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Decreased expression of the vitamin D receptor in women with recurrent pregnancy loss

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ABSTRACT

The multiple functions of vitamin D_3 have stimulated interest in the role that this vitamin may play during pregnancy. The present study investigated the expression of the vitamin D receptor (VDR) in women during the first trimester of pregnancy in order to determine whether VDR is associated with recurrent pregnancy loss (RPL). Forty women at 7–10 weeks gestation with RPL and 40 women of similar gestational age with a healthy pregnancy were recruited. VDR mRNA and protein in chorionic villi and decidua were evaluated by immunohistochemistry, confocal laser scanning microscopy (CLSM), western blot, and quantitative real-time polymerase chain reaction. The serum levels of VDR were measured by an enzyme-linked immunosorbent assay. Women with RPL had a significantly weaker expression of VDR mRNA in villi and decidual tissues compared with the control women (both $p < 0.0001$). Western blot analysis showed an approximately 46% decrease in VDR expression in villi and a 52% decrease in decidua in the RPL vs. the controls. Serum VDR levels were also significantly lower in the RPL group than in the control group ($p = 0.003$). Compared with the controls, immunohistochemical and CLSM analysis revealed significantly lower VDR expression in villous cytotrophoblasts and stromal cells, as well as in decidual glandular epithelial and stromal cells (all $p < 0.05$). In conclusion, these observations show that women with RPL have lower levels of VDR expression in chorionic villi, decidua and serum compared with normal pregnant women, suggesting that decreased VDR expression in the first trimester pregnancy may be associated with RPL.

1. Introduction

Several pathogenic mechanisms associated with recurrent pregnancy loss (RPL) have previously been described, including chromosomal anomalies, congenital uterine anomalies, acquired and inherited thrombophilia, endocrine problems, infections, autoimmune disorders, and male factors. Furthermore, up to 50% of cases of RPL have no clearly identifiable etiology [1]. Immunological mechanisms have been proposed to explain some cases of RPL. Women with RPL exhibit what is considered a generalized exaggerated inflammatory immune response during pregnancy and show signs of a disruption in the tolerance of autoantigens and fetal antigens [2].

It is well established that 1, 25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$), a pleiotropic steroid hormone, has classic effects on bone metabolism and mineral homeostasis. However, the discovery that multiple functions of vitamin D_3 are important for growth and development, including immune system development [3], has stimulated interest in the role that this vitamin may play during pregnancy. Several studies have suggested that vitamin D carries out important functions in a large number of immune-mediated responses [4–8]. Vitamin D_3 inhibits adaptive immunity and cell proliferation and simultaneously promotes innate immunity and stimulates cellular differentiation [4]. Th17 cells, characterized by IL-17 production, play a critical role in the pathogenesis of autoimmune diseases and are inhibited by $1,25(OH)_2D_3$ in vitro [5].

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Vitamin D_3 deficiency or insufficiency is associated with an increased prevalence of autoimmune diseases, such as rheumatoid arthritis [6], systemic lupus erythematosus (SLE) [7], and multiple sclerosis [8]. Vitamin D_3 may also have potential utility in the treatment of asthma by suppressing TNF-α expression [9]. Studies have shown that vitamin D_3 acts as a potent modulator of both innate and acquired immune responses and is also an immunoregulatory hormone with beneficial effects on T-helper 1 (Th1) cell-mediated inflammatory diseases.

Vitamin D_3 exerts its effects by binding to a single vitamin D receptor (VDR) in the cell nucleus, which is present in multiple tissues [10]. The presence of VDR in human placenta, decidua, and endometrium [10–12] suggests that the cells of these tissues are targets of vitamin D_3 action. However, the level of VDR expression in the decidua and chorionic villi in women with RPL has received very limited attention. Thus, the purpose of our study was to test the hypothesis that VDR expression is decreased in the fetal-maternal interface in women with RPL. In this study, we investigated VDR expression in the chorionic villous, decidual tissues and serum of women with RPL and normal pregnant women in the first trimester of pregnancy.

2. Materials and methods

2.1. Subjects

In this study, research subjects were recruited from the First Affiliated Hospital of Xi'an Jiaotong University, Shaanxi, China, between October 2013 and October 2014. Forty women (aged 25–32 years) with an intact gestational sac and no visible cardiac activity in the embryo were diagnosed with a pregnancy loss by transvaginal ultrasound (RPL group). The average gestational age was 64.8 ± 7.9 days (range, 7–10 weeks). In addition, these women had a history of at least two consecutive pregnancy losses at 7–10 weeks gestation without any prior normal pregnancies. Those women who were diagnosed with uterine anomalies; chromosomal abnormalities; thyroid dysfunction; infections with rubella, toxoplasma, cytomegalovirus, and herpes virus; hypertension; diabetes mellitus; and autoimmune disorders were excluded. The control group consisted of forty women (aged 24–32 years) with viable pregnancies between 7 and 10 weeks gestation (63.7 ± 9.4 days) who were gestationally matched to the study group and who underwent voluntary pregnancy termination. The control group had a history of one or more prior normal pregnancies, no history of pregnancy loss and were generally in good health. Women in the control and RPL groups did not differ significantly in average maternal and average gestational age.

The study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University and was conducted according to Declaration of Helsinki principles. An informed consent was obtained from all participants.

2.2. Human chorionic villus, decidua, and serum analysis

Chorionic villous and decidual tissues were obtained during the surgical evacuation of uterus in women with RPL within the first 24 h after diagnosis or elective termination of normal uncomplicated pregnancy. Additionally, a blood sample (3 mL) was collected into 5-mL sterile plain tubes without anticoagulanton the same day in 40 women with RPL and 40 women in the control group. For RNA and protein extraction, a total of 40 villous and decidual samples, including 20 RPL and 20 control samples, were collected and snap-frozen in liquid nitrogen (−80 °C). For immunohistochemistry and confocal laser scanning microscopy (CLSM), 60 villous and decidual samples (30 RPL and 30 control samples) were routinely processed and frozen at −70 °C.

2.3. Streptavidin-peroxidase immunohistochemistry for VDR

VDR expression in decidua and chorionic villi was analyzed using a streptavidin-peroxidase immunohistochemistry kit (Zymed Laboratories, Inc., San Francisco, CA, USA) in accordance with the manufacturer's instructions. Briefly, frozen sections were incubated with 30% hydrogen peroxide (1:10) to block endogenous peroxidase activity for 5–10 min at room temperature and then washed in distilled water. The sections were then incubated in normal goat serum blocking solution, followed by anti-VDR monoclonal antibody (1:400; Abcam Inc., Cambridge, MA) for 2 h at 37 °C. After rinsing with phosphate-buffered saline (PBS) (pH 7.4) for 15 min, the biotinylated goat anti-mouse IgG was added to sections for 30 min at 37 °C, followed by horseradish peroxidase-conjugated avidin for 30 min at 37 °C. Staining was developed using diaminobenzidine, followed by counterstaining with hematoxylin and washing with cold running water (DAB-Stock Stain box; Boster Biological Technology, Ltd., Wuhan, China). The primary antibody was omitted in the negative controls. Digital images were acquired using a section microscope scanner (Leica MP SCN400, German). The software was used to semi-automatically estimate the volume of immunopositive cells within a tissue sample. In the final quantification, the average gray-scale value of five fields in each slide was estimated. To further exclude operator bias, observations were performed on coded samples in a blinded manner following the same procedure.

2.4. Confocal laser scanning microscopy for VDR

The fluorescent intensity detection of VDR immunostaining was performed using previously described methods [13]. Briefly, air-dried cryosections immersed in hydrogen peroxide were incubated in normal goat serum blocking solution, followed by anti-VDR monoclonal antibody (1:500; Abcam Inc., Cambridge, MA) overnight at 4 °C. After rinsing with PBS (pH 7.4) for 15 min, the sections were exposed to fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G for 2 h at 37 °C, and their nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1:5000) for 15 min at room temperature. The fluorescence signal from VDR immunostaining was observed and quantified using CLSM. The images were analyzed by IMAGE PLUS software (Leica, Microsystems, Wetzlar, Germany), and the mean fluorescence intensity within a specific population was calculated. Fluorophores were excited using a 488-nm argonlaser beam, and fluorescence emission was assessed at 543 nm.

2.5. Western blot for VDR

Samples of villi and decidual tissues (200 mg) were treated with 500 μL of cold lysis buffer containing 2 μL of protease inhibitor mix. Lysates were subsequently centrifuged at 12,000 g for 20 min at 4 °C; the supernatant was collected; and the protein concentration was determined using a commercial protein assay kit (BCA Protein Assay Kit, Beijing Tiangen Co., China). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). For VDR detection, after briefly washing with TBST (25 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1% Tween 20), the membranes were blocked with 5% skim milk for 2 h at room temperature. The membranes were incubated overnight at 4 °C with anti-VDR monoclonal antibody (Abcam Inc., Cambridge, MA) diluted in 1000 mL of TBST. After washing with TBST, the membranes were incubated for 2 h at 37 °C with peroxidase-conjugated