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

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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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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.