Int J Clin Exp Pathol 2015;8(2):1973-1978
www.ijcep.com /ISSN:1936-2625/IJCEP0004345

Original Article

Galectin-1 reduction and changes in T regulatory cells may play crucial roles in patients with unexplained recurrent spontaneous abortion

Minjun Wu, Ping Liu, Licun Cheng

Shanghai Changning District Obstetrics Maternal and Child Health Hospital, Shanghai 200051, China

Received December 2, 2014; Accepted January 28, 2015; Epub February 1, 2015; Published February 15, 2015

Abstract: To investigate the changes of Galectin-1 and T-lymphocyte phenotypes in unexplained recurrent spontaneous abortion (URSA). Totally 60 participants were recruited and divided into 3 groups in average: pregnant patients with URSA (URSA group), normal early pregnant women with induced abortion (IA group) and normal non-pregnant women (control group). After the tissue and blood sample were collected, Galectin-1 was measured using enzyme-linked immunosorbent assay. Then the proportion of T regulatory cells was determined by flow cytometry. The expression levels of Galectin-1 in IA group and URSA group was significantly higher than that in the control group (24.30 ± 3.06 and 6.23 ± 2.41 vs. 1.30 ± 0.66, $P < 0.05$). Besides, the expression level of Galectin-1 in URSA group was lower than that in IA group ($P < 0.05$). The percentage of $CD4^+CD25^+Foxp3^+$ Tregs was lower in URSA group than IA group (0.77 ± 0.31 vs. 1.00 ± 0.35, $P < 0.05$) and the ratio of $CD4^+CD25^+Foxp3^+/CD4^+$ in URSA group was also obviously lower than that in IA and control group ($P < 0.05$). Galectin-1 and $CD4^+CD25^+Foxp3^+$ may play essential roles in maintaining a normal pregnancy and their reduction may involve in the pathogenesis of URSA.

Keywords: T cells, decidual tissue, $CD4^+CD25^+Foxp3^+$

Introduction

Recurrent spontaneous abortion (RSA) is defined as two or more consecutive losses at ≤ 20 weeks of gestation from the last menstrual period [1, 2]. It occurs in approximately 1% to 5% of women at reproductive age [1, 3]. The aetiology of unexplained recurrent spontaneous abortion (URSA) remains partially unknown and may be multi-factorial [4]. Although reasonably accepted aetiological causes include genetic, infectious, endocrinologic and anatomic abnormalities have been associated with URSA, the specific cause still remains unclear [5]. Recent studies have shown that URSA is involved in the failure of feto-maternal immunologic tolerance [6]. Successful pregnancy is primarily associated with the immunologic status of the gestational woman and immunoregulatory capability of the embryo [7, 8]. Galectin-1 has been previously found in fetal-maternal interface [9], embryo during early pregnancy [10] and placenta tissue [11, 12]. Galectin-1 is initially synthesized in the trophectoderm of the expanded blastocyst immediately prior to implantation, suggesting a role in the attachment of the embryo to the uterine epithelium [13].

T regulatory cells (Tregs) are essential for the maternal immune system to tolerate an aggressive allogeneic response against the fetus [14]. Besides, $CD4^+CD25^+$ Tregs play important roles in the mechanisms mediating maternal immune tolerance of conceptus antigens and maintenance of pregnancy [15]. Forkhead box p3 (Foxp3) has been described as an essential transcription factor for induction and development of $CD4^+CD25^+$ Tregs [16]. Mutations of Foxp3 cause immune dysregulation, enteropathy and polyendocrinopathy, characterized by high incidences of autoimmune diseases [17]. Thus, as a specific maker of Tregs, Foxp3 is essential in the development and function of these cells.

In this retrospective study, to explore the cause of URSA in the immunological aspect, we inves-

Table 1. Baseline characteristic of participants (mean ± SD)

Terms	control group (n = 20)	IA group (n = 20)	URSA group (n = 20)
Age (years)	28.10 ± 3.55	29.30 ± 3.21	28.85 ± 3.05
Gestation (days)	0.00	53.25 ± 9.00	59.95 ± 12.41

P > 0.05.

tigated the expression of Galectin-1 in decidual tissue and the percentage of $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$ Tregs in peripheral blood in URSA patients, normal early pregnant women with induced abortion (IA) and non-pregnant women.

Materials and methods

Patients

All the participants were recruited at the Department of Reproductive Immunology of our hospital from June 2010 to December 2011. Total 20 patients who had at least three successive spontaneous early miscarriages (7-12 weeks of gestation) of unexplained etiology were enrolled. Patients were excluded if they had any infectious, metabolic, anatomic, endocrine or autoimmune diseases, and no fetal or maternal chromosomal abnormalities were found by laboratory tests. The two control groups: 20 normal early pregnant women with IA (IA group) and 20 non-pregnant women who asked for the examination of endometrium by hysteroscopy (control group). There were no significant differences in the age and pregnancy duration between the three groups (**Table 1**). The participants in IA group were excluded if they had any endocrine or autoimmune diseases, infectious or organic disease of the reproductive system, threatened abortion symptom and abnormal embryos confirmed by type-B ultrasound. The healthy non-pregnant women were excluded if they had a history of previous abortion, endocrine abnormality, autoimmune or genetic diseases.

The ethical committee of Shanghai Changning District Obstetrics Maternal and Child Health Hospital approved the study protocol and detailed informed was obtained from all patients or their family members for the procedures.

Tissue and blood sample collection

Decidual samples were obtained from patients in URSA and IA groups and endometrium samples were obtained from control group. The samples were washed by 0.25% precooled phosphate-buffered saline (PBS) solution within 20 minutes after tissue detached. After that, samples underwent homogenate in normal saline (1:9, W/V) and centrifugation of 12000 g for 15 min at 4°C. The liquid supernatant was preserved and used for enzyme-linked immunosorbent assay (ELISA) of Galectin-1 using commercial kits (CSB-EL012882HU, Cusabio Biotech Co., Wuhan, China) in accordance with the manufactures' instructions.

Total 5 ml heparinized peripheral venous blood was obtained by venipuncture immediately from all participants who were undergoing a dilation and evacuation procedure for the isolation of peripheral blood mononuclear cells (PBMCs). PBMCs was then isolated for analysis by flow cytometry and centrifuged on Lymphoprep (Nycomed Pharma, Oslo, Norway) at 840 × g for 15 min at room temperature. The serum was separated from the samples and stored at -70°C till use.

Galectin-1 measurement

Decidual tissues of patients in URSA and IA groups and endometrial samples of control were detached. The optical density of Galectin-1 in each sample was determined at 450 nm using a microplate ELISA reader Model 450 (Bio-Rad, Miinchen, Germany) according to the manufacturer's instructions.

Flow cytometry detection for tregs

To each tube, 100 μl prepared PBMCs (1×10^7/ml) were added into two tubes, respectively. For one tube, 100 μl cell suspensions were mixed with anti-CD4-FITC monoclonal antibody (Pharmingen), anti-CD25-APC monoclonal antibody (Pharmingen) and anti-human Foxp3-PE monoclonal antibody (eBioscience), and incubated in the dark for 30 minutes at 4°C. As control, the other tube did not add any reagents. Flow cytometry was performed with a BD LSK flow cytometer (BD Biosciences). Data were then collected and analyzed using using CellQuest Pro software (Becton Dickinson) to investigate percentages of cells with different fluorescent antibodies. The percentages of $CD4^+$, $CD4^+CD25^+$, $CD4^+CD25^+Foxp3^+$ T-lymphocytes were calculated, followed by the ratio of $CD4^+CD25^+Foxp3^+/CD4^+$ was obtained.

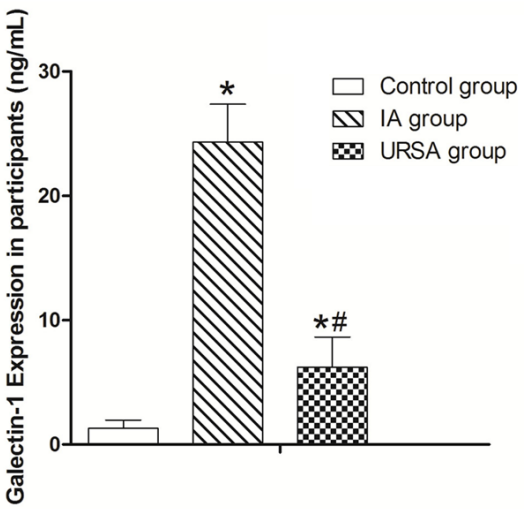

Figure 1. Galectin-1 expression in participants detected by Galectin-1 ELISA commercial kit. *$P < 0.05$ vs. control group; #$P < 0.05$ vs. IA group.

Statistical analysis

All statistical analyses were performed using software SPSS 17.0 (SPSS Inc., Chicago, USA). The data were presented as mean ± standard deviation. As determined by the Student's t-test or one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Levels of Galectin-1 expression

The expression levels of Galectin-1 in different groups were determined in the decidual tissue or endometrium by ELISA. As shown in **Figure 1**, the expression levels of Galectin-1 in IA group and URSA group was significantly higher than that in the control group (24.30 ± 3.06 and 6.23 ± 2.41 vs. 1.30 ± 0.66, $P < 0.05$). Besides, the expression level of Galectin-1 was lower in URSA group than IA group (6.23 ± 2.41 vs. 24.30 ± 3.06, $P < 0.05$).

T-lymphocyte subpopulations analyzed by flow cytometry

The flow cytometry results demonstrated that the percentage of $CD4^+$ T-lymphocytes and $CD4^+CD25^+$ Tregs had no differences among the three groups ($P > 0.05$, **Figure 2**). The percentage of $CD4^+CD25^+Foxp3^+$ Tregs was lower in URSA group than IA group (0.77 ± 0.31 vs. 1.00 ± 0.35, $P < 0.05$). Moreover, the ratio of $CD4^+CD25^+Foxp3^+/CD4^+$ in URSA group was also significantly lower than that in IA group (0.029 ± 0.012 vs. 0.044 ± 0.020, $P < 0.05$) and control group (0.029 ± 0.012 vs. 0.040 ± 0.015, $P < 0.05$).

Discussion

URSA belongs to an alloimmunity disease associated with the failure of materno-fetal immunologic tolerance [18]. The immunologic status of the pregnant woman and immunoregulatory capability with the embryo affect the development of the fetus [19]. In the present study, after detected the expression of Galectin-1 and proportion of different T-lymphocyte subgroups, we found that the expression levels of Galectin-1 in IA group and URSA group were significantly higher than that in the control group (24.30 ± 3.06 and 6.23 ± 2.41 vs. 1.30 ± 0.66, $P < 0.05$). Besides, the percentage of $CD4^+CD25^+Foxp3^+$ was lower in URSA group than IA group (0.77 ± 0.31 vs. 1.00 ± 0.35, $P < 0.05$).

The importance of Galectin-1 has been reported in many studies during gestation. In humans, Galectin-1 expression greatly increased in the latesecretory-phase endometrium and decidual tissue [20]. It has been expressed in pathological placenta [21] and is involved in immune-mediated fetal tolerance during pregnancy, inducing IL-10 expression T regulatory cells, and provoking apoptosis of susceptible Th1 cells [22, 23]. In addition, Galectin-1 has been reported to induce trophoblast and fusion during the placenta formation, and play an important role in regulating trophoblast differentiation [24, 25]. Our study further provided the evidence on the importance of Galectin-1 as a predictive factor for pregnancy, given that the level of Galectin-1 decreased in healthy pregnant women compared to IA and URSA group. Liu *et al.* [26] have reported a down-regulation of Galectin-1 expression in placental villous tissues from early pregnancy loss patients, and this finding is consisted with our study of low expression level of Galectin-1 in URSA group compared with IA group (**Figure 2**). Together with the influence of Galectin-1 on the immune evasion in trophoblast cells, the data suggested that Galectin-1 may be important in the maintenance of pregnancy.

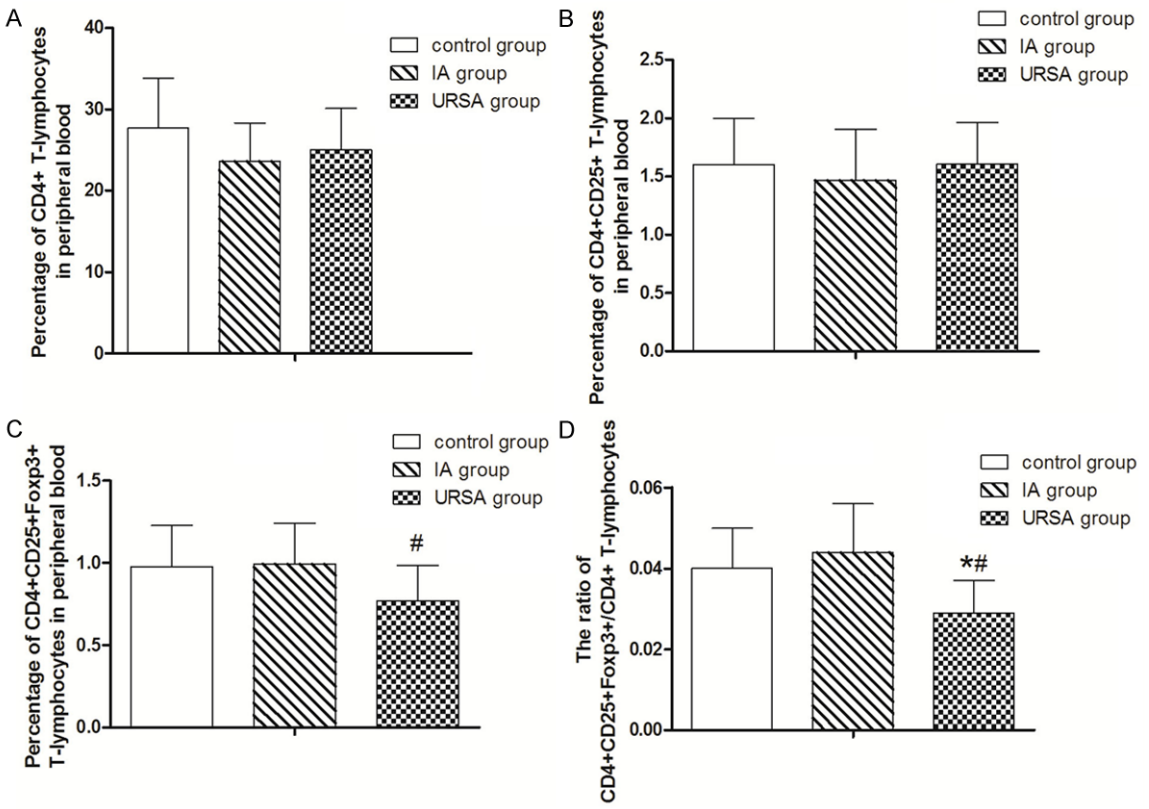

Figure 2. T-lymphocyte subpopulations detected by flow cytometry. (A-C) were the percentage of $CD4^+$, $CD4^+CD25^+$, or $CD4^+CD25^+Foxp3^+$ Tregs in peripheral blood. (D) was the ratio of $CD4^+CD25^+Foxp3^+/CD4^+$ Tregs. $^*P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. IA group.

Evidence has demonstrated that $CD4^+CD25^+$ Tregs play a central role in the development of maternal tolerance to fetus during pregnancy [27]. Shi *et al*. [28] have found that frequency and immunosuppressive capacity of $CD4^+CD25^+CD127$ dim/-regulatory Tregs was decreased in URSA decidua. Mei *et al*. [29] also investigated the proportion of $CD4^+CD25^+Foxp3^+$ T cells in pregnant women with URSA and found that the proportion of $CD4^+CD25^+Foxp3^+$ was statistically significantly lower in URSA patients compared with normal early pregnant women in the decidua. In addition, $CD4^+CD25^+Foxp3^+$ T regulatory cells have been served as a superior pregnancy marker for assessing miscarriage risk in newly pregnant women [30]. Together with our study, it is suggested that $CD4^+CD25^+Foxp3^+$ Tregs may be important in maintaining a normal pregnancy, and the reduction of $CD4^+CD25^+Foxp3^+$ Tregs may be involved in the pathogenesis of URSA.

In conclusion, we found that the proportion of Galectin-1 and $CD4^+CD25^+Foxp3^+$ Tregs was lower in URSA group than IA group. These findings suggest that Galectin-1 and $CD4^+CD25^+Foxp3^+$ may play essential roles in maintaining a normal pregnancy and their decrease may involve in the pathogenesis of URSA. Our data may help us explore the pathology of URSA and find new therapies.

Acknowledgements

We wish to express our warm thanks to Dr. Liang Xu for his technical support to this project.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Minjun Wu, Shanghai Changning District Obstetrics Maternal and Child Health Hospital, 773 Wuyi Road, Changning District, Shanghai 200051, China. Tel: +86-21-62288686*2501; Fax: +86-21-62288686*2501; E-mail: minjunwuu@163.com

References

[1] Kim JW, Park SY, Kim YM, Kim JM, Han JY, Ryu HM. X-chromosome inactivation patterns in Korean women with idiopathic recurrent spontaneous abortion. J Korean Med Sci 2004; 19: 258-262.

[2] Pildner Von Steinburg S, Schneider K. Recurrent Spontaneous Abortions-An Update on Diagnosis and Management. Journal für Reproduktionsmedizin und Endokrinologie-Journal of Reproductive Medicine and Endocrinology 2009; 6: 11-16.

[3] Gao L, Zhang JP, Chen H, Zhang SN, Chen LB, Tan JP, Liu ML, Meng LL, Wang YH, Zhang R, Liu YL, Cai WB. Characteristics of immune cell changes before and after immunotherapy and their clinical significance in patients with unexplained recurrent spontaneous abortion. Genet Mol Res 2014; 13: 1169-1178.

[4] Khadem N, Poorhoseyni A, Jalali M, Akbary A, Heydari ST. Sperm DNA fragmentation in couples with unexplained recurrent spontaneous abortions. Andrologia 2014; 46: 126-130.

[5] Cramer DW, Wise LA. The epidemiology of recurrent pregnancy loss. in Seminars in reproductive medicine. 2000. Copyright© 2000 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York. NY 10001. USA.

[6] Christiansen OB, Nybo Andersen AM, Bosch E, Daya S, Delves PJ, Hviid TV, Kutteh WH, Laird SM, Li TC, Van Der Ven K. Evidence-based investigations and treatments of recurrent pregnancy loss. Fertil Steril 2005; 83: 821-839.

[7] Guleria I, Sayegh MH. Maternal acceptance of the fetus: true human tolerance. J Immunol 2007; 178: 3345-3351.

[8] Chen SJ, Liu YL, Sytwu HK. Immunologic regulation in pregnancy: from mechanism to therapeutic strategy for immunomodulation. Clin Dev Immunol 2012; 2012: 258391.

[9] Than NG, Romero R, Erez O, Weckle A, Tarca AL, Hotra J, Abbas A, Han YM, Kim SS, Kusanovic JP. Emergence of hormonal and redox regulation of galectin-1 in placental mammals: Implication in maternal–fetal immune tolerance. Proc Natl Acad Sci U S A 2008; 105: 15819-15824.

[10] Van Den Brule FA, Fernandez PL, Buicu C, Liu FT, Jackers P, Lambotte R, Castronovo V. Differential expression of galectin-1 and galectin-3 during first trimester human embryogenesis. Dev Dyn 1997; 209: 399-405.

[11] Phillips B, Knisley K, Weitlauf KD, Dorsett J, Lee V, Weitlauf H. Differential expression of two beta-galactoside-binding lectins in the reproductive tracts of pregnant mice. Biol Reprod 1996; 55: 548-558.

[12] Vicovac L, Jankovic M, Cuperlovic M. Galectin-1 and -3 in cells of the first trimester placental bed. Hum Reprod 1998; 13: 730-735.

[13] Poirier F, Timmons PM, Chan CT, Guenet JL, Rigby PW. Expression of the L14 lectin during mouse embryogenesis suggests multiple roles during pre- and post-implantation development. Development 1992; 115: 143-155.

[14] Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat Immunol 2004; 5: 266-271.

[15] Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and peripheral blood CD4+ CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. Mol Hum Reprod 2004; 10: 347-353.

[16] Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet 2001; 27: 68-73.

[17] Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, Von Boehmer H, Bronson R, Dierich A, Benoist C. Projection of an immunological self shadow within the thymus by the aire protein. Science 2002; 298: 1395-1401.

[18] Bulletti C, Flamigni C, Giacomucci E. Reproductive failure due to spontaneous abortion and recurrent miscarriage. Hum Reprod Update 1996; 2: 118-136.

[19] Lohr J, Knoechel B, Nagabhushanam V, Abbas AK. T-cell tolerance and autoimmunity to systemic and tissue-restricted self-antigens. Immunol Rev 2005; 204: 116-127.

[20] Von Wolff M, Wang X, Gabius HJ, Strowitzki T. Galectin fingerprinting in human endometrium and decidua during the menstrual cycle and in early gestation. Mol Hum Reprod 2004; 11: 189-194.

[21] Božić M, Petronijević M, Milenković S, Atanacković J, Lazić J, Vićovac L. Galectin-1 and galectin-3 in the trophoblast of the gestational trophoblastic disease. Placenta 2004; 25: 797-802.

[22] Blois SM, Ilarregui JM, Tometten M, Garcia M, Orsal AS, Cordo-Russo R, Toscano MA, Bianco GA, Kobelt P, Handjiski B. A pivotal role for galectin-1 in fetomaternal tolerance. Nat Med 2007; 13: 1450-1457.

[23] Kopcow HD, Rosetti F, Leung Y, Allan DS, Kutok JL, Strominger JL. T cell apoptosis at the maternal-fetal interface in early human pregnancy, involvement of galectin-1. Proc Natl Acad Sci U S A 2008; 105: 18472-18477.

[24] Tirado-González I, Freitag N, Barrientos G, Shaikly V, Nagaeva O, Strand M, Kjellberg L, Klapp BF, Mincheva-Nilsson L, Cohen M, Blois SM. Galectin-1 influences trophoblast immune

evasion and emerges as a predictive factor for the outcome of pregnancy. Mol Hum Reprod 2012; 19: 43-53.
[25] Kolundžić N, Bojić-Trbojević Ž, Kovačević T, Stefanoska I, Kadoya T, Vićovac L. Galectin-1 is part of human trophoblast invasion machinery-a functional study in vitro. PLoS One 2011; 6: e28514.
[26] Liu AX, Jin F, Zhang WW, Zhou TH, Zhou CY, Yao WM, Qian YL, Huang HF. Proteomic analysis on the alteration of protein expression in the placental villous tissue of early pregnancy loss. Biol Reprod 2006; 75: 414-420.
[27] Guleria I, Sayegh MH. Maternal acceptance of the fetus: true human tolerance. J Immunol 2007; 178: 3345-3351.
[28] Bao SH, Wang XP, De Lin Q, Wang WJ, Yin GJ, Qiu LH. Decidual CD4+ CD25+ CD127dim/- regulatory T cells in patients with unexplained recurrent spontaneous miscarriage. Eur J Obstet Gynecol Reprod Biol 2011; 155: 94-98.
[29] Mei S, Tan J, Chen H, Chen Y, Zhang J. Changes of CD4+ CD25high regulatory T cells and FOXP3 expression in unexplained recurrent spontaneous abortion patients. Fertil Steril 2010; 94: 2244-2247.
[30] Winger EE, Reed JL. Low circulating CD4+ CD25+ Foxp3+ T regulatory cell levels predict miscarriage risk in newly pregnant women with a history of failure. Am J Reprod Immunol 2011; 66: 320-328.