Vitamin D_3 receptor is expressed in the endometrium of cycling mice throughout the estrous cycle

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Objective: To investigate the expression and localization of vitamin D_3 receptor (VDR) in reproductive organs of cycling mice.
Design: Experimental animal study.
Setting: Academic research center.
Animal(s): Mature (8 to 12 weeks old) cycling female Balb/c mice.
Intervention(s): Reproductive tissue, including endometrium, ovary, and fallopian tubes, were collected at each phase of estrous cycle to examine VDR expression.
Main Outcome Measure(s): Expression of VDR messenger (mRNA) was determined by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). The presence and localization of VDR was assessed by immunohistochemistry, and the intensity of VDR expression was quantified with U.S. National Institutes of Health image-analysis software.
Result(s): The VDR mRNA was expressed in the endometrium throughout the estrous cycle. The relative expression of VDR mRNA at the estrus phase was more prominent compared with the other phases. Immunohistochemical analysis revealed that dendritic cells, macrophages, and luminal and glandular epithelial cells of the endometrium, granulosa, and cumulus oophorus cells of the ovary and fallopian epithelial cells strongly express VDR, particularly during the estrus phase.
Conclusion(s): Our findings have demonstrated, for the first time, that VDR is present and differentially expressed in murine reproductive organs throughout the estrous cycle. Further studies are required to evaluate the functional immunologic role of VDR. (Fertil Steril® 2010;93:2738–43. ©2010 by American Society for Reproductive Medicine.)

Key Words: Endometrium, estrous cycle, fallopian tube, mice, ovary, vitamin D3 receptor

The active form of vitamin D3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) has been well characterized as an essential regulator of bone and mineral homeostasis and can also have a major influence on the growth and differentiation of a variety of tissues. Most of the biological effects of vitamin D_3 are mediated through the intracellular 1,25-dihydroxyvitamin D receptor (VDR) (1). Regulation of expression of the VDR gene in target cells is the primary determinant of biological responses of tissues to vitamin D_3 (2, 3).

Steroid hormones are the main regulators of VDR expression. Available data indicate that estrogen up-regulates VDR expression in a variety of cell types. Menopause (4) and ovariectomy (5) are associated with a decreased circulating level of estrogen and a concomitant decrease in intestinal calcium absorption. Apart from the primary target organs for vitamin D_3 action—the intestine and kidneys—vitamin D_3 performs functions in many other tissues through VDR (1, 6–11). Recent studies have demonstrated the functional role of vitamin D_3 in female reproduction. Mice deficient in 1α-hydroxylase enzyme, the key renal enzyme responsible for production of the biologically active form of vitamin D_3, or VDR were infertile and exhibited uterine hypoplasia and absent corpora lutea (12, 13). Lack of vitamin D_3 reduces reproductive capacity in rodents as well (14).

Vitamin D_3 has also pronounced immunoregulatory properties and affects the growth and differentiation of many immune cell types, including dendritic cells (DC), macrophages, natural killer (NK) cells, and T lymphocytes. Our previous report showed that DCs play a protective role at the fetomaternal interface throughout gestation (15) and function in the maintenance of pregnancy (16), and that reproductive failure may result from increased concentrations of specific populations of uterine NK cells and from cytokine secretion imbalance toward a T_H1 response (17, 18); thus, vitamin D_3 can play a pivotal role in protecting pregnancy

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via its immunomodulatory effects. A recent study by Bubanovic (19) has shown that vitamin D_3 administration can be potentially effective in the treatment of recurrent spontaneous abortion.

Taking into account the central role of vitamin D_3 in fertility and in the induction of tolerance at the fetomaternal interface, and the involvement of steroid hormones in regulation of VDR, we investigated the expression of VDR in the uterus, ovaries, and fallopian tubes of Balb/c mice at different stages of the estrous cycle.

MATERIALS AND METHODS

Animals

Mature (8 to 12 weeks) female Balb/c mice were obtained from a breeding colony at Pasteur Institute, Tehran, Iran. All procedures were conducted according to the guidelines of the Animal Care and Ethics Committee at Avicenna Research Institute.

Estrous Cycle Stage Determination

Stages of the estrous cycle were determined by analysis of wet vaginal smears, according to the protocol we published elsewhere (20). Each animal was classified according to its stage of the estrous cycle as proestrus, estrus, metestrus, or diestrus.

RNA Extraction from the Tissue

Mice were killed by cervical dislocation, and uterine horns were removed under sterile conditions. The left uterine horn was washed with sterile phosphate-buffered saline (PBS) and cut open on the antimesometrial line. The endometrial tissue was carefully scraped with a scalpel, immediately transferred into microtubes containing RNA-Bee solution (Nordic Biosite, Täby, Sweden), and homogenized by Pellet Pestle (Sigma, St. Louis, MO). Total RNA was extracted according to the standard method. Briefly, chloroform (10% v/v) was added to the sample and shaken vigorously. After 5 minutes' incubation on ice, the samples were centrifuged at 18,000 × *g* for 10 minutes at 4°C. The aqueous phase was then transferred to a clean tube, and an equal volume of isopropanol was added. After resting for 1 hour at −20°C, the sample was centrifuged at 18,000 × *g* for 5 minutes at 4°C to precipitate the RNA. The supernatant was removed, and the RNA pellet was washed once with 75% ethanol, centrifuged, and briefly air dried. The RNA pellet was dissolved in distilled water, and samples were stored at −20°C until further analysis. The RNA quality was assessed by agarose gel electrophoresis. Homogenates of mouse kidney in RNA-Bee solution were used as the positive control.

Complementary DNA Synthesis

A 10-μL volume of RNA was denatured for 7 minutes at 65°C and immediately placed on ice. A mixture containing 5× reaction buffer (Fermentase, Vilnius, Lithuania), 1 mM dNTP mix (Roche, Penzberg, Germany), 1 μM Random-Hexamer (Cybergene, Stockholm, Sweden), and 20 IU/μL of Reverse Transcriptase M-MuLV (Fermentase) in a final volume of 10 μL of distilled water was added to the denatured RNA and mixed thoroughly. After incubation for 1 hour at 42°C in a thermocycler (Eppendorf AG, Hamburg, Germany), the resulting cDNA (20 μL/microtube) was aliquoted and stored at −20°C.

Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The PCR reaction mix contained PCR buffer (10×), $MgCl_2$ (2.5 mM for GAPDH as internal control and 2 mM for VDR), 0.4 mM dNTP mix, and Taq DNA Polymerase at 0.04U/μL final concentration (Roche). We added 1 μL of each pair of primers, equivalent to the final concentrations of 0.4 μM for GAPDH and 0.2 μM for VDR. We then performed PCR as follows: GAPDH with the program 94°C for 2 minutes (1 cycle), 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds (30 cycles), and 72°C for 7 minutes (1 cycle); and VDR with the program 94°C for 2 minutes (1 cycle), 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds (35 cycles), and 72°C for 7 minutes (1 cycle). The following primers were used for GAPDH and VDR amplification: GAPDH: F: 5'-CAG-GAG-CGA-GAC-CCC-ACT-A-3'; R: 5'-GGC-ATG-GAC-TGT-GGT-CAT-GA-3'; VDR: F: 5-GAG-GTG-TCT-GAA-GCC-TGG-AG-3'; R: 5'-ACC-TGC-TTT-CCT-GGG-TAG-GT-3'. The primers were designed to generate a 309 base pair (bp) fragment for GAPDH and a 155 bp fragment for VDR. For VDR expression, complementary DNA (cDNA) prepared from mouse kidney tissue served as the positive control. In negative control samples, deionized water was substituted for the cDNA.

Agarose Gel Electrophoresis and Densitometry

The PCR products for GADPH and VDR genes were electrophoresed on agarose gel. The gels were photographed with ultraviolet transilluminator Gel Doc (UVP, LLC, Upland, CA). Densities of DNA bands were quantified, and the VDR/GADPH density ratio was calculated using Alpha Ease software (Alpha Innotech Corporation, San Leandro, CA).

Immunohistochemistry

Mouse reproductive tissue samples including uterus, fallopian tubes and ovaries were collected, placed in optimal cutting temperature (OCT) medium Tissue-Tek (Sakura, Zoetenwoude, The Netherlands), flash frozen in liquid nitrogen, cut at 5-μm thicknesses, mounted on glass slides, and processed for immunohistochemistry. Briefly, cryostat sections were air dried at room temperature (1 hour), fixed in cold acetone (2 minutes), and washed in Tris-buffered saline 0.1% bovine serum albumin (TBS-BSA) (3 × 3 minutes). Sections were then blocked in 5% normal goat serum (10 minutes) and incubated with Avidin/Biotin blocking solutions (Dako, Glostrup, Denmark) to block endogenous biotin activity (20 minutes). After they were washed with TBS-BSA, the sections were incubated with monoclonal anti-VDR primary antibody (2.5 μg/mL; NeoMarkers, Fremont, CA) at room temperature (90 minutes), washed, and treated with 0.3% H_2O_2 (10 minutes) to block endogenous peroxidase activity, followed by rinsing and incubation (45 minutes) with biotinylated goat anti-rat secondary antibody (5 μg/mL; BD Biosciences Pharmingen, San Diego, CA) at room temperature. The slides were rinsed, incubated in streptavidin-horseradish peroxidase (Biosource, Camarillo, CA) (1:500 dilution, 30 minutes) at room temperature, rinsed again, and then incubated in 3,3'-diaminobenzide tetrahydrochloride (DAB) chromogen (Roche) as the substrate. After a final rinse with H_2O, the sections were counterstained with Harris hematoxylin, ethanol dehydrated, and mounted using Entellan glue (Merck, Darmstadt, Germany). Intestinal tissue was processed as previously described and used as the positive tissue control. In the negative control sections, the primary antibody was omitted or substituted by an appropriate dilution of rat serum.

Semiquantitative Digital Image Analysis

The level of VDR expression was quantified with a U.S. National Institutes of Health ImageJ digital image analysis program (National Institutes of Health, Bethesda, MD). Immunostained tissues were viewed under high power (magnification ×400) by light microscope (Olympus BX51; Olympus, Tokyo, Japan), and photos were captured as digital images with a DP70 digital camera (Olympus). A total of 10 to 17 images each containing 200 to 400 cells were analyzed at each stage of the estrous cycle.

Briefly, the first step was blind scoring of all cells from 0 to 3+ according to their intensity of VDR expression with the use of the manual cell-counter in the NIH image software. The next step was calculating the mean percentage of each level of expression. Because the total number of endometrial cells and the size of the VDR positive cells (which is an indirect measure of total VDR protein) vary considerably during each stage of the estrous cycle, the intensity profiling could not give a precise picture of VDR expression at each stage. So a correction method based on the grey level was applied. Each digital image was deconvoluted into the three blue, green, and brown (representing DAB) colors. Brown images were converted to a 16-bit black-and-white image, and the total number of black pixels representing the DAB color was measured. The mean percentage of each score (0 to