Antifibrogenic effects of vitamin D derivatives on mouse pancreatic stellate cells

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AIM
To study the molecular effects of three different D-vitamins, vitamin D2, vitamin D3 and calcipotriol, in pancreatic stellate cells (PSCs).

METHODS
Quiescent PSCs were isolated from mouse pancreas and activated in vitro by seeding on plastic surfaces. The cells were exposed to D-vitamins as primary cultures (early-activated PSCs) and upon re-culturing (fully-activated cells). Exhibition of vitamin A-containing lipid droplets was visualized by oil-red staining. Expression of \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), a marker of PSC activation, was monitored by immunofluorescence and immunoblot analysis. The rate of DNA synthesis was quantified by 5-bromo-2'-deoxyuridine (BrdU) incorporation assays. Real-time PCR was employed to monitor gene expression, and protein levels of interleukin-6 (IL-6) were measured by ELISA. Uptake of proline was determined using \( ^{18}F \)-proline.
cell proliferation, loss of lipid droplets and exhibition of stress fibers, indicating cell activation. When added to PSCs in primary culture, all three D-vitamins diminished expression of α-SMA (to 32%-39% of the level of control cells; P < 0.05) and increased the storage of lipids (scores from 1.97-2.15 on a scale from 0-3; controls: 1.49; P < 0.05). No such effects were observed when D-vitamins were added to fully-activated cells, while incorporation of BrdU remained unaffected under both experimental conditions. Treatment of re-cultured PSCs with D-vitamins was associated with lower expression of IL-6 (-42% to -49%; P < 0.05; also confirmed at the protein level) and increased expression of the vitamin D receptor gene (209%-321% vs controls; P < 0.05). There was no effect of D-vitamins on the expression of transforming growth factor-β1 and collagen type 1 (chain α1). The lowest uptake of proline, a main component of collagen, was observed in calcipotriol-treated PSCs.

CONCLUSION
The three D-vitamins inhibit, with similar efficiencies, activation of PSCs in vitro, but cannot reverse the phenotype once the cells are fully activated.

Key words: Pancreatic stellate cells; Fibrosis; Vitamin D2; Vitamin D3; Calcipotriol

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Core tip: Modulation of the stroma response by vitamin D has been suggested as a concept to treat chronic pancreatitis and pancreatic cancer. Here we show that three derivatives, vitamin D2, vitamin D3 and calcipotriol, with similar efficiencies prevented pancreatic stellate cell (PSC) activation in vitro. Once the cells were fully activated, vitamin D failed to induce a reversal of the myofibroblastic phenotype, but still exerted antifibrotic effects by diminishing the uptake of proline and secretion of interleukin-6, an autocrine mediator of PSC activation. Our findings encourage further studies on the potential of vitamin D derivatives as antifibrotic drugs.

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INTRODUCTION
Pancreatic stellate cells (PSCs) were identified as the main source of extracellular matrix (ECM) proteins in the diseased pancreas, specifically in the context of cancer and chronic pancreatitis (CP), two decades ago. Since then, cellular interactions of PSCs and extracellular as well as intracellular regulators of PSC function have been studied in great detail (reported by Erkan et al[1]). In the healthy pancreas, PSCs exist in a quiescent state and are phenotypically characterized by the presence of abundant vitamin A-containing lipid droplets in their cytoplasm[2,3]. In response to mitogens (such as platelet-derived growth factor) and profibrogenic mediators (e.g., transforming growth factor-β1), PSCs undergo an activation process that involves cell proliferation and an enhanced synthesis of ECM proteins[4]. The cells also lose their vitamin A storages and exhibit stress fibers containing α-smooth muscle actin (α-SMA) protein. In vitro, contact of PSCs with plastic surfaces induces phenotypic changes that mimic the activation process under in vivo conditions[2,3].

In the past years, it has become clear that PSCs play an active and complex role in the progression of pancreatic cancer (PC), a stroma-rich tumor with the worst prognosis of all common human malignancies[5-8]. Most recent publications in the field have shifted the main focus of attention from a stroma deletion, which even displayed deleterious effects in experimental models of PC[9,10], towards a modulation of the stroma response, with the aim to interrupt tumor-promoting interactions between PSCs and PC cells on one hand while preserving the barrier function of the stroma on the other hand[11]. In this regard, vitamin D and its receptor VDR have gained significant interest as master regulators of PSC function and mediators of a transcriptional reprogramming of the tumor stroma that enable an enhanced chemotherapeutic response[12]. Recently, suppression of cell proliferation and inhibition of ECM synthesis have been proposed as important mechanisms of vitamin D action in PSCs[13]. Nevertheless, to this end the precise molecular actions of vitamin D in stellate cells are not fully understood. Furthermore, relative efficiencies of different vitamin D derivatives have not been studied yet.

Here, we have compared the effects of vitamin D2, vitamin D3 and the synthetic vitamin D3 derivative calcipotriol on primary cultures of originally quiescent PSC as well as on fully-activated re-cultured cells. All D-vitamins inhibited PSC activation, but did not reverse the phenotype once the cells were fully activated. Interleukin-6 (IL-6) was identified as an important target of vitamin D action. Although the three D-vitamins displayed slightly different molecular effects in our studies, their general efficiency was found to be similar.

MATERIALS AND METHODS

Cell culture
Quiescent PSCs were isolated from the pancreas of healthy C57BL/6 mice (approximately 3 months old) by collagenase digestion followed by Nycodenz® (Nycomed, Oslo, Norway) density gradient centrifugation[14]. Afterwards, they were resuspended in cryopreservation
medium [fetal calf serum (FCS) supplemented with
10% dimethyl sulfoxide] and stored at -150 °C until
required as previously described[15]. After thawing,
the cells were cultured in Iscove's modified Dulbecco's
medium supplemented with 17% FCS, 1% non-
essential amino acids (dilution of a 100 × stock
solution), 105 U/L penicillin and 100 mg/L streptomycin
(all reagents from Merck Millipore, Darmstadt,
Germany). Approximately on day 3 of primary culture,
PSCs started to proliferate and reached subconfluency
around day 8. Afterwards, the cells were harvested by
trypsinization and re-cultured according to the
experimental requirements.

**Histochemical staining of intracellular fat**

PSCs were grown on glass coverslips as primary
cultures or cells of the first passage for the indicated
periods of time. Subsequently, they were fixed for
30 min in 2.5% paraformaldehyde, and intracellular
fat droplets were visualized by oil red O staining as described before[16]. Briefly, the coverslips were incubated with dye solution [three parts of an oil red O stock solution (1% wt/vol dissolved in isopropanol, mixed with two parts of distilled water)] for 15 min followed by counterstaining with Mayer’s hemalum solution (Merck Millipore). The samples were evaluated by light microscopy and assessed, in a blinded manner, by two independent investigators on a semiquantitative scale from 0 (absence of lipid droplets) to 3 (large and numerous lipid droplets).

**Quantification of DNA synthesis**

DNA synthesis was quantified employing the 5-bromo-2′-deoxyuridine (BrdU) labelling and detection enzyme-linked immunosorbent assay kit (Roche Diagnostics, Mannheim, Germany). Therefore, quiescent or activated (proliferating) PSCs were plated in 96-well plates at equal seeding densities and allowed to adhere overnight. Afterwards, the cells were exposed to vitamin D2, vitamin D3 (both from Santa Cruz Biotechnologies, Heidelberg, Germany) or calcipotriol (Sigma-Aldrich, Deisenhofen, Germany) for the indicated periods of time. Twenty-four hours prior to cell harvesting, BrdU labelling was initiated by adding labelling solution at a final concentration of 10 μmol/L. Afterwards, labelling was stopped, and BrdU uptake was measured according to the manufacturer’s instructions.

**Immunoblotting**

PSCs in primary culture were pretreated as indicated, and protein extracts were prepared and subjected to immunoblot analysis as described before[19], using polyvinylidene fluoride membrane (Merck Millipore) for protein transfer. Primary antibodies were obtained from the following sources: anti-α-SMA; Sigma-Aldrich (#A2547), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH); New England Biolabs, Frankfurt am Main, Germany (#M869B). To develop the blots, LI-COR reagents for an Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, United States) were used as described before[21]. Signal intensities were quantified by means of the Image Studio Lite Version 5.2, and normalized to GAPDH by calculating the ratio α-SMA/GAPDH.

**Immunofluorescence detection of α-SMA**

Quiescent PSCs or cells of the first passage were seeded onto glass coverslips and allowed to adhere overnight before they were exposed to D-vitamins at 100 nmol/L. After the indicated periods of time, the cells were fixed with ice-cold methanol, followed by staining of the DNA with 4',6-diamidino-2-phenylindole. Next, the cells were incubated with a mouse monoclonal antibody to α-SMA (#A2547; Sigma-Aldrich). Antibody binding was determined by a fluorescein-labelled goat anti-mouse IgG (MoBiTec, Göttingen, Germany) and visualized employing a fluorescence microscope (Leica DFC320, Leica Microsystems, Wetzlar, Germany). For further evaluation, α-SMA expression in PSCs and organization of the protein in stress fibers were assessed in a semiquantitative manner, using a scoring system as previously established[24]. Therefore, blinded samples were scored by two independent investigators on a scale from 0 (low or undetectable α-SMA expression; absence of stress fibers) to 3 (high expression levels; extensive stress fiber bundles).

**Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology**

PSCs of passage 1 were grown in 12-well plates and treated as indicated, before total RNA was isolated with TriFast reagent (PEQLAB Biotechnologie, Erlangen, Germany). Unless indicated otherwise, reagents from Thermo Fisher Scientific (Karlsruhe, Germany) were used for all subsequent procedures. Traces of genomic DNA were removed employing the DNA-free kit, and 250 ng of RNA per sample was reverse transcribed into cDNA by means of TaqMan™ Reverse Transcription Reagents and random priming. Target cDNA levels were quantified by real-time PCR, using a Viia 7 sequence detection system (Thermo Fisher Scientific). Therefore, qPCR MasterMix (Eurogentec, Seraing, Liège, Belgium) and the following mouse-specific TaqMan™ gene expression assays with fluorescently labelled MGB probes were employed: Mm00446190_m1 (Il6), Mm01178820_m1 (Tgfβ1), Mm00801666_m1 (α1 type I collagen, Col1a1), Mm00437297_m1 (vitamin D receptor, Vdr) and Mm01545399_m1 (hypoxanthine guanine phosphoribosyl transferase; Hprt; house-keeping gene control). PCR conditions were: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C/1 min at 60 °C. The relative amount of target mRNA in untreated PSCs (control) and all other samples was expressed as 2-ΔΔCt, where ΔΔCt = ΔCt sample – ΔCt control.

**IL-6 ELISA**

IL-6 protein levels were determined using a mouse IL-6-specific ELISA (Thermo Fisher Scientific). Therefore, activated PSCs (passage 1) were grown in 24-well plates and treated with D-vitamins as indicated. Cell culture
Statistical analysis
All data were stored and analyzed using the IBM SPSS Statistics 22.0. Values were expressed as mean ± SE for the indicated number of samples (n) per experimental protocol. Mean group differences were checked using analysis of variance. If data did not meet the assumptions for ANOVA, nonparametric analysis of variance was performed employing the Kruskal-Wallis test or, in case of dependency of samples, the Friedman test, before subgroups were tested pairwise using the Mann-Whitney U test and the Wilcoxon rank sum test, respectively. Here, Bonferroni-adjusted P-values, otherwise P < 0.05 were considered to be statistically significant.

RESULTS

D-vitamins inhibit exhibition of a myofibroblastic PSC phenotype
In a first set of experiments, we analyzed the effects of D-vitamins on the activation status of cultured PSCs in vitro. Therefore, PSCs in early primary culture and cells of the first passage were incubated with vitamin D2, vitamin D3 or calcipotriol as indicated, and cytoplasmic lipid droplets (Figure 1) as well as stress fiber bundles of α-SMA (Figure 2) were used as surrogate markers of

**Figure 1** D-vitamins prevent loss of lipid droplets in quiescent pancreatic stellate cells. Pancreatic stellate cells in primary culture (days 1-4) and of passage 1 (days 1-3) were exposed to the indicated D-vitamins at 100 nmol/L each. Afterwards, intracellular lipids were stained with oil-red, and size/number of lipid droplets were assessed on a semiquantitative scale from 0 to 3. A: Exemplary stains for scores 1-3 (original magnification, × 400); B: Shown are the mean scores of n ≥ 14 independent samples (mean ± SE) for primary cultures (left panel) and passaged cells (right panel). *P < 0.05 vs untreated controls.
the cellular phenotype. At the time of analysis, originally quiescent PSCs had undergone 4 d of primary culture on plastic and were considered as a model of early activated stellate cells. Accordingly, re-cultured PSCs corresponded to mature activated cells.

As shown in Figure 1, primary cultures of PSCs contained more lipid droplets after incubation with any of the three vitamin D derivatives than without this treatment. In passaged cells, no such effect was observed. In primary culture as well as upon re-culturing, untreated PSCs expressed high levels of α-SMA protein that was organized in stress fibers (Figure 2). All three D-vitamins significantly diminished the formation of stress fiber bundles in primary cultures, but not in re-cultured cells. The effects of D-vitamins on cells in primary culture were also confirmed by Western blot analysis of α-SMA expression (Figure 3).

High levels of lipid droplets and absence of stress fibers are characteristics of quiescent PSCs [2,3]. Therefore, our data suggest that D-vitamins support the maintenance of a quiescent state when added early in the course of primary culture, but cannot reverse the myofibroblastic phenotype (with high levels of α-SMA and a decline of lipid droplets) once the cells are fully activated.

Effects of D-vitamins on DNA synthesis
At concentrations of 100 nmol/L (Figure 4) and below, D-vitamins had no effect on the rate of DNA synthesis, independent of whether primary cultures or passaged
cells were exposed to the drugs.

**D-vitamins reduce IL-6 expression and secretion**

Effects of D-vitamins at the level of gene expression were studied in passaged PSCs only, since the mRNA yield from the small number of cells in primary culture proved too low for reproducible results. As expected, all D-vitamins enhanced the expression of VDR as a known target gene\(^\text{[21]}\) (Figure 5A). Interestingly, vitamins D2 and D3 significantly diminished the mRNA and protein levels of IL-6, an autocrine mediator of PSC activation and major proinflammatory cytokine\(^\text{[22,23]}\) (Figures 5B and 6). Calcipotriol showed similar effects, but only the inhibition of mRNA expression was statistically significant. None of the investigated derivatives inhibited the expression of collagen type 1 (\(\alpha1\)-chain) and TGF-\(\beta\) as the main profibrogenic cytokine\(^\text{[4,24]}\) substantially (Figure 5C and D).

**Effects of D-vitamins on proline uptake**

In subsequent experiments, uptake of proline, a main component of collagen, by PSCs was measured. The lowest rate of proline incorporation was observed for calcipotriol-treated cells (Figure 7). Although a Friedman test yielded a \(P\) value of 0.007, the differences between treated cells and controls did not reach statistical significance.
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DISCUSSION

To this end, antifibrotic drugs that effectively inhibit the stroma response in the context of CP and PC are largely missing. Undoubtedly, their availability would also facilitate experimental studies on the role of fibrosis in the progression of both diseases, which remains unknown (CP) or controversial (PC). Currently, drugs that block pro-tumorigenic functions of the stroma but maintain, or even enhance, its antitumorigenic properties are considered most interesting with respect to their clinical potential. Recently, the vitamin D derivative calcipotriol has been suggested to exert such effects by inducing a transcriptional reprogramming of the tumor stroma in PC, and PSCs have been suggested as important targets of vitamin D action\[12,13\].

The results of this study provide for the first time insights into the relative efficiencies of different vitamin D derivatives in the context of pancreatic fibrogenesis. Using primary cultures of quiescent murine PSCs, we found that vitamin D2, vitamin D3 and calcipotriol all significantly reduced the expression of α-SMA and prevented the loss of vitamin A-containing fat droplets. Once the cells were fully activated (upon re-culture), no such effects were detected anymore. Together, these data suggest that all three vitamin D derivatives inhibit activation of PSCs in vitro, but cannot reverse the myofibroblastic phenotype of fully activated PSCs. Nevertheless, the latter cells remained vitamin D-responsive. Specifically, all D-vitamins reduced the mRNA levels of IL-6. Moreover, vitamins D2 and D3 also significantly diminished IL-6 protein secretion. Here, the effect of calcipotriol was not significant, although this might be due to the small sample size. Furthermore, uptake of proline was affected by vitamin D-treatment. Somewhat unexpectedly, we did not observe significant effects of any vitamin D derivative on DNA synthesis, a finding that is in contrast to a previous report of antiproliferative effects of vitamin D3 on murine PSCs[20,21].

The inability of D-vitamins to reverse the activated PSC phenotype does not necessarily limit their potential as antifibrotic drugs, since (1) they may prevent the further recruitment of still quiescent PSCs; and (2) diminished secretion of the autocrine PSC activator IL-6[20,21]. The reduced uptake of proline, a main compound of collagen, is compatible with a preserved antifibrotic action of D-vitamins even upon completion of PSC activation. We therefore suggest follow-up studies in animal models not only of PC but also of CP to further evaluate the antifibrotic effects of vitamin D under in vivo conditions.

ARTICLE HIGHLIGHTS

Research background
Chronic pancreatitis and pancreatic cancer are accompanied by an extended fibrosis that plays an active role in disease progression. Pancreatic stellate cells (PSCs) are the main source of extracellular matrix proteins in the diseased organ. To this end, there is a lack of specific antifibrotic agents for preclinical evaluation and potential clinical applications.

Research motivation
Vitamin D has recently been suggested to modulate the pancreatic stroma in a way that pancreatitis is suppressed and pancreatic cancer therapy is enhanced. PSCs were identified as a target of vitamin D action. The molecular mechanisms of vitamin D action in PSCs are only partially understood, and the relative efficiencies of different vitamin D derivatives have not been elucidated yet.

Figure 6  D-vitamins reduce interleukin-6 secretion. Pancreatic stellate cells of the first passage were incubated for 24 h with D-vitamins (at 100 nmol/L each) as indicated. Subsequently, interleukin-6 (IL-6) protein levels in cell culture supernatants were analyzed by ELISA. Mean values and SE were calculated from 6 independent samples. \(^* P < 0.05\) vs untreated controls. For calcipotriol, the Bonferroni-adjusted \(P\)-value was 0.141.

Figure 7  Effects of D-vitamins on \(^{18}\)F-proline incorporation. Pancreatic stellate cells were treated for 48 h with the indicated D-vitamins at 100 nmol/L each. Afterwards, incorporation of \(^{18}\)F-proline was determined as described in the materials and methods section. Raw data were normalized to the protein content of the samples. One hundred percent \(^{18}\)F-proline incorporation corresponds to untreated PSCs. Data are expressed as mean ± SE (\(n = 5\) samples). \(P = 0.007\), but no statistically significant differences between treated cells and the control group.
Research objectives
The objective of this study was to analyze and to compare the biological and molecular effects of three different D-vitamins, vitamin D2, vitamin D3 and calcipotriol, in pSCs.

Research methods
Murine PSCs were exposed to D-vitamins as primary cultures (early activated PSCs) and upon re-culturing (fully-activated cells). Exhibition of vitamin A containing lipid droplets and expression of α-smooth muscle actin were used as surrogate markers of PSC activation. Therefore, oil red staining, immunofluorescence studies and immunoblot analyses were performed. Gene expression was monitored by real-time PCR, and interleukin-6 (IL-6) protein levels were quantified by ELISA. Furthermore, 1F-proline was employed to measure the cellular uptake of proline.

Research results
The results of this study show for the first time that vitamin D exerts distinct effects on quiescent and activated PSCs in vitro. In quiescent PSCs, vitamin D prevented the exhibition of a myofibroblastic phenotype. Once the cells were fully activated, vitamin D failed to induce a complete reversal of the myofibroblastic phenotype, but still exerted antifibrotic effects on PSCs by inhibiting uptake of proline and expression of IL-6. Three vitamin D derivatives, vitamin D2, vitamin D3 and calcipotriol, displayed very similar biological effects.

Research conclusions
D-vitamins are efficient inhibitors of PSC activation in vitro, but cannot reverse the phenotype once the cells are fully activated. In line with other publications in the field, our findings encourage a further evaluation of vitamin D effects in pancreatic cancer and chronic pancreatitis. A modulation of the stroma response by vitamin D might hold potential as part of a multimodal concept for the treatment of both diseases.

Research perspectives
These investigations have shown that vitamin D2, vitamin D3 and calcipotriol are similarly effective with respect to the inhibition of PSC activation in vitro. Directions of future research should include both mechanistic studies on the molecular basis of vitamin D action in PSCs, and experimental studies on vitamin D efficiency in the context pancreatic cancer and chronic pancreatitis. Therefore, advanced cell culture models such as human stellate cells and animal models of pancreatic fibrosis need to be employed.

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