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Cytokine Gene Polymorphisms and Early Pregnancy Loss

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Abstract—A disbalance of pro- and antiinflammatory cytokines can negatively affect the early stages of human embryogenesis. The association between polymorphism of cytokine genes (−31*C-T IL1*β, −174*G-C IL6*, −308*G-A TNF*α, and −592*C-A* and −819*C-T IL10*) and pregnancy loss was studied. The analysis was performed on DNA samples from two groups of women with pregnancy loss: those with a missed abortion (MA) ($n = 62$) and those with a spontaneous abortion (SA) ($n = 62$). The control group included 114 women with normal pregnancy. Cytokine genotyping was performed using PCR with sequence-specific primers with the CNP-express kit (Lytech, Russian Federation). The increase in the frequency of heterozygotes for the −31*C-T* polymorphism of the *IL*1β gene among women with MA (58.1%) compared to those with SA (36.7%) was found. In the SA group, the frequency of heterozygotes for −591*C-T* of the *IL*10 gene was higher (56.7%) than in the control group (32.5%). The frequency of the −819*T* allele of *IL*10 gene among women with SA was higher than in the control group (0.33 vs 0.23). At the same time, the frequency of the −308*A* allele of the *TNF*α gene was the lowest in the SA group compared to those in the MA and the control group. Our data show that the risk of spontaneous abortion increases if the −592*A* and −819*T* alleles of the *IL*10 and −308*G* allele of *TNF*α are present in the genotype.

Keywords: cytokines; polymorphism; pregnancy; spontaneous abortion; missed abortion

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INTRODUCTION

The problem of human reproductive disorders remains one of the most topical in modern biology and medicine. Pregnancy loss as a multifactorial condition results from the additive effect of many factors both environmental and genetic. Reproductive losses in humans account for up to 50% of the conceiving number (Alipov, Golovachev, 1983). Spontaneous pregnancy loss in the first trimester accounts for 15–20% of the total number of registered pregnancies (Dobrokhotova et al., 2010). Alloimmune reactions against fetal cells and tissues belong to the immune factors associated with a pregnancy loss. Immunological disorders can be due to the genotype features, including cytokine genes which are the endogenous mediators of intercellular interactions. The cytokines are the mediators of intercellular and intersystem interactions causing cascade reactions inducing the activity of many genes; they regulate cell survival and stimulate or suppress cells growth, differentiation, functional activity, and apoptosis. Due to the effect of cytokines the coordinated functioning of an organism's systems and organs is ensured. The cytokines participate in the formation and regulation of an organism's defensive reaction against pathogens and in violation of tissue integrity. When systemic inflammation develops the cytokines show a huge range of biological activity and influence all systems of the organism (Ketlinskii, Simbirtsev, 2008).

The risk of fetal loss can result from the change of the functional activity of cytokines playing an important role in pregnancy development and mediating the maternal immune response against the fetus (Simbirtsev, 2004; Bombell, McGuire, 2008; Kaur, 2011; Agrawal et al., 2012). According to modern ideas, the stages of oocyte development, blastocyst adhesion and implantation, and placental formation and growth are cytokine-dependent processes, which are controlled by the immune system. When the pregnancy is normal the maternal immunotolerance against the embryo as allograft is provided by the complex interaction of regulatory molecules synthesized by the maternal and fetal cells. During the trophoblast invasion the immune cells are activated leading to the synthesis of a wide range of cytokines. For mice from *Mus musculus* of the BALB/c IFN$\gamma^{-/-}$ line, it was shown that the production by the *Th*1 cells of typical proinflammatory cytokines is necessary for the normal passing of the early stages of embryogenesis and implantation (Ashkar, Croy, 2001). *IL1β*, being a typical proinflammatory cytokine, activates the expression of the integrin β3 subunit, providing the adhesion processes; it helps fetal development and stimulates cell proliferation, forming a placental barrier. Besides, *IL1β* activates the expression of the *IL6* family of cytokines in the endometrium, which also promotes the implanta-

tion (Chistyakova et al., 2005; Dimitriadis et al., 2005; Levkovich, 2008; Fitzgerald et al., 2008). *IL6* is a typical early inducible cytokine. The cytokines of the *IL6* family regulate the expression of the cell cycle's control genes and components of the extracellular matrix, increase the adhesion of the endometrial epithelial cells to collagen on the blastocyst surface, and increase the expression of integrines by the endometrial epithelial cells (Marwood et al., 2009).

One of the key regulators of the immune response is the family of *IL10* cytokine, which inhibits secretion of proinflammatory cytokines by the lymphocytes and activated macrophages. The level of this cytokine increases gradually in the first trimester of pregnancy, inducing a decrease of the level of proinflammatory cytokines and activating angiogenesis (Thaxton, Sharma, 2010).

Besides the adhesion and implantation, the cytokines provide the formation and growth of new blood vessels. The enhanced cytokine release has an activating effect on endothelium which, in turn, produces several growth factors required for angiogenesis (Amchislavskii et al., 2003). The activated endotheliocytes themselves begin producing proinflammatory cytokines that can lead to the violation of endothelium-dependent mechanisms such as the regulation of vascular tonus and the permeability and maintenance of balance between the thrombogenic potential of the vascular wall and its thromboresistance. As a result the microcirculation can be disrupted, which has a negative influence on the embryo. A high content of proinflammatory cytokines leads to the activation of thrombosis in fetoplacental tissues; and blood flow in the vessels, supplying the developing embryo, stops. In the case of spontaneous abortion (SA), the immune events take place with a multiple increase of inflammatory markers. Missed abortion (MA), being an attempted abortion, is characterized by the suppression of cellular immunity and the uterus's immunological reactivity, which prevents rejection of the ovum (Dobrokhotova et al., 2010).

The deregulation in cytokine functioning, including the one caused by genotype, can be a negative factor for the early stages of embryogenesis (Daher et al., 2012). This work aims to investigate the frequency of allelic variants of cytokine genes among women with missed abortions and spontaneous abortions in the first trimester of pregnancy.

MATERIALS AND METHODS

For the molecular-genetic analysis, the DNA samples were extracted from leucocytes of peripheral blood of 122 women with pregnancy loss in the first trimester (mean age 29.2 years). They include 62 women with missed abortions and 60 women with spontaneous abortions. One hundred and fourteen women (mean age 30.3 years) with physiological pregnancy and no spontaneous abortions and/or missed abortions in anamnesis were included in the control group. Their informed consent was obtained in each case.

Blood samples and anamnesis were collected and the studied groups were formed by the fellows of the Department of obstetrics and gynecology of Rostov State Medical University, as well as by obstetrician-gynecologists of city hospital no. 8 and maternity hospital no. 5 of Rostov-on-Don. Missed abortion was diagnosed based on the dynamics of the level of the human chorionic hormone in the peripheral blood of women and the cessation of the fetal heart detected by ultrasound examination.

The exclusion criteria were previously diagnosed arterial hypertension, diabetes, thyroid disease and autoimmune pathology, as well as infections during pregnancy. Besides, women with uterus abnormalities and polycystic ovary syndrome (diagnosed by transvaginal ultrasound) were excluded. In the compared groups there were no women with exogenous risk factors—alcohol abuse and contact with harmful factors of production (electromagnetic radiation, noise, vibration, and chemicals).

For the comparative analysis of three groups of women the confidence interval for proportion was calculated (Glants, 1999). It was shown that in the MA group there was the largest proportion of women without delivery in anamnesis (Table 1). At the same time, fewer women delivered a live-born child in this group than in the other two groupss.

DNA was extracted using the DIAtom™ DNA Prep 100 kit (Ltd Center of Molecular Genetics). Allelic variants *−31C-T* of *IL1β* (MIM *147720), *−174G-C* of the *IL6* gene (MIM *147620), *−592C-A* and *−819C-T* of the *IL10* gene (MIM *124092), and *−308G-A* of the *TNFα* gene (MIM *191160) were investigated using the SNP-express kit (Litekh, Moscow). The analysis is based on the amplification reaction with two pairs of allele-specific primers. The amplification products were visualized by horizontal electrophoresis in 3% agarose gel using transilluminator GelDoc (BioRad).

The correspondence of the distribution of the genotype frequencies to the Hardy-Weinberg equilibrium was detected using the Hardy-Weinberg equilibrium calculator in the www.oege.org/software/Hardy-Weinberg software (Rodriguez et al., 2009). The estimation of differences in the distribution of allelic variants in the groups was performed with the χ^2 criterion in BIOSTAT software (Biostatistica…, 1998). The risk of pregnancy loss was based on the odds ratio (OR) calculated as $OR = (A/B) \times (D/C)$, where A and C are the number of mutant genotype women with pregnancy loss and normal pregnancy, respectively; B and D are the number of women from both groups without mutant genotype. OR is shown with a 95% confidence interval (CI) (Petrie et al., 2003).

Table 1. Description of women groups

Criteria			Control			
	Abs.	% (95% CI)	Abs.	% (95% CI)	Abs.	% (95% CI)
The proportion of patients with a first pregnancy, %	9	8.8 (2.9–12.8)	20	32.2 (20.6–43.9)	4	6.6 (0.35–12.9)
The proportion of women without delivery in anamnesis, %	20	17.5 (10.6–24.5)	34	54.8 (40.4–67.2)	4	6.6 (0.35–12.9)
The proportion of women with a pregnancy in anamnesis ended with a life birth, %	93	81.5 (74.5–88.7)	28	45.2 (32.8–57.5)	53	88.3 (80.2–96.5)
The proportion of women with a missed abortion in the first trimester in anamnesis, %	0	0	6	9.7 (2.3–17.0)	4	6.6 (0.35–12.9)

MA—missed abortion; SA—spontaneous abortion.

Table 2. The frequency of genotypes and alleles of *IL1*β and *IL6* genes in women's blood cells

Genotype	Control, abs., (%)	Pregnancy failure					
		SA, abs., %	OR (95% CI)	χ_1^2 (*P*)	MA, abs., %	OR (95% CI)	χ_1^2 (*P*)
IL-1*β *–31 C-T							
CC	42 (36.8)	32 (53.3)	1.96 (1.04–3.69)	4.38 (0.11)	18 (29.0)	0.7 (0.36–1.37)	1.37 (0.5)
CT	56 (49.1)	22 (36.7)	0.6 (0.32–1.14)		36 (58.1)	1.43 (0.77–2.68)	
TT	16 (14.0)	6 (10.0)	0.68 (0.25–1.84)		8 (12.9)	0.91 (0.36–2.26)	
Allele –31 *T*	0.386	0.283	0.63 (0.39–1.01)	3.64 (0.06)	0.419	1.15 (0.74–1.79)	0.37 (0.54)
χ_2^2 (*P*)	Genotypes: 7.55 (0.02)				Alleles: 4.94 (0.03)		
Il-6 –174 G-C							
GG	21 (18.4)	11 (18.3)	0.99 (0.44–2.23)	1.84 (0.4)	15 (24.2)	1.41 (0.67–2.99)	4.79 (0.09)
GC	69 (60.5)	31 (51.7)	0.7 (0.37–1.31)		27 (43.5)	0.5 (0.27–0.94)	
CC	24 (21.1)	18 (30.0)	1.61 (0.79–3.28)		20 (32.3)	1.79 (0.89–3.59)	
Allele –174 *C*	0.513	0.558	1.2 (0.77–1.87)	0.64 (0.42)	0.54	1.12 (0.72–1.73)	0.24 (0.63)
χ_2^2 (*P*)	Genotypes: 0.96 (0.62)				Alleles: 0.08 (0.78)		

SA—spontaneous abortion; MA—missed abortion; χ_1^2—comparison of genotype and allele frequencies with control; χ_2^2—comparison between SA and MA groups.

RESULTS

It was shown that in the control group allele –31*C* of the *IL1β* was homozygous in 36.5% of the women and –31*C*-*T* was heterozygous in 49.1% of the women (Table 2). The distribution of genotype frequencies for the investigated polymorphism among women with a missed abortion did not differ from the control. Among women with spontaneous abortion homozygotes for allele –31*C* of *IL1*β prevailed (53.3%). However, these differences were not statistically significant.

At the same time, the differences in genotype frequencies for polymorphism –31*C*-*T* of *IL1*β between two groups of women with pregnancy failure in the first trimester were statistically significant. The frequency of allele –31*T* of *IL2β* in the MA group was higher than in the SA group (0.419 and 0.283 respectively) (Table 2).

The pattern of distribution of genotype frequencies for polymorphism –174*G*-*C* of the *IL6* gene among women with spontaneous or missed abortions corresponded to the control (Table 2). There were no statis-

Table 3. The frequency of genotypes and alleles of *IL10* and *TNFα* genes in women's blood cells

Genotype	Control, abs., (%)	Pregnancy failure					
		SA, abs., %	OR (95% CI)	χ^2_1 (*P*)	MA, abs., %	OR (95% CI)	χ^2_1 (*P*)
IL-10 –592 C-A							
CC	65 (57.0)	24 (40.0)	0.5 (0.27–0.95)	10.4 (0.006)	37 (59.7)	1.12 (0.6–2.09)	
CA	37 (32.5)	34 (56.7)	2.72 (1.43–5.18)		19 (30.6)	0.92 (0.47–1.79)	0.12 (0.94)
AA	12 (10.5)	2 (3.3)	0.29 (0.06–1.36)		6 (9.7)	0.91 (0.32–2.56)	
Allele –592 *A*	0.268	0.317	1.27 (0.78–2.06)	0.93 (0.33)	0.25	0.91 (0.55–1.51)	0.13 (0.72)
χ^2_2 (*P*)	Genotypes: 8.99 (0.01)				Alleles: 1.34 (0.25)		
IL-10 –819 C-T							
CC	69 (60.5)	26 (43.3)	0.5 (0.26–0.94)	4.69 (0.1)	40 (64.5)	1.19 (0.62–2.25)	
CT	37 (32.5)	28 (46.7)	1.82 (0.96–3.46)		15 (24.2)	0.66 (0.33–1.34)	1.89 (0.39)
TT	8 (7.0)	6 (10.0)	1.47 (0.49–4.46)		7 (11.3)	1.69 (0.58–4.89)	
Allele –819 *T*	0.232	0.333	1.65 (1.01–2.69)	4.09 (0.04)	0.234	1.01 (0.6–1.69)	0 (0.98)
χ^2_2 (*P*)	Genotypes: 6.95 (0.03)				Alleles: 2.97 (0.08)		
TNFα –308 G-A							
GG	72 (63.2)	49 (81.7)	2.6 (1.22–5.54)	6.96 (0.03)	36 (58.1)	0.81 (0.43–1.52)	
GA	39 (34.2)	11 (18.3)	0.43 (0.2–0.92)		24 (38.7)	1.21 (0.64–2.31)	0.45 (0.8)
AA	3 (2.6)	0	0.26 (0.01–5.18)		2 (3.2)	1.23 (0.2–7.59)	
Allele –308 *A*	0.197	0.092	0.41 (0.2–0.83)	6.51 (0.01)	0.226	1.19 (0.7–2.02)	0.4 (0.53)
χ^2_2 (*P*)	Genotypes: 8.79 (0.01)				Alleles: 8.17 (0.004)		

SA—spontaneous abortion; MA—missed abortion; χ^2_1—comparison of genotype and allele frequencies with control; χ^2_2—comparison between SA and MA groups.

tically significant differences in the frequencies of genotypes and alleles between two groups of women with early pregnancy pathology.

It was shown that in the control group 57% of the women were homozygous for the allele –592*C* of *IL10* gene (Table 3). In the group of women with spontaneous abortions the number of homozygotes –592 *CC* of the *IL10* gene was 40% and the proportion of heterozygotes for polymorphism –592*C-T* was higher than in the control (56.7%) (Table 3). These differences are statistically significant. For heterozygotes – 592*C-T* of the *IL10* gene, the increase of the risk of a possible spontaneous abortion was shown (OR = 2.72). Statistically significant differences were also shown between two groups of women with the disorders of the early stages of embryo development. Among the women with SA, the frequency of heterozygotes for this polymorphism was increased compared to the group of women with a missed abortion (Table 3).

A similar situation was also observed for polymorphism –819*C-T* of the *IL10* gene (Table 3). In the control group and among women with a missed abortion the homozygotes for allele –819 *C* of the *IL10* gene prevailed. The number of heterozygotes –819 *C-T* of *IL10* in these two groups of women did not exceed 32%. At the same time, among women with a spontaneous abortion, the number of both homozygotes – 819*CC* and heterozygotes –819*CT* of the *IL10* gene was 43–46%. And the distribution of genotypes' frequencies was not statistically significantly different from the control. However, the frequency of allele – 819*T* of the *IL10* gene among women with a spontaneous abortion in the first trimester was 0.333. It is statistically significantly different from the control value.

Between two groups of women with a pregnancy failure in the first trimester significant differences were found: when the number of homozygotes –819*TT* of the *IL10* gene among women with a missed abortion were the same, the frequency of heterozygotes –

819*CT* was 24.2%, while in the group of women with SA it was 46.7% (Table 3).

In the control group and in the group of women with a missed abortion the homozygotes for allele –308*G* of *TNFα* were somewhat less (63.2% and 58.1% respectively) than in the group of women with SA—81.7% (Table 3). The heterozygotes –308*G-A* of the *TNFα* gene were least likely to be met among women with a spontaneous abortion. Interestingly, the number of homozygotes for polymorphism –308 *A* in two groups (the control and in the group of women with MA) was almost the same and they were absent from the group of women with SA (Table 3). The distribution of genotype frequencies for the investigated polymorphism among women with MA did not differ from the control group. However, in the SA group, the increase of the number of homozygotes for allele –308*G* with the simultaneous decrease of the proportion of heterozygotes –308*GA* in comparison to the control group was detected. Also, the statistically significant differences between two groups of women with a pregnancy failure in the first trimester were shown (MA and SA). In the group of women with a spontaneous abortion the frequency of allele –308*A* was 0.092 which is the lowest value for the three groups.

DISCUSSION

The causes of early embryonic loss are numerous and diverse. Embryo karyotype abnormalities are most frequently detected when the embryonic development is delayed both in natural and assisted pregnancies (Simon et al., 1998; Liunger et al., 2005; Kirillova et al., 2006; Baranov, Kuznetsova, 2007; Morales et al., 2008; Chiryaeva et al., 2012). Immune and autoimmune factors contribute significantly to the pathogenesis of early embryo loss. If in population samples antibodies against phospholipids are detected in 2% of women, then among women with a recurrent pregnancy loss, this indicator is significantly higher (Rai et al., 1995; Kerchelaeva, 2003). The antiphospholipid syndrome is accompanied by thrombophlebitis, which is also observed in heritable forms of thrombophilia (Younis et al., 2000; Makatsaria, Bitsadze, 2001). A change of the gonadotropins' secretion level, abnormal functioning of their receptors, hyperprolactinemia, hyperandrogenism, and thyroid diseases are among the endocrine reasons of pregnancy failure (Li et al., 2000; Bellver et al., 2008; Prakash et al., 2006; 2008). The role of infections in pregnancy failure is discussed (Summers, 1994; Ralph et al., 1999; Podzolkova et al., 2003). We investigated women without autoimmune, endocrine, and infectious diseases. However, the karyotype analysis of embryos was not performed.

Almost all the etiological factors of pregnancy failure mentioned above influence the morphological and functional properties of the developing placenta and the level of proliferation and apoptosis of cells (Qumsiyeh et al., 2000). Intercellular interactions with the participation of several cytokine families are mandatory components of the processes of implantation and placenta development. The genotype peculiarities for cytokine genes can cause an increased production of proinflammatory cytokines or an imbalance between the levels of pro- and antiinflammatory cytokines, which can become one of the reasons of the failure of the early stages of human embryo development. The analyzed allelic variants of the *IL1β* and *TNFα* genes are characterized by the increased level of expression, while the allelic variants of the *IL6* and *IL10* genes cause a decrease of the level of the corresponding mRNA (Huizinga et al., 1997; Grimaldi et al., 2000; Bennermo et al., 2004). The data in the literature about a possible link between the presence of the analyzed allelic variants of cytokine genes and the risk of failure of the early stages of human embryogenesis are contradictory (Hefler et al., 2001; Wang et al., 2002; Prigoshin et al., 2004; Costeas et al., 2004; Kamali-Sarvestani et al., 2005; Bombell, McGuire, 2008; Zammiti et al., 2009; Liu et al., 2010; Kaur, 2011; Agrawal et al., 2012; Ma et al., 2012).

We showed that the groups of women with missed and spontaneous abortion differ from each other by the genotype peculiarities for cytokine genes. Thus, among women with a missed abortion the frequency of allele –31*T* of the *IL1β* gene was significantly higher than in the group of women with a spontaneous abortion. At the same time, it was shown that heterozygotes –592*CA* of *IL10* gene have an increased risk of spontaneous pregnancy loss. It is known that *IL10* actively participates in ensuring the immunotolerance of the maternal organism to the developing embryo. Besides, *IL10* ensures a decrease of the level of expression by the decidual cells of several molecules—coagulation activators (Cochery-Nouvellon et al., 2009). The presence in the genotype of alleles –31*T* of *IL1β* or –592*A* of *IL10* assumes an increase of the level of proinflammatory cytokines in the target tissues. Proinflammatory cytokines are synthesized at the early stages of pregnancy in small amounts, providing a dynamic equilibrium between the processes of trophoblast invasion and rejection. However, the production of proinflammatory cytokines higher than the optimal level can lead to thromboses and ischemic necroses in the structure of fetoplacental tissues. Proinflammatory cytokines are able to produce the direct embryotoxic effect and limit the trophoblast invasion, disrupting its normal formation, which leads to a delay or interruption of embryogenesis. For example, the hyperproduction of *IL1β* can induce a hypercute inflammatory response and, accompanied by an intrauterine infection, can finally lead to the embryo's rejection (Gromova, Simbirtsev, 2005).

The embryo's implantation during a normal pregnancy is associated with the shift of the cytokine balance towards the excess of factors with immunosuppressive activity. During the pregnancy in the uterus

the production of *Th*1 cytokines is suppressed, together with the increase of the synthesis of the *Th*2 cytokines. The disruption of the *Th*1/*Th*2 balance is one of the reasons of pregnancy failure.

However, it was shown that with the stopping of the embryo development the concentration and ratio of pro- and antiinflammatory cytokines (particularly, *TNFα* and *IL10*) in the peripheral blood of women and in the chorionic villi depend on the embryo karyotype (Calleja-Agius et al., 2012). The authors assume the existence of different mechanisms for the elimination of embryos with a normal and abnormal karyotype. According to the authors, the loss of pregnancy with a normal embryo karyotype is associated with systemic inflammatory processes in the mother's organism. When the karyotype of an embryo is abnormal the local reactions (in the placenta) matter. The cytokines' role in these reactions is undeniable.

The detected differences in the frequencies of the genotypes and alleles of cytokines' genes between the groups of women with SA and MA suggest a change of different links of the cytokine system. The primary link in the disruption of the balance of the cytokines can be the increase of the level of expression of proinflammatory cytokines (particularly, *IL1β*). The following disruptions of the functioning of the placenta can lead to the interruption of embryo development and, therefore, to missed abortion. If the primary disruption of the cytokine balance is a decrease of the concentration of proinflammatory cytokines (particularly, *IL10*), then the rejection of the embryo is possible as genetically alien for the maternal organism.

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