Vitamin D inhibits myometrial and leiomyoma cell proliferation in vitro

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Objective: To determine the effect of 1,25(OH)2D3 and 25(OH)D3 vitamin D derivates on the growth of leiomyoma and myometrial cells in vitro.

Design: In vitro study.

Setting: Cell biology research laboratory.

Patient(s): Six premenopausal women with uterine leiomyomas undergoing hysterectomy.

Intervention(s): Samples of leiomyomas and normal myometrial tissue were obtained, and paired cultures were established.

Main Outcome Measure(s): A colorimetric crystal violet assay to determine the effect of 1,25(OH)2D3 and 25(OH)D3 on cell growth.

Result(s): In both myometrial and leiomyoma cells, 0.1 nM physiologic level of 1,25(OH)2D3 inhibited growth by 12% when compared with controls. The growth inhibition was concentration dependent; the highest concentration of 1,25(OH)2D3 (100 nM) inhibited growth by 62% in both cell types. All the differences were statistically significant. A slight stimulation (<4%) of cell proliferation was observed with the lowest 25(OH)2D3 concentrations. When treated with either a 500 nM or 1000 nM concentration of the compound, the growth of both cell types fell to approximately 50% of that of the control cultures, and the level of inhibition with the latter concentration was statistically significant.

Conclusion(s): Both myometrial and leiomyoma cell growth in vitro was effectively inhibited by 1,25(OH)2D3. Vitamin D may play a role in the growth of uterine leiomyomas. (Fertil Steril 2009;91:1919–25. ©2009 by American Society for Reproductive Medicine.)

Key Words: Uterine leiomyoma, vitamin D, hypovitaminosis, cell growth, in vitro

Vitamin D is a potent antiproliferative and immunomodulatory secosteroid hormone with a well-established role in the maintenance of calcium homeostasis. Synthesis of 1,25(OH)2D3, the active form of vitamin D, is through sunlight-induced conversion of 7-dehydrocholesterol to vitamin D3 and its successive hydroxylations to 25(OH)D3 and 1,25(OH)2D3 in the liver and the kidney. The signaling of 1,25(OH)2D3 is mediated via its ubiquitously expressed nuclear receptor, the vitamin D receptor (VDR), which is expressed in both the myometrium and endometrium of the human uterus throughout the menstrual cycle (6–8).

The most bioactive form of vitamin D [1,25(OH)2D3] modulates cell proliferation, differentiation, cancer invasion, and angiogenesis (9–12). Vitamin D deficiency may stimulate cell proliferation (12). The vitamin D [1,25(OH)2D3]-induced antiproliferative action is mediated predominantly through a G1/S phase block of the cell-cycle. Because 1,25(OH)2D3 regulates many of the cell cycle regulatory genes and reduces or increases the kinase activities of cyclin-dependent kinases (CDKs), this results in a decreased number of cells in the S phase and an accumulation of cells in

Uterine leiomyomas (fibroids), the most common benign clonal tumors in the female reproductive system, arise from smooth muscle cells of the uterus and contain extracellular matrix. Many fibroids are asymptomatic, but their presence may be associated with pressure effects, menstrual problems, pain, miscarriage, and infertility (1). Leiomyoma is a leading cause of hysterectomy in the Western world (2).

The precise pathophysiology of leiomyoma remains unknown, although estrogen and progesterone are recognized as promoters of tumor growth. Chromosomal abnormalities, hormonal deregulation, and growth and angiogenic factors have all been implicated in the etiology of these clonal smooth muscle cell proliferations (3–5).
the G0–G1 phase (13–15). The cyclin-dependent kinase inhibitors p21 and/or p27 are genomic targets of the 1,25(OH)2D3–VDR complex in many cell types (16, 17). Also, 1,25(OH)2D3 blocks mitogenic signaling, including that of estrogen, epidermal growth factor (EGF), and insulin-like growth factor 1 (IGF-1), and up-regulates growth inhibitors such as transforming growth factor β (TGF-β) (18). In addition, 1,25(OH)2D3 activates VDR-mediated apoptosis (19).

Despite the extensive research on the role of vitamin D in the inhibition of cell growth in a large variety of tissues, there have been no reports concerning the possible growth modulatory effects of vitamin D on benign uterine tumor cells. In the present study, we evaluated the potential effects of 1,25(OH)2D3 and 25(OH)D3 on the growth of leiomyoma and myometrial cells in vitro.

MATERIALS AND METHODS

Tissue Collection

The present study was conducted with the approval of the ethics committee of Tampere University Hospital, and fully informed consent was obtained from each patient before surgery. Samples of benign leiomyomas and of adjacent matched myometrial tissue were obtained from six premenopausal women undergoing hysterectomy during the proliferative phase of the endometrium. The mean age of the patients was 44 years (range: 38 to 49 years), and none of them had received any hormone therapy for at least 6 months before the surgery. Immediately after removal of the uterus, tissue specimens were taken from intramural leiomyoma (size range: 3 to 5 cm) and from myometrial tissue. Tissue specimens were taken into ice-cold DMEM/F12 medium (GIBCO BRL, Paisley, Scotland) supplemented with 100 IU/mL of penicillin, 100 μg/mL of streptomycin (penicillin/streptomycin solution, GIBCO BRL), and 2.5 μg/mL of amphotericin B (Sigma Chemical, St. Louis, MO) for transport to the laboratory. A portion of each specimen was fixed in 10% neutral buffered formalin before routine processing in paraffin blocks.

Cell Culture

The tissue specimens were cut into small pieces and digested in 0.1% collagenase A (Roche, Mannheim, Germany) in culture medium (phenol red-free DMEM/F12) (GIBCO BRL) supplemented with 10% dextran-coated-charcoal-treated (DCC) fetal calf serum (GIBCO BRL) and antibiotic/antimycotic agents as already described) at 37°C overnight. The digest was passed through a wire sieve, and the cells were collected by centrifugation at 1000 rpm × g for 5 minutes and washed several times with fresh culture medium. The isolated cells were seeded in 75-cm² flasks in culture medium supplemented with 1 nM estradiol (Sigma Chemical) and propagated at 37°C in a humidified atmosphere containing 5% CO2 in air. Cells from subconfluent primary or secondary cultures were used in all the experiments.

Immunohistochemistry

For the immunohistochemical analysis, cells were seeded in four-chamber cell culture slides (Lab-Tek II Chamber slide, Nalge Nunc, Naperville, IL) at 5000 cells/chamber and cultured until subconfluence. Thereafter, the cells were fixed in 2% paraformaldehyde for 20 minutes at room temperature and were permeabilized with prechilled 94% ethanol for 10 minutes on ice. The following mouse monoclonal antibodies were used at the dilutions indicated: anti-estrogen receptors (ER; 1:100) and progesterone receptor (PR; 1:300) (NovoCastra Laboratories, Newcastle upon Tyne, United Kingdom), antivimentin (1:500), anti-smooth muscle actin (SMA; 1:100), and antidesmin (1:100) (Dako, Glostrup, Denmark). Rat monoclonal anti-VDR antibody (1:200) was obtained from Neo Markers (Fremont, CA). In the controls, the primary antibodies were omitted, and staining done with nonimmunized mouse or rat IgG. The staining was performed with a broad-spectrum Zymed Histostain-Plus kit (Zymed Laboratories, South San Francisco, CA) with the following modifications to the manufacturer’s protocol: primary antibodies were incubated overnight at 4°C and biotinylated secondary antibodies for 20 minutes at room temperature. All washings were repeated three times, 5 minutes each. The cells were counterstained with hematoxylin.

The percentage of cells expressing SMA, desmin, or vimentin was determined by calculating the number of immunopositive and immunonegative cells in five randomly selected microscopic fields at a magnification of ×200.

We visualized VDR in paraffin-embedded tissue samples by immunohistochemical staining of deparaffinized 5-μm sections with the rat monoclonal anti-VDR antibody (1:20). For unmasking of the epitope, the sections were boiled in a microwave oven (1000 W) in 0.01 mol/L citrate buffer (pH 6.0) for 10 minutes before application of the primary antibody. The staining procedure has already been described.

Cell Growth Assay

Leiomyoma cells and matched normal myometrial cells were seeded into 96-well microtiter plates at a density of 2000 cells per 200-μL well (in two cases, 1000 and 1500 cells/well) and were allowed to attach for 48 hours in culture medium supplemented with 1 nM estradiol. The medium was then replaced, and the cells were subjected to treatment with the indicated concentrations of 1,25(OH)2D3 and 25(OH)D3 (Fluka, Buchs, Switzerland) in culture medium supplemented with 1 nM estradiol. Ethanol vehicle was included in the control treatment. The media were renewed every 3 days.

Relative cell numbers were assessed at 0, 3, 6, 9, 12, and 15 days using the colorimetric crystal violet assay (20). The cells were fixed on the bottom of the wells by the addition of 10 μL of 11% glutaraldehyde solution to 100 μL of medium. After 15 minutes, the plates were washed with deionised water, air-dried, and stained with 0.1% solution of crystal violet for 20
minutes. Thereafter, excess dye was washed out with deionised water, and the plate was air-dried. The bound dye was then dissolved with 10% acetic acid, and the optical densities of the extracts were measured at 590 nm using a Victor 1420 Multilabel Counter (Wallac, Turku, Finland).

Eight determinations were used to calculate the mean optical density at each time point. The absorbance value of day 0 was set to 0 by subtracting the value for that day from each value obtained from the subsequent timepoint measurements (days 3 to 15). Growth curves were created based on the basis of the resultant absorbance values. Day 9 was used to compare the effect of the hormone treatments.

Mean ± standard error of the mean (SEM) was calculated for each treatment, and statistical differences were assessed using the Wilcoxon signed-rank test. \( P \leq 0.05 \) was considered statistically significant.

**RESULTS**

**Immunohistochemical Characterization**

Characterization of the cell cultures was done by immunohistochemical means with antibodies against two proteins associated with the smooth muscle phenotype, SMA and desmin, and the intermediate filament protein distinctive of the fibroblastic phenotype, vimentin. Antibodies specific to VDR, ER, and PR were employed to assess steroid receptor expression in the cultivated cells.

Smooth muscle actin (Fig. 1A) was shown to be expressed in 74% ± 8% (mean ± SEM) of normal myometrial and in 67% ± 12% of leiomyoma cells; the corresponding percentage values for the desmin staining (see Fig. 1B) were 9% ± 2% and 8% ± 2%, respectively. An intense vimentin immunoreactivity was observed in all cells (see Fig. 1C). Whereas all the intact tissue samples expressed ER and PR (data not shown), the overall ER staining in cultured cells proved negative with the exception of a few immunopositive nuclei (see Fig. 1D, arrow). We did not detect PR (see Fig. 1E). Virtually all myometrium and leiomyoma cells were positive for VDR (see Fig. 1F), and its staining appeared characteristically in discrete foci in the nucleus.

The steroid receptor status was shared with all the six pairs of myometrium and leiomyoma cultures studied. Control stainings were negative (data not shown). Immunohistochemical analysis of tissue samples showed that VDR was present in all myometrial cultures (Fig. 2A) and their matched leiomyoma specimens (see Fig. 2B). No clear differences in staining intensities between samples could be demonstrated.

**Growth Pattern of Cell Cultures**

The growth curves of normal myometrial and leiomyoma cell cultures from six patients are illustrated in Figure 3. The curves show exponential growth of both cell types with no statistically significant differences in their mean rates of proliferation. The growth rates varied markedly between individuals, and no consistent differences were detected between the growth rates of paired myometrial and leiomyoma cell cultures originating from the same patient. The initial cell number (1000 to 2000 cells) seemed not to have an impact on the final shape of the growth curves.

**Regulation of Myometrial and Leiomyoma Cells by 1,25(OH)\(_2\)D\(_3\) and 25(OH)D\(_3\)**

To test the effects of 1,25(OH)\(_2\)D\(_3\) and 25(OH)D\(_3\) on the growth of normal myometrial and leiomyoma cells, the cells were treated for 9 days with increasing doses of the two compounds, as indicated in Figure 3. In both myometrial and leiomyoma cells, a similar concentration-dependent growth inhibition with 1,25(OH)\(_2\)D\(_3\) was detected (Fig. 4A and B). In both cell types, the lowest amount of 1,25(OH)\(_2\)D\(_3\) tested (0.1 nM) inhibited growth by 12% when compared with controls.

As illustrated in Figures 4C and D, a slight stimulation of cell proliferation was observed with the lowest 25(OH)D\(_3\) concentrations (10 nM and 100 nM) in some myometrial and leiomyoma cultures. When treated with the compound in a 1000 nM concentration, the growth of both cell types fell to approximately 50% of that of the control cultures, and the size of the inhibition was statistically significant \((P<.05)\). In the case of 500 nM 25(OH)D\(_3\), where the number of cultures tested was four, statistical significance was not reached \((P=.068)\).

**DISCUSSION**

Our preliminary results show that primary or secondary cultures of both normal myometrial and leiomyoma cells are highly sensitive to the growth-inhibiting effect of 1,25(OH)\(_2\)D\(_3\). We found that 1,25(OH)\(_2\)D\(_3\) was able to statistically significantly suppress the growth of both cell types at a concentration as low as 0.1 nM, which lies within the physiologic concentration range (48–156 pM) of 1,25(OH)\(_2\)D\(_3\) (21). These results add to previous data on the inhibitory effect of 1,25(OH)\(_2\)D\(_3\) on primary cultures of human cells from various organs including the prostate (21, 22) and epidermis (23).

In many cell types, low concentrations of 1,25(OH)\(_2\)D\(_3\) have been shown to stimulate rather than inhibit cell proliferation (23–26). Here, however, the 0.1nM concentration of 1,25(OH)\(_2\)D\(_3\) inhibited cell growth by as much as 12%, and the percentage gradually increased to over 60% with 100 nM 1,25(OH)\(_2\)D\(_3\). Myometrial and leiomyoma cells are clearly target cells of 1,25(OH)\(_2\)D\(_3\). The data are consistent with the observed expression of VDR protein in the myometrial and leiomyoma tissues and cultivated cells and with our previous description of VDR mRNA expression in myometrial biopsies (8). The punctuate pattern of expression within
the nuclei of cultured cells has also been observed in other cell types and may display specific binding sites of VDR to target genes (21).

By contrast, the effects of the two lowest concentrations of 25(OH)D₃ investigated here were growth promoting, as previously reported in primary cells of human prostatic epithelium (22) and epidermis (23). The growth-inhibitory effect detected when a 500 nM or 1000 nM concentration of the metabolite was used may indicate local conversion of 25(OH)D₃ to the active metabolite 1,25(OH)₂D₃ (27, 28). Alternatively, we have previously found evidence that 25(OH)D₃ itself may act as an active hormone, its growth-modulating effects being mediated via direct binding to VDR (21). As has been earlier reported by Shushan et al. (29), no statistically significant differences in the growth patterns of leiomyoma and myometrial cells were detected in our study. Fibroid transformation in uterine smooth muscle cells did not seem to affect their susceptibility to growth inhibition by vitamin D, as determined.

**FIGURE 1**

Immunohistochemical characterization of myometrial and leiomyoma cells in culture. The pictures are representative of leiomyoma cells, but both cell types displayed similar staining patterns. (A) The majority of the cultivated cells were smooth muscle actin (SMA) positive. Arrows point to immunonegative cells. (B) Fewer than 10% of cells were positive for desmin. (C) The intermediate filament vimentin was strongly expressed in all cells. (D) A few scattered nuclei (arrow) were immunopositive for estrogen receptor (ER). (E) No progesterone receptor (PR) labeling was detected. (F) Vitamin D receptor (VDR) immunoreactivity was localized in discrete foci within the nuclei. Bar = 50 μm.
here by the colorimetric crystal violet assay specifically developed and validated for the measurement of cell number in monolayer cultures (20, 30).

We chose to use cell number as an end point because it sums up all potential effects of vitamin D on cell growth: cell cycle arrest, apoptosis, and regulation of growth factor signaling, thereby giving a straightforward insight into the efficacy of vitamin D action (12). The use of additional studies is required to determine which abundant regulatory mechanisms are responsible for the precise growth-inhibitory effects of 1,25(OH)2D3 and 25(OH)D3 in human myometrial and leiomyoma cells.

The cultures established here consisted predominantly of cells expressing smooth muscle actin. However, the expression of desmin, another marker of smooth muscle phenotype, was detected only in a small number of cells. Immunohistochemical staining for desmin has previously been shown to decrease in uterine smooth muscle cells within a few days in culture, concomitant with an increase in vimentin expression (31). This is in accordance with our present finding of a sparse desmin but an extensive vimentin staining, the latter most likely reflecting an adaptation of the cells to living in an in vitro environment (32). Yet probably because of differences in culture conditions, this mode of desmin expression has not been unanimously reported (33, 34). Both ER and PR were below the detection limit of the immunohistochemical method. Depending on the in vitro environment, either maintenance of ER and PR or a rapid loss of the two receptor proteins has previously been observed in human leiomyoma and myometrial cultures (29, 35).

The inhibitory effect of vitamin D on uterine smooth muscle cells has not been reported previously. We found that both leiomyoma and myometrial tissues are sensitive target organs for vitamin D and that their cell growth was effectively inhibited by physiologic vitamin D concentrations in vitro. Whether this also takes place in vivo remains to be studied. A link between vitamin D status and proliferative disorders of the myometrium in vivo is an intriguing clinical possibility. More research is needed to find out whether women with hypovitaminosis D also have more uterine leiomyomas than women with efficient vitamin D supplies.
Vitamin 1,25(OH)₂D₃ inhibits in a concentration-dependent manner the growth of myometrial and leiomyoma cells. Both myometrial and leiomyoma cells were sensitive to 1,25(OH)₂D₃, as suppression was already detected in the physiologic vitamin D levels. Hypovitaminosis D may have an important role in the etiology and growth of leiomyoma cells.

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REFERENCES


