Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Relationship between expression of COX-2, TNF-α, IL-6 and autoimmune-type recurrent miscarriage

Fu Hua, Chang-Hua Li*, Hong Wang, Hong-Ge Xu

Department of Gynaecology and Obstetrics, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu 223300, China

ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form 15 October 2013
Accepted 15 November 2013
Available online 20 December 2013

Keywords:
Autoimmune
Recurrent miscarriage
Animal model
Maternal-fetal interface

ABSTRACT

Objective: To investigate the roles of COX-2, TNF-α, IL-6 in the pathogenesis of autoimmune-type recurrent spontaneous abortion (RSA). **Methods:** RT-PCR was used to detect the mRNA of COX-2, TNF-α, IL-6 in the trophoblast cells of murine RSA and normal pregnant models. The COX-2, TNF-α, IL-6 protein expressions were determined by using immunohistochemisry staining method. The COX-2, TNF-α, IL-6 protein expressions were determined by ELISA. **Results:** The embryo loss rates in experiment group was significantly higher than that in normal pregnancy control group,the expression of COX-2, TNF-α, IL-6 in the trophoblast cells of murine RSA and normal pregnant models. The expression of COX-2 in autoimmune-type recurrent spontaneous abortion was significantly lesser than in normal pregnant models. The expression of TNF-α, IL-6 in autoimmune-type recurrent spontaneous abortion was significantly higher than in normal pregnant models. There was a positively correlation between TNF-α and IL-6. There was no relationship between COX-2, TNF-α and IL-6. **Conclusions:** The abnormal expression of COX-2, TNF-α and IL-6 may result in RSA.

1. Introduction

The incidence of recurrent spontaneous abortion (RSA) showed an increasing trend, its pathogenesis is still unclear. At present, it is considered that the pathogenic mechanisms were related to antiphospholipid antibodies (APA) syndrome, which was mediated by the APA induced autoantibody and cause implantation failure. Cyclooxygenase-2 (COX-2) is an important rate-limiting enzyme of the conversion from arachidonic acid to prostaglandins (PGs), and the PGs plays a vital role in the embryonic circulation. Therefore we speculated the COX-2 was the key factor which can affect the pathogenesis of RSA, and the expression of tumor necrosis factor-α (TNF-α) and interleukin -6 (IL-6) also play an important role in cell-mediated immunity. The expression of COX-2, TNF-α and IL-6 in RSA have not been reported. We used human β2 glycoprotein I (β2GPI) sub-bureaucratic injection method to establish a mouse model of autoimmune RSA, and explore the relationship between the COX-2, TNF-α, IL-6 and autoimmune type RSA.

2. Materials and methods

Mouse were kept in 12-hour light/dark period isolation cabinet, with a constant humidity (40% -50%) and a constant temperature (25 ℃-27 ℃), and autoclaved water and standard feed as free food.

2.1. Animals and model establishment

Six to eight weeks SPF level CBA/J female mice and Balb/

*Corresponding authors: Chang-Hua Li, Chief Physician, Huai'an First People's Hospital, Nanjing Medical University, 6 Beijing Road West Huai'an, Jiangsu Province 223300, China.
Tel: 13770385968
E-mail: lchlqq@126.com

c male mice which were purchased from the Experimental Animal Center of XX University were selected. The SPF level mice were randomly divided into the experimental group and the control group (female:male=2:1). In the experimental group, mice were injected with 50 μL PBS containing 1 Oug human β2GP1 and complete Freund's adjuvant (CFA) with the ratio of 1:1[1]. Then CFA was changed into incomplete Freund's adjuvant (IFA) as a booster immunization, with the same dose of previous immunization. After treatment for 10 days, normal pregnancy control group received a normal saline (NS) injection intraperitoneally. All mice were kept in 12-hour light/dark period isolation cabinet, with a constant humidity (40%-50%), a constant temperature (25 ℃ -7 ℃), autoclaved water and standard feed as free food. The differences in age and body weight between two groups were not statistically significant (P>0.05). The first day of pregnancy was termed as the pessary fell off. Embryos absorption and the average mass of placenta was observed and calculated in each group .

2.2. Reagents and primers

COX-2, TNF-α, IL-6 were all rabbit anti-mouse monoclonal antibody, which were purchased from Sigma (USA). COX-2, TNF-α, IL-6 SP immunohistochemistry kit and DAB chromogenic kit were purchased from Wuhan Boster Biological Engineering Co., Ltd..COX-2, TNF-α, IL-6 ELISA kit were purchased from R & D company,USA. Horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody was purchased from Sigma, USA.COX-2 (357 bp) upstream primers:5′-CCAACCTCTCCTACTACACCAGGG-3′,downstream:5′-ACACCTCTCCACCAATGACCTGAT-3′; IL-6 (441 bp) upstream primers: 5′-GTCTATACCACTTCACAAGTCGGA-3′, downstream 5′-TTGGATGGTCTTGGTCCTTAGCCA-3′; β-actin (478 bp) upstream primers 5′-AGGGAAATCGTGGGTGACATCAAA-3′,downstream:5′-ACTCATCGTACTCCTGCTTGCTGA-3′,all synthesized by Shanghai Sangon Biological Engineering Co., Ltd.

2.3. Experimental methods

2.3.1. Drawing materials

The blood of all pregnant rats was collected by extirpating eyeballs on the 14th day of pregnancy for determination of serum COX-2, TNF-α and IL-6. Mice were laparotomize to obtain the uterus and the embryo, then they were divided into two parts: one part was fixed with 10% formalin and then underwent COX-2, TNF-α, IL-6 immunohistochemical staining, the remaining part were frozen at -80 ℃ and then underwent COX-2, TNF-α, IL-6 mRNA detection.

2.3.2. ELISA detection

Serum cytokines COX-2, TNF-α, IL-6 was detected by ELISA, which was carried out strictly according to the manual of the kit.

2.3.3. RT-PCR

The specimens were removed from -80 ℃, total RNA was extracted by TRIzol and synthesized to cDNA by reverse transcription, and amplified by PCR, with β-actin as an internal reference. PCR reaction volume was 25 μL: Reverse transcription production 5 μL,10 pmol upstream and downstream 1 μL of each primer, 5mmol/L dNTP 1 μL, 10×reaction buffer 2.5 μL, 25 mmol/L $MgCl_2$ 1.5 μL, *Taq* DNA polymerase 1 U (0.5 μL), PCR H_2O 12.5 μL. PCR amplification program: 95 ℃ 5 min, after 1 cycle adding *Taq* DNA polymerase; 94 ℃ 1 min, 62 ℃ (COX-2), 63 ℃ (TNF) or 65 ℃ (IL-6) 45 s and 72 ℃ 45 s, 35 cycles; extension at 72 ℃ for 5 min. Five ul of the PCR product was obtained and underwent agarose electrophoresis.

2.3.4. Immunohistochemical assay

The embryo was embedded in paraffin, and was added with first antibody (COX-2, TNF-α, IL-6 antibody, both diluted 1:100) after dewaxing. Horseradish peroxidase-labeled secondary antibody was added for incubation, then color developing was conducted, with PBS instead of primary antibody as a negative control.

2.4. Results analysis

Embryo resorption rate=Absorption embryo number/(Absorption embryo number+surviving embryo number). Semi-quantitative determine of RT-PCR results was as follows: analyzing the light intensity of each band by density scanning and Quantity One software, with β-actin expression as control. Relative expression levels of each factor was calculated and compared. Semi-quantitative immunohistochemical assay was as follows: Randomly selected 5 fields (200 ×), and the color range was divided into 4 kinds:

+ Color range <1/4 vision; + + Color range from 1/4 to 1/2 vision; + + + Color range from 1/2 to 3/4 vision; + + + + Color range> 3/4 vision. + was 1, + +was 2, + + + was 3 , + + + + was 4.

2.5. Statistical analysis

The data was analyzed with SPSS 13.0 software. t-test, χ^2 analysis and Spearman rank correlation analysis were applied. P<0.05 was regarded as statistical significance.

3. Results

3.1. Pregnancies of female mice in two groups

The absorption rate of the female mice in the experimental groups was 27.9% (15/54), significantly higher than that of control group (6/7, 8.3%) ($P<0.05$); the average quality of the embryo in the experimental group was (87.7±8.8) mg, significantly lower than of the control group [(95.9±7.9) mg] ($P<0.05$).

3.2. Effect on release level of COX-2, TNF-α, IL-6

COX-2 mass concentration of the experimental serum levels was (28.6±1.8) pg/mL, significantly lower than the control group ($P<0.05$). TNF-α and IL-6 concentrations were were significantly higher than that of control group (Table 1).

Table 1

Comparison of cytokine expression between two groups.

Groups	*n*	COX-2 (pg/mL)	TNF-α (ng/mL)	IL-6 (pg/mL)
Experimental group	8	28.6±1.8*	1.5±0.6*	38.4±2.5*
Control group	8	43.4±2.8	0.7±0.3	22.8±1.6

Note: * Compared with control group, $P<0.05$.

3.3. RT-PCR detection of COX-2, TNF-α and IL-6 expression in embryonic tissues

COX-2 could be tested in placental tissue of 3 cases in experimental group, and 6 cases in control group. The relative COX-2 expression of the embryonic tissues in the experimental group was significantly lower than the control group ($P<0.05$); TNF-α could be tested in 2 cases of control group, and 5 cases in experimental group. The relative expression of TNF-α of the embryos tissues in the experimental group was significantly higher than that of control group ($P<0.05$); IL-6 could be tested in 3 cases in control group and 5 cases in experimental group. The relative IL-6 expression of the embryonic tissues in the experimental group was significantly higher than the control group ($P<0.05$) (Table 2, Figure 1).

Table 2

Relative expression of COX-2, TNF-α and IL-6 mRNA in embryonic tissues.

Groups	*n*	COX-2 mRNA	TNF-α mRNA	IL-6 mRNA
Experimental group	8	0.334±0.068*	0.486±0.168*	0.543±0.189*
Control group	8	0.525±0.187	0.276±0.088	0.283±0.077

Note: * Compared with control group, $P<0.05$.

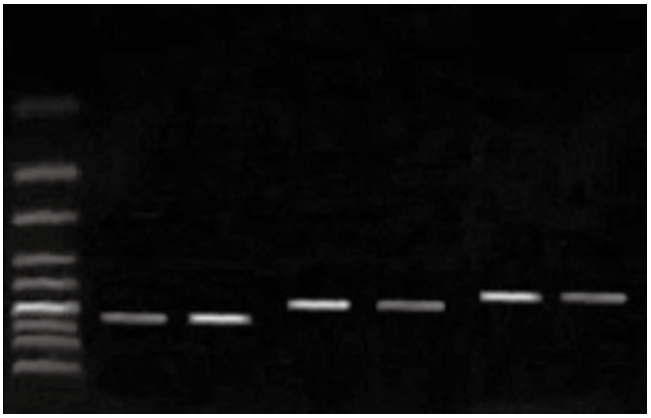

Figure 1. COX-2, TNF-α and IL-6 mRNA expression in two groups.

3.4. Correlation analysis of COX-2, TNF-α and IL-6 mRNA in the embryos tissues of experimental group

The Spearman rank correlation analysis showed that among the COX-2, TNF-α and IL-6 mRNA expression in embryonic tissues, IL-6 and TNF-α expression was positively correlated (Correlation coefficient: 0.676, $P<0.05$). There was no correlation between COX-2 and TNF-α (Correlation coefficient: −0.738, $P>0.05$), or COX-2 and IL-6 expression (Correlation coefficient: −0.782, $P>0.05$).

3.5. COX-2, TNF-α and IL-6 protein expression

Immunohistochemical results showed that positive parts of COX-2 protein were expressed in the nucleus and cytoplasm of the pregnant mice decidua, while the COX-2 expression in the experimental group were lower than the control group, the difference was statistically significant ($P<0.05$). The TNF-α positive protein was expressed in the cytoplasm of the pregnant rat decidua, the TNF-α expression in the decidua of the experimental group was significantly higher than in the control group ($P<0.05$). The IL-6 positive protein was expressed in the cytoplasm of the pregnant rat decidua, there was no significant difference between experimental group and the control group ($P>0.05$) (Table 3).

Table 3

Protein expression of COX-2, TNF-α and IL-6 in abortion embryos tissues and normal control embryonic tissues.

Groups	*n*	COX-2	TNF-α	IL-6
Experimental group	8	0.857±0.075*	1.568±0.158*	1.123±0.118
Control group	8	2.965±0.566	0.797±0.085	0.986±0.098

Note: * Compared with control group, $P<0.05$.

4. Discussion

Autoimmunity is the failure of an organism in recognizing its own constituent parts as self, which allows an immune response against its own cells and tissues. Any disease that results from such an aberrant immune response is termed an autoimmune disease, which the autoantibodies and/or autoreactive T cells attack its own tissues and cells. RSA refers to more than three times of spontaneous abortion before 20 weeks of gestation, the incidence rate is about 0.5%-1%[2-4], and with the increase of the abortion the incidence of RSA will be increased. Pregnancy failure may related to the immune rejection of the maternal to the embryo. Therefore, it is the current research focus to clarifies the mechanism of local immune regulation of the maternal-fetal interface and how to improve the abortion phenomenon. In this study, we established a mouse model of autoimmune RSA, detected the relationship of COX-2, TNF-α, IL-6 and autoimmune recurrent spontaneous abortion.

PGs play a vital role in the embryonic circulation. COX-2 is a key enzyme in the formation of PGs. As the only cyclooxygenase which expressed during the embryo sac implantation, and can not be affected by steroid hormones, we speculated that COX-2 may play a role in the process of embryo implantation. Previous studies[5-8] showed that COX-2 play a role in the mammalian embryo implantation process, which has a direct impact on the success of implantation. The results of Xiao[9-11] and other studies suggest that COX-2 and its signal transduction pathway related molecules may play an important role in the pathogenesis of autoimmune RSA. ELISA results showed COX-2 expression serum levels in the experimental group was significantly lower than the control group. In this study, we detected the COX-2 expression on the transcription level, the result showed the relative COX-2 mRNA expression was significantly decreased. And the immunohistochemical assay showed that the positive parts of the COX-2 protein were expressed in the nucleus and cytoplasm, the COX-2 expression of the experimental group was significantly lower than the control group. We speculated that abnormal expression of COX-2 may be associated with the pathogenesis of autoimmune RSA. It maybe because the decreased COX-2 expression in maternal-fetal interface of the patients can lead to decreased PGs expression, and then resulting in the loss of embryo which can cause autoimmune RSA.

There were macrophages in decidua during each period of the pregnancy. The abnormalities of the activity and function of the macrophage can cause the imbalance of maternal-fetal immune tolerance, which can lead to the occurrence of autoimmune RSA.TNF-α, IL-6 is a monocyte/macrophage-derived cytokines, which plays a role to maintaining the uterus in a quiet state[13-15], when the balance is upset it can eventually lead to miscarriage. Our experimental results showed that TNF-α and IL-6 concentrations were significantly higher than the expression in the control group. The TNF-mRNA expression in embryonic tissue of the experimental group is relative higher, and the IL-6 mRNA expression in embryos tissues of the experimental group was higher than that in the control group. Immunohistochemical results showed that both the expression were higher in the experimental group. So we can speculate that TNF-α, IL-6 may play an important role in the embryonic development, its high expression may be associated with autoimmune recurrent spontaneous abortion. TNF-α, IL-6 as an inflammatory factor have double effects, which may have immunomodulatory and anti-inflammatory and other effects in maintaining the immune tolerance of maternal-fetal. Its overexpression can induced a variety of diseases and cause the miscarriage[16-23].

Spearman rank correlation analysis the COX-2, TNF-α and IL-6 mRNA expression in embryos tissue, which showed that the COX-2 expression was not correlated to the TNF-α, IL-6 expression, while the IL-6 and TNF-α expression was positively correlated. The continued high expression of the IL-6 and TNF-α can lead to the increase and the aggregation of the inflammatory cytokine concentration, and the interaction between cells can also cause cytotoxic effect, which lead to the imbalance of immune tolerance and then induced autoimmune RSA. IL-6 and TNF-α can induce the expression of COX-2 and then causing increased PGs. Therefore, when the IL-6 and TNF-α expression were in the normal level, the COX-2 can form the dynamic equilibrium, which can ensure the safe implantation of embryo sac and avoid abortion[24-30].

In summary, the COX-2, TNF-α and IL-6 are considered to play an important role in the maintenance of pregnancy, but the exact mechanism between them and the mutual regulation relationship were not been fully understood, which need our further study.

Conflict of interest statement

We declare that we have no conflict of interest.

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