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Changes in Regulation of Oxidase Activity of Peripheral Blood Granulocytes in Women with Habitual Abortions

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Generation of active oxygen forms by blood granulocytes was studied in women with a history of habitual abortions (2-3 spontaneous abortions in the first trimester, undeveloped pregnancies). The level of spontaneous luminol-dependent chemiluminescence of nonfractionated peripheral blood was increased in this patient population (study group) in comparison with women with normal reproductive function (reference group). The two groups differed by the level of activation of respiratory burst induced by opsonized zymosan and by activity of isolated granulocytes in response to chemotactic peptide N-formyl-methionyl-leucyl-phenyl-alanine (1-50 μ M). Differences in the effects of inhibitor of tyrosine protein kinases and protein phosphatases and inhibitor of mitogen-activated protein kinase p38 MAPK were detected. The results attest to predisposition to oxidative stress and poor cytotoxic functions of granulocytes in women with habitual abortions, which can be due to specific features of regulation of oxidase activity by tyrosine protein kinases and protein phosphatases and by p38 MAPK.

Key Words: *granulocyte; active oxygen forms; intracellular signaling; habitual abortion*

Changes in the immune system parameters were detected in women with habitual abortions. The involvement of phagocytes in spontaneous loss of pregnancy was described previously [11,14,15]. Early miscarriages are associated with inflammatory reaction in the maternal part of *d. parietalis* and *d. basalis*, in which numerous maternal macrophages, large granular lymphocytes, and T cells are detected [14]. The functional activity of peripheral blood leukocytes is changed in women with habitual miscarriages [11,15].

The relationship between oxidative stress and miscarriage was previously discussed [7,8,10]. Spontaneous abortion is associated with the prooxidant/

antioxidant imbalance, which leads to oxidative stress [10]. Oxidative burst develops in normal placenta when maternal circulation is established; it can play a physiological role in the stimulation of normal differentiation of the placenta, but can also contribute to the pathogenesis of gestosis and early spontaneous abortion in case of antioxidant exhaustion [8].

The role of signal molecules in the mother-fetus relationships is little studied [2,4]. It was shown that high activity of the cytosol low-molecular-weight phosphotyrosine phosphatase (ACP1) in the mother, associated with low or moderate activity in the fetus, is more preferable for fetal growth and development [2]. When ACP1 activity is lower in the mother, the probability of miscarriage is higher and the survival of the fetus is lower in comparison with cases when maternal ACP1 activity is higher than in fetus. The role of S- and F-isoforms of this enzyme can be different: no

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significant differences between the groups were detected for F-isoform, while a specific decrease of ACP1 S-isoform concentration was detected in women with repeated miscarriages in comparison with women with normal course of pregnancy [4].

We compared functional activity of peripheral blood granulocytes in healthy nonpregnant women with a history of several normal pregnancies and in nonpregnant women with a history of habitual abortions. Differences in the functions of the signal pathway inhibitors involved in the regulation of the granulocyte respiratory burst were investigated.

MATERIALS AND METHODS

Thirty-five women aged 21-35 years were examined: 12 of these with normal reproductive function (group 1, control) and 23 patients with a history of habitual abortions (2-3 spontaneous abortions during the I trimester and undeveloped pregnancies, group 2).

Oxidase activity was evaluated by the production of active oxygen forms (AOF) measured by luminol-dependent chemiluminescence. The measurements in whole blood samples and isolated granulocytes were carried out using a Chemilum-2001 chemiluminometer developed at Institute of Cell Biophysics, Russian Academy of Sciences.

The samples were prepared from whole blood stabilized with heparin (20 U/ml), diluted 1:1 with Ca^{2+} -free Hanks' solution, and incubated 1 h at 4°C. Blood (20 μl) was put into measuring well (working volume 200 μl). The medium for measurements contained complete Hanks' medium with 0.35 mM luminol. The measurements were carried out in 3 blood samples from each patient: the level of spontaneous chemiluminescence was measured in one sample and chemiluminescence induced by opsonized zymosan (0.25 mg/ml) in two others.

Granulocytes were isolated by standard centrifugation of the blood in Ficoll-Urografin (1.077 and 1.118 g/ml). Cell viability assessed by Trypan blue exclusion was at least 95%, the suspension contained 98% granulocytes, which was shown by cell staining with fluorescent stain (acridine orange). The cell suspension was kept at 4°C and used in experiment 1 h after isolation. Isolated cells were activated with 0.1-50.0 μM N-formyl-methionyl-leucyl-phenylalanine

(FMLP). The following reagents were used: 10 μM tyrphostine 51 (specific inhibitor of tyrosine protein kinase), 1 μM SB 203580 (specific inhibitor of p38 mitogen-activated protein kinase — p38 MAPK), and 0.1 mM orthovanadate (tyrosine protein phosphatase inhibitor). The volume of sample for measurements was 200 μl with cell density of $10^6/\text{ml}$. Intact cells and cells treated with the inhibitor were incubated for 30 min at 37°C, after which chemiluminescence of all samples was recorded in parallel with registration of 12 wells in succession within 2.5 sec.

Basal chemiluminescence and the amplitude of the maximum response to opsonized zymosan (OZ) were measured for whole blood and isolated cell samples. Activation index for the whole blood was estimated as the ratio of amplitude to basal level. Effects of inhibitors were evaluated by the ratio of parameters for cells treated with the inhibitor and intact cells (100%). Differences between the groups were detected using Student's *t* test for analysis of specified number of independent measurements.

RESULTS

Comparison of blood chemiluminescence parameters in nonpregnant women of groups 1 and 2 showed that in group 2 the basal chemiluminescence level and the maximum amplitude of response to OZ were notably higher, while activation index was significantly lower (Table 1).

After activation of blood cells with OZ, AOF are generated mainly by phagocytes [1]. OZ promotes phagocytosis and stimulates activity of membrane-bound enzyme NADPH-oxidase. However the effect of OZ is associated with activation of many receptors and can be directed not only towards phagocytes, which impedes analysis of intracellular signaling. That is why in the next series of experiments we compared the reactions to a chemotactic peptide FMLP acting through a specific receptor, which is well studied [5].

Basal level of isolated granulocyte chemiluminescence (spontaneous chemiluminescence) was significantly higher in patients with habitual miscarriages (Table 2), while the maximum amplitude of responses to chemotactic peptide in concentrations 1 and 50 μM was lower in this group in comparison with controls (Table 2). A lower response to FMLP in patients with

TABLE 1. Parameters of Chemiluminescence of Whole Blood in Nonpregnant Women ($M \pm m$)

Group	Basal level, arb. units	Maximum amplitude of response to OZ, arb. units	Activation index
1	0.17 \pm 0.06	5.17 \pm 0.61	95.5 \pm 21.8
2	0.93 \pm 0.39*	8.35 \pm 0.74*	28.3 \pm 4.9*

Note. * $p < 0.001$ compared to group 1.

elevated basal level indicates an imbalance between the systems of AOF generation and elimination, which is considered to be a sign of oxidative stress [12]. Phosphorylation of cytoplasmic and membrane components of NADPH oxidase leads to its activation and release of AOF. Phosphorylation by tyrosine residues of components of the signal pathway from FMLP receptor to NADPH oxidase is a mechanism regulating NADPH oxidase activity [5]. The ratio of protein kinase to protein phosphatase activities determines the balance between the phosphorylated and dephosphorylated status of proteins. AOF act as the signal molecules in different cells [6,12,13], and phosphorylation status of blood cells changes during oxidative stress [3]. It was hypothesized that signs of oxidative stress in the blood of nonpregnant women with a history of habitual abortions are associated with changes in the functioning of protein kinases and protein phosphatases. We found no reports on this problem. Some investigations evaluated the involvement of protein phosphatases in miscarriages [2,4]. Analysis of the effects of tyrosine kinases and phosphatases on respiratory burst induced by FMLP was carried out using specific inhibitors of tyrosine protein kinases, tyrosine protein phosphatases, and p38 MAPK, whose activity can be regulated via phosphorylation of tyrosine, serine, and threonine residues.

Incubation of cells with tyrosine protein kinase and protein phosphatase inhibitors modified their response to 1 and 50 μ M FMLP (Table 2).

Tyrphostine 51 (10 μ M), a specific inhibitor of tyrosine protein kinases, markedly suppressed basal chemiluminescence of granulocytes in group 1, while its effect on granulocytes of women from group 2 was less pronounced (Table 2). Inhibition of the response to FMLP by tyrphostine 51 virtually did not depend on FMLP concentration in the cells of group 1 women; there was a trend to attenuation of the effect of the inhibitor on respiratory burst induced by 50 μ M FMLP in comparison with its effect in response to 1 μ M FMLP

(Table 2). The effect of tyrphostine 51 was weaker in granulocytes from group 2 women irrespective of FMLP concentration. Hence, the regulatory effect of tyrosine protein kinases on respiratory burst was markedly reduced in the peripheral blood granulocytes of women with habitual abortions.

Sodium orthovanadate (0.1 mM), a tyrosine protein phosphatase inhibitor, increased the basal level of AOF generation by granulocytes in both groups; no differences between the groups were detected (Table 2). Responses of granulocytes of healthy nonpregnant women to 1 and 50 μ M FMLP also markedly increased under the effect of orthovanadate, its effect virtually not depend on FMLP concentration used for cell activation. Granulocyte response in women with habitual abortions was also stimulated by orthovanadate. When FMLP was applied in a concentration of 1 μ M the effect of orthovanadate was less pronounced compared to the control group, while after 50 μ M FMLP the inhibitory effect was the same in both groups. Phosphorylation/dephosphorylation processes were weaker in granulocytes of women with habitual abortions, which seems to limit the possibility of balance between these two processes. Activity of enzymes in the MAPK cascade, which can serve as the point of the signal route interactions [5,9], is regulated via phosphorylation of the serine, threonine, and tyrosine residues [2]. Hence, alteration of the role of tyrosine phosphorylation in the regulation of respiratory burst in granulocytes from women with habitual abortions can modulate activity of MAPK.

SB 203580 (1 μ M), a specific inhibitor of p38 MAPK, significantly reduced basal chemiluminescence of cells from women of both groups, but its effect was weaker in group 2 (Table 2). The inhibitor stimulated the response to FMLP in all cases. Its effect on respiratory burst induced by 1 μ M FMLP was significantly lower in group 2, while its effect on the response to 50 μ M FMLP was more pronounced in granulocytes from group 1 women.

TABLE 2. Effects of Inhibitors on Isolated Blood Granulocytes of Nonpregnant Women ($M \pm m$)

Parameters	Group 1			Group 2		
	basal level	maximum amplitude of response		basal level	maximum amplitude of response	
	0	1 μ M	50 μ M	0	1 μ M	50 μ M
Parameters of intact cells, arb. units	4.31 \pm 0.78	48.3 \pm 13.7	48.6 \pm 13.6	6.4 \pm 2.2**	28.8 \pm 8.3**	31.3 \pm 7.7**
Sodium orthovanadate, 0.1 mM, %	132 \pm 10	177 \pm 20	1.2 \pm 16	117 \pm 17	152 \pm 37***	173 \pm 32
Tyrphostine 51, 10 μ M, %	45 \pm 11	61 \pm 17	54 \pm 09	89 \pm 09*	81 \pm 12**	79 \pm 12*
SB 203580, 1 μ M, %	75 \pm 7	161 \pm 20	122 \pm 12	85 \pm 6**	133 \pm 25**	172 \pm 17*

Note. * $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$ compared to group 1.

These data give us grounds to consider that signaling from the chemotactic peptide receptor to NADPH oxidase responsible for AOF generation is changed in granulocytes from women with habitual abortion, which can lead to changes in the course of the inflammatory process.

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