expression in SZ95 sebocytes is demonstrated as a characteristic band at 52 kD, using mAb 9A7γ as primary antibody. VDR protein expression was induced by treatment with $1,25(OH)_2D_3$ (10^{-7} M) after 48 (lane 2) and 72 (lane 4) hrs as compared to respective untreated controls (lanes 1 and 3). (B). Several protein variants of 1αOHase (B) can be detected by western analysis using pAb 462 in cultured SZ95 sebocytes *in vitro*. Intensity of individual bands was only marginally altered following treatment with $1,25(OH)_2D_3$ (10^{-7} M) after 48 h (lane 2) and 72 h (lane 4) as compared to respective untreated controls (lanes 1 and 3). GAPDH served as loading control.

Moreover, we demonstrated that human sebocytes possess the complete enzymatic machinery for the extrarenal production of $1,25(OH)_2D_3$ from vitamin D or $25(OH)D_3$, as it has already been published for other cell types [27–29]. We detected low mRNA levels of 25OHase and high ones of 1αOHase in SZ95 sebocytes using RTqPCR. Therefore, an autocrine or paracrine production of $1,25(OH)_2D_3$ is likely to represent a key regulator of proliferation and differentiation of sebaceous gland cells.

Another finding of clinical relevance is the high mRNA levels of 24OHase in SZ95 sebocytes, the enzyme been responsible for $1,25(OH)_2D_3$ metabolism. The mRNA levels of 24OHase and VDR were regulated in a time- and dose-dependent manner under treatment with $1,25(OH)_2D_3$ or EB 1089. In various cell types including human keratinocytes, $1,25(OH)_2D_3$ has been shown to enhance VDR expression at the mRNA and protein levels *in vitro* [30,31]. The increase of VDR protein levels following $1,25(OH)_2D_3$ administration is due to an increased transcription of the VDR gene and/or an increased receptor protein lifetime [31]. In contrast to the expression of 24OHase that abrogates $1,25(OH)_2D_3$-mediated growth control, strong expression of functionally intact 25OHase and 1αOHase results in synthesis of biologically active $1,25(OH)_2D_3$ and should thereby exert anti-proliferative and prodifferentiating effects. Additionally, the expression of 1αOHase and 25OHase genes in SZ95 sebocytes indicates that precursors of biologically active $1,25(OH)_2D_3$ may be used for the prevention or treatment of acne and other sebaceous gland-associated disorders.

In conclusion, our findings demonstrate that local synthesis or metabolism of vitamin D metabolites may be of importance for various cellular functions of sebaceous gland cells including growth regulation. Sebaceous glands represent promising targets for therapy with vitamin D analogues or for pharmacological modulation of $1,25(OH)_2D_3$ synthesis/metabolism.

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