1,25-Dihydroxyvitamin D3 Reduces Extracellular Matrix-Associated Protein Expression in Human Uterine Fibroid Cells

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ABSTRACT

Uterine fibroids (leiomyomas) are the most common benign tumors associated with excessive deposition of extracellular matrix (ECM)-associated proteins that increase fibroid tumorigenicity. Herein, we determined the expression levels of vitamin D receptor (VDR) protein in human uterine fibroids and compared these levels to those in adjacent normal myometrium. Using Western blot analysis, we found that more than 60% of uterine fibroids analyzed (25 of 40) expressed low levels of VDR. We also found that the biologically active 1,25-dihydroxyvitamin D3 (1,25(OH)\(_2\)D3), which functions via binding to its nuclear VDR, induced VDR in a concentration-dependent manner and reduced ECM-associated fibrotic and proteoglycans expression in immortalized human uterine fibroid cell line (HuLM). At 1–10 nM concentrations, 1,25(OH)\(_2\)D3 significantly induced (\(P < 0.05\)) nuclear VDR, which was further stimulated by higher concentrations of 1,25(OH)\(_2\)D3 in HuLM cells. 1,25(OH)\(_2\)D3 at 10 nM also significantly reduced (\(P < 0.05\)) the protein expression of ECM-associated collagen type 1, fibronectin, and plasminogen activator inhibitor-1 (PAI-1) in HuLM cells. We also found that 1,25(OH)\(_2\)D3 reduced mRNA and protein expressions of proteoglycans such as fibromodulin, biglycan, and versican in HuLM cells. Moreover, the aberrant expression of structural smooth muscle actin fibers was reduced by 1,25(OH)\(_2\)D3 treatment in a concentration-dependent manner in HuLM cells. Taken together, our results suggest that human uterine fibroids express reduced levels of VDR compared to the adjacent normal myometrium and that treatment with 1,25(OH)\(_2\)D3 can potentially reduce the aberrant expression of major ECM-associated proteins in HuLM cells. Thus, 1,25(OH)\(_2\)D3 might be an effective, safe, nonsurgical treatment option for human uterine fibroids.

1,25-dihydroxyvitamin D3 (1,25(OH)\(_2\)D3), extracellular matrix (ECM), fibroids, vitamin D receptor (VDR)

INTRODUCTION

Uterine fibroids (leiomyoma) are the most common benign tumors associated with excessive symptoms, including vaginal bleeding, pelvic pain, recurrent miscarriage, and preterm labor [1, 2]. They are the most commonly cited reason for hysterectomy in the United States [3]. Although the initiating factors that lead to the development of uterine fibroids are not well understood, results of numerous studies support the idea that estrogen and progesterone play important roles in the growth of uterine fibroids [4, 5]. African-American women are at 3- to 4-fold greater risk of developing uterine fibroids than Caucasian women [6]. In addition, hypovitaminosis D, a condition in which the vitamin D levels are lower than the normal physiological levels, is approximately 10-fold more prevalent in African-American women (40%–45%) compared to Caucasian women (4%) [7]. However, the exact reasons for this higher occurrence of hypovitaminosis D in African-American women are not well understood. Moreover, the correlation between vitamin D3 and uterine fibroids is also not well documented.

Uterine fibroids are characterized by the deposition of excessive extracellular matrix (ECM)-associated proteins, and the process is known as fibrosis [8]. Studies have demonstrated genes that encode ECM proteins are expressed abnormally in uterine fibroids [9, 10]. The transforming growth factor-beta (TGF-\(b\)) family are multifunctional peptides that regulate diverse biological functions, including cell growth, differentiation, inflammation, apoptosis, and tissue remodeling, and these processes are important to tissue fibrosis [11–13]. TGF-\(b\) has been identified in a variety of normal and transformed mammalian cells and tissues [11]. These profibrotic cytokines are overexpressed in a wide range of fibrotic tissues, including uterine fibroids [13–15]. TGF-\(b\) has been shown to upregulate the synthesis of many ECM proteins associated in the process of fibrosis [16]. The ECM-associated genes such as collagen type 1 and fibronectin were overexpressed in uterine fibroids as compared to normal myometrium [17, 18]. TGF-\(b\) stimulates ECM production in uterine fibroids by inducing the expression of collagen type 1, fibronectin, laminin, and proteoglycans [11, 19–21]. These multifunctional peptides promote fibroproliferative and tumorigenic processes in different organ systems, including uterine fibroids [22].

Vitamin D3 is known as the primary form of dietary vitamin D. Vitamin D3 itself is biologically inactive and must be metabolized to its biologically active forms. After it is consumed in the diet or synthesized in the epidermis, vitamin D3 enters the circulation and is transported to the liver, where it is hydroxylated to form 25-hydroxyvitamin D3, the major circulating form of vitamin D3. The 25-hydroxyvitamin D3 is then enzymatically catalyzed in the kidney to form the biologically active 1,25-dihydroxyvitamin D3 (1,25(OH)\(_2\)D3), which exerts the physiological effects within the body [23]. A previous study has demonstrated that 1,25(OH)\(_2\)D3 can act as a
strong growth inhibitor and induce apoptosis in human breast cancer cells [24]. 1,25(OH)2D3 can suppress proliferation of normal and malignant cells by inducing differentiation and apoptosis [25]. An analog of 1,25(OH)2D3 exhibits potential antitumor activity in vivo in a murine squamous cell carcinoma model [26]. Recently, we and others have shown a growth inhibitory function of 1,25(OH)2D3 on human uterine fibroid cells [27, 28]. We have also shown that 1,25(OH)2D3 reduces fibroid tumor growth in vivo in an Eker rat model [29]. Moreover, we have shown that 1,25(OH)2D3 can attenuate TGF-β-induced protein expression in human uterine fibroid cell line [30]. Our recent study also demonstrated the risk of lower levels of serum vitamin D3 (both 25-hydroxyvitamin D3 and 1,25(OH)2D3) in the development of human uterine fibroids [31]. In addition, several recent epidemiological studies demonstrated the risk of lower levels of serum vitamin D3 in the development of human uterine fibroids [32, 33]. To our knowledge, however, no study has yet established the lower levels of vitamin D receptor (VDR) in human uterine fibroids compared to the adjacent normal myometrium and the risk associated in the development of human uterine fibroids. In addition, the biological function of 1,25(OH)2D3 in the regulation of major ECM-associated proteins, which are involved in the process of fibrosis, is also not known. Thus, the present study was undertaken to evaluate the risk associated with reduced levels of VDR protein in human uterine fibroid tumors and to determine the biological function of 1,25(OH)2D3 in the regulation of ECM-associated proteins, which are key in the process of fibrosis.

MATERIALS AND METHODS

Cell Lines and Cultures

Both immortalized human uterine fibroid cell line (HuLM) and human normal uterine smooth muscle cell line (UTSM) were a generous gift from Dr. Darlene Dixon (National Institute of Environmental Health Sciences, Research Triangle Park, NC) [34]. These cell lines were cultured and maintained as described previously [27, 30].

Reagents and Antibodies

The bioactive 1,25(OH)2D3 was purchased from Sigma Biochemicals. Smooth muscle cell culture medium (SbmBm) was purchased from Lonza. Antibodies were purchased as follows: anti-collagen type I (catalog no. 70R-CR007X) from Fitzgerald; anti-plasminogen activator inhibitor-1 (PAI-1; catalog no. sc-9797), anti-fibromodulin (catalog no. sc-33772), anti-biglycan (catalog no. sc-33788), anti-vascular (catalog no. sc-28381), and anti-VDR (catalog no. sc-1008) from Santa Cruz Biotechnology; and anti-fibronectin (catalog no. F3648), anti-fibronectin (catalog no. A5229), fibronectin isocitohy- anate (FITC)-conjugated phalloidin (F-actin; catalog no. P5282), and monoclonal anti-beta-actin (catalog no. A5441) from Sigma Biochemicals.

Protein Extraction from Tissue Samples

Uterine fibroid tumors and samples of the adjacent normal myometrium tissue were collected from 40 individuals who underwent hysterectomy at clinics in Texas and Tennessee to surgically remove their uterine fibroids. Tissue samples were collected from different ethnic groups, including African-American, Caucasian, and Hispanic patients who underwent hysterectomy (abdominal, vaginal, and laparoscopic). All subjects were from 35 to 53 years old, and their uterine fibroid sizes were from 2.5 to 14 cm. Tissue samples were also collected from the Meharry Clinic under an approved IRB protocol (Protocol 090630WJR246 11). All patients received no hormone supplements, including vitamin D3, for at least 3 mo before the hysterectomy was performed. Extraction of proteins from tissue samples was performed as described previously [35, 36] with minor modifications. Briefly, a portion (3–5 mm3) of each uterine fibroid and the adjacent normal myometrium tissue samples was chopped into small pieces, homogenized in lysis buffer containing 50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 10 mM ethylenediaminetetra-acetic acid, 0.5% (wt/vol) Nonidet P-40, 0.5 mM dithiothreitol, 0.1% (wt/vol) SDS, 0.5% (wt/vol) sodium deoxycholate, 5 mM sodium fluoride, 1.0 mM phenylmethylsulfonyl fluoride, and 3 μg/ml of each protease inhibitor such as leupeptin, pepstatin, and apro tinin. Tissue homogenates were sonicated to solubilize proteins and left on ice for approximately 30 min, vortexed intermittently, and then centrifuged at 14,000 rpm for 20 min at 4°C. Clean supernatants were collected into new Eppendorf tubes, and then the protein concentration of each sample was determined using Bradford Protein Assay reagent (Bio-Rad) according to the manufacturer’s instructions. To determine the expression of VDR in uterine fibroids and the adjacent normal myometrium, equal amounts of each protein lysate were examined by Western blot analysis using anti-VDR and anti-beta-actin (loading control) antibodies.

Western Blot Analysis

For analysis of protein expressions, HuLM and UTSM cells were serum starved overnight and then treated with increasing concentrations of 1,25(OH)2D3 (0, 1, 10, 100, and 1000 nM) for 48 h, as indicated in the figure legends. The 0 nM concentration of 1,25(OH)2D3 in each individual experiment served as the vehicle control. Cell lysates were prepared using the above-described lysis buffer, and Western blot analysis was performed as previously described [30, 37]. Equal amounts of solubilized proteins from either tissue or cultured cells (30–40 μg) were resolved in 8% and 10% SDS-PAGE. The proteins were transferred onto polyvinylidene fluoride membranes (Bio-Rad) and incubated with specific primary antibodies for 2 h at room temperature, followed by a 1-h incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. For detection of proteoglycan proteins such as fibromodulin, biglycan, or versican in Western blots, we used Santa Cruz antibodies. The anti-fibromodulin antibody has previously been used to show the protein expression in uterine fibroids and myometrium [38]. This antibody has also been used to examine the expression of fibromodulin in fibroproliferative disorders such as hypertrophic scarring [39]. On the other hand, the anti-biglycan antibody has also recently been used to examine the expression of biglycan in hypertrophic scarring [39], and the anti-versican antibody has been used to examine the expression of versican protein in overused supraspinatus tendon [40] and atherosclerosis disorders [41]. Moreover, the anti-fibronectin antibody has previously been used to determine fibronectin protein in Western blots [29, 42]. The antigen-antibody complexes in Western blots were detected with an enhanced chemiluminescence detection system (Amer sham Biosciences). Specific protein bands were visualized after exposure of autoradiography films and developing the films using an automatic x-ray developer. The intensity of each protein band was quantified using image-analysis software and normalized against beta-actin, and then the normalized ratio was used to generate data graphs.

RNA Isolation and First-stand cDNA Synthesis by Reverse Transcription

To examine the effects of 1,25(OH)2D3 on mRNA levels of proteoglycans such as fibromodulin, biglycan, and versican, HuLM cells were plated in 60-mm dishes, serum starved for 20 h, and then treated with increasing concentrations of 1,25(OH)2D3 (0, 1, 10, 100, and 1000 nM) for 48 h. Cells were collected from the plates, and total cellular RNA was isolated using RNasey Protect kit (Qiagen) as described previously [43]. RNA concentration was determined using a NanoVue system (GE Healthcare). First-strand cDNA was synthesized using the RT synthesis kit (GoScript Reverse Transcription System, Catalog no. A5000, Promega). First-strand cDNA was prepared by reverse transcriptase using 1 μg of total RNA in a 20-μl reaction volume containing oligo-dT primers, dNTPs, MgCl2, RNase inhibitor, and reverse transcriptase according to the manufacturer’s instruction. The reaction mixture was incubated for 1 h at 42°C and stopped by incubation at 70°C for 10 min.

Quantitative Real-time PCR

Quantitative real-time PCR was performed to determine the mRNA expression of fibromodulin, biglycan, and versican using total RNA from 1,25(OH)2D3-treated HuLM cells as described above. Primer sequences were obtained from the published literature [44, 45]. Primer sequences were as follows: fibromodulin, 5′-TTTTTATCATCCTTGCTCCTGACTG-3′ (sense) and 5′-TGTGTGGCAGGACCTTAAAGGA-3′ (antisense); biglycan, 5′-TTGCCCAAAACCTGTACTG-3′ (sense) and 5′-AAAAACGGTTGTGGACTCT-3′ (antisense); versican, 5′-TTGGAATGATTGCCTCTGACCA-3′ (sense) and 5′-AAGGTTCGGCACCTTTCACAACAG-3′ (antisense); and glyceroldehyde 3-phosphate dehydrogenase (GAPDH), 5′-ATGGGGCAAGTGAAGGTCG-3′ (sense) and 5′-TAAAAGAAGCCTTGTTGAC-3′ (antisense). Both sense and antisense primers for fibromodulin, biglycan, versican, and GAPDH were purchased from Qiagen. Amplification reactions were

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performed using a SYBR Green PCR Master Mix reagent kit (Qiagen/SA Bioscience). Briefly, 50 ng of each cDNA were added to the Master Mix containing appropriate primer sets (200 nM each) and SYBR Green in a 25-µl reaction volume. All samples were analyzed in triplicate measurements. RT-PCR analysis was performed using a MyiQ5 machine (Bio-Rad). Amplification of all genes was performed under the following cycling conditions: denaturation at 95°C for 10 min and then 45 cycles of 95°C for 15 sec and 60°C for 1 min. Synthesis of a DNA product of the expected size was confirmed by melting-curve analysis. Relative quantitation of target gene expression was calculated by the comparative cycle threshold (CT) method, which normalizes the copy number of the target gene to that of an endogenous reference gene. For each cDNA sample, the CT value of target sequence was used. GAPDH was chosen as the reference housekeeping gene based on the majority of the previous studies. The CT values of each gene were normalized to those of GAPDH, and the resultant ratios were used to express data.

Immunofluorescence Analysis

Both HuLM and UTSM cells were cultured on sterile glass cover slips, serum starved for 20 h, and treated with increasing concentrations of 1,25(OH)2D3 (0, 10, 100, and 1000 nM) for 48 h. The 0 nM concentration of 1,25(OH)2D3 in each individual experiment was considered as the vehicle control. Cells were fixed in a 3.7% formaldehyde solution at room temperature for 15 min. After washing in PBS, cells were permeabilized for 10 min using 0.2% Triton X-100/PBS, and then nonspecific binding was inhibited by blocking for 30 min in blocking/incubation solution containing 10% (wt/vol) fetal bovine serum (FBS)/0.1% (wt/vol) Triton X-100/PBS. Incubation with either primary anti-fibronectin or anti-collagen type I or anti-VDR antibody (1:50 dilution each) and Cyt3- or FITC-conjugated secondary antibody (1:150 dilution) were performed for 1 h at room temperature. Cells were washed for 15 min (three washes of 5 min each) with the above-described incubation solution, air-dried, and mounted onto microscopic slides with one drop of VECTASHIELD solution (Vector Laboratories) containing 4',6-diamidino-2-phenylindole. For staining with smooth muscle f-actin, cells were incubated with 2 µg/ml of FITC-conjugated phalloidin followed by washing with PBS for 15 min (three washes of 5 min each). Fluorescent images were taken using an Axiovert 100 M inverted microscope. Analyses of images were performed using image-analysis software (MCY-INST-10, MVIA, Inc.). Staining signals for structural smooth muscle f-actin (green) were visualized by fluorescent microscopy. Intensities of the signals were visually compared between the control and 1,25(OH)2D3-treated cells.

Laser Confocal Microscopy

Both HuLM and UTSM cells were cultured on sterile glass cover slips and treated with increasing concentrations of 1,25(OH)2D3 (0, 10, 100, and 1000 nM) for 48 h. Cells were fixed, permeabilized, and processed for immunofluorescence analysis using anti-collagen type I and anti-fibronectin antibodies and FITC-conjugated phalloidin as described above. Staining signals for collagen type I (red), fibronectin (red), and structural smooth muscle f-actin (green) were visualized using laser confocal fluorescent microscopy. A TE2000-U C1 confocal laser scanning microscope (Nikon) was used to collect fluorescence emissions with the 505- to 550-nm filter set. Confocal images were captured at 400X magnification under conditions similar to those described previously [30]. The EZ.C1 confocal software (Nikon) was then used to reconstruct serial z-plane images into a three-dimensional (3D) rendering, which was then transferred to PowerPoint (Microsoft) as described previously [30].

Statistical Analysis

All statistical data analyses were performed using Student t-test. The Student t-test was used to assess the significance of differences in levels of VDR in human uterine fibroids and the adjacent normal myometrium (M) samples. The Student t-test was also used to assess the significance of differences between both untreated control versus 1,25(OH)2D3-treated data points and within the 1,25(OH)2D3-treated data points. Values were considered to be statistically significant at the 95% confidence level for P < 0.05. Data are presented as the mean ± SD.

RESULTS

Human Uterine Fibroids Expressed Lower Levels of VDR than Adjacent Normal Myometrium

We recently demonstrated an association of lower levels of serum vitamin D3 were also lower in women with uterine fibroids as compared to the healthy counterpart. The biologically active vitamin D3, 1,25(OH)2D3, exerts its function in the cell system through interacting with the VDR [23]. The VDR is a nuclear receptor that functions as a transcription factor and plays a major role in the modulation of gene expression by interacting with the VDR-response element (VDRE) in the promoter region of target genes. We hypothesized that reduced levels of VDR might be an important risk factor for the pathogenesis of human uterine fibroids due to inadequate function of 1,25(OH)2D3. To test this hypothesis, we performed Western blot analysis for VDR expression using protein lysates that were prepared from human uterine fibroids and the adjacent normal myometrium tissues. We used rabbit polyclonal anti-VDR antibody from Santa Cruz Biotechnology that recognized approximately 56-kDa VDR protein. This anti-VDR antibody has previously been used successfully [29, 46]. We found that more than 60% of uterine fibroid tumors analyzed (25 of 40) showed reduced levels of VDR as compared to the adjacent normal myometrium (Supplemental Fig. S1; available online at www.bioreprod.org). The total Western blot data for VDR expression are shown in the Supplemental Data (Supplemental Fig. S1). To further evaluate whether the reduced levels of VDR in these 25 uterine fibroids were statistically significant, we determined the mean values of VDR levels in both uterine fibroids and the adjacent normal myometrium. These mean values of VDR were used to generate the graph shown in Figure 1. The reduced levels of VDR in those fibroid tumors were statistically very significant (P = 0.0002) compared to levels in the adjacent normal myometrium. These results suggest that reduced levels of VDR might be an important risk factor for the pathogenesis of human uterine fibroids.

1,25(OH)2D3 Treatment Induced VDR Expression in Cultured HuLM Cells

The 1,25(OH)2D3 has been shown to exerts its biological function by interacting with and inducing/activating VDR [23]. 1,25(OH)2D3 has also been shown to inhibit proliferation and promote differentiation of human cancer cells through the activation of VDR, which is a transcription factor of the nuclear receptor superfamily [47]. To test whether 1,25(OH)2D3 can sensitize HuLM cells via the induction of VDR, we performed Western blot analysis using lysates from cultured HuLM cells treated with increasing concentrations of 1,25(OH)2D3 (0, 1, 10, 100, and 1000 nM) for 8, 24, and 48 h. Equal amounts of each cell lysates were analyzed by Western blots using anti-VDR antibody. We found that 1,25(OH)2D3 treatment induced VDR expression in a concentration-dependent manner in HuLM cells (Fig. 2A). At low concentrations (1–10 nM), 1,25(OH)2D3 significantly induced VDR expression at 48 h compared to untreated control (P < 0.05) (Fig. 2A). Because 1,25(OH)2D3 showed a concentration-dependent effect at the 48-h time point, we used only the 48-h time point for the following experiments with HuLM cells. To further test the subcellular localization of VDR protein, we performed immunofluorescence analysis after treating HuLM cells with increasing concentrations of 1,25(OH)2D3 for 48 h. At the 10 nM concentration, 1,25(OH)2D3 induced nuclear VDR staining, which was further stimulated by higher concentrations of 1,25(OH)2D3 in HuLM cell (Fig. 2B). We further examined the levels of VDR in lysates from UTSM cells treated with 1,25(OH)2D3 for 48 h. Similar to HuLM cells, equal amounts of each lysate from UTSM cells were analyzed for VDR expression. We found that 1,25(OH)2D3 induced VDR levels...
in a concentration-dependent manner in UTSM cells, whereas the induction was not as strong as that in HuLM cells (Fig. 2C, left panel). We also analyzed VDR expression in UTSM cells after the treatment with 1,25(OH)\(_2\)D\(_3\) for the 8- and 24-h time points. However, 1,25(OH)\(_2\)D\(_3\) was enabled to induce VDR levels consistently (data not shown). Moreover, we compared the endogenous levels of VDR in both cell types and observed that HuLM cells expressed comparatively lower levels of VDR than UTSM cells (Fig. 2C, right panel). To further test whether 1,25(OH)\(_2\)D\(_3\) can also induce nuclear VDR in UTSM cells, we performed similar immunofluorescence analysis. We found that 1,25(OH)\(_2\)D\(_3\) induced nuclear VDR staining in UTSM cells. These results suggest that 1,25(OH)\(_2\)D\(_3\) can potentially sensitize HuLM cells while not markedly affecting normal UTSM cells. Thus, these results suggest that 1,25(OH)\(_2\)D\(_3\) potentially induces VDR protein expression in HuLM cells.

1,25(OH)\(_2\)D\(_3\) Reduced Collagen Type 1 Protein Expression in Cultured HuLM Cells

Collagen type 1 is the most abundant collagen subtype within human, and it plays an important role in the process of fibrosis. We tested whether 1,25(OH)\(_2\)D\(_3\) can affect collagen type 1 protein expression using HuLM cells treated with increasing concentrations of 1,25(OH)\(_2\)D\(_3\) for 48 h. Cell lysates were examined by Western blot analysis using anti-collagen type 1 antibody, which has negligible (<1%) cross-reactivity with collagen types 2–6. We have previously used this anti-collagen type 1 antibody in Western blot analysis [29]. In the present study, we found that at the 10 nM concentration, 1,25(OH)\(_2\)D\(_3\) significantly reduced collagen type 1 protein expression in HuLM cells compared with the untreated control \((P < 0.05)\) (Fig. 3A). We observed that the anti-collagen type 1 antibody recognized approximately 140 kDa as a major protein band by reducing SDS-PAGE (Fig. 3, A and E). 1,25(OH)\(_2\)D\(_3\) also reduced collagen type 1 protein levels in a concentration-dependent manner in HuLM cells. To further evaluate the pattern of collagen type 1 protein expression in uterine fibroid cells and compare it to that in normal myometrial cells, both HuLM and UTSM cells were treated with 1,25(OH)\(_2\)D\(_3\) for 48 h and then analyzed by immunofluorescence. Collagen type 1 staining was positive in both HuLM and UTSM cells. However, more intense and disorganized collagen type 1 staining was observed in HuLM cells compared with UTSM cells (Figs. 3, B and C). UTSM cells expressed considerably higher amount of endogenous VDR (Fig. 2C, right). Treatment with 1,25(OH)\(_2\)D\(_3\) reduced those disorganized staining of collagen type 1 in HuLM cells, whereas no obvious changes of collagen type 1 occurred in UTSM cells (Fig. 3E). Confocal microscopy revealed a similar staining pattern of collagen type 1 in UTSM cells, whereas disorganized collagen type 1 was present in HuLM cells (Fig. 3D). Furthermore, 1,25(OH)\(_2\)D\(_3\) reduced the presence of disorganized collagen type 1 in HuLM cells (Fig. 3D). To further examine the effect of 1,25(OH)\(_2\)D\(_3\) on collagen expression in UTSM cells, we also performed Western blot analysis using lysates from UTSM cells treated with 1,25(OH)\(_2\)D\(_3\). We found that 1,25(OH)\(_2\)D\(_3\) did not markedly reduce collagen type 1 expression in UTSM cells (Fig. 3E). These results suggest a potential role of 1,25(OH)\(_2\)D\(_3\) in the reduction of excessive disorganized production of collagen type 1 in HuLM cells.

1,25(OH)\(_2\)D\(_3\) Reduced Fibronectin Protein Expression in Cultured HuLM Cells

Fibronectin is a key component in ECM, which plays an important role in the process of fibrosis. To test whether 1,25(OH)\(_2\)D\(_3\) treatment affects the levels of fibronectin protein, we performed Western blot analysis using lysates from HuLM cells treated with increasing concentrations of 1,25(OH)\(_2\)D\(_3\). We found that at the 10 nM concentration, 1,25(OH)\(_2\)D\(_3\) significantly reduced fibronectin expression compared to control \((P < 0.05)\) (Fig. 4A). 1,25(OH)\(_2\)D\(_3\) also reduced fibronectin levels in a concentration-dependent manner in HuLM cells (Fig. 4A). To examine the effect of 1,25(OH)\(_2\)D\(_3\) on UTSM cells, we also performed similar Western blot analysis using lysates from UTSM cells treated with 1,25(OH)\(_2\)D\(_3\). We found that 1,25(OH)\(_2\)D\(_3\) reduced fibronectin expression in UTSM cells as compared to the untreated control. However, fibronectin expression in UTSM cells was not consistently reduced with 1,25(OH)\(_2\)D\(_3\) doses (Fig. 4E). To further evaluate the pattern of fibronectin expression in uterine fibroid cells and compare it to that in normal myometrial cells, both HuLM and UTSM cells were treated with 1,25(OH)\(_2\)D\(_3\) for 48 h and then analyzed by immunofluorescence. Fibronectin staining was observed in both HuLM and UTSM cells, whereas more intense and disorganized staining signals were observed in HuLM cells compared to UTSM cells (Fig. 4, B and C). We found that treatment with 1,25(OH)\(_2\)D\(_3\) reduced those disorganized staining of fibronectin in HuLM cells, whereas no obvious changes occurred in UTSM cells (Fig. 4, B and C). Additionally, confocal microscopy demonstrated parallel and organized fibronectin staining in UTSM cells, whereas more disorganized signals were observed in HuLM cells (Fig. 4D). Furthermore, treatment with 1,25(OH)\(_2\)D\(_3\) reduced excessive fibronectin staining in HuLM cells. These results suggest that 1,25(OH)\(_2\)D\(_3\) can effectively reduce the excessive production of fibronectin in HuLM cells.

1,25(OH)\(_2\)D\(_3\) Reduced PAI-1 Protein Expression in Cultured HuLM Cells

A TGF-β target gene, PAI-1 is known to be associated with the process of tissue fibrosis. To test whether the PAI-1 protein levels are affected by 1,25(OH)\(_2\)D\(_3\) in uterine fibroid cells, we
performed Western blot analysis using lysates from HuLM cells treated with different concentrations of 1,25(OH)\(_2\)D\(_3\) for 48 h. Equal amounts of each lysate from control and 1,25(OH)\(_2\)D\(_3\)-treated HuLM cells were analyzed for PAI-1 protein expression using anti-PAI-1 antibody. We found that at the 10 nM concentration, 1,25(OH)\(_2\)D\(_3\) significantly reduced PAI-1 protein expression in HuLM cells compared to control \((P<0.05)\) (Fig. 5A). Furthermore, 1,25(OH)\(_2\)D\(_3\) reduced PAI-1 protein expression in HuLM cells in a concentration-dependent manner (Fig. 5A). To further test the effect of 1,25(OH)\(_2\)D\(_3\) on PAI-1 protein expression, we performed similar Western blot analysis using lysates from 1,25(OH)\(_2\)D\(_3\)-treated UTSM cells. We found that 1,25(OH)\(_2\)D\(_3\) reduced PAI-1 protein in a concentration-dependent manner in UTSM cells, whereas this reduction was not as strong as HuLM cells (Fig. 5B). These results suggest the potential of 1,25(OH)\(_2\)D\(_3\) in regulating the TGF-\(\beta\) target PAI-1 gene expression in HuLM cells.

1,25(OH)\(_2\)D\(_3\) Reduced mRNA and Protein Expressions of Proteoglycans in Cultured HuLM Cells

Proteoglycans are glycosylated proteins, which are important components in the ECM of connective tissues [48]. The small, leucine-rich proteoglycans such as fibromodulin, biglycan, and versican play major roles in ECM organization, and these proteins are overexpressed during the development of diseases. To test whether 1,25(OH)\(_2\)D\(_3\) can reduce expression of those proteoglycans, we performed Western blot analysis using lysates from HuLM cells treated with increasing concentrations of 1,25(OH)\(_2\)D\(_3\) (0, 10, 100, and 1000 nM) for 48 h. Treated cells were fixed, permeabilized, and incubated with anti-VDR antibody, followed by staining with FITC (green)-conjugated rabbit secondary antibody. Staining signals for VDR expression was monitored using a fluorescent microscopy. Nuclei of cells were staining with 4',6 diamidino-2-phenylindole. Original magnification \(\times\)200 (B and D).
FIG. 3. Effect of 1,25(OH)₂D₃ on protein expression of collagen type 1 (collagen) in HuLM and UTSM cells. A) HuLM cells were serum starved and treated with increasing concentrations of 1,25(OH)₂D₃ (0, 1, 10, 100, and 1000 nM) for 48 h. Equal amounts of each cell lysate were examined by Western blot analysis using anti-collagen type 1 antibody. Western blot analysis with anti-β-actin antibody was used as a loading control. The intensity of each protein band was quantified and normalized to the corresponding β-actin, and then the signal ratio (collagen:β-actin) was used to generate the graph. *P < 0.05 when compared to control, **P < 0.05 when compared between 1,25(OH)₂D₃-treated data points. Data are presented as the mean ± SD (n = 3 independent measurements). B and C Immunofluorescence analysis was performed using HuLM (B) and UTSM (C) cells cultured on sterile glass cover slips. Cells were serum starved and treated with increasing concentrations of 1,25(OH)₂D₃ for 48 h. Treated cells were fixed, permeabilized, and then incubated with anti-collagen type 1 antibody, followed by staining with CY3 (red)-conjugated specific secondary antibody. Staining signals for collagen type 1 (red) were monitored using a fluorescent microscopy. Nuclei of cells were stained with 4',6-diamidino-2-phenylindole. D) Immunofluorescence analysis was performed as described above, and pictures were captured using laser confocal microscopy. E) UTSM cells were treated with 1,25(OH)₂D₃ as described above, and then cell lysates were examined by Western blot analysis for collagen type 1 protein expression. In addition, protein lysates from human uterine fibroid tumor (fibroid) and the adjacent myometrium (Myo) were also analyzed for collagen type 1 protein expression. Data were analyzed as described above. Original magnification ×200 (B–D).
The levels of biglycan in HuLM cells were also verified by Western blot analysis, and these levels were significantly reduced by 1,25(OH)₂D₃ compared to untreated control (P < 0.05) (Fig. 6C). Moreover, we observed concentration-dependent reduction of biglycan protein in HuLM cells after 1,25(OH)₂D₃ treatment. On the other hand, versican is a large ECM proteoglycan that acts as a structure molecule in ECM and plays a role in key events during the
development of uterine fibroids. We found that versican expression was significantly reduced at the 10 nM concentration of 1,25(OH)₂D₃ in HuLM cells. Moreover, 1,25(OH)₂D₃ reduced versican expression in a concentration-dependent manner (P < 0.05) (Fig. 6E). To further examine the effects of 1,25(OH)₂D₃ on mRNA expression of the above-mentioned proteoglycans, we performed quantitative real-time PCR analysis using total RNA from HuLM cells treated with increasing concentrations of 1,25(OH)₂D₃. Similar to protein expressions, 1,25(OH)₂D₃ treatment reduced mRNA expression of fibromodulin (Fig. 6B), biglycan (Fig. 6D), and versican (Fig. 6F) in cultured HuLM cells. These results suggest that 1,25(OH)₂D₃ has the potential to reduce the expression of proteoglycans in cultured HuLM cells.

1,25(OH)₂D₃ Reduced Disorganized Structural Actin Fibers in Cultured HuLM Cells

We next evaluated whether 1,25(OH)₂D₃ has any effect on expression of structural smooth muscle actin fibers by performing immunofluorescence analysis using HuLM and UTSM cells treated with or without increasing concentrations of 1,25(OH)₂D₃. Cells were then fixed in formaldehyde solution, permeabilized, and stained with FITC-conjugated phalloidin to stain structural smooth muscle f-actin. Staining for f-actin was visualized by a fluorescent microscopy. We observed homogeneous parallel staining of actin fibers in UTSM cells, whereas disorganized accumulation of actin staining was visualized in HuLM cells (Fig. 7, A and B). Treatment with 1,25(OH)₂D₃ reduced the excessive deposition of disorganized actin fibers in HuLM cells but not in UTSM cells (Fig. 7, A and B). Laser confocal microscopy also revealed a significant reduction in staining of actin fibers by 1,25(OH)₂D₃ (Fig. 7C). We quantified the fluorescent staining using image-analysis software and found that 1,25(OH)₂D₃ significantly reduced (1.6- and 3.5-fold) fluorescent intensity of actin fibers in a concentration-dependent manner (P < 0.05) (Fig. 7C). We further used Western blot analysis to quantitatively measure the effect of 1,25(OH)₂D₃ on actin expression using lysates from HuLM and UTSM cells treated with and without increasing concentrations of 1,25(OH)₂D₃. At the 10 nM concentration, 1,25(OH)₂D₃ significant reduced the expression of actin fibers in HuLM cells (P < 0.05) (Fig. 7D). On the other hand, 1,25(OH)₂D₃ did not markedly reduced α-actin levels in UTSM cells, and reduction of α-actin was not concentration-dependent (Fig. 7E). These results suggest that 1,25(OH)₂D₃ has the potential to reduce the synthesis and accumulation of disorganized actin fibers in HuLM cells.
DISCUSSION

In the present study, we examined the risk of reduced expression of VDR for the pathogenesis of human uterine fibroids. VDR is a nuclear receptor that modulates gene expression when interacting with its ligand, 1,25(OH)\textsubscript{2}D\textsubscript{3}, the biologically active form of vitamin D\textsubscript{3}. The cellular effects of VDR signaling include growth arrest, differentiation, and/or induction of apoptosis, demonstrating the participation of vitamin D signaling in the inhibition of cell growth. Herein, we determined the protein levels of VDR in human uterine fibroid tumors, and we compared these levels with those in the adjacent normal myometrium. Although we analyzed a total of 40 uterine fibroids and their adjacent normal myometrium, more than 60% of cases (25 of 40) showed reduced levels of VDR, as shown in the Supplemental Figure S1. We further determined that the reduced levels of VDR in these 25 uterine fibroid tumors were statistically significant (\(P = 0.0002\)) (Fig. 1). These results demonstrate that loss or inadequate expression of VDR might be a risk for the development of human uterine fibroids. The bioactive 1,25(OH)\textsubscript{2}D\textsubscript{3} is known to function via binding to nuclear VDR for activation of further downstream signaling in cell systems. The reduced levels of VDR in uterine fibroid tumors suggest an association with the pathogenesis of uterine fibroids. On the other hand, we also observed that in approximately 40% of uterine fibroid cases, the levels of VDR protein were either upregulated or unchanged (Supplemental Fig. S1). However, it is unknown how the upregulation of VDR might be involved in uterine fibroid pathogenesis.

In the present study, we next verified the biological function of 1,25(OH)\textsubscript{2}D\textsubscript{3} through the induction of VDR expression in both HuLM and UTSM cells. HuLM cells serve as a suitable model of human uterine fibroids because these well-character-
FIG. 7. Effect of 1,25(OH)\(_2\)D3 on expression and accumulation of structural actin fibers in HuLM and UTSM cells. A and B) Immunofluorescence analysis was performed after culturing HuLM (A) and UTSM (B) cells on sterile glass cover slips and treatment with increasing concentrations of 1,25(OH)\(_2\)D3 (0, 10, 100, and 1000 nM) for 48 h. Treated cells were fixed, permeabilized, and stained with FITC-conjugated phalloidin. The smooth muscle f-actin staining (green) was monitored by a fluorescent microscopy. Nuclei of cells were stained with 4',6 diamidino-2-phenylindole (blue). A high-magnification of the region is shown (bottom right). C) Immunofluorescence analysis was also performed as described above. The f-actin signals were captured and visualized using fluorescent laser confocal microscopy (top). Fluorescent staining was quantified by image-analysis software, and relative fluorescent intensity is shown. *\(P < 0.05\) compared to control (bottom). D and E) Equal amounts of lysates from HuLM (D) and UTSM (E) cells treated with increasing concentrations of 1,25(OH)\(_2\)D3 (0, 1, 10, 100, and 1000 nM) were examined by Western blot analysis using anti-\(\alpha\)-actin antibody. The intensity of each protein signal was quantified and normalized to the corresponding \(\beta\)-actin, and then the signal ratio (\(\alpha\)-actin: \(\beta\)-actin) was used to generate the graphs. *\(P < 0.05\) compared to control, **\(P < 0.05\) compared between 1,25(OH)\(_2\)D3-treated data points. Data are presented as the mean ± SD (n = 3 independent measurements). Original magnification \(\times200\) (A and B) and \(\times400\) (inset in A, inset in B, and C).
1,25(OH)\(_2\)D3 REDUCES ECM-ASSOCIATED PROTEINS

Our results demonstrated that HuLM cells expressed detectable levels of VDR, whereas treatment with 1,25(OH)\(_2\)D3 significantly induced nuclear VDR expression in a concentration-dependent manner (P < 0.05) (Fig. 2, A and B). Our Western blot data also showed that HuLM cells expressed lower levels of VDR compared to UTSM cells (Fig. 2C, right). Although UTSM cells are somewhat responsive to 1,25(OH)\(_2\)D3 treatment to induce VDR levels, the response is quite weak compared to HuLM cells (Fig. 2C, left). Because UTSM cells were derived from normal human uterus [34] and these cells expressed comparatively higher levels of VDR (Fig. 2C, right), further treatment with 1,25(OH)\(_2\)D3 could not considerably induce VDR levels in those cells. These results suggest that 1,25(OH)\(_2\)D3 treatment can potentially sensitize HuLM cells while not significantly affecting UTSM cells. Moreover, 1,25(OH)\(_2\)D3 exerts its cellular function by inducing nuclear VDR levels in HuLM cells. This result is consistent with our previous observation that 1,25(OH)\(_2\)D3 can induce VDR expression in vitro cell culture [46] and in uterine fibroids tumors in an in vivo Eker rat model [29]. Moreover, this result is also consistent with previous observations where an analog of 1,25(OH)\(_2\)D3 functions via the induction of VDR in both breast and prostate cancer cells [51]. An analog of vitamin D3 (paricalcitol), also known as Zemplar, has also been shown to function through the activation/induction of VDR and to inhibit cardiac fibrosis in a mouse model [52]. Our findings suggest a possible mechanism through which 1,25(OH)\(_2\)D3 can function in uterine fibroid cells by inducing/activating the nuclear VDR levels, which can further interact with retinoid X receptor alpha (RXR-\(\alpha\)) to form a VDR-RXR-\(\alpha\) complex. This VDR-RXR-\(\alpha\) heterodimeric complex further binds to specific VDREs in the promoter regions of vitamin D target genes, including genes that are associated with the process of fibrosis.

Next, we examined the effects of 1,25(OH)\(_2\)D3 on ECM-associated proteins because the ECM plays an important role in the process of tissue fibrosis. It has been demonstrated previously that the excessive synthesis and accumulation of ECM leads to the process of tissue fibrosis [53]. Herein, we evaluated the role of 1,25(OH)\(_2\)D3 in the regulation of ECM-associated protein expression in human uterine fibroid cells. We observed significant reduction of collagen type 1 and fibronectin proteins following 1,25(OH)\(_2\)D3 treatment (Figs. 3 and 4), suggesting a potential role of 1,25(OH)\(_2\)D3 in the regulation of key fibrotic proteins. Using immunofluorescence staining, we observed that collagen type 1 was localized primarily in the cytoplasm of both HuLM and UTSM cells (Figs. 3 and 4). However, fibronectin was localized around UTSM cells, whereas it was localized primarily in the cytoplasm of HuLM cells (Fig. 4). These findings are, to some extent, consistent with the observation by Stewart et al. [17] that fibronectin was localized primarily around individual smooth muscle cells and that collagen type 1 was distributed across the ECM and in the cytoplasm of smooth muscle cells. The limitation of this study is that we analyzed ECM using a 2D rather than a 3D cell culture model system that can mimic the actual living tumor environment and can produce abundant ECM [54]. Thus, it is important to use an established 3D cell culture system that produces abundant ECM. Nevertheless, in the present study, we examined the role of 1,25(OH)\(_2\)D3 on reduction of ECM production in cultured HuLM cells. Our results support the view that uterine fibroid cells can undergo molecular alterations that may increase the fibrotic phenotype of these tumor cells by overproduction and accumulation of fibrotic proteins. These alterations in ECM-associated protein expression can be effectively suppressed by 1,25(OH)\(_2\)D3 in uterine fibroid cells.

Collagen is a family of proteins that are the main components of the ECM. Although several different types of collagenic proteins exist, type I and type III comprise approximately 90% of total collagen [55]. The overall balance of ECM deposition can be affected by TGF-\(\beta\) through a variety of biological activities. Studies have shown that TGF-\(\beta\) expression is elevated in a wide array of fibrotic processes, including pulmonary fibrosis, liver cirrhosis, scleroderma, and keloids [56–59].

The TGF-\(\beta\) has been shown to induce ECM production and to stimulate expression of type 1 collagen (COL1A1) and PAI-1 [18]. TGF-\(\beta\) directly increases the expression of collagen type 1 and collagen type 2 transcripts [60, 61]. Uterine fibroids exhibit increased levels of ECM gene expression, and the ECM collagen fibril structure and orientation have been found to be disoriented and loosely packed in a nonparallel manner compared to matched normal myometrium [62, 63]. The deregulated accumulation of the ECM represents an imbalance between synthesis and dissolution and produces a fibroproliferative condition called keloids [9]. Figure 3 shows a concentration-dependent inhibition of collagen type 1 expression, demonstrating an important role of 1,25(OH)\(_2\)D3 in the regulation of ECM deposition and that reduction of ECM deposition may ultimately lead to reduce uterine fibroid cell proliferation.

Another ECM-associated protein, fibronectin, forms fibrils that are associated with matrix components and promotes cell adhesion and migration, cytodifferentiation, phagocytosis, and cell growth [64]. It has been demonstrated that in adult lung, fibronectin is specifically localized at the basal membrane, around the smooth muscle cells and in the alveolar lining fluid [65]. The excessive synthesis and deposition of the collagenic protein was found in fibroplasia, a condition where fibronectin production is elevated [65]. We tested the effect of 1,25(OH)\(_2\)D3 on fibronectin expression in uterine fibroid cells. Figure 4 shows that 1,25(OH)\(_2\)D3 at 1–10 nM concentrations significantly reduced fibronectin expression in HuLM cells but not in UTSM cells, suggesting its regulatory role in ECM deposition in uterine fibroid cells. Moreover, PAI-1 is a TGF-\(\beta\) target gene known to be associated with the process of fibrosis [66]. Our results showed significant reduction of PAI-1 protein expression by 1,25(OH)\(_2\)D3 treatment in HuLM cells (P < 0.05) (Fig. 5A), which demonstrates the potential role of 1,25(OH)\(_2\)D3 in the regulation of fibrosis process.

Proteoglycans are important components in all ECM and are known to be elevated in uterine fibroids, so we also addressed whether 1,25(OH)\(_2\)D3 can affect the expression of proteoglycans such as fibromodulin, biglycan, and versican in HuLM cells. Fibromodulin is a collagen-binding proteoglycan that plays a key role in fibrogenesis by regulating collagen fibril spacing and thickness. It has been shown that fibromodulin can promote tumor cells motility and invasion [67]. Fibromodulin mRNA and protein levels were found to be upregulated in uterine fibroids [38]. Figure 6, A and B, shows that 1,25(OH)\(_2\)D3 reduced mRNA and protein levels of fibromodulin in HuLM cells, which suggests the potential role of 1,25(OH)\(_2\)D3 in regulating the ECM. Furthermore, biglycan is a small, leucine-rich proteoglycan in ECM that influences differentiation and proliferation processes [68], and we tested whether 1,25(OH)\(_2\)D3 can regulate biglycan expression in HuLM cells. Our results (Fig. 6, C and D) showed that mRNA and protein levels of biglycan were reduced by 1,25(OH)\(_2\)D3 in a concentration-dependent manner in HuLM cells, suggesting ECM regulation by 1,25(OH)\(_2\)D3. It has been demonstrated...
that versican is a large proteoglycan in ECM and is associated with cell proliferation and apoptosis [69]. The mRNA and protein levels of versican are overexpressed in uterine fibroids [19], and it has also been demonstrated that the aberrant production of excessive and disorganized ECM involves in the activation of the TGF-β signaling and that the excessive production of versican can lead to the fibroid phenotype [19]. Therefore, we next tested the effect of 1,25(OH)2D3 on the regulation of versican expression in both mRNA and protein levels in uterine fibroid cells. Our results showed reduction of mRNA and protein levels of versican in HuLM cells by 1,25(OH)2D3, and these results further indicate the role of 1,25(OH)2D3 in the regulation of ECM deposition.

The smooth muscle actin fibers act as structural support proteins for uterine fibroid cells. A previous study has shown that uterine fibroid cells produce excessive amounts of actin molecules and that structural changes in cell size occur due to the deposition and disorganization of structural actin fibers [70]. In the present study, we examined the effect of 1,25(OH)2D3 on expression of those smooth muscle actin proteins in HuLM cells. Figure 7 shows a significant reduction of structural smooth muscle f-actin by 1,25(OH)2D3 in HuLM cells but not in UTSM cells. Moreover, we observed a concentration-dependent reduction of α-actin in 1,25(OH)2D3-treated HuLM cells but not in UTSM cells (Fig. 7, D and E). These findings further demonstrate that the function of 1,25(OH)2D3 is reducing the excessive synthesis and deposition of disorganized structural smooth muscle actin fibers, which ultimately can lead to the reduction of uterine fibroid phenotype.

In conclusion, we observed reduced levels of VDR in human uterine fibroids as compared to the adjacent normal myometrium. Treatment with 1,25(OH)2D3 significantly induced nuclear VDR expression in a concentration-dependent manner in HuLM cells. 1,25(OH)2D3 also significantly reduced the expression of ECM-associated collagen type I, fibronectin, and PAI-1 in HuLM cells but not in UTSM cells. Moreover, 1,25(OH)2D3 reduced mRNA and protein levels of proteoglycans such as fibromodulin, biglycan, and versican in HuLM cells. These proteoglycans are the major components of ECM and are known to be upregulated in uterine fibroids. Furthermore, 1,25(OH)2D3 reduced excessive deposition and disorganized structural actin fibers in HuLM cells. Taken together, our findings suggest that human uterine fibroids expressed reduced levels of VDR and that reduction of VDR might be a risk factor for fibroid pathology. In addition, treatment with 1,25(OH)2D3 can effectively reduce the expression of major ECM-associated proteins and structural actin fibers in HuLM cells. Thus, 1,25(OH)2D3 may have potential as an effective therapeutic agent for a safe, nonsurgical treatment option for human uterine fibroids.

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