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Short communication

The role of human chorionic gonadotropin in regulation of naïve and memory T cells activity *in vitro*

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A B S T R A C T

The role of human chorionic gonadotropin (hCG) in the regulation of molecular genetics factors determining the functional activity of human naïve and memory T cells *in vitro* was studied. It was found that hCG (10 and 100 IU/ml) inhibited CD28 and CD25 expression on the naïve T cells (CD45RA +) and CD25 expression on the memory T cells (CD45R0 +). hCG didn't affect the CD71 proliferation marker expression in total. Nevertheless, hCG reduced the percentage of proliferating memory T cells with simultaneous suppression of CD71 expression on proliferating CD45R0 + cells.

In parallel, expression of U2af1l4, Gfi1, and hnRNPLL genes, which are Ptprc gene alternative splicing regulators was evaluated. It was established that hCG stimulated the expression of U2af1l4 and hnRNPLL genes, responsible for the assembly of CD45R0 in memory T cells, but reduced the expression of Gfi1 in these cells. In general, hCG promotes the differentiation of memory T cells by increasing of CD45R0 expression, but inhibits proliferation and CD25 expression which reflects their functional activity.

1. Introduction

In pregnancy the maternal immune system undergoes changes that lead to maternal-fetal tolerance. During the first pregnancy there are short-term changes in immunological memory: increasing of $CD4^+$ effector memory T cell (T_{EM}) portions and activation of $CD4^+$ memory T cells. The long-term effects are resulted in much higher percentages of $CD4^+$ T_{EM}, $CD8^+$ T_{EM}, $CD4^+$ central memory cells (T_{CM}) and activated $CD4^+$ cells in blood of delivered women [1]. In addition, there are data suggesting that fetal antigens exposure during pregnancy induces the differentiation of long-lived maternal $CD8^+$ T cells with selective effector dysfunction [2].

Changes in immune system responsible for the successful establishment and maintenance of pregnancy are mediated also by pregnancy hormones [3]. Human chorionic gonadotropin (hCG), the placental analogue of luteotropic hormone, is produced by trophoblast cells following fertilization. Hormone has many biological functions, main of which is placental and fetal steroidogenesis regulation. Furthermore it possesses immunomodulatory properties and is considered to be one of major factors of immune tolerance formation in pregnancy [4]. Nowadays, this hormone is known to regulate functions of conventional and regulatory T cells (Treg) [3]. Nevertheless, the effect of pregnancy-associated proteins and hormones in general and hCG in particular on functional activity of naïve and memory T cells remains poorly explored.

Among other things, hCG is widely used in clinic, primarily in treatment of dysmenorrhea, ovarian dysfunction, anovulation, corpus luteum deficiency syndrome, and ovarian hyperstimulation syndrome after the *in vitro* fertilization (IVF) [5,6].

Distinguishing of naïve and memory T cells is based on the CD45 isoforms detection. CD45 is a transmembrane protein tyrosine phosphatase encoded by *Ptprc* gene. Upon T cell activation skipping of the CD45 variable exons happens. Three genes, *Gfi1* (growth factor independent 1), *hnRNPLL* (heterogeneous nuclear ribonucleoprotein L-

Abbreviations: Ac/Exp, T Cell Activation/Expansion Kit human; APC, antigen-presenting cell; CD45, cluster of differentiation 45, leukocyte common antigen; CD45RA, high molecular weight isoform of CD45 receptor; CD45R0, low molecular weight isoform of CD45 receptor; cDNA, complementary deoxyribonucleic acid; DNA, deoxyribonucleic acid; Gfi1, growth factor independent 1; hCG, human chorionic gonadotropin; hnRNPLL, heterogeneous nuclear ribonucleoprotein L-like; MHC, major histocompatibility complex; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; PTPRC, protein tyrosine phosphatase, receptor type, C; RNA, ribonucleic acid; Th, helper T cell; TCR, T cell receptor; U2af1l4, U2 small nuclear RNA auxiliary factor 1 like 4

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like) and *U2af1l4* (U2 small nuclear RNA auxiliary factor 1 like 4) are responsible for this process, known as *Ptprc* gene alternative splicing [7]. Eight different isoforms of CD45 molecule can be generated by alternative splicing of *Ptprc* gene exons (4, 5, and 6), five of which are present on lymphocytes (R0, RA, RB, RBC, RABC) and linked with their differentiation stages. Moreover, CD45 isoforms are involved in T cell receptor (TCR) signaling [8], as «shortening» of this surface molecule leads to homodimerization at the cell surface, which inhibits phosphatase activity and decreases signaling through the TCR [9].

CD25, CD28, and CD71 surface markers expression is well known to be tightly linked with functional activity of T lymphocytes [10].

Our purpose was to evaluate hCG effect on membrane costimulatory and activation molecules expression by naïve ($CD45RA^+$) and memory ($CD45R0^+$) T cells with parallel assessment of relative expression of genes *U2afl14*, *Gfil*, and *hnRNPLL* which regulate the *Ptprc* gene alternative splicing in T cell subsets under study.

2. Materials and methods

The research was performed according to World Medical Association's Declaration of Helsinki and Council of Europe Protocol to the Convention on Human Rights and Biomedicine, and approved by Ethics Committee of the Institute of Ecology and Genetics of Microorganisms, Ural Branch of the Russian Academy of Sciences (IRB00010009) on 12.06.2016. Written informed consent was obtained from all participants.

2.1. Study group

Venous blood samples were collected from healthy donors (non-pregnant women, n = 13, 21–36 years old) by venipuncture with vacuum tubes (BD Vacutainer™, Greiner-bio-one, Austria). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Urografin (Pharmacia, Sweden, Bayer Pharma AG, Germany) density gradient centrifugation ($\rho = 1.077\ g/cm^3$).

2.2. Isolation and cultivation of $CD45RA^+$ and $CD45R0^+$-cells

The experiment included several steps: isolation of naïve and memory T cells, T cell cultivation in the presence of hCG and T cell activator and subsequent flow cytometric detection of activation markers and qRT-PCR of target genes (Fig. 1, supplementary).

Naïve T cells ($CD45RA^+$) and memory T cells ($CD45R0^+$) were obtained from PBMCs using immunomagnetic separation with MACS® MicroBeads and MS Columns (Miltenyi Biotec, Gmbh, Germany). The percentage of $CD3^+CD45RA^+CD14^-CD19^-$ and $CD3^+CD45R0^+CD14^-CD19^-$ T cells in the experiment cell cultures was 98,5 ± 1,5%. Isolated cells with $CD45RA^+$ or $CD45R0^+$ phenotype (1×10^6 cells/ml, 500 μl) were cultured in 48-well plates in complete medium (CM): RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10% FBS (Sigma, USA), 10 mM HEPES, 2 mM L-glutamine (both from ICN Pharmaceuticals, USA), 5×10^{-5} M β-mercaptoethanol (AcrosOrganics, USA) and 30 μg/ml of gentamycin (KRKA, Slovenia) in a humidified CO_2 incubator at 37 °C and 5% of CO_2 for 48 h.

Physiological concentrations of hCG (Moscow Endocrine Plant, Russia) were used (10 and 100 IU/ml) which correspond to second-third and first trimesters of pregnancy, respectively [11]. Sample with CM instead of hormone served as a negative control.

T Cell Activation/Expansion Kit human (Ac/Exp), Anti-Biotin MACSiBead™ particles loaded with biotinylated antibodies against human CD2, CD3 and CD28 (Miltenyi Biotec, Germany) was used for activation and subsequent proliferation of T cells. Flow cytometric analysis of costimulatory and activation markers on $CD45RA^+$ and $CD45R0^+$ T cells was performed with MACS Quant flow cytometer (Miltenyi Biotec, Germany) using CD71 FITC, CD25 PE-Cy 5.5, CD28 PE-Cy7 monoclonal antibodies (Miltenyi Biotec, Germany). Mouse IgG1 were used for isotype control and human FcR blocking reagent (both reagents from Miltenyi Biotec, Germany) was used to avoid non-specific Ab binding. Flow cytometry data were processed using KALUZA Analysis Software (Beckman Coulter, USA).

2.3. Multiplex qRT-PCR assay

Total RNA from samples was isolated with ExtractRNA kit (Eurogen, Russia) according to the manufacturer's recommendations. Reverse transcription reaction was performed with oligo(dT)23-primer (20 mkM) (Beagle, Russia) and reverse transcriptase MMLV (Eurogen, Russia). Multiplex PCR assay was made with qPCRmixHS PCR mixture (Eurogen, Russia), TaqMan specific probes and primers in 10 pM concentration (Beagle, Russia). 5 μl of cDNA were used as matrix, and GAPDH gene – as reference housekeeping gene. PCR was performed in triplicates using the LightCycler 480 Real-Time PCR amplifier (Roche, Switzerland) in the following mode: 95 °C, 5 min; 95 °C, 20 s; 60 °C, 30 s; 72 °C, 60 s – 45 cycles, 72 °C, 5 min. mRNA expression levels in control and test samples were normalized to GAPDH gene expression using the method of $2^{-\Delta Ct}$, calculating the value $2^{-(Ct\ of\ target\ gene\ -\ Ct\ of\ reference\ gene)}$, where Ct – threshold cycle of reaction [12].

2.4. Evaluation of interleukin-2 (IL-2) concentration

IL-2 concentration in supernatants was evaluated by enzyme-linked immunosorbent assay (ELISA) kit (Vector-Best, Russia) according to manufacturer's instructions using multichannel spectrophotometer Biohit BP 800 (Finland).

2.5. Proliferation analysis

Differential gating [13] in author's modification was used to determine proliferative status of cells. This method could be named flow cytometric variant of «classic» microscopic evaluation of blast transformation by determining the proportion of large cells. Gating strategy is shown in Fig. 4 (supplement). Three regions were determined at the FSC/SSC dot plots of CD45RA+- and CD45RO+ - T-cell cultures in accordance with their light scatter characteristics: the first one – non-proliferating living lymphocytes (small size and low granularity); the second – proliferating living lymphocytes (size is bigger and granularity is higher); the third – apoptotic and dead cells (smaller size and different granularity). The percentage of cells in each gate from their total number was determined. In addition percentage of $CD71^+$ T cells in proliferating and nonproliferating gates was assessed.

2.6. Statistics

Statistical analyses of flow cytometry and ELISA data were performed using Student's *t*-test. Results are presented as mean with standard deviation, M ± SD. Multiplex qRT-PCR data were analyzed using Mann-Whitney *U* test and are presented as median with first and third quartiles (Q1 − Q3). Differences were considered significant when $P < 0,05$.

3. Results

CD28 is a T cell primary costimulatory receptor that takes part in immune synapse formation through the interaction with antigen-presenting cells (APC) surface CD80/86. CD25 (α-chain of IL-2 receptor) is an activation marker reflecting cell ability to differentiate and proliferate. This molecule expression is functionally related to IL-2 production. CD71 is a receptor for transferrin, which required for iron import into cells by endocytosis, and is expressed on proliferating cells [10].

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