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Vitamin D inhibition of pro-fibrotic effects of transforming growth factor β 1 in lung fibroblasts and epithelial cells

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Abstract

The mechanisms that control fibroproliferation and matrix deposition in lung fibrosis remain unclear. We speculate that vitamin D deficiency may contribute to pulmonary fibrosis since vitamin D deficiency has been implicated in several diseases. First, we confirmed the presence of vitamin D receptors (VDR) in cultured NIH/3T3 and lung fibroblasts. Fibroblasts transfected with a vitamin D response element – reporter construct and exposed to the active vitamin D metabolite, 1,25(OH)₂D₃, showed increased promoter activity indicating VDR functionality in these cells. Testing the effects of 1,25(OH)₂D₃ on fibroblasts treated with transforming growth factor β 1 (TGF β 1), considered a driver of many fibrotic disorders, we found that 1,25(OH)₂D₃ inhibited TGF β 1-induced fibroblast proliferation in a dose-dependent fashion. 1,25(OH)₂D₃ also inhibited TGF β 1 stimulation of α -smooth muscle actin expression and polymerization and prevented the upregulation of fibronectin and collagen in TGF β 1-treated fibroblasts. Finally, we examined how 1,25(OH)₂D₃ affects epithelial-mesenchymal transformation of lung epithelial cells upon exposure to TGF β 1. We showed that the TGF β 1-induced upregulation of mesenchymal cell markers and abnormal expression of epithelial cell markers were blunted by 1,25(OH)₂D₃. These observations suggest that under TGF β 1 stimulation, 1,25(OH)₂D₃ inhibits the profibrotic phenotype of lung fibroblasts and epithelial cells.

Keywords

Vitamin D; VDR; TGF β ; myofibroblast differentiation; epithelial mesenchymal transformation; fibrosis

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(2) INTRODUCTION

Dysregulated synthesis and tissue accumulation of matrix molecules are the hallmarks of fibrotic diseases and are directly responsible for organ destruction and abnormal physiology. In the lung, these changes may be seen in two anatomically distinct diseases: idiopathic pulmonary fibrosis (IPF) and post-transplant obliterative bronchiolitis (OB). Both IPF and OB carry poor prognoses, with mortality rates upwards of fifty percent at 3 years [1–3], and to date, there are no safe and effective treatments capable of halting and/or reversing the fibrotic processes in these diseases.

While not enough is known about the mechanisms responsible for these events, some hypotheses implicate an aberrant wound healing process in response to some known or unspecified injurious stimulus. Characteristically, there is fibroblast activation with a cellular phenotype typified by a resistance to apoptosis, the overproduction of connective tissue matrices, and the appearance of a contractile apparatus [4]. What triggers the induction of these putative myofibroblasts likely involves TGF β , a pro-fibrotic cytokine consistently found in fibrotic tissues [5]. Interestingly, the changes in fibroblasts promoted by TGF β also seem to be mimicked by epithelial cells under similar conditions [6]. Thus, a greater understanding of host and environmental factors influencing fibroproliferation and matrix expression in the lung is needed in order to identify novel targets for the development of effective therapies.

Vitamin D3 is a steroid pre-pro hormone that requires sequential enzymatic modification, 25- and 1 α -hydroxylation, in the liver and kidney, respectively, to have maximal biological activity as the hormone, 1,25(OH) $_2$ D. 1,25(OH) $_2$ D acts in an endocrine fashion on target organs (i.e. parathyroid, bone, intestine and kidney) to regulate bone, calcium, and phosphate metabolism; and its effects are mediated through a specific protein, the Vitamin D Receptor (VDR).

VDR is a ligand-dependent transcription factor belonging to the super-family of nuclear hormone receptors. VDR binds to its ligand, 1,25(OH) $_2$ D, dimerizes with the retinoic acid receptor (RXR), and attaches to specific genomic sequences termed Vitamin D response elements (VDRE). Like others in the nuclear hormone receptor class, VDRs are modular in structure, with an N-terminal transcriptional activation domain and C-terminal ligand-binding domain. The mid-region DNA binding domain, which consists of two zinc fingers, specifically recognizes DR3-type DNA motifs in target promoters to modulate gene expression. In point of fact, the expression of over two-hundred genes are controlled via 1,25(OH) $_2$ D / VDR – dependent pathways, either directly or indirectly, including genes that regulate proliferation, differentiation, and apoptosis, amongst others [7].

There does appear to be a role for 1,25(OH) $_2$ D in the “non-calcemic” homeostasis of extra-skeletal organs as suggested in a recent clinical review on Vitamin D [7]. Evidence for this is provided by data demonstrating the expression of VDRs and 1 α -hydroxylases in brain, prostate, breast, colon tissues, and circulating leukocytes. However, the exact role of vitamin D in such organs remains unelucidated and the biological consequences of vitamin D deficiency remain unclear. Of note, epidemiologic studies suggest a role for Vitamin D deficiency in the pathogenesis of numerous chronic illnesses [7]. For one, there is circumstantial evidence that living at higher latitudes (where decreased solar UVB radiation is a cause of Vitamin D deficiency) increases the risk of multiple sclerosis, Crohn’s disease, hypertension and cardiovascular disease, hematological and solid organ malignancy, and asthma. The strongest data come from the cancer literature linking Vitamin D deficiency with increased incidence of and mortality from colon, prostate, and breast cancer. Moreover, higher maternal intake of vitamin D during pregnancy was found in one study to be associated with a lower risk of recurrent wheeze in children at 3 years of age. Interestingly, a recent meta-analysis of 18

randomized clinical trials enrolling over 57,000 subjects suggested that intake of vitamin D supplements may decrease all-cause mortality [8].

Little is known about the role of vitamin D in lung or its impact on the function of lung cells. In particular, vitamin D status has not been systematically studied in patients with fibrotic lung disease. Unfortunately, such data are only found in studies of end-stage lung disease patients on a transplant waiting list containing a subgroup of pulmonary fibrotics [9,10]. We speculate that vitamin D may influence lung fibrosis through direct effects on lung fibroblasts. Specifically, we hypothesize that vitamin D opposes intracellular pro-fibrogenic signals. To begin to address this hypothesis, we studied NIH3T3 fibroblastic cells and primary lung fibroblasts and found that they express functional vitamin D receptors. In these cells, vitamin D opposed the effects of transforming growth factor β 1 (TGF β 1), a well-studied pro-fibrotic factor. Interestingly, vitamin D seems to have similar anti-TGF β 1 effects in lung epithelial cells thereby extending its protective functions to several pulmonary cells.

(3) METHODS

(4) General reagents

The following antibodies were used against E-cadherin (BD Transduction Laboratories, San Jose, CA), α -smooth muscle actin (α SMA) (Sigma, St. Louis, MO), VDR (Affinity BioReagents, Golden, CO), Pro-Collagen Type I (Novus Biologicals, Littleton, CO and Developmental Studies Hybridoma Bank, University of Iowa), fibronectin (Sigma), ZO-1 (Invitrogen, Carlsbad, CA), cytokeratin (DSHB, Iowa and Santa Cruz Biotechnology, Santa Cruz, CA), and PCNA (Cell Signal Technology, Danvers, MA). 1,25(OH) $_2$ D $_3$ and rhTGF β (10 ng/ml) were purchased from Alexis Biochemicals (San Diego, CA) and R&D Systems (Minneapolis, MN), respectively. The 1,25(OH) $_2$ D $_3$ antagonist, ZK159222, was obtained from Bayer Schering Pharma AG (Berlin, Germany).

(5) Expression Plasmids

The expression plasmid ECFP VDR(119-C) [11], which lacks residues 1–118 that encode the DNA binding domain of the VDR, was kindly provided by Dr. A. Norman (University of California, Riverside, CA). pSPPx3-TK-Luc [12], which consists of the Vitamin D response element from the mouse osteopontin promoter upstream of a luciferase reporter, was a generous gift from Dr. D.W. Russell (University of Texas Southwestern Medical Center, San Antonio, TX). 3TP-Lux, purchased from Addgene (#11767, Cambridge, MA) is a luciferase, TGF β -responsive reporter construct [13,14]. The Renilla luciferase plasmid, pRL-CMV (Promega, Madison, WI), was included in all transfections for normalization.

(6) Cell culture

Primary lung fibroblasts were isolated from C57BL/6 mice as previously described [15] and used between passage two and ten. Primary murine lung and NIH/3T3 fibroblasts (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L of glucose supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% antibiotic-antimycotic solution (100 U/mL penicillin G sodium, 100 U/mL streptomycin, and 0.25 g/mL amphotericin B). The rat type II alveolar epithelial line (RLE-6TN, ATCC) was maintained in DMEM with 4.5 gm/l glucose, 10% FBS, 40 mM HEPES and 1% antibiotic-antimycotic solution. Where indicated, fibroblasts or RLE cells were serum-starved, pre-treated for 1 hour with the stated concentration of 1,25(OH) $_2$ D $_3$ (or ethanol vehicle) and incubated with TGF β (10 ng/ml) for the specified amount of time. Since the maximal concentration of 1,25(OH) $_2$ D $_3$ achievable in lung tissue is not known, 1 μ M was chosen as the highest dose based on several *in vitro* studies of Vitamin D in fibroblasts and cancer cells [16].

(7) Cell transfection and luciferase assays

A calcium-phosphate transfection based method was used [17]. NIH/3T3s were plated in fibroblast media at a cell density of 1×10^5 cells per well in a 12-well dish the day prior to cell transfection. On the day of transfection, cells were incubated for one hour in fresh media, and DNA precipitate was made by adding 40 μg of plasmid (5 $\mu\text{g}/\mu\text{l}$) in 30 μl of 2.5M CaCl_2 to 500 μl of bubbling 2 $\times$ HEPES buffered saline (pH 7.05). After 20 min at room temperature, 45 μl of the precipitate was applied to each well. Cells were incubated with DNA overnight under standard growth conditions, washed twice with PBS the following day, and recultured with complete serum free media for 8 hours prior to treatment. After the appropriate time, treated cells were lysed with 5 $\times$ Passive Lysis Buffer (Promega) and analyzed for Firefly and Renilla luciferase activity on a Thermo Luminoskan Ascent Luminometer (Waltham, MA) as described by Dyer *et al* [18].

(8) Reverse-Transcription and Polymerase Chain Reaction

Cells were cultured onto 6 well plates and incubated in complete serum free media for 24 hours prior to the addition of TGF β 1. Total RNA was isolated with a TRI reagent as previously described [19]. The reverse transcription reactions of the extracted RNA were performed by combining the following reagents: 0.625 μM dNTPs, 16 nmol random hexamer oligonucleotides (Roche Diagnostics, Indianapolis, IN), 5 μl First Strand Buffer (Invitrogen), 20 mM DTT, 200 units reverse transcriptase enzyme, 0.5 μl RNasin (Promega, Madison, WI), and 1 μg extracted RNA in a total volume of 25 μl . Murine primers for PCR reactions were based on GenBank published sequences and are as follows: *α SMA* (5'-ATG GAG TCA GCG GGC ATC-3'; 5'-AAC TGG AGG CGC TGA TCC-3'), *VDR* (5'-CAG CCA GCA CCT CCC TGC-3'; 5'-AGA AAC CCT TGC AGC CTT CA -3'), *fibronectin* (5'-GTT ATG ACG ATG GGA AGA-3'; 5'-ACT GGT TGT AGT TGT GGC-3'), *type I collagen* (5'-CTG CTG TTG GTG CTG CTG-3'; 5'-CAG GAG CAC CAG CAA TAC-3'), *type III collagen* (5'-TCT CAC CCT TCT TCA TCC CA-3'; 5'-GGC AGT CTA GTG GCT CCT CA -3'), *PAI-1* (5'-TCA TCA GAC AAT GGA AGG GC-3'; 5'-ACT GTG CCG CTC TCG TTT AC-3'), *18s* (5'-TTG AAA ATC CGG GGG -3'; 5'-ACA TTG TTC CAA CAT GCC AG -3').

Reactions containing 10 $\times$ PCR buffer (Denville Scientific, South Plainfield, NJ), 1 unit Taq polymerase (Denville), 10 μM each primer, and 800 ng cDNA in 7.5% glycerol, were performed using an optimized, primer-specific PCR thermocycling protocol. Amplicons were resolved on 1% agarose gels, stained with SYBR Green (Invitrogen, Carlsbad, CA), and visualized with a SafeImager transilluminator (Invitrogen).

(9) Western blotting

Fibroblasts were plated at near confluence, serum starved for 24 hours, and treated as indicated. Cells were washed with ice-cold PBS, incubated in 0.1 ml modified RIPA lysis buffer with protease inhibitors, and sonicated. Protein concentration was determined with the Bio-Rad protein assay reagent (Hercules, CA) according to the manufacturer's directions. Equal aliquots of protein were fractionated by electrophoresis in 12% SDS-polyacrylamide gels and transferred onto nitrocellulose paper. The nitrocellulose paper was blocked with 10% milk in TBS-0.1% Tween-20 buffer and incubated overnight at 4°C with diluted primary antibody followed by a horseradish peroxidase-conjugated secondary antibody (Sigma). Immunoreactivity was visualized by chemiluminescence (Pierce, Rockford, IL). Membranes were stripped and reprobed for GAPDH (Abcam, Cambridge, MA).

(10) Immunofluorescence

Treated cells in chamber slides (Nalge Nunc International, Rochester, NY) were fixed in 4% paraformaldehyde for 15 minutes and permeabilized with 0.5% Triton X-100 in Tris buffered

saline (TBS). Slides were washed in TBS, blocked with 5% bovine serum albumin and 2% goat serum in 0.1% Triton X-100/TBS, and incubated with primary antibodies at 4 °C overnight. An Alexa Fluor® - labeled secondary antibody (Invitrogen) was applied for 1 hour to slides, which were then counterstained with DAPI (Sigma), briefly air-dried, and cover-slipped with ProLong® Gold mounting medium (Invitrogen). Slides were viewed under epifluorescence microscopy (Olympus BX41, Melville, NY) and images were captured using Magnafire 2.1 digital image acquisition software (Goleta, CA) using optimized exposure times for each fluor within each experimental condition. Brightness and contrast enhancement was applied equally to all images using a batch processing algorithm in Adobe Photoshop v7.0.

(11) Proliferation Assay

Cells were plated at a density of 2×10^3 cells per well for NIH/3T3s and 4×10^3 for primary lung fibroblast in a 96-well tissue culture dish. At the indicated time points, cells were rinsed with PBS, fixed with 100% ice-cold methanol for 5 minutes, washed with several changes of PBS, and incubated with 0.2% (w/v) crystal violet (Sigma) in 2.5% acetic acid for 30 minutes at room temperature. Culture plates were gently rinsed with tap water until clear, and allowed to dry. To extract the cell-bound dye, crystal violet stain was solubilized with 1% SDS for 1 hour at room temperature with shaking. The absorbance at 590 nm was determined on a AD 340C Beckman Coulter Absorbance Detector (Beckman Coulter, Fullerton CA).

(12) Cytotoxicity Assay

NIH/3T3s were plated at a density of 2×10^4 cells per well in a 96-well tissue culture dish and treated as indicated. After 24 hours, 100 μ L of cell culture media was analyzed for adenylate kinase activity using a bioluminescent cytotoxicity assay kit (Biovision, Mountain View, CA). The background luminescence of water (in the absence of cells) was used as a negative control. The positive control consisted of supernatants from cells treated with toxic doses of H_2O_2 .

(13) Collagen Gel Contraction Assay

Serum-starved primary lung fibroblasts were treated $1,25(OH)_2D_3$ and/or $TGF\beta$ as indicated for 72 hours, harvested with trypsin, and diluted in collagen type I (Sigma) to achieve a final collagen concentration of 0.65 mg/ml with 1.25×10^5 cells/ml. 250 μ L of the collagen cell suspension were again treated $1,25(OH)_2D_3$ and/or $TGF\beta$, placed in a 35mm tissue culture dish in triplicates, allowed to polymerize for 2 hours, and covered in fresh culture media supplemented with the indicated treatments. Diameters of the collagen gels were measured daily.

(14) Data analysis

Western blotting and RT-PCR experiments were performed in duplicates and repeated at least three times to ensure consistency. Reporter and proliferation data were also repeated thrice each with 3–4 replicates per experiment. All results are presented as mean $\pm$ S.E. GraphPad Prism v3.0 was used to analyze data by one-way ANOVA computation with Tukey's multiple comparisons test. A '*p*' value of 0.05 was considered significant.

(15) RESULTS

(16) Lung fibroblasts express functional Vitamin D receptors

To date, it remains unclear whether lung cells are capable of recognizing $1,25(OH)_2D_3$ through the classic vitamin D receptor (VDR). To test this, we began by evaluating the expression of VDRs in lung tissue, and since fibroblasts are thought to be the predominant effector cell in organ fibrosis, we also assessed the expression of VDRs in this cell type. As depicted in Figure 1, VDR mRNA and protein expression were detected in the NIH/3T3 cell line (confirming

previous reports [20]), primary lung fibroblasts, and whole lung homogenates, via RT-PCR (Figure 1A) and western blotting (Figure 1B), respectively. As expected, immunofluorescence staining (Figure 1C) in NIH/3T3 (left panel) and lung fibroblasts (right panel) revealed fibroblast nuclei as the main site of localization of vitamin D nuclear receptors.

As a test of VDR functionality in fibroblasts, NIH/3T3 cells were transfected with a Vitamin D response element (VDRE) connected to a luciferase reporter gene and treated with 1,25(OH)₂D₃. We found that 1,25(OH)₂D₃ stimulated the expression of the gene with a maximal effect observed at 100 nM followed by a subsequent dose-dependent decline (Figure 2A). Next, fibroblasts were either pretreated with a specific antagonist of the VDR, ZK159222, or transfected with a truncated VDR capable of binding 1,25(OH)₂D₃ without affecting gene transcription and thereby competing with endogenous VDR. Both of these interventions were able to block the VDRE promoter activity in fibroblasts stimulated by 1,25(OH)₂D₃ back to baseline indicating that the effects of 1,25(OH)₂D₃ on lung fibroblasts were indeed dependent on VDR (Figure 2B).

(17) Vitamin D opposes the effects of TGFβ1 in lung fibroblasts

Having established the presence of functional VDRs in lung fibroblasts, we turned our attention to TGFβ. TGFβ1 is considered a master switch in fibrotic lung disorders and is known for its ability to stimulate cell proliferation and matrix expression in lung fibroblasts [5]. Therefore, we set out to test if active vitamin D influences these effects by treating fibroblasts with TGFβ1 in the presence or absence of 1,25(OH)₂D₃. As shown in Figure 3, A and B, TGFβ1 stimulated the proliferation of primary lung fibroblasts and NIH/3T3 fibroblasts, respectively. 1,25(OH)₂D₃ inhibited this effect. The highest concentrations of 1,25(OH)₂D₃ reduced the proliferation of TGFβ1-treated fibroblasts lower than that observed for unstimulated fibroblasts, and 100 pM 1,25(OH)₂D₃ was able to blunt TGFβ-mediated proliferation to near-baseline levels. Also, the expression of PCNA, proliferating cell nuclear antigen, by fluorescence immunocytochemistry was examined as a marker of actively replicating cells (Figure 3C). 1,25(OH)₂D₃ at 1 μM was able to block the upregulation of PCNA by TGFβ to levels even below that at baseline. Most notably, the inhibitory effect of 1,25(OH)₂D₃ was not associated with cytotoxicity as data were not meaningfully different than negative controls (Figure 3D).

1,25(OH)₂D₃ also opposed the effects of TGFβ1 on myofibroblast transdifferentiation as demonstrated by its ability to inhibit TGFβ1-induced expression of α-smooth muscle actin (αSMA), a well known marker of myofibroblast transdifferentiation [21]. Immunofluorescence staining of NIH/3T3 fibroblasts also revealed that TGFβ1 stimulated the organization of αSMA into the tubular structure of the actin cytoskeleton, a process that was inhibited by 1,25(OH)₂D₃ (Figure 4A) as a function of concentration. RT-PCR analysis revealed that 1,25(OH)₂D₃ inhibited TGFβ1-induced αSMA mRNA expression in a dose-dependent manner in these cells (Figure 4B). Similarly, 1,25(OH)₂D₃-treated primary lung fibroblasts displayed diminished TGFβ-related expression of αSMA at the transcript and protein level (Figure 4, C and D).

We next tested the effects of 1,25(OH)₂D₃ on the myofibroblast phenotype induced by TGFβ1. This phenotype is characterized by augmented matrix expression, and consistent with this, TGFβ1 is known to stimulate the expression of type I collagen and fibronectin in fibroblasts as depicted in Figure 5, A–D. In NIH/3T3 and primary lung fibroblasts, 1,25(OH)₂D₃ inhibited this effect in a dose-dependent manner by preventing induction of matrix mRNA (Figure 5, A and B) and protein (Figure 5, C and D) expression in response to TGFβ1. In similar fashion, 1,25(OH)₂D₃ blocked the upregulation of the TGFβ-dependent genes, type III collagen and PAI-1 (Figure 5E). Additionally, the myofibroblastic phenotype induced by TGFβ1 is also distinguished by increased contractility of collagen gels. Therefore,

we tested the effect of $1,25(\text{OH})_2\text{D}_3$ on gel contractility induced by $\text{TGF}\beta 1$ -treated fibroblasts. As shown in Figure 5F, $\text{TGF}\beta 1$ enhanced gel contractility, whereas $1,25(\text{OH})_2\text{D}_3$ eliminated the effect.

To begin studying the mechanisms by which active Vitamin D inhibits $\text{TGF}\beta$ effects in lung fibroblasts, $\text{TGF}\beta$ signal transduction was studied using the 3TP-Lux plasmid. $\text{TGF}\beta$ drives luciferase reporter expression by activating promoter elements of this construct, taken from the human collagenase and PAI-1 genes. For this purpose, NIH/3T3 cells were transiently transfected with 3TP-Lux, treated with $1,25(\text{OH})_2\text{D}_3$, and exposed to increasing concentrations of $\text{TGF}\beta$. The effects of high (15 ng/ml) and medium (7 ng/ml) concentrations of $\text{TGF}\beta$ were completely abolished by $1,25(\text{OH})_2\text{D}_3$. Moreover, $1,25(\text{OH})_2\text{D}_3$ suppressed $\text{TGF}\beta$ activity below that of basal levels even under stimulation by low (2 ng/ml) amounts of $\text{TGF}\beta$.

(18) Vitamin D opposes the effects of $\text{TGF}\beta 1$ in epithelial cells

The above data suggest that $1,25(\text{OH})_2\text{D}_3$ opposes the pro-fibrogenic effects of $\text{TGF}\beta 1$ in lung fibroblasts. In addition to its effects on fibroblasts, $\text{TGF}\beta 1$ has also been implicated in the transdifferentiation of epithelial cells into myofibroblasts, a process termed epithelial-mesenchymal transformation. This concept has received much attention lately due to its potential role in lung fibrosis [6]. To study this, we began by evaluating the expression of VDRs in a cultured rat lung epithelial cell line by immunofluorescence and as expected, found it localized in their nuclei (Figure 6A; left image). EMT is characterized by an increase in the expression of mesenchymal markers and a loss of epithelial cell markers, which, in this epithelial cell line, may take up to 10 days of incubation with $\text{TGF}\beta 1$ [6]. We examined the early events (at 3 days) driven by $\text{TGF}\beta$ in lung epithelial cells and demonstrated that $\text{TGF}\beta 1$ greatly stimulated the expression of mesenchymal markers, αSMA and procollagen I, whereas $1,25(\text{OH})_2\text{D}_3$ opposed this effect (Figure 6, B and C). Under our experimental conditions, the total expression of the epithelial markers, E-cadherin, cytokeratin, and ZO-1, in the presence or absence of $1,25(\text{OH})_2\text{D}_3$ or $\text{TGF}\beta$, was marginally affected when detected within 72 hours by Western blot in whole cell lysates (Figure 6C). Immunofluorescence microscopic analysis was also used as a complementary approach to discern any phenotypic changes of EMT associated with $1,25(\text{OH})_2\text{D}_3$ and $\text{TGF}\beta$. We demonstrated that quiescent epithelial cells negligibly express αSMA , collagen, and fibronectin (Figure 6D, left panels), yet upon stimulation by $\text{TGF}\beta$, cells were noted to organize αSMA into stress fibers, express collagen, and secrete fibronectin (middle panels). These changes mediated by $\text{TGF}\beta$ were, however, mitigated by $1,25(\text{OH})_2\text{D}_3$ (right panels). $\text{TGF}\beta$ did, interestingly, induce derangements as quickly as 3 days in the pattern of cellular localization of epithelial markers. Specifically, when compared to control, $\text{TGF}\beta$ treatment resulted in the disruption of cell membrane E-cadherin contiguity, the failure of cytokeratin filamentous organization, and the shifting of crisp, perinuclear and membrane ZO-1 staining to a diffuse, cytoplasmic staining. These changes suggested dysregulation of protein expression early on in lung epithelial cells after stimulation by $\text{TGF}\beta 1$, even before a decrement in expression. More importantly, prior exposure of epithelial cells to $1,25(\text{OH})_2\text{D}_3$ was able to abrogate these processes.

(19) DISCUSSION

The role of vitamin D in lung remains largely unexplored. Specifically, it is unclear whether lung cells recognize vitamin D and whether vitamin D levels affect lung cell functions. We hypothesized that vitamin D influences pro-fibrogenic processes by targeting lung fibroblasts and tested this hypothesis in NIH/3T3 cells and primary murine lung fibroblasts. We demonstrated VDR expression in lung tissue homogenates and in lung fibroblasts. Furthermore, we found that the VDR localizes to the nucleus, consistent with its role as a nuclear receptor, and that it is functional since NIH/3T3 fibroblasts transfected with a VDRE

fused to a luciferase reporter showed increased expression of the transfected gene when exposed to $1,25(\text{OH})_2\text{D}_3$. Having demonstrated the expression of functional VDRs in lung fibroblasts, we proceeded to test the effect of $1,25(\text{OH})_2\text{D}_3$ in cells exposed to TGF β 1, a well known pro-fibrotic growth factor. As expected, TGF β 1 stimulated the proliferation of lung fibroblasts and their myofibroblastic transdifferentiation as highlighted by increased expression and organization of α SMA, increased secretion of matrix proteins, and increased contractility of collagen gels. All of these effects were inhibited in a dose-dependent fashion by $1,25(\text{OH})_2\text{D}_3$. Of interest, a similar effect was noted in epithelial cells since $1,25(\text{OH})_2\text{D}_3$ inhibited their TGF β 1-induced EMT.

These observations are important for the following reasons. First, they confirm that lung fibroblasts and epithelial cells are indeed capable of recognizing $1,25(\text{OH})_2\text{D}_3$ through functional VDRs. VDRs have long been described in the *fetal* lung, as the immature lung is a known target organ for $1,25(\text{OH})_2\text{D}_3$. Owing to its pro-differentiation role, it is not surprising then that Vitamin D promotes lung development and alveolarization in the prenatal and neonatal lung. However, in order to regulate lung maturation properly, its effects on the developing lung sit in stark contrast to our findings in mature lung cells. For instance, fetal lung fibroblasts proliferate more in the presence of $1,25(\text{OH})_2\text{D}_3$ than in its absence [22,23]. Additionally, $1,25(\text{OH})_2\text{D}_3$ may actually contribute to the EMT in the lung that is a necessary, programmed process for organogenesis [24].

In the mature lung, data are only emerging as to the cellular targets for Vitamin D. VDRs were recently detected in the epithelia of normal and malignant human bronchial tissue [25], while VDR responsive genes were found to be upregulated in lung epithelial cells treated with vitamin D metabolites [26]. Similarly, airway smooth muscle cells express functional VDRs, and under stimulation by $1,25(\text{OH})_2\text{D}_3$ demonstrate differential gene expression [27].

Secondly, another important observation relates to the fact that $1,25(\text{OH})_2\text{D}_3$ was capable of inhibiting the TGF β -mediated tissue remodeling responses in cultured lung fibroblasts. Though quite limited, much of the work on the role of vitamin D in TGF β -related fibrosis has been studied, not unexpectedly, in cells or organs known to be vitamin D targets. For instance, similar to our data, *in vitro* myofibroblastic transformation of kidney interstitial fibroblasts by TGF β , as measured by α SMA and collagen expression, was also blocked with $1,25(\text{OH})_2\text{D}_3$ [28], while cultured normal murine dermal fibroblasts are less efficient in contracting collagen gels [29]. More recently, $1,25(\text{OH})_2\text{D}_3$ was shown in mesenchymal multipotent cells to prevent their differentiation into a pro-fibrotic cellular phenotype in addition to induce the expression of anti-fibrotic soluble factors [30].

Correspondingly, treatment of animals with $1,25(\text{OH})_2\text{D}_3$ one of its analogues appears to confer protection against fibrosis in various animal models. Activation of Vitamin D pathways have been observed to decrease matrix expression and/or affect TGF β signaling in experimental renal fibrosis [31,32], UV-induced skin fibrosis [33], and viral myocarditis [34]. As a curiosity, mice lacking VDRs begin to display evidence of cardiac remodeling, even in the absence of concomitant injury [35].

A third observation that deserves highlighting relates to our findings in lung epithelial cells. Several recent studies have pointed to epithelial cell dysfunction as a potential early event in tissue fibrosis. Furthermore, epithelial cells are now considered a source of myofibroblasts through EMT, a process by which epithelial cells transform into smooth muscle/fibroblast-like cells capable of enhancing fibroproliferative responses driven by TGF β 1. We found that lung epithelial cells express VDR and that $1,25(\text{OH})_2\text{D}_3$ greatly upregulated the expression of mesenchymal markers in these cells while inhibiting the expression of epithelial cell markers, suggesting inhibition of TGF β 1-induced EMT. In fibrogenic EMT, nothing has been

previously reported *vis á vis* Vitamin D in the lung. Tan et al. were able to inhibit EMT in a murine model of renal fibrosis with a 1,25(OH)₂D₃ analogue via an inhibition of TGFβ signals [31]. They reported that activation of Vitamin D signals also prevented TGFβ-mediated EMT in cultured renal tubular epithelial cells. Furthermore, it has been suggested that Vitamin D may induce the differentiation of carcinoma cells thereby potentially affecting oncogenic EMT by stimulating the expression of E-cadherin and blocking upregulation of mesenchymal genes [36,37].

The relevance of the *in vitro* findings reported here to the situation *in vivo* is uncertain and further studies are required to confirm them in humans. However, our work in lung fibroblasts and epithelia suggests a pathway, previously unexplored in cells of this organ, capable of influencing TGFβ1-related pro-fibrotic responses. If confirmed, this would unveil a potential new target for fibrotic lung disorders.

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FIG 1.

VDRs are expressed in whole lungs and lung fibroblasts. Extracts of NIH/3T3 cells and primary lung fibroblasts and homogenates of whole mouse lung were analyzed for Vitamin D receptor expression by (A) RT-PCR and (B) Western blotting. VDRs in NIH/3T3 and murine lung fibroblasts were detected using indirect immunofluorescence (E).

FIG 2.

VDRs are functional in fibroblasts. (A) NIH/3T3 fibroblasts were transfected with a luciferase reporter driven by three copies of a Vitamin D response element taken from the mouse osteopontin promoter (pSPPx3-Luc). Transfected fibroblasts were then treated with the indicated concentration of 1,25(OH)₂D₃ for 24 hours followed by testing for luciferase activity. (B) 3T3 fibroblasts were either co-transfected with pSPPx3-Luc and a mutated VDR (119-C) or treated with the VDR antagonist, ZK159222, after transfection with pSPPx3-Luc. Afterwards, cells were incubated overnight with 1 μM 1,25(OH)₂D₃ and analyzed for luciferase expression. Data are expressed as the average normalized fold change over control ± SE. * = $p < 0.001$. ^a = not statistically significant compared to control

FIG 3.

Effects of Vitamin D on fibroblast proliferation and viability. (A) Primary lung and (B) NIH/3T3 fibroblasts were treated with increasing doses of 1,25(OH)₂D₃ for 1 hour followed by exposure to TGF β 1 (10 ng/ml). Cell counts were determined colorimetrically at days 2 and 5. (C) 3T3 fibroblasts were stimulated with 1,25(OH)₂D₃ (1 μ M) and/or TGF β (10 ng/ml) and analyzed for PCNA expression by indirect immunofluorescence. (D) NIH/3T3s were incubated for 48 hours with the indicated concentrations of 1,25(OH)₂D₃ in the presence or absence of TGF β 1. Cytotoxicity was determined using a bioluminescent assay. Background luminescence, i.e. negative control, was measured using plain water. Complete cell lysis with 15 mM H₂O₂ was used as a positive control. Data shown are means $\pm$ SE. * = $p < 0.001$ compared to control. ** = $p < 0.01$ compared to control.

FIG 4.

1,25(OH)₂D₃ inhibits myofibroblast transdifferentiation. NIH/3T3 fibroblasts were treated for 1 hour with the indicated amount of 1,25(OH)₂D₃, stimulated with TGFβ1 (10 ng/ml). (A) Immunofluorescence microscopy was used to assess the degree of αSMA (green) and f-actin (red) cytoskeletal organization after 72 hours. (B) αSMA expression by RT-PCR was detected after 24 hours. Primary lung fibroblasts were treated similarly. (C) RNA and (D) protein were extracted and analyzed for αSMA.

FIG 5.

Matrix expression and collagen gel contraction are inhibited by 1,25(OH)₂D₃. NIH/3T3 (A and C) and primary lung fibroblasts (B and D) were exposed to 1,25(OH)₂D₃ at the specified concentrations and incubated with TGFβ (10 ng/ml) for 24 hours for RT-PCR experiments (A and B) and 72 hours for immunoblot studies (C and D). Densitometric analyses for the immunoblots are shown. Samples were analyzed for procollagen I and fibronectin expression. (E) RNA expression of type III collagen and PAI-1 were determined by RT-PCR in 3T3 fibroblasts exposed to 1,25(OH)₂D₃ (1 μM) and/or TGFβ (10 ng/ml). (F) 1,25(OH)₂D₃ / TGFβ - treated primary lung fibroblasts were encased in collagen gels containing the same

treatments. Diameters of the gels were measured daily for 4 days. Data were obtained in triplicate and are expressed as mean fold change in diameter over control $\pm$ SE.

FIG 6.

1,25(OH)₂D₃ interrupts TGFβ signaling. (A) NIH/3T3 fibroblasts were transiently transfected with a luciferase reporter under the control of a TGFβ-sensitive promoter (3TP-Lux). Transfected fibroblasts were then treated with the 1,25(OH)₂D₃ (1 μM) for 1 hour, exposed to TGFβ at the indicated concentration (ng/ml) for 24 hours, followed by quantification of luciferase activity. Data are expressed as the average normalized fold change over control ± SE. * = $p < 0.001$ compared to control. *a* = not statistically significant compared to control

FIG 7.

TGFβ1 induction of epithelial-mesenchymal transformation is antagonized by 1,25(OH)₂D₃. Indirect immunofluorescence for VDRs (A, left panel) was applied to cultured lung epithelial cells. An IgG control is also shown (A, right panel). Lung epithelial cells were pre-treated with 1,25(OH)₂D₃ (1μM) for 1 hour and incubated with TGFβ1 (10 ng./ml) for 72 hours. Western blotting of protein extracts was performed for (B) αSMA, (C) ZO-1, Pro-collagen I, and E-cadherin. Densitometric analyses of the immunoblots are shown. Double label immunofluorescence (D) was performed in untreated (left panels), TGFβ-treated (middle panels) and 1,25(OH)₂D₃/TGFβ- treated (right panels) lung epithelial cells. Epithelial/

mesenchymal pairs that were studied included E-cadherin and α SMA (top panels), cytokeratin and procollagen I (middle panels), and ZO-1 and fibronectin (bottom panels).