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The Influence of Chorionic Gonadotropin and Estriol on NK Cell Phenotype and Functional Activity

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Abstract—The influence of chorionic gonadotropin (CG) and estriol (E_3) at the concentrations typical of pregnancy on the expression of phenotypic markers and cytokine production by separated NK cells has been studied. It has been found that these hormones increase the percentage of $CD56^{bright}$ L-selectin$^+$ NK cells, but also stimulate the expression of the inhibitory molecule NKG2A in the lymphocytes. In addition, E_3 and CG stimulate the production of TGF-β, inhibiting the secretion of all other cytokines by separated NK cells. In general, these hormones contribute to the formation of the phenotype and cytokine spectrum characteristic of the regulatory NK3 subpopulation of NK cells during pregnancy.

Keywords: NK cells, chorionic gonadotropin, estriol, cytokines

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Pregnancy is a phenomenon of semi-allogenic transplantation, because the fetus is genetically half alien to the mother. The new endocrine interactions formed during pregnancy provide the normal coexistence of the maternal and fetal organisms. The placenta not only determines the biochemical processes maintaining fetal growth and development, but also synthesizes new hormones from maternal and fetal precursors. The hormones of the greatest physiological significance in this process, which appear in the female organism mainly during pregnancy, are chorionic gonadotropin (CG) and estriol (E_3). These placental hormones not only efficiently regulate fetal differentiation and development, but also have a substantial effect on functional activity of the cells of the maternal immune system [1].

The process of gestation affects many systems of the organism, including the immune system, followed by the changes in the functional activity of different populations of leukocytes aimed at maintenance of the fetus. Natural killer (NK) cells are a population of lymphocytes of innate immune system exhibiting cytotoxic and regulatory activities. NK cells dominate among the lymphoid cells localized in the decidua during pregnancy. They produce cytokines, chemokines, enzymes, and vascular growth factors, thereby contributing to the formation and modeling of spiral arteries, placentation and regulation of trophoblast invasion [2]. The fetotrophic functions of NK cells are displayed under the influence of specific regulatory signals including, in the first place, the hormones of the fetoplacental unit. The presence of receptors for both E_3 [3] and CG, which exerts its regulatory effect via the alternative mannose receptor [4], has been shown for peripheral NK cells.

The objective of this study was to estimate the effects of CG and E_3 on the expression of phenotypic markers and the production of cytokines by NK cells.

METHODS

The peripheral venous blood taken from healthy nonpregnant women of childbearing age in the follicular phase of the menstrual cycle ($n = 10$) was used in the study; mononuclear cells were isolated from the blood in the ficoll–verografin density gradient (1.077 g/mL). Then, the enriched suspension of NK cells was obtained by negative selection using Dynabeads Untouched Human NK Cells (Invitrogen, United States). The purity of isolation assessed by the expression of NKp46 marker (Anti-human CD335-PC5, Beckman Coulter, United States) was 90–95%. The hormones were used at physiological concentrations corresponding to trimester I and III of pregnancy, respectively: CG (Profasi, Italy), 100 and 10 IU/mL [5]; E_3 (Biomedicals, Germany), 2 and 20 ng/mL [6]. Since the study was performed in separated NK cells, the cell cultures were supplemented with the cytokines necessary for the growth and development of the lymphocytes population (GIBCO, United States): IL-2, 1 ng/mL; IL-12, 2 ng/mL; IL-15, 10 ng/mL [7].

NK cells (500 μL) were cultured in a complete nutrient medium (the RPMI-1640 medium contain-

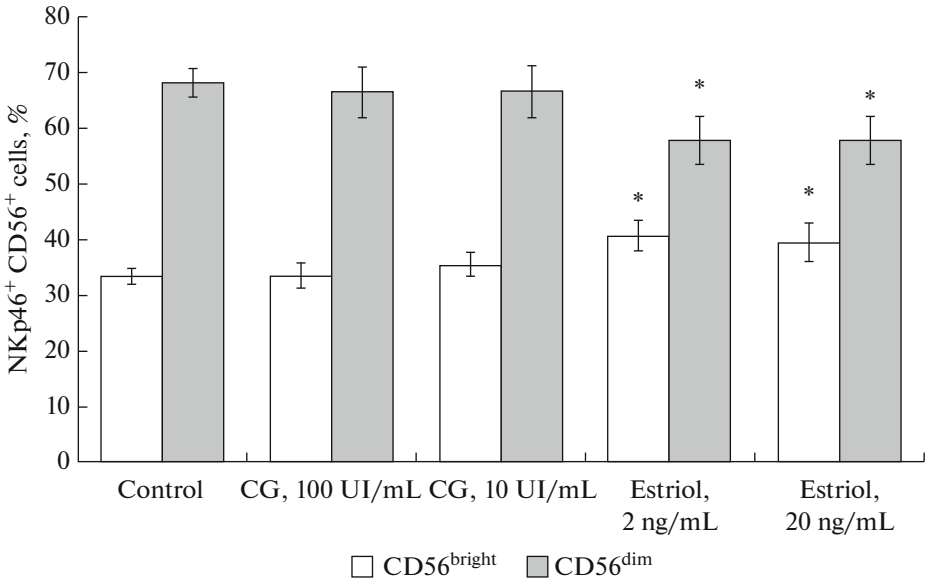

Fig. 1. The effects of CG and E_3 on the degree of expression of CD56 molecules by separated NK cells. Hereinafter, * are significant ($p < 0.05$) differences from the control.

ing 10% fetal calf serum (Charcoal Stripped FBS, Sigma, United States), 10 mM Hepes (ICN Pharmaceuticals, United States), 2 mM *L*-glutamine (ICN Pharmaceuticals, United States), and 100 μg/mL gentamycin (KRKA, Slovenia)) in the presence of cytokines and hormones for three days; then the supernatants were collected and phenotype of NK cells were estimated in the lymphocyte gate by flow cytometry (Becton Dickinson, United States). The cell staining technique was proposed by the manufacturer of monoclonal antibodies (Beckman Coulter, United States). No less than 10^5 cells were counted for analyzing the results. The respective isotype controls were used for controlling the nonspecific binding and for distinguishing a lymphocyte gate with negative fluorescence.

In the isolated NK cells, the expression of inhibitory molecule NKG2A (Anti-human CD159-PE), L-selectin (Anti-human CD62L-FITC), and CD56 marker (Anti-human CD56-PE) was estimated.

The concentrations of IL-4, IL-10, IL-17A, IFN-γ and TGF-β (Cytokin, Russia; R&D, United States) in the supernatants of cell cultures were estimated by the method of ELISA.

Since the distribution by the Fisher's test was normal, the reliability of differences between the mean values was assessed by the pair Student's *t* test.

RESULTS AND DISCUSSION

It is known that the number and lytic potential of peripheral NK cells decrease during normal pregnancy, mainly due to the decreased percentage of $CD16^+CD56^{dim}$ lymphocytes [8]. At the same time, the enhanced content of $CD16^+56^{dim}$ NK cells in the peripheral blood of women is associated with pregnancy miscarriage [9]. It has been established that the incubation of NK cells with E_3, regardless of concentration, reliably increases the percentage of $CD56^{bright}$ cells relative to the control with the parallel decrease in the level of $CD56^{dim}$. In contrast to E_3, CG had no effect on the of CD56 expression level (Fig. 1).

The decidual NK cells maturate from the peripheral $CD16^-CD56^{bright}$ lymphocytes migrating into uterus due to the expression of L-selectin (CD62L) molecules on their surface [10]. Estimation of the influence of reproductive hormones on the L-selectin expression has shown that both E_3, regardless of the concentration, and CG at a concentration typical of trimester III of pregnancy reliably increase the percentage of $CD56^{bright}$ $CD62L^+$ NK cells. Simultaneously, E_3 at a high concentration decreases the percentage of $CD56^{dim}$ $CD62L^+$ NK cells (Fig. 2). It seems that these effects of the hormones are associated with an enhanced expression of this molecule, which is of considerable importance for L-selectin-dependent adhesion of peripheral $CD56^{bright}$ lymphocytes to vascular endothelial cells and, consequently, may play a key role in the migration of $CD56^{bright}$ cells to the decidua during pregnancy [10].

The important indicator of NK cells functional activity is the presence of inhibitory receptors on their surface. It is known that the expression of such receptors, especially NKG2A, by the peripheral NK cells increases in the first weeks of pregnancy, reaching the maximum by the third month of gestation [11].

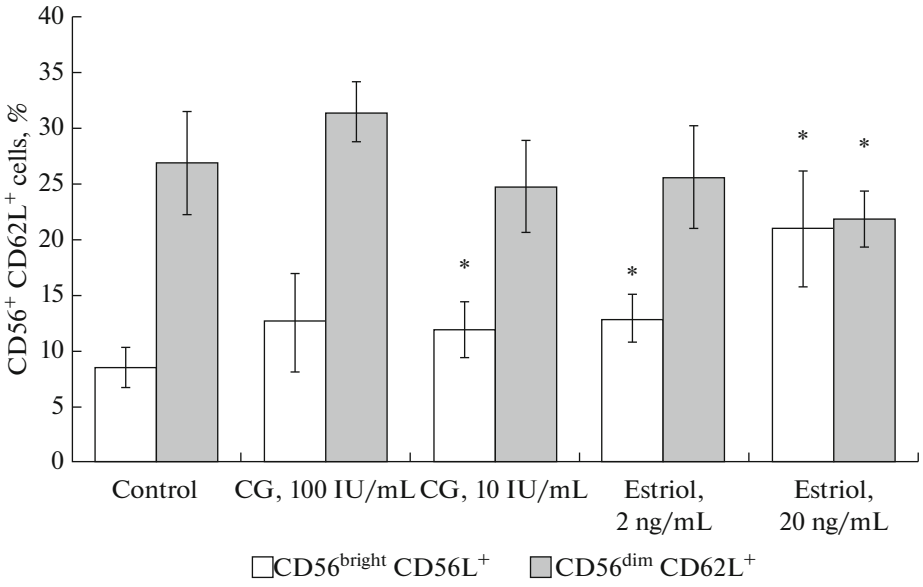

Fig. 2. The effects of CG and E_3 on the expression of L-selectin by separated NK cells.

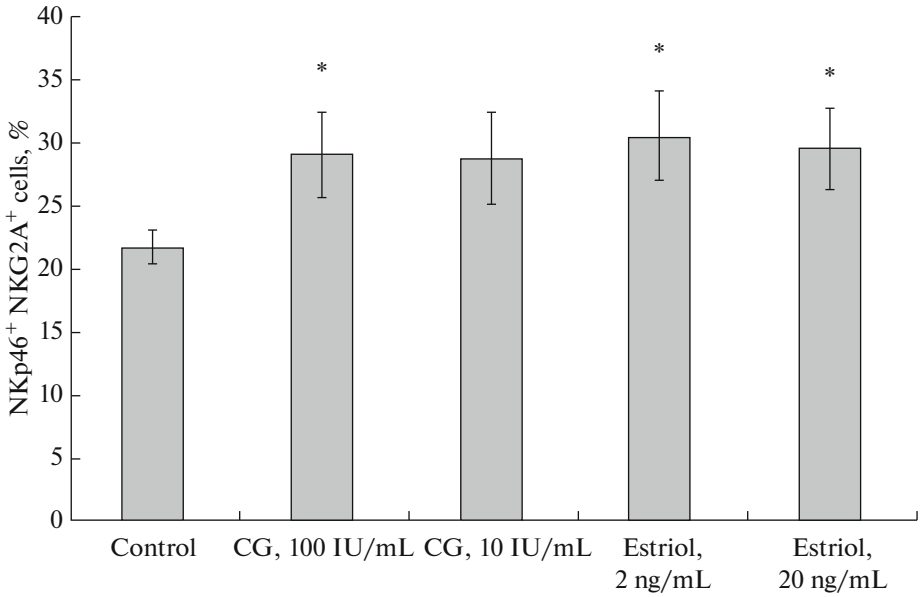

Fig. 3. The effects of CG and E_3 on the expression of NKG2A by separated NK cells.

Assessment of the effects of hormones on the expression of these molecules has shown that CG at the concentration corresponding to trimester I of pregnancy and E_3, regardless of the concentration, significantly increase the percentage of NK cells expressing the NKG2A inhibitory molecule (Fig. 3). The enhanced expression of CD56 ($CD56^{bright}$), L-selectin and NKG2A is considered to reflect the transformation of NK cells into a regulatory type incapable of cytotoxic action [8, 12].

Thus, in trimester I of pregnancy, E_3 increases the percentage of $CD56^{bright}$ NK cells and simultaneously intensifies the expression of L-selectin on them. At the same time, CG, not influencing the level of $CD56^{bright}$, increases only the expression of L-selectin. In contrast to CG, E_3 additionally decreases the number of $CD56^{dim}$ cells and stimulates the expression of the inhibitory molecule of NKG2A. In trimester III, the high level of $CD56^{bright}CD62L^+$ cells are also maintained by the hormones. In addition, E_3 inhibits

The effects of E_3 and CG on secretion of different types of cytokines by separated NK cells, pg/mL ($M \pm m$)

Group	IL-4	IL-10	TGF-β	IL-17A	IFN-γ
Control	8.69 ± 0.188	25.62 ± 1.247	263.08 ± 7.843	239.36 ± 4.736	506.45 ± 26.403
CG (100 IU/mL)	7.06 ± 0.489*	21.81 ± 1.431*	282.38 ± 12.196*	218.47 ± 8.444*	344.68 ± 43.732*
CG (10 IU/mL)	7.92 ± 0.762	24.59 ± 1.754	277.07 ± 13.010	225.02 ± 10.928	372.06 ± 48.037*
E_3 (2 ng/mL)	7.00 ± 0.323*	21.08 ± 1.358*	284.98 ± 10.882	213.40 ± 10.019*	321.25 ± 38.747*
E_3 (20 ng/mL)	7.28 ± 0.218*	21.51 ± 1.590*	287.53 ± 5.165*	212.65 ± 9.968*	374.38 ± 52.381*

the formation of $CD56^{dim}$ lymphocytes, including those carrying L-selectin, but stimulates the expression of NKG2A similar to CG.

It is known that the $CD56^{bright}$ NK cells are able to produce considerable amounts of various cytokines, which is the basis for distinguishing the subtypes of peripheral and decidual NK cells. Thus, NK1 produce IFN-γ and TNF-α; NK2 produce IL-4, IL-5, IL-6, and IL-13; NK3 produce TGF-β; and NKr1 produce IL-10 [8, 13]. It has been shown that IFN-γ is predominant in the supernatants of peripheral NK cells (table), which is in agreement with the literature data [13]. At the same time, CG and E_3, regardless of the concentration, reliably inhibit its production and simultaneously, at high concentrations, intensify the secretion of anti-inflammatory cytokine TGF-β. Thus, hormones are physiological inducers of differentiation of NK1 cells into NK3. Currently there are few works demonstrating the ability of NK cells to produce IL-17 [14, 15]; however, in our studies, the level of this cytokine has been sufficiently high. It is probably a consequence of additional influence of IL-2 and IL-15 on the separated NK cells, since the above cytokines have been shown capable of stimulating the production of IL-17 by lymphocytes including NK cells [16]. E_3, regardless of the concentration, as well as CG at a concentration typical of trimester I of pregnancy, decrease the level of IL-17 in the supernatant of NK cells. The production of IL-4 and IL-10 was initially at a low level and was suppressed by the hormones (table). Since it is known that the stimulation of NK cells leads to the enhanced synthesis of proinflammatory cytokines [17], it is not surprising that the subpopulation of these lymphocytes secreting IL-4 and IL-10 proved to be minor.

Thus, in trimester I of pregnancy, E_3 and CG can be important regulators of cytokine production by NK cells, reducing the levels of IL-4, IL-10, IFN-γ and IL-17A, while CG additionally stimulates the production of TGF-β. In trimester III, E_3 can be a factor that maintains the enhanced secretion of TGF-β by suppressing the production of other cytokines. It seems that the hormones of pregnancy contribute to both the formation of decidual NK cells and their differentiation into the regulatory subtype, NK3, actively producing TGF-β.

CONCLUSIONS

The peripheral and uterine $CD16^{-}CD56^{brigh}$ NK cells have mainly an immunoregulatory effect and, as a consequence, prevent trophoblast lysis, as well as maintain the implantation and growth of the placenta. They express the high-affinity inhibitory receptor, NKG2A, the ligand for which is the "nonclassical" major histocompatibility complex (MHC) class I molecule HLA-*E* [18]. As the synthesis of both hormones under study is associated with pregnancy, it is quite natural that CG and E_3 enhance the expression of this receptor. Since we have established that E_3 can increase the percentage of $CD56^{bright}$ NK cells and to decrease the quantity of $CD56^{dim}$, this hormone may be one of the factors modulating the functional activity of NK cells by increasing their regulatory potential and decreasing cytotoxic activity throughout the gestation. In addition, the increase in the number of $CD56^{bright}CD62L^{+}$ cells under the influence of CG and E_3 is indicative of their ability to enhance the migration of NK cells into the placenta during normal pregnancy [10, 19].

The decidual NK cells are characterized primarily by the fact that they are represented mainly by the TGF-β-producing NK3 lymphocytes, while the increase in the percentage of NK1 cells is observed in case of spontaneous abortions [13]. At the same time, TGF-β not only has an immunosuppressive effect on many lymphocyte populations, but also promotes angiogenesis [20]. Our experiments have shown that the hormones under study shift the cytokine balance towards the production of TGF-β, considerably reducing the levels of IFN-γ and IL-17*A*, thereby contributing to the formation of a regulatory subpopulation of NK cells, NK3. In view of the fact that TGF-β is one of the factors involved in the transformation of peripheral NK cells into decidual ones [21], the role of hormones as differentiation factors essentially increases. It is important to note that the intensified secretion of TGF-β, with the simultaneous decrease in the levels of IFN-γ and IL-17*A*, occurs under the influence of hormones at the concentrations extrapolated from their levels in trimester I of pregnancy. Spontaneous recurrent abortions of immune genesis are most frequent in this period of gestation [22]. It seems that CG and E_3 play a key role in modulation of

the functional activity of NK cells and can be considered as inducers and factors of maintenance of immune tolerance during pregnancy.

Solely as a subject for discussion, it should be mentioned that the effects of CG revealed in our research can occur only during pregnancy, in spite of the fact that the luteinizing hormone (LH) interacts with the homologous receptor (the LH/CG receptor). First, the concentration of LH at the peak of its secretion in the absence of pregnancy is several orders of magnitude lower compared to CG that we used. Second, it has been shown that CG has a regulatory effect on NK cells not via the classical LH/CG receptor, but via the alternative mannose receptor [4].

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