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Original article

# Physiological and clinically attainable concentrations of 1,25-dihydroxyvitamin $D_3$ suppress proliferation and extracellular matrix protein expression in mouse pancreatic stellate cells

Merja Bläuer [a], Juhani Sand [a, b], Johanna Laukkarinen [a, b, *]

[a] Tampere Pancreas Laboratory, Tampere University Hospital, Teiskontie 35, FIN-33521 Tampere, Finland
[b] Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Teiskontie 35, FIN-33521 Tampere, Finland

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ABSTRACT

*Background/objectives:* Vitamin D is an antiproliferative and differentiation-promoting secosteroid hormone with pleiotropic homeostatic functions in bone and extraskeletal tissues. Signaling of vitamin D is mediated via its ubiquitously expressed nuclear receptor, the vitamin D receptor (VDR). Pancreatic stellate cells have recently been identified as targets of vitamin D action. Our aim was to elucidate the effectiveness of the most potent endogenous vitamin D metabolite, 1,25-dihydroxyvitamin $D_3$ [$1,25(OH)_2D_3$] on the proliferation and extracellular matrix (ECM) protein expression in pancreatic stellate cells (PSCs) using concentrations of the compound from the physiological and clinically attainable range in humans.

*Methods:* Culture-activated mouse PSCs were exposed to $1,25(OH)_2D_3$ concentrations ranging from 0.1 nM to 10 nM for 7 days and subjected to colorimetric crystal violet assay for cell growth assessment and to Western blot and immunohistochemical analyses of VDR, fibronectin and collagen I using protein-specific antibodies. Immunohistochemical localization of VDR was performed on mouse pancreatic tissue and on a set of human specimens obtained at pancreatic surgery.

*Results:* A low basal level of VDR was detected in PSCs that was strongly induced in the presence of ligand. Cell growth was suppressed dose-dependently by $1,25(OH)_2D_3$, the mean percentages of inhibition ranging from 24% at the physiological 0.1 nM concentration to around 60% at 10 nM. Significant 48% and 40% reductions in fibronectin expression were seen at 0.5 nM and 1 nM $1,25(OH)_2D_3$. A minor decrease in collagen I expression was detected at 5 nM. VDR was predominantly localized in the islets of Langerhans in mouse and human tissues. In the latter VDR was expressed also in the exocrine tissue showing individual variation in its cellular distribution.

*Conclusions:* Mouse PSCs express VDR protein and are sensitive $1,25(OH)_2D_3$ target cells with low levels of $1,25(OH)_2D_3$ exerting antiproliferative and antifibrotic effects on activated PSCs *in vitro*.


## Introduction

The role of pancreatic stellate cells (PSCs) as key modulators of the extracellular matrix (ECM) in pancreatic tissue is well established [1]. In the healthy pancreas PSCs help to maintain a balanced turnover of the ECM through their ability to synthesize and secrete ECM proteins as well as matrix metalloproteinases and their inhibitors. PSCs respond to tissue injury by undergoing a transition from a quiescent phenotype to an activated myofibroblast-like stage characterized by a loss of the retinoid-containing lipid droplets of quiescent cells and an increased potential to proliferate and to express ECM proteins. Persistent PSC activation leading to dysregulated synthesis and accumulation of ECM proteins is the major cause of the development of fibrosis in chronic pancreatitis and pancreatic cancer. PSC-mediated fibrogenesis is known to be directly involved in the progression of these diseases, proposing the fibrogenic process as a potential target for therapeutic strategies [1,2].

* Corresponding author. Tampere Pancreas Laboratory, Tampere University Hospital, Teiskontie 35, FIN-33521 Tampere, Finland. Tel.: +358 3 311611; fax: +358 3 3116 4358.
*E-mail address:* johanna.laukkarinen@fimnet.fi (J. Laukkarinen).

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Candidates for potential treatment modalities to alleviate pancreatic fibrosis have emerged from the group of fat-soluble vitamins. Consistent with the idea of restoring PSC quiescence by replenishing the retinoid stores lost upon activation, vitamin A and its metabolites have been shown to efficiently deactivate PSCs *in vitro* [3–5]. Experimental evidence has also been provided for the ability of members of the vitamin E family to induce apoptosis and autophagy in activated PSCs, suggesting targeted cell death as a possible means to limit fibrosis [6].

In contrast to vitamins A and E, the primary target for vitamin D action in the pancreas has previously been considered to be the endocrine compartment of the tissue [7,8]. As we were running the investigations presented here, however, Sherman et al. [9] reported a high level of vitamin D receptor (VDR) mRNA expression in mouse and human PSCs and showed a marked reduction in markers of inflammation and fibrosis in pancreatitis and human tumor stroma after treatment with the synthetic VDR ligand, calcipotriol.

Vitamin D is a pluripotent secosteroid hormone produced by sunlight-induced conversion of 7-dehydroxycholesterol to vitamin $D_3$ and its successive hydroxylations to $25(OH)D_3$ and the most potent endogenous metabolite $1,25(OH)_2D_3$ in the liver and the kidney [10]. Besides its well-known role in maintaining calcium homeostasis in bone, a diversity of effects have been ascribed to $1,25(OH)_2D_3$ in a vast range of VDR-expressing target tissues because of its ability to modulate master regulatory networks involving cell proliferation, differentiation and apoptosis [10,11]. Physiological levels of $1,25(OH)_2D_3$ in humans range from 0.05 to 0.16 nM, while clinical studies have demonstrated that under a weekly administration schedule, $1,25(OH)_2D_3$ may reach peak blood levels of 3–16 nM with little toxicity [12–14].

Regulation of PSC behavior by the natural VDR ligand $1,25(OH)_2D_3$ has not been previously examined. In the present study we have investigated the effects of physiological and clinically attainable levels (0.1–10 nM) of $1,25(OH)_2D_3$ on mouse PSCs that were maintained in activation-promoting culture conditions. Our data show that cultured mouse PSCs express the VDR protein and respond to low doses of ligand with significant growth inhibition and reduction in ECM protein expression. In an additional immunohistochemical study VDR was localized in the endocrine compartment of mouse and human pancreatic tissue samples and in some human specimens also VDR-positive acinar and stromal cells could be detected. Our data support the newly discovered role of PSCs as vitamin D target cells.

## Materials and methods

### *Primary PSC culture*

PSCs were obtained from healthy mice of the strain c57BL/6JOlaHsd (Harlan, the Netherlands) by the explant outgrowth method [15,16]. The use of mice as donors of pancreatic tissue for cell culture purposes was approved by the Institutional Animal Welfare Committee.

Whole mouse pancreata were minced into small pieces that were pipetted into T-75 culture bottles in 7 ml culture medium consisting of DMEM/F12 supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (P/S) and L-glutamine. All culture reagents were from Invitrogen, Grand Island, NY. After 7–9 days of culture the outgrown PSCs were collected, reseeded in T-75 bottles and propagated to near confluence before passaging. After two 1:3 passages the cultures were detached and cryopreserved in liquid nitrogen. The isolation method was standardized so as to produce PSCs of high purity. The obtained cells displayed the characteristic stellate-like or spindle-shaped morphology of PSCs with long cytoplasmic extensions clearly visible in low-density cultures. Translucent cytoplasmic lipid droplets could be seen in primary out-migrating cells. After extended culture on plastic, α-smooth muscle actin expression typical of activated PSCs was detected. Vitamin D experiments were performed in phenol red-free DMEM-F12 supplemented with 10% dextran-coated-charcoal-treated FCS (DCC-FCS), P/S and L-glutamine with cells at passage numbers 4–6.

### *Cell growth assay*

Stellate cells were seeded in 96-well culture plates at 1000 cells/well and allowed to attach for 72 h prior to medium change and addition of vehicle or 0.1, 0.5, 1, 2, 5, and 10 nM concentrations of $1,25(OH)_2D_3$ (Sigma–Aldrich, St. Louis, MO) in octuplicate. The plates were incubated for 7 days with medium renewal at days 2 and 4. Relative cell numbers in three independent experiments were determined at day 7 using the colorimetric crystal violet assay as previously described [17].

### *SDS-PAGE and Western blot*

For Western blot analyses PSCs were seeded in 6-well plates at 30 000 cells/well. After three days the medium was replaced and the cells were subjected to treatment with $1,25(OH)_2D_3$ as above. The cells were lysed with Mammalian Protein Extraction Reagent (Thermo-Scientific, Rockford, IL) supplemented with protease inhibitors (Complete Mini Protease Inhibitory Cocktail Tablets; Roche Diagnostics GmbH, IN) according to the protocol of the manufacturer. The lysates were centrifuged at 12 000 g for 5 min at 4 °C and protein concentrations of the supernatants were determined using BCA Protein Assay (Thermo-Scientific).

Thirty μg of total protein were mixed 1:1 with 2× Laemmli sample buffer (Sigma–Aldrich), boiled for 5 min and separated in 7.5% polyacrylamide gels (PAGE). The separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes with a pore size of 0.2 μm (Macherey–Nagel GmbH, Düren, Germany) at room temperature using NuPage transfer buffer (Invitrogen, Carlsbad, CA). The membranes were blocked with TBS Blotto A (Santa Cruz Biotechnology, Dallas, TX) and probed with primary antibodies against VDR (1:1000; Cell Signaling Technology, Danvers, MA), fibronectin (1:2000; Abcam, Cambridge UK) and collagen I (1:500; Novus Biologicals, Cambridge, UK), followed by horseradish peroxidase-conjugated secondary antibody (HRP-linked anti-Rabbit IgG; Cell Signaling Technology). The antigens were detected with enhanced chemiluminescence (ECL) using the ECL Western Blotting Reagents of GE Healthcare, Buckinghamshire, UK. Densitometric analysis of the immunoreactive protein bands was performed using the ImageJ software (provided by the National Institutes of Health, Bethesda, MD). Protein densities were equalized with reference to β-actin (Sigma–Aldrich).

### *Immunocytochemistry*

Immunocytochemical analysis of VDR, fibronectin and collagen I expression was performed using their corresponding antibodies at 1:200 dilutions. Anti-mouse Ki67 (1:200; Dako, Glostrup, Denmark) was employed to identify cells at active phases of proliferation. For analyses, PSCs were grown on coverslips placed in the wells of 24-well culture plates (1500 cells per well) and subjected to 0, 0.1, 1 and 10 nM $1,25(OH)_2D_3$ for 7 days. Procedures for cell fixation and staining were as described in Bläuer et al. [17].

Tissue localization of VDR immunoreactivity was studied by staining paraffin-embedded mouse and human pancreatic tissue samples after antigen unmasking in citrate buffer (pH 6.0). Healthy mouse tissue and a preliminary set of five human specimens obtained at pancreatic surgery were analyzed. The use of human

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