РЕГУЛЯЦИЯ КИССПЕПТИНОМ-54 АКТИВНОСТИ ИНДОЛАМИН-2,3-ДИОКСИГЕНАЗЫ И АПОПТОЗА ЛИМФОЦИТОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ

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Резюме. Беременность представляет собой феномен естественной полуаллогенной трансплантации, поскольку плод наполовину чужероден в силу экспрессии отцовских антигенов. Установлено, что гипоталамический гормон кисспептин в период беременности вырабатывается синцитиотрофобластом плаценты и участвует в формировании нового специфического гормонального фона. В крови беременных женщин циркулируют несколько форм гормона: кисспептин-10, кисспептин-14 и кисспептин-54 (по количеству аминокислотных остатков в молекуле гормона), однако основной активной формой является кисспептин-54. Основным механизмом формирования иммунной толерантности во время беременности является индукция экспрессии фермента индоламин-2,3-диоксигеназы (IDO) антигенпрезентирующими клетками периферической крови, вследствие чего происходит катализ триптофана (Trp) до кинуренинов (KYN), блокирующих активацию и вызывающих апоптоз цитотоксических $CD8^+$Т-лимфоцитов в зоне соприкосновения материнских иммунных клеток с антигенами плацентарно-фетального комплекса. Кроме этого, в период беременности важная роль отводится процессу апоптоза, поскольку активированные клетки могут быть потенциально опасными для развивающегося плода. Иммунокомпетентные клетки крови экспрессируют специфический мембранный рецептор кисспептина (KISS-1R). Поскольку кисспептин-54 поступает в системный кровоток только во время беременности, то гормон оказывает действие на иммунные клетки только в этот период.

Целью данной работы была оценка влияния кисспептина-54 в концентрациях, сопоставимых с его уровнем во время физиологической беременности, на активность IDO и апоптоз лимфоцитов периферической крови.

В качестве объекта исследования использовались мононуклеарные клетки периферической крови (PBMC) полученные от 10 здоровых небеременных женщин репродуктивного возраста (от 23 до 32 лет). Апоптоз лимфоцитов оценивали в суспензии PBMC путем окрашивания аннексином-V и йоди-

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стым пропидием. Определение количества клеток на ранней и поздней стадиях апоптоза проводили в изолированном гейте лимфоцитов. Активность IDO в PBMC определяли спектрофотометрически по изменению концентрации KYN – первого стабильного метаболита пути распада Trp.

Выявлено, что кисспептин-54 в концентрации 4,6 pM, соответствующей II триместру беременности, достоверно усиливает активность IDO, увеличивает количество клеток, находящихся в ранней и поздней стадиях апоптоза. Таким образом, кисспептин-54 является важным механизмом контроля этих процессов в период беременности, направленным на защиту полуаллогенного плода от неблагоприятных иммунных реакций матери и благоприятным развитием беременности.

Ключевые слова: кисспептин-54, беременность, апоптоз, индоламин-2,3-диоксигеназа, мононуклеарные клетки периферической крови, лимфоциты

REGULATION OF KISSPEPTIN-54 ACTIVITY OF INDOLAMINE-2,3-DIOXYGENASE AND APOPTOSIS OF PERIPHERAL BLOOD LYMPHOCYTES

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Abstract. Pregnancy is a phenomenon of natural semi-allogeneic transplantation, since the fetus is half alien due to the expression of paternal antigens. It was found that the hypothalamic hormone kisspeptin during pregnancy is produced by the syncytiotrophoblast of the placenta and participates in the formation of a new specific hormonal background. Several forms of the hormone circulate in the blood of pregnant women: kisspeptin-10, kisspeptin-14 and kisspeptin-54 (according to the number of amino acid residues in the hormone molecule), but the main active form is kisspeptin-54. The main mechanism for the formation of immune tolerance during pregnancy is the induction of the expression of the enzyme indolamine-2,3-dioxygenase (IDO) by antigen-presenting cells of peripheral blood, resulting in the catalysis of tryptophan (Trp) to kynurenins (KYN) blocking the activation and causing apoptosis of cytotoxic $CD8^+$T lymphocytes in the zone of contact of maternal immune cells with placental-fetal complex antigens. In addition, during pregnancy, an important role is assigned to the process of apoptosis, since activated cells can be potentially dangerous for the developing fetus. Immunocompetent blood cells express a specific membrane receptor of kisspeptin (KISS-1R). Since kisspeptin-54 enters the systemic circulation only during pregnancy, the hormone has an effect on immune cells only during this period.

The aim of this work was to evaluate the effect of kisspeptin-54 in concentrations comparable to its level during physiological pregnancy on IDO activity and apoptosis of peripheral blood lymphocytes.

Peripheral blood mononuclear cells (PBMC) obtained from 10 healthy non-pregnant women of reproductive age (from 23 to 32 years) were used as the object of the study. Lymphocyte apoptosis was assessed in PBMC suspension by staining with annexin-V and propidium iodide. The determination of the number of cells in the early and late stages of apoptosis was carried out in the isolated gate of lymphocytes. IDO activity in PBMC was determined spectrophotometrically by changes in the concentration of KYN, the first stable metabolite of the Trp decay pathway.

It was found that kisspeptin-54 at a concentration of 4.6 pM corresponding to the second trimester of pregnancy significantly enhances the activity of IDO, increases the number of cells in the early and late stages of apoptosis. Thus, kisspeptin-54 is an important mechanism for controlling these processes during pregnancy, aimed at protecting the semi-allogeneic fetus from adverse immune reactions of the mother and the favorable development of pregnancy.

Keywords: kisspeptin-54, pregnancy, apoptosis, indolamine-2,3-dioxygenase, peripheral blood mononuclear cells, lymphocytes

This work was carried out within the framework of the state task, the state topic registration number: AAAA-A19-119112290007-7.

Introduction

Pregnancy is a phenomenon of natural semi-allogeneic transplantation since the fetus is half foreign due to the expression of paternal antigens [12]. During pregnancy, the mother's immune system is restructured due to the formation of a specific immune tolerance aimed at preserving the fetus from adverse immune reactions of the mother and, simultaneously, protecting the mother and fetus from pathogens [8]. The expression of indolamine-2,3-dioxygenase (IDO) by antigen-presenting peripheral blood cells is one of the mechanisms for the formation of peripheral tolerance during pregnancy. IDO catalyzes tryptophan (Trp) to kynurenines (KYN), which block the activation and cause apoptosis of cytotoxic $CD8^+$T lymphocytes in the zone of contact of maternal immune cells with placental-fetal complex antigens [8]. Also during pregnancy, an important role is assigned to the process of apoptosis, programmed cell death of cells. In addition to the elimination of altered – damaged, defective, mutant or infected cells, through apoptosis, the processes of differentiation and morphogenesis are realized during the formation of tissues and organs, the cellular homeostasis of an already formed organism is maintained, as well as its protection from pathogens during the implementation of protective reactions [15].

Pregnancy hormones play an important role in this restructuring, having a regulating effect on the cells of the mother's immune system [10]. It has recently been established that the hypothalamic hormone kisspeptin is also produced by the placental syncytiotrophoblast [5], and can have systemic effects on the leukocytes of a pregnant woman since they express a specific membrane kisspeptin receptor (KISS–1R) [9]. KISS-1R is a membrane protein that belongs to the class of Gaq-associated receptors (GPCR) [4]. However, there are few data on the immunomodulatory effect of kisspeptin on the cells of the immune system. We have previously shown that the interaction of kisspeptin-54 with $CD4^+$T lymphocytes causes their transformation into suppressor type regulatory cells (Treg) with simultaneous inhibition of Th17 differentiation and their functional activity [3].

The aim of this work was to evaluate the effect of kisspeptin-54 in concentrations comparable to its level during physiological pregnancy on IDO activity and apoptosis of peripheral blood lymphocytes.

Materials and methods

Hormone

Kisspeptin (Kisspeptin-54, Metastin, Synthetic, CALBIOCHEM, USA) was used in physiological concentrations corresponding to its level in peripheral blood in the first, second and third trimesters of pregnancy: 1,3 pM, 4,6 pM and 9,6 pM, respectively [5].

Objects of research

The object of the study was peripheral blood mononuclear cells (PBMC), as well as separated monocytes and neutrophils obtained from 10 healthy non-pregnant women of reproductive age (from 23 to 32 years). Venous peripheral blood was collected in the follicular phase of the menstrual cycle (day 5-11) since the expression of KiSS-1R has a maximum during this period [2]. The study was conducted in accordance with the Helsinki Declaration of the BMA of 2000 and the Protocol of the Council of Europe Convention on Human Rights and Biomedicine of 1999. The approval was received from the Ethics Committee of the Institute of Ecology and Genetics of Microorganisms of the Ural Branch of the Russian Academy of Sciences. Voluntary informed consent to the examination was required to be included in the study. The individuals taking hormonal drugs were excluded from participation.

Isolation and cultivation of cells

PBMC of blood was obtained by centrifugation at 350×g for 40 minutes on a Ficoll-Urographin density gradient (1,077 g/cm^3) (Pharmacia, Sweden; Bayer Schering Pharma AG, Germany). After that, the cells were washed with RPMI 1640 (Sigma-Aldrich, USA). Then PBMC were divided into two parts. The first part was used to determine the activity of IDO. The second part was used to determine apoptosis of lymphocytes. The viability of PBMC determined by the inclusion of the vital dye eosin (0,01%) (Sigma, USA) was 95-98%.

Culturing of PBMC (10^6 cell/mL) with kisspeptin-54 was performed in a complete nutrient medium containing RPMI 1640 (Sigma-Aldrich, USA) with the addition of 10% FBS (Sigma, USA), 10 mM Hepes (ICN Pharmaceuticals, USA), 2 mM L-glutamine (ICN Pharmaceuticals, USA) and 30 μg/mL gentamicin (KRKA, Slovenia) at 37 °C and 5% CO_2 for 1 h. A hormone solvent (0,9% NaCl) was added to the control samples.

Determination of lymphocyte apoptosis

Lymphocyte apoptosis was evaluated in PBMC suspension by staining with annexin-V (AnV-FITC, Caltag, USA) and propidium iodide (PI, eBioscience, USA). This method makes it possible to identify cells in the early (AnV^+/PI^-) and late (AnV^+/PI^+) stages of apoptosis [13]. Dexamethasone (10^{-6} M, "KRKA", Slovenia) was used to induce apoptosis, which was introduced into cultures 30 minutes before the hormone. The control was samples to which only an apoptosis inducer was added. Incubation was carried out for 24 hours in a full nutrient medium, at 37 °C and 5% CO_2. The results were taken into account

TABLE 1. EFFECT OF KISSPEPTIN-54 ON LYMPHOCYTE APOPTOSIS

Experimental impact	Apoptosis, %		
	n	An^+Pr^-	An^+Pr^+
Control	10	10.60±0.82	15.69±0.96
Kisspeptin-54, 1,3 pM	10	12.65±0.50 p < 0.05	18.12±0.18 p < 0.05
Kisspeptin-54, 4,6 pM	10	17.88±1.23 p < 0.05	17.20±0.56 p < 0.05
Kisspeptin-54, 9,6 pM	10	15.48±0.84 p < 0.05	19.71±0.99 p < 0.05

on a FACSCalibur flow cytofluorimeter (Becton Dickinson, USA). The determination of the number of cells in the early and late stages of apoptosis was carried out in the isolated gate of lymphocytes.

Determination of IDO enzymatic activity

IDO activity in PBMC was determined spectrophotometrically by changes in the concentration of KYN, the first stable metabolite of the Trp degradation pathway [1]. For this purpose, PBMC stimulated with lipopolysaccharide (LPS) (100 ng/mL, Sigma, USA) was cultured in HBSS containing 100 μM of Trp (Sigma, USA) for 4 hours. Then, 50 μL of 30% $C_2HCL_3O_2$ was added to 100 μL of the cellular supernatant, shaken and centrifuged for 5 min. Then 75 μL the resulting reaction mixture was added to the wells of the 96-well plate, mixed with an equal volume of Ehrlich reagent (100 mg of p-dimethylbenzaldehyde, 50 mL $C_2H_4O_2$). Optic density was measured at 492 nm using a microplate reader (Synergy H1, BioTek, USA).

Statistical analysis

The obtained experimental data were processed using variational statistics. For the variables representing the analyzed sample, the arithmetic mean and the error of calculating the average (M±m) were calculated. For statistical verification of compliance with the law of normal distribution, the Fisher criterion was used. Given that the sample distribution was normal in all tests, the reliability of the differences between the mean values was evaluated according to the Student's paired t-test.

Results and discussion

The effect of kisspeptin-54 on lymphocyte apoptosis

Kisspeptin-54 increases the number of cells in the stage of early apoptosis in concentrations characteristic of the II and III trimesters of pregnancy. Introduction of kisspeptin-54 into PBMC culture, regardless of concentration, leads to an increase in

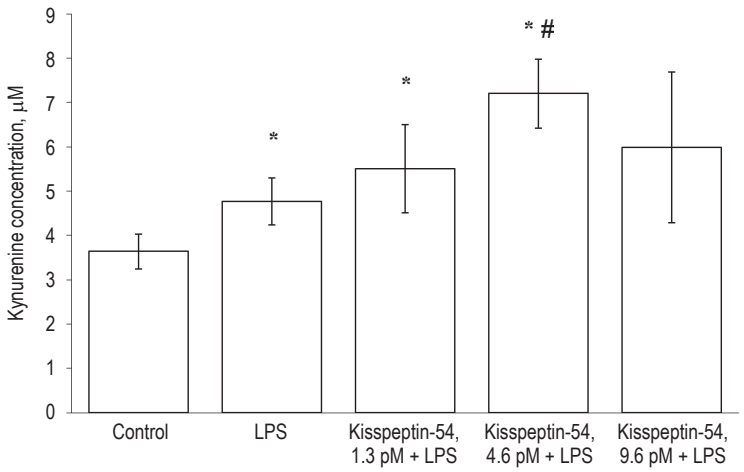

Figure 1. Kisspeptin-54 influence on IDO activity

Note. *, p < 0.05 compared with control, pair Student's t-test. #, p < 0.05 compared with LPS, pair Student's t-test.

the percentage of cells in the late stage of apoptosis (Table 1).

Modulation of IDO activity by kisspeptin-54

When evaluating the LPS-induced activity of IDO into PBMC, it was found that kisspeptin-54 at a concentration of 4,6 pM corresponding to the second trimester of pregnancy significantly increases the activity of IDO (Figure 1).

Thus, the hormone initiates immune tolerance at the level of adaptive immune responses. It is important to emphasize that the concentration of kisspeptin-54, observed only in the second trimester of pregnancy, the most vulnerable to immunocompromising conditions, has such plasticity of the regulatory potential [11]. Considering that IDO is produced only by antigen presenting cells, which are mainly represented by monocytes in PBMC, it can be assumed that kisspeptin-54 enhances LPS-stimulating signaling, leading to the expression of active IDO in these cells.

Summarizing the results obtained, it can be argued that kisspeptin-54 is directly involved in the regulation of PBMC apoptosis, which is obviously an important mechanism for controlling the activation of these cells during pregnancy. Stathaki, M. and co-authors also showed that kisspeptin-54 induces apoptosis of lymphocytes *in vitro* [14]. In addition, we have shown that kisspeptin-54 promotes the formation of peripheral tolerance by stimulating IDO activity. In turn, kininurinins – IDO products block activation and cause apoptosis of cytotoxic $CD8^+$T lymphocytes [6]. Thus, it can be assumed that kisspeptin-54 acts on cytotoxic $CD8^+$T lymphocytes, causing their apoptosis, through increased production of IDO by antigen-presenting cells.

It was found that at the beginning of physiological pregnancy, activation of apoptosis of peripheral lymphocytes and monocytes, a shift in the differentiation of T helper cells towards Th2 cells is observed. At the end of pregnancy, the process of apoptosis stabilizes, but the high level of cells in its later stages remains [7]. Most likely, the elimination of activated Th2 cells increases in the placenta at the beginning of pregnancy, and cytotoxic lymphocytes accumulate in the later stages of pregnancy. Apparently, the elimination of activated cell clones due to apoptosis is a protective mechanism, since activated cells can be potentially dangerous for the developing fetus.

Conclusion

The data obtained by us indicate that kisspeptin-54 is directly involved in the control of these processes during pregnancy, which is aimed at protecting the semi-allogeneic fetus from adverse immune reactions of the mother and the favorable development of pregnancy.

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