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Original Article

Different expression of NOD2 in decidual stromal cells between normal and unexplained recurrent spontaneous abortion women during first trimester gestation

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Abstract: The NOD2 gene, encoding intracellular paternal recognition receptor (PRR) also called caspase activation and recruitment domain 15 (CARD15), is mutated in Crohn's disease, an autoimmune-disorder. Unexplained recurrent spontaneous abortion (URSA) involved in complex auto-immune disorder. However, little is known about the expression of NOD2 protein at maternal-fetal interface with URSA patients. Our aim was to compare the expression levels of NOD2 in the decidual stromal cells (DSCs) from patients with normal pregnancy to those with unexplained recurrent spontaneous abortion (URSA) during first trimester pregnancy. Tissues and DSCs were collected from 12 patients with URSA and 26 patients with normal pregnancies that required abortion. DSCs in the normal pregnancy group showed significantly higher mRNA and protein levels of NOD2 than those isolated from the URSA group using real time PCR and in cell western. The appropriate expression of NOD2 in normal DSCs suggests that this protein may be required to sustain normal pregnancy.

Keywords: NOD2, decidual stromal cells (DSCs), unexplained spontaneous recurrent abortion

Introduction

Spontaneous miscarriages (abortions) occur in approximately 14% to 16% of naturally conceived pregnancies [1]. Abortions may arise from an abnormal uterine cavity, parental karyotypes, endocrine factors, infection, autoimmunity [2, 3], and unknown mechanisms [4]. A loss of three or more consecutive pregnancies before the 20th week of gestation with unknown etiology is called unexplained recurrent spontaneous abortion (URSA) and occurs in approximately 1-3% of fertile women [5]. Some of these abortions may arise from the loss of maternal immuno-tolerance or abnormal maternal responses to the fetus.

In full term pregnancies, the maternal immune responses remain tolerant from early implantation of the semi-allogeneic trophoblast to the full development of the fetus, while protecting it from infection. Known mechanisms involved in implantation include interaction of the CXCL12/CXCR4 axis between the trophoblast and the decidual stromal cells (DSCs) [6]. However, the molecular mechanisms of DSCs in maintaining normal pregnancy are not completely understood. Female hormones help modulate the activities of dendritic cells [7] and local DSCs in the uterus, which influence the immune milieu [8]. Immune tolerance towards the fetus also includes regulatory T cells [9, 10], finely tuned activity of natural killer (NK) cells [11] and macrophages [12], and a complex orchestrated fluctuation of cytokines/chemokines with a balance of Th1 and Th2 responses [4]. When the immunological balance is disturbed, the abnormal immune response can adversely affect the

outcome (e.g., spontaneous abortion) [2, 3, 13, 14].

In human reproductive system, NOD2 proteins are expressed in first trimester trophoblasts [15], endometrial stroma cells (late secretory phase of the menstrual cycle), decidualized stromal cells and the glandular epithelium at the first trimester [8]. NOD2 gene mutation affects various immune-related disorders and infectious diseases, such as Crohn's disease [16], asthma [17] and NOD2-associated autoinflammatory disease (NAID) [18].

DSC as one of the most important elements at maternal-fetal interface plays essential role for immunologic protection of the fetus, but the expression of NOD2 in DSCs (normal and URSA) have not been elucidated. In this study, we compared the expression levels of NOD2 in DSCs during normal pregnancy with URSA patients. Our study suggests that loss of function of NOD2 may be responsible for the URSA.

Materials and methods

Samples

Human first trimester pregnancy decidual samples (26 cases normal pregnancy women and 12 cases URSA patients) were obtained with informed consent from first trimester voluntary termination of gestation in Sun Yat-Sen Memorial Hospital and Red-House hospital of Fudan University from January 21st 2012 to October 6th 2012. The inclusion criteria for URSA group: genetics, anatomy, endocrine, hormone, certain coagulation and serum levels of immune regulatory proteins were normal. The patients with infection, smoking and alcohol consumption, environmental factors, psychological trauma, and stressful life event were excluded. All patients had signed consent forms of the tissue collection, and the study was approved by Human Research Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University and Sun Yat-Sen University's Human Investigations Committee.

Immunohistochemistry

Immunohistochemistry was performed essentially as described previously [19]. Briefly, all sections were fixed in 4% paraformaldehyde over night, then deparaffinized, rehydrated through a graded alcohol series, and subjected to antigen retrieval using citric acid antigen repair fluid (Beyotime, ShangHai, China). NOD2 expression was detected by AB complex (streptavidin/peroxidase) method (ZymedHistostain plus Kit, Zymed, USA) according to manufacturer's instructions. Slides were kept at 4°C overnight incubating with mouse anti-human CK7 mAb (Santa Cruz, Heidelberg, Germany), mouse anti-human vimentin mAb (Santa Cruz, Heidelberg, Germany), mouse anti-human NOD2 mAb (Abcam, Cambridge, UK) or PBS, and then stained by haematoxylin (Sigma), dehydrated with ethanol and mounted from xylene.

DSCs isolation

Decidual stromal cells were isolated from fresh tissue which collected in ice-cold DMEM/F12 (Gibco, Grand Island, NY, USA). The individual patient tissues were washed in calcium- and magnesium-free Hanks balanced salt solution (HBSS), then subjected to dissociate by collagenase IV/DNase-I digestion (Sigma, Saint Louis, Missouri, USA) and isolated by discontinuous Percoll gradient centrifugation, as described [20]. DSCs of density between 1.042 and 1.062 g/ml were collected and cultured in DMEM/F12 (Gibco, Grand Island, NY, USA) complete medium (10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin) in 5% CO_2 at 37°C. After 30 min, non-adherent hematopoietic and immune cells were washed away, leaving 98% pure DSCs (confirmed by staining of mouse anti-Cytokeratin7 mAb and mouse anti-vimentin mAb (ZSGB-BIO, Beijing, China).

RNA extraction and QPCR

The purified DSCs were seeded in 12-well plates at a density of 5×10^5 cells/well. After 24 hrs incubation in complete medium (DMEM/F12 with 10% FBS) in 5% CO_2 at 37°C. Cells were harvested and total RNA was extracted using trizol (Invitrogen, Carlsbad, CA, USA) protocol. Total cellular RNA (1 μg) was reverse transcribed into cDNA by cDNA Synthesis Kit (Fermentas, Waltham, USA) in a Mastercycler personal PCR machine (Eppendorf AG, Hamburg, Germany). SYBR Premix ExTaq II (2 ×) (Takara, Dalian, China) were used for Real time PCR , Primers of NOD2 and primers of GAPDH (all Applied Sangon Biotech, Shanghai, China)

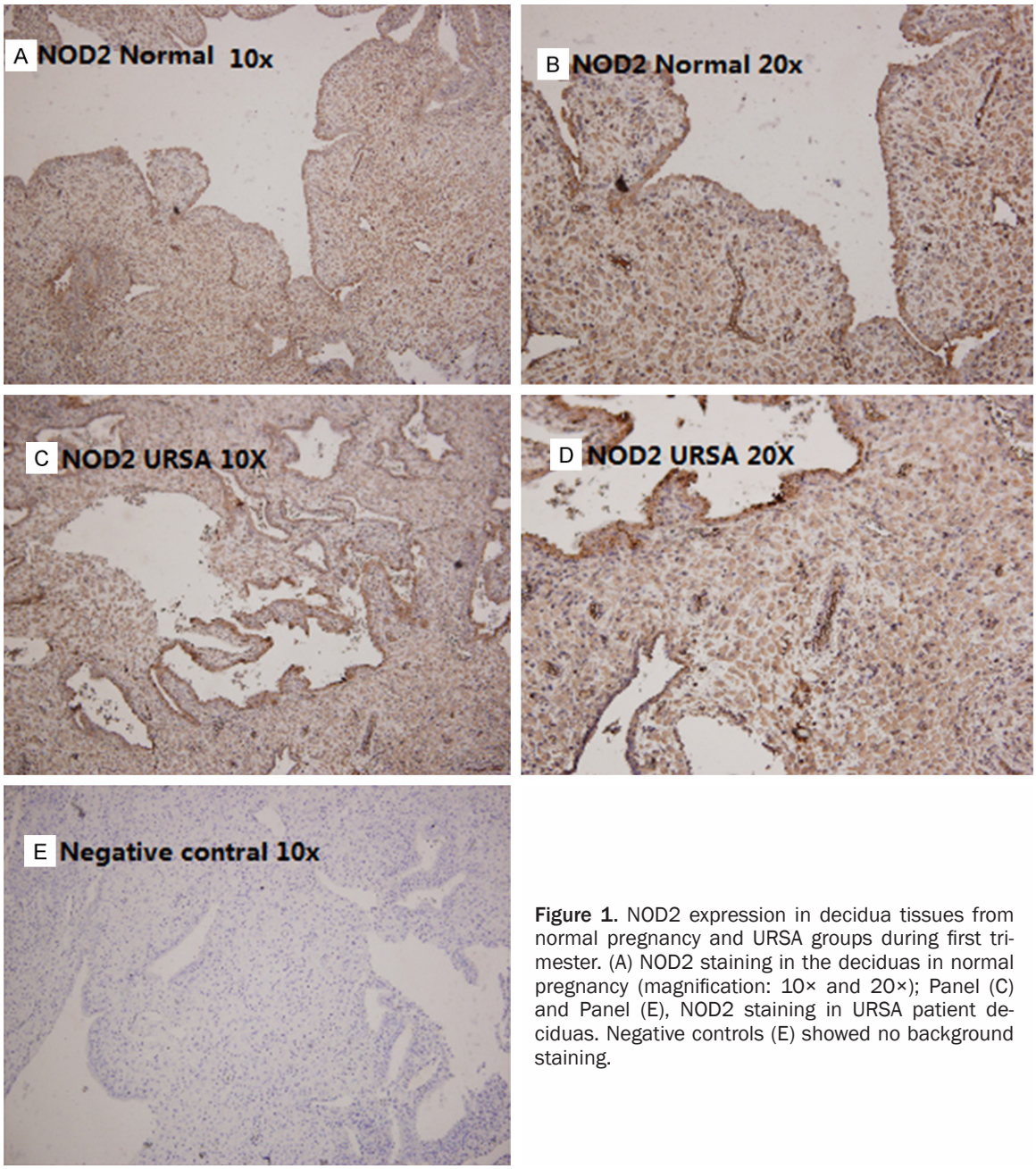

Figure 1. NOD2 expression in decidua tissues from normal pregnancy and URSA groups during first trimester. (A) NOD2 staining in the deciduas in normal pregnancy (magnification: 10× and 20×); Panel (C) and Panel (E), NOD2 staining in URSA patient deciduas. Negative controls (E) showed no background staining.

as follows: 5'-TGCGGACTCTACTCTTTGAGC-3' (forward) and 5'-CCGTGAACCTGAACTTGAACT-3' (reverse); for human glyceraldehyde-3-phosphatedehydrogenase (GAPDH): 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGA-3' (reverse). 3 μl cDNA was diluted into 12 μl, and 2 μl dilution was added for each PCR reaction. PCRs were run on an ABI 7900HT (Perkin-Elmer Applied Biosystems, USA). Each cDNA was amplified in triplicate and the corresponding sample without reverse transcriptase (ddH_2O) was included as the negative control. The expression of the NOD2 was normalized to that of GAPDH. The replicates were then averaged, and fold induction was determined in a ΔΔCt-based fold-change calculations.

In-cell Western

In-cell Western was carried out to determine the protein level of NOD2 in the DSCs, as described [19]. Purified isolated DSCs were seeded (1.5×10^4/well) in 96-well plate and

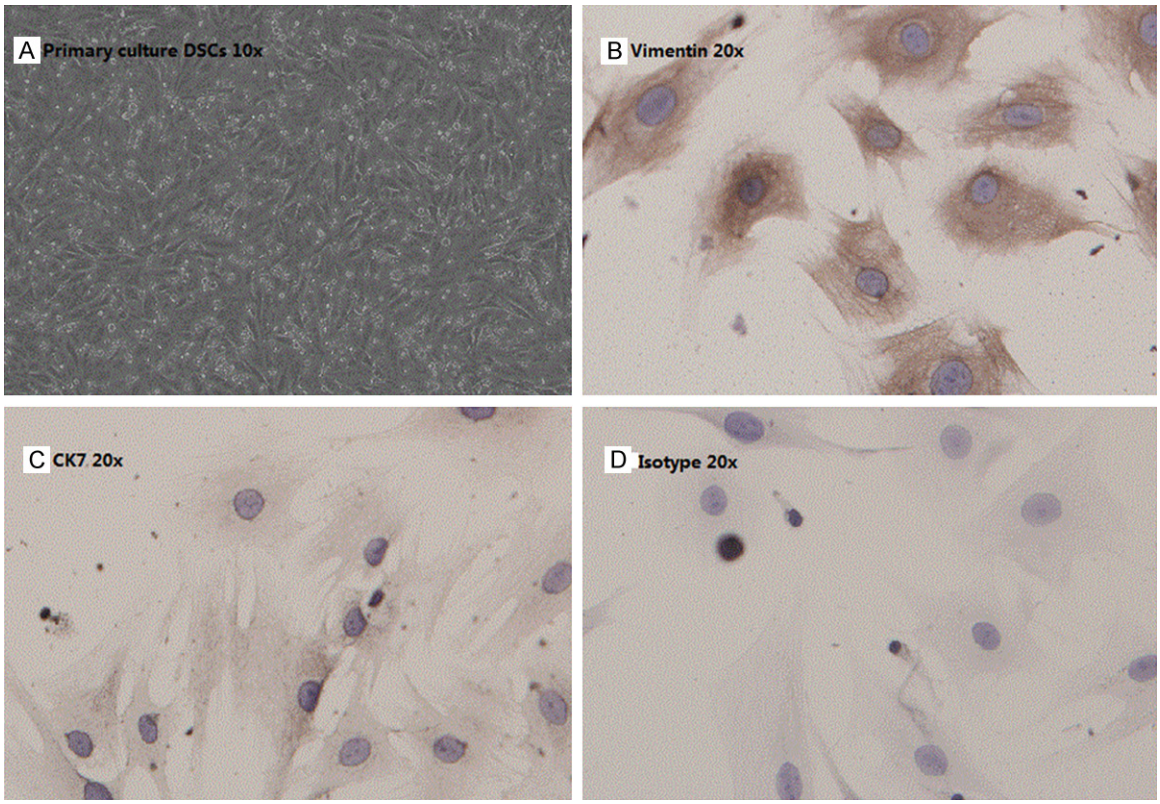

Figure 2. Primary culture of DSCs (A) were characterized by positively staining for vimentin (B) but not CK7 (C) in the cytoplasm, while negative controls (D) showed no staining.

cultured 24 hrs, then Cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with 0.1% Triton 3 times, cells were blocked by adding 100 μl of LI-COR Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, Nebraska, USA) for 90 min at room temperature. Cells were incubated with mouse anti-human mAb NOD2 (25 μg/ml, Abcam, Cambridge, UK) and the rabbit anti-human β-actin (1:80, Santa Cruz, CA, USA) which detected the control reference protein overnight at 4°C. Cells were washed 3 times with 1 × PBS, and incubated with the corresponding secondary antibodies: anti-mouse IRDyeTM-700DX conjugated (1:60, affinity purified, Red fluorescence) and anti-rabbit IRDyeTM800DX conjugated (1:80, affinity purified, green fluorescence) in darkness (secondary antibodies: Rockland, Inc, Gilbertsville, PA, USA). Images of NOD2 were analyzed by Odyssey Infrared Imaging System (LI-COR Biosciences GmbH). The protein level of NOD2 was analyzed by the ratio of the intensity of NOD2 (green fluorescence) to that of β-actin (red fluorescence). Each experiment was repeated three times.

Statistics

All values were expressed as the mean ± SEM and differences were compared by Student *t*-test with GraphPad Prism 5.0. The differences were accepted as significant at $P < 0.05$.

Results

To investigate if the NOD2 expression level in DSCs is altered in URSA, we compared the NOD2 expression in DSCs from normal pregnant women with that from URSA women at the first trimester. As shown in **Figure 1**, positive immunoreactivity for NOD2 was observed in DSCs and adenoepithelial cells of both URSA and normal pregnancy group (**Figure 1** panels A, B, C and D), the structures of decidual tissue from URSA patients were irregular. NOD2 expression level seems more intense in decidual tissue of normal pregnancy group than that of URSA group (**Figure 1**). To confirm our observation, First, we isolated DSCs from decidual tissue and characterized cells by staining CK7 (-) and Vimentin (+), the purify of DSCs was 98%

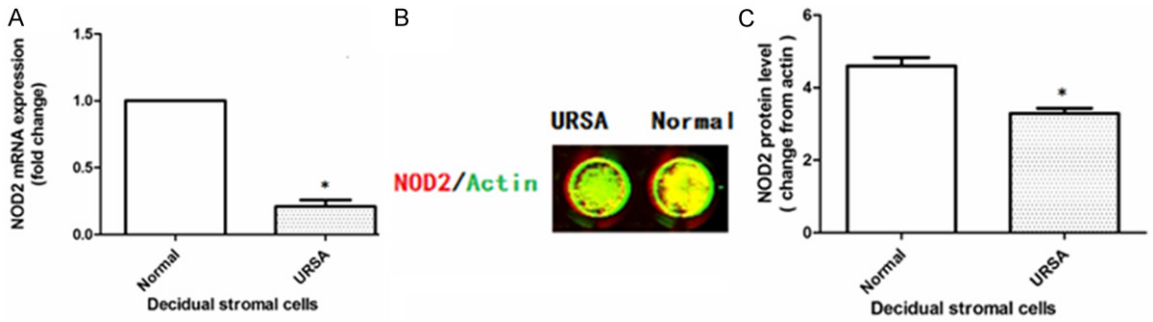

Figure 3. NOD2 expression in DSC primary culture was confirmed by RT-PCR (A) and in-cell Western blot analysis (B and C). NOD2 protein expression indicated by the ratio of the intensity of NOD2 protein to that of β-actin (C). The pictures were from one representative experiment of three, *P < 0.05.

(**Figure 2**). Second, the expression of NOD2 in DSCs from the primary cultures was evaluated by real time PCR and In-cell Western blot analysis. Consistent with the up regulation of the mRNA of NOD2, the DSCs from each woman in the normal pregnancy group expressed higher NOD2 protein than DSCs in each individual URSA patient (**Figure 3**).

Discussion

Till now, in human gestation system, NOD2 expression has been observed in first trimester placenta, specifically in the trophoblast cell [15], and in endometrial epithelial cell [8]. NOD2 acts by regulating chemokins/cytokines secretions, at least in part by protecting maternal-fetal interface from pathogens evading, For example, MDP stimulation of endometrial epithelial cells significantly increased expression of IL-8 and TNF-α [8], MDP stimulation of trophoblasts from the first trimester up-regulated secretion of GRO-α, IL-6, IL-8, and MCP-1 [15, 20]. This data suggested that NOD2 is functional at maternal-fetal interface. Defective NOD2 expression affects the cytokines secretion baseline, for example ectopic expression of NOD2 in cell line H8 (a first trimester trophoblast cell line), produced higher levels of cytokines than that with vehicle treatment alone [20]. The appropriate level of cytokines aids the tight regulation of cytokines and chemokines necessary to maintain the maternal tolerance towards the fetus. Interestingly, pretreatment of a murine macrophage cell line by NOD2 ligand: MDP, the cell releases its tight chaperone, heat shock protein 90, and degrades the activated Nod2 protein, which blocks Nod2's continual activation [21]. Based on this data, if MDP stimulation also down regulates expression of NOD2 in human DSCs, the short burst of NOD2 activation can contribute to the tightly regulated temporal and spatial expression of chemokines/cytokines at maternal-fetal interface.

In this study, we demonstrated for the first time that different expression of NOD2, an important cytosolic pattern recognition receptor (PRR), was lower in decidual stromal cells from patients with unexplained recurrent spontaneous abortion in first trimester than those from patients with normal pregnancies. The physiological roles of NOD2 in DSCs during normal pregnancy are not well understood but appear to be broader than only surveillance for intracellular pathogens.

We propose 5 potential effects of lower NOD2 levels in URSA patients. First, low NOD2 levels may disrupt the balance of chemokines/cytokines signaling at the maternal-fetal interface which are essential during implantation and placentation [4, 22-24]. Costello et al demonstrated that ectopic expression of NOD2 in the first trimester trophoblast cell line H8 cells produced higher levels of cytokines than the vehicle treated H8 cells [15], suggesting that intracellular NOD2 expression levels affect baseline production of cytokines. Second, studies of graft-versus-host disease demonstrated that NOD2 can negatively regulate the activity and function of host antigen-presenting cells [25], since NOD2-/- APCs exhibited a greater ability to trigger inflammatory T-cell responses than normal APCs [25]. The embryo is a semi-allogeneic implantation, and may encounter the same threat from the maternal immune response

[26]. Normal NOD2 levels can play a protective role in the embryo, helping it evade the maternal immune response by maintaining cytokines and chemokines at an appropriate level for the successful embryo implantation. Third, low NOD2 expression levels may indicate a previous infection and subsequent downregulation of NOD2 after activation by MDP, as observed in a murine macrophage cell line [21]. Fourth, low NOD2 levels in DSCs may be insufficient to induce an adequate immune response to thwart the pathogenic invader in sufficient time. Growing evidence suggests that obligate intracellular bacteria, such as Chlamydia, may be responsible for reproductive tract infections, and Chlamydia infection is associated with an increased risk of miscarriage [27]. Fifth, activation of NOD2 requires sufficient intracellular NOD2 levels [15]. Since many URSA patients show an inflammatory cytokine milieu rather than the finely tuned cytokine orchestra observed during normal pregnancy [4], this may help explain why URSA patients have lower NOD2 expression.

Limitations of this study were the lack of data on the effects of deficiency of Nod2 in animal model may induce recurrent spontaneous abortion. Next, we will set up the model of Nod2-dificiency mouse to observe the affection during mouse pregnancy.

In summary, to our knowledge this is the first report demonstrating a difference in the expression of NOD2 in DSCs between participants with normal pregnancy and those with URSA. These findings suggest that an appropriate level of NOD2 is necessary for pregnancy maintenance.

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Disclosure of conflict of interest

None.

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