ISSN: 0975-766X
CODEN: IJPTFI
Available Online through *Research Article*
www.ijptonline.com

EXPRESSION OF INDOLEAMINE 2,3-DIOXYGENASE mRNA IN PATIENTS WITH SPONTANEOUS MISCARRIAGES AT EARLY STAGES OF PREGNANCY

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Received on 03-06-2016 *Accepted on 27-06-2016*

Abstract

mRNA expression of indoleamine 2,3-dioxygenase (IDO) and Toll-like receptors (TLR) 1-4 in epithelial cells of cervix uteri in 57 patients with early stage miscarriages (6-10 weeks of pregnancy) and 57 patients with artificial abortion (control group) was examined using quantitative polymerase chain reaction (PCR). It was determined, that in patients with early miscarriages significant increase of mRNA expression of TLR1, TLR2 and TLR4 , and 3-fold increase of IDO. Expression of IDO had moderate correlation with expression of TLR2, TLR3 and TLR4. Therefore, the patients with spontaneous miscarriage at early pregnancy demonstrate positive increase in mRNA expression of Toll-like receptors 1, 2 and 4, the ligands of which are bacterial structures, which probably can facilitate hyperactivation of innate immunity system and rejection of gestational sac. Such increase is accompanied by activation of indoleamine 2,3-dioxygenase synthesis which can be compensatory or facilitate excessive immune suppression of T-cell immunity.

Key words: indoleamine 2,3-dioxigenase, innate immunity, Toll-like receptors, miscarriages.

Introduction

Inflammatory diseases are one of the main causes of spontaneous miscarriages (Carp, 2007; Kitaya, 2011; Soloveva *et al.*, 2013). The success rate of embryo implantation is statistically significantly lower in women with chronic endometritis than in women with normal endometrium (11% and 58%, respectively) (Romero *et al.*, 2004). In the majority of cases, when identifying chronic endometritis that causes spontaneous miscarriages, it is impossible to identify a certain agent, which points at the non-specific character of inflammation due to changes in local immune reactivity (Cakmak and Taylor, 2011).

Therefore, the study of the role of indoleamine-2,3-dioxygenase enzyme (IDO) having immunosuppressive and antibacterial effect is of interest in pathogenesis of recurrent pregnancy loss. IDO is an enzyme that catabolizes tryptophane on a kynurenine pathway and thus facilitates suppression of a T-cell component of immune system due to formation of toxic catabolism products (Fallarino *et al.*, 2002; Bauer *et al.*, 2005; King and Tomas, 2007). IDO is shown to be able to inhibit T-cells proliferation, suppress complement activation and prevent dendritic cell activation (Aylamazyan *et al.*, 2010; Mellor *et al.*, 2001; Munn *et al.*, 1999).

IDO is widely expressed in the majority of organs and tissues, including chorion, placenta and decidua (Curti *et al.*, 2009; Ligam *et al.*, 2005). It was established in vitro that IDO activation in trophoblast cells promotes suppression of cell immunity and development of immune tolerance to an embryo (Doherty *et al.*, 2011). Cell culture identified that IDO expression is stimulated by γ-interferone, with its synthesis depending on Toll-like receptor 3 (Wang *et al.*, 2011). Besides, IDO is the major intracellular mechanism of protection against some pathogens, including but not limited to chlamydia, since it deprives them of their essential amino acid – tryptophan (Entrican G. et al., 2009; Roshick C. *et al.*, 2006).

However, the role of IDO in pathogenesis of recurrent pregnancy loss in vivo, and its interrelation with Toll-like receptors expression, has almost never been studied.

Therefore, the study of peculiarities of IDO expression in patients with spontaneous miscarriage at early stages of pregnancy is of much interest.

Objective: to study IDO expression in epithelium of cervix uteri in women with spontaneous miscarriage at early stages of pregnancy and its interrelation with Toll-like receptors (TLR) 1-4 expression.

Materials and Methods

The main group included 57 patients admitted to Belgorod Municipal City Clinical Hospital № 1 with the symptoms of spontaneous miscarriage with duration of gestation from 6 to 10 weeks; the control group included 57 women who applied for legal abortion at the same stage of pregnancy. Epithelial cells were obtained from cervix uteri and placed into preservative solution RNA later (“Ambion”, USA). Quantitative polymerase chain reaction (PCR) method was used to identify mRNA TLR 1-10 and IDO expression. RNA was obtained by phenol-chloroform extraction using Trisol agent («Invitrogen», USA). The obtained RNA was treated with DNAse using a set of DNAse I RNAse free (“Fermentas”, USA). In order to perform reverse transcription, a reverse transcriptase Mint kit and oligoDT (“Evrogen”, Russia) were used. 500 ng of RNA were added to the mixture for reaction. Reverse transcription was

made according manufacturer's instruction in amplifier "Tercyc" ("DNA-technology", Russia). Specific primers for PCR were selected in real-time mode from the database Blast (www.ncbi.nlm.nih.gov) (table 1). As housekeeping genes β-actin and peptidyl prolyl isomerase A (PPIA) were used. Amplification was carried out by CFX96 ("Bio-Rad", USA) device using qPCR-mix HS SYBR ("Evrogen", Russia). Amplification was performed using the following cycling conditions: 5 minutes at 95°C, and 45 three-step cycles of 15 seconds at 95°C, 30 seconds of appropriate gene annealing according to the table 1 and 30 seconds at 68°C.

The obtained results were expressed in relative units, which were calculated by the formula:

$R= 2^{-(Cq\ target - (Cq\ ref_1 + Cq\ ref_2)/2)}$,

where R is a normalized mRNA expression of genes under investigation; Cq ref_1, and Cq ref_2 – Cq housekeeping genes; Cq target – Cq of the gene under investigation (Pffafl, 2006).

Table 1: Primers for quantative PCR.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Annealing temperature, ºC
TLR1	CAGGCACCAGGGCGTGATGG	GATGGAGGGGCCGGACTCGT	57
TLR2	ATCCTGCTCACGGGGGTCCTG	TGCTGGGAGCTTTCCTGGGC	57
TLR3	ACTGATGCTCCGAAGGGTGGC	TGCGTGTTTCCAGAGCCGTGC	56
TLR4	GGAGCCCTGCGTGGAGGTGGTT	GTTGAGAAGGGGAGGTTGTCGGGGA	57
IDO	TGCTAAACATCTGCCTGATC	GGAGCAATTGACTCAAATCA	61
β-actin	CAGGCACCAGGGCGTGATGG	GATGGAGGGGCCGGACTCGT	64
PPIA	CCGCCGAGGAAAACCGTGTACT	TGGACAAGATGCCAGGACCCGT	64

Statistical processing of the data obtained was carried out using Statistica 10.0 program (Statsoft, license number №AXXR505C705306FAN12). Reliability of differences was evaluated according to Mann-Whitney test, the results of which were represented as a median (lower quartile; upper quartile). The Spearman's rank criterion was used for the correlation analysis.

Results and Discussion

It was established, that IDO mRNA expression level in patients with spontaneous miscarriage was positively higher than that in the control group (33.61 (0.69; 314.08) and 11.47 (0.39; 1230.47), respectively) (p=0.001) (Table 2).

TLR3 mRNA expression in the main group had also the tendency for increase – 0.2483 (0.0828; 0.7196) versus 0.1472 (0.0502; 0.3763) in the control group, but was unreliable (p=0.28).

Table 2: Expression of Toll-like receptors and indoleamine-2,3-dioxygenases in patients with spontaneous miscarriages and in the control group.

	Gene	Group with miscarriages (n=57)	Control group (n=57)
1.	TLR1	**0.00051 (0.000005; 0.00130)****	0.00001 (0.000001; 0.000005)
2.	TLR2	**0.00266 (0.00061; 0.00923)****	0.00024 (0.00005; 0.00123)
3.	TLR3	0.2483 (0.0828; 0.7196)	0.1472 (0.0502; 0.3763)
4.	TLR4	**0.000021(0.000002; 0.000078)***	0.000003(0.000001; 0.000046)
5.	IDO	**33.61 (0.69; 314.08)****	11.47 (0.39; 1230.47)

*- $p<0.05$; ** - $p<0.01$

According to special literature, the IDO level increase facilitates the development of immune tolerance to gestational sac antigens. Therefore, the IDO level increase in patients with spontaneous miscarriage is likely to be a protective mechanism aimed at fighting with intracellular pathogens and promoting suppression of cell component of immune system.

Moderate, highly-reliable, positive correlation (R=0.31; p=0.009) was observed between expression of mRNA IDO and TLR3. This correlates with the data provided for in the special literature, according to which the IDO expression in the trophoblast cells in vitro depends on activation of Toll-like receptor 3, leads to increase in γ-interferon production being the IDO synthesis inducer [14]. Since dsRNA of viruses is a ligand for TLR3, the IDO activation mechanism described above is specific for virus infections (Adams *et al.*, 2004; Mao *et al.*, 2011). However, since no significant differences in TLR3 mRNA expression in the main and the control group were observed, one may suppose that the specified above mechanism of IDO activation during pregnancy is not the main one.

For this reason, the influence of TLR on IDO expression, with bacterial ligands TLR1, TLR2 and TLR4 being stimulators was studied. Patients with spontaneous miscarriage had positively higher expreR1 mRNA (0.00051 (0.000005; 0.00130) versus 0.00001 (0.000001; 0.000005) in the control group ($p<0.01$)), as well as TLR2 (0.00266 (0.00061; 0.00923) compared to 0.00024 (0.00005; 0.00123) in the control group ($p<0.01$)). Besides, the positive increase in expression level of mRNA TLR4 (0.000021(0.000002; 0.000078) versus 0.000003(0.000001; 0.000046) in the control group ($p<0.05$) was revealed.

The positive strong correlation was distinguished between the expression level of TLR1 mRNA and TLR2 (R=0.76; p=0.000028) (Table 3). This correlates with the data provided for in the special literature, according to which TLR1 and TLR2 form the heterodimeric complex necessary for identification of triacetylated lipopeptides (Sandor *et al.*, 2003).

Table 3: Correlation between expression of Toll-like receptors and IDO(Spearman criterion).

Item	Description	Spearman criterion	p
1.	IDO/TLR1	0.36	0.080
2.	IDO/TLR2	**0.39**	**0.002**
3.	IDO/TLR3	**0.31**	**0.009**
4.	IDO/TLR4	**0.30**	**0.013**
5.	TLR1/TLR2	**0.76**	**0.000028**

The IDO expression was reliably correlated with levels of TLR2 (R=0.39; p=0.002) and TLR4 (R=0.30; p=0.013).

In some works with the cell culture it was shown that IDO depends on stimulation of TLR2 and TLR4. So, the possibility of IDO expression stimulation by means of ligands TLR2 and TLR4 was demonstrated in the cell culture during the experiment with the human gingival fibroblasts (Mahanonda *et al.*, 2007). During the work with fibroblasts, M. Park et al. (2011) demonstrated the possibility of IDO synthesis stimulation by activation of not only TLR3, but also TLR4. At that, TLR4 activation is carried out in a MyD88-dependent pathway that does not lead to γ-interferon synthesis (Park *et al.*, 2011).

However, the correlation between IDO mRNA expression and expression of TLR activated by bacterial ligands can be explained not only by direct stimulation of IDO expression through TLR2 and TLR4. Described was the mechanism of TLR3 stimulation by viral ligands, which leads to improvement of IDO expression, which, in its turn, facilitates decrease in IL-10 and TNF-α levels. During the experiments with mice, the improvement of IDO expression induced by influenza virus has led to development of the secondary bacterial pneumonia due to immune suppression (Siljuis *et al.*, 2006). That is why the increase in TLR2 and TLR4 expression can be secondary, in response to the bacterial number increase.

Conclusion

Therefore, the patients with spontaneous miscarriage at early pregnancy demonstrate positive increase in mRNA expression of Toll-like receptors 1, 2 and 4, the ligands of which are bacterial structures, which probably can

facilitate hyperactivation of innate immunity system and rejection of gestational sac. Such increase is accompanied by activation of indoleamine 2,3-dioxygenase synthesis which can be compensatory or facilitate excessive immune suppression of T-cell immunity. However, the mechanism of secondary hyperactivation of Toll-like receptors in response to the increase in bacterial flora growth due to immune suppression induced by excessive IDO synthesis is not improbable.

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