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Effect of Estriol, Chorionic Gonadotropin, and Oncostatin M on the Expression of Recombinase RAG-1 in Regulatory T Lymphocyte Subpopulations

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We studied the effect of estriol, chorionic gonadotropin, oncostatin M, and hormone-cytokine combinations on the expression of recombinase RAG-1 in T-regulatory (Treg) and T helper 17 (Th17) lymphocytes. It was found that estriol in a concentration corresponding to the first trimester of pregnancy increased the level of Treg ($CD4^+FoxP3^+$) cells and suppressed the formation of Th17 ($CD4^+RORC^+$) lymphocytes. This effect was nor observed after individual administration of chorionic gonadotropin and oncostatin M, but some combinations of the studied hormones with oncostatin M increased the percentage of $CD4^+FOXP3^+$ cells. In the presence of oncostatin M, the studied hormones enhanced the expression of RAG-1 in $CD4^+FoxP3^+$ cells, but not in $CD4^+RORC^+$ cells, thereby initiating of Treg T-cell receptor (TCR) revision. The mechanisms of hormone cytokine control of induction of the immune tolerance during pregnancy are discussed.

Key Words: *estriol; chorionic gonadotropin; oncostatin M; regulatory T cells; recombinase-1*

Pregnancy is associated with the formation of new endocrine interactions maintaining normal coexistence and development of two genetically different organisms. The placenta being a fetoprotective structure provides the growth and development of the fetus and protects the semiallogenous fetus from attack of the maternal immune system. This function is provided by hormones and cytokines produced by the placenta. Of these, chorionic gonadotropin (CG) and estriol (E_3) detected in the woman body only during pregnancy are most physiologically important. These hormones are critical factors regulating the differentiation and growth of the fetoplacental complex. At the same time, they produce a pronounced effect on functional activity of the maternal immune system. Oncostatin M (OSM) secreted by trophoblast cells [12] is one of the key cytokines determining survival of the implanted blastocyst [9]. It is known that OSM under different conditions can act as a pro- or as an anti-inflammatory factor [13] and can induce extrathymic differentiation of T lymphocytes [7] that is intensified during pregnancy [4,6]. Revision of antigenic receptor of T lymphocytes (TCR) in mature peripheral $CD4^+$T cells due to expression of recombinases (RAG-1 and 2) is the basis for extrathymic differentiation of $CD4^+$T cells αβTCR in pregnancy [1]. Repeated rearrangement of αβTCR genes results in expression of new TCR molecules with changed specificity on the membrane [1]. This mechanism contributes to the formation of peripheral immune tolerance [1] and can create additional conditions for survival of the semiallogenic fetus [2].

Here we studied the effect of E_3, CG, and OSM as well as hormone and cytokine combinations on the expression of recombinase RAG-1 in Treg and Th17 taking into account the effect of hormones on $CD4^+$T cell differentiation into these regulatory subpopulations.

MATERIALS AND METHODS

Peripheral venous blood from healthy non-pregnant women of reproductive age (n=9) was used in the study. Mononuclear cell suspension was obtained by

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centrifugation in Ficoll-verografin density gradient (ρ=1.077 g/cm^3) and then, $CD4^+$T lymphocytes were isolated from this suspension by immunomagnetic separation (R&D Systems). The purity of isolated fraction controlled by the expression of CD4 molecule (BioLegend) was >95%. The isolated cells (5×10^6/ml) were incubated in a complete culture medium RPMI-1640 (Gibco) supplemented with 10% fetal calf serum, 1 mM HEPES, 2 mM L-glutamine, and 1 mg/ml gentamicin for 48 h at 37°C and 5% CO_2 in the presence of OSM and hormones. OSM (R&D Systems) was used in a concentration of 2 ng/ml extrapolated from the level of its secretion by the decidual tissue [12]. Hormones were used in concentrations corresponding to their blood levels during the first and third trimesters, respectively: 2 and 20 ng/ml E_3 (Biomedicals) [10] and 10 and 100 IU/ml CG (Profasi, I.F. Serono S.p.A.) [8]. Officinal hormone solvents were used as the control.

After 48-h incubation with hormones, the phenotype of T lymphocytes was assessed by flow cytometry (Becton Dickinson). As the expression of the transcription factor FoxP3 is the main marker of Treg, they were defined as $CD4^+FoxP3^+$ cells (Novus Biologicals); IL-17-producing lymphocytes (Th17) were identified as $CD4^+RORC^+$ (Novus Biologicals). Expression of recombinase RAG-1 (Recombination Activating Gene 1) [15], the marker of antigen receptor gene rearrangement, was determined separately in Treg and Th17 cells according to the manufacturer's instructions (Cell Signaling Technology).

As the results did not fit normal distribution, significance of differences between the groups was evaluated using the Wilcoxon's test for paired dependent samples. Quantitative parameters were presented as the median with the lower and upper quartiles: Me (LQ; UQ). The differences were significant at $p<0.05$.

RESULTS

When studying the effect of E_3 on Treg induction, a significant increase in the percentage of $CD4^+FoxP3^+$ T lymphocytes (iTreg) was revealed, but only at hormone concentration corresponding to the first trimester (2 ng/ml). As no additional stimulation of $CD4^+$T cells with Treg-polarizing cytokines was used, we can conclude that E_3 is an independent factor of differentiation of $CD4^+$T lymphocytes towards Treg. In contrast to

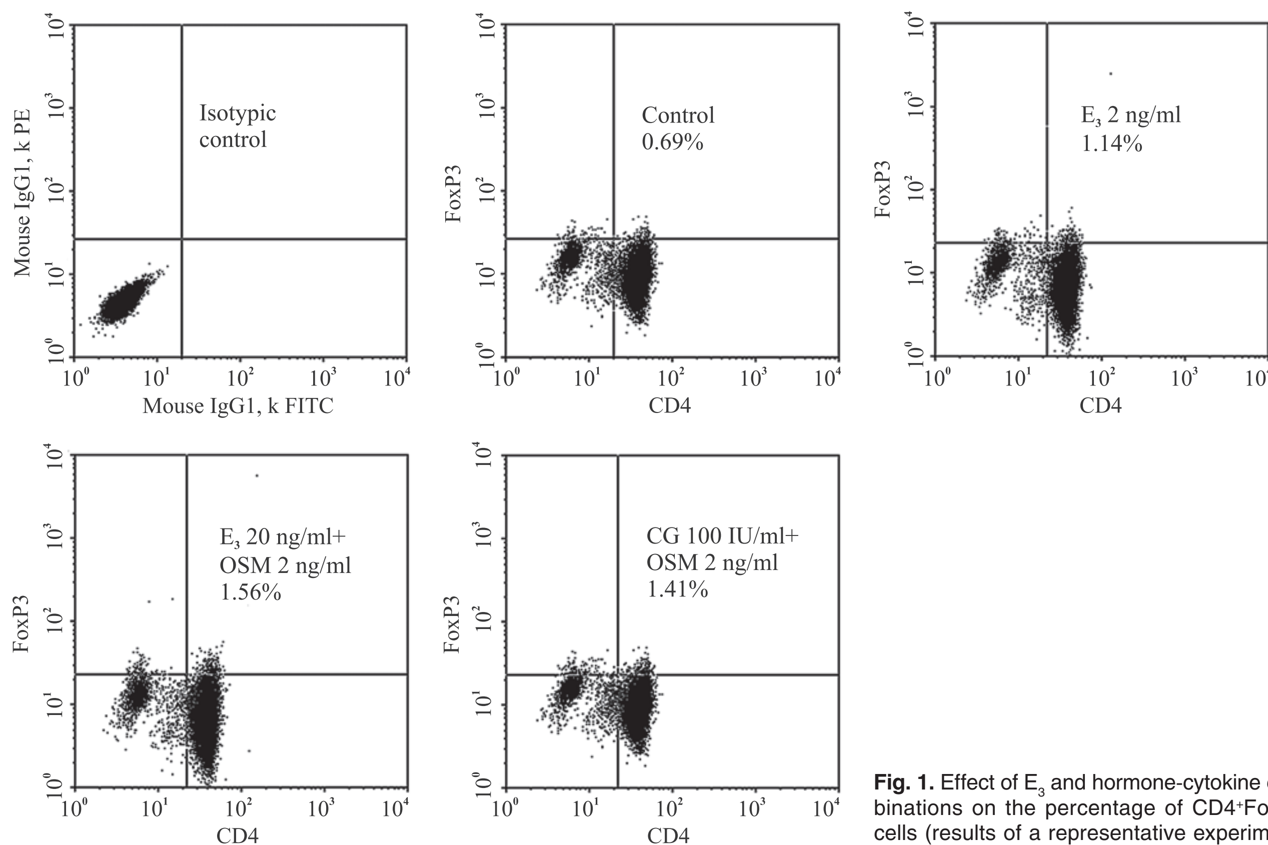

Fig. 1. Effect of E_3 and hormone-cytokine combinations on the percentage of $CD4^+FoxP3^+$ cells (results of a representative experiment).

E_3, OSM and CG in all concentrations had not effect on induction of CD4+FoxP3+ cells. Combined application of OSM with CG (100 IU/ml) or E_3 (20 ng/ml) extrapolated from hormone levels during the first and third trimesters on CD4+T cells significantly increased the percentage of CD4+FoxP3+ T lymphocytes (Table 1; Fig. 1). Thus, OSM helps placental hormones to realize their Treg-inducing action, acting as a co-activator. It should be noted that this co-activating effect of OSM appears when the hormone does not induce Treg differentiation (CG) or when this induction depends on hormone concentration (2 ng/ml E_3). The observed phenomenon should be taken into account when evaluating the immunomodulating potencies of reproduction hormones.

Revision of the antigen-recognizing receptor iTreg usually enhances their suppressor functions with respect to fetal antigens recognized in complex with molecules of major histocompatibility complex (MHC) [14]. Therefore, at the next stage we analyzed changes in the expression of recombinase RAG-1 in CD4+FoxP3+ T cells under the influence of hormones and OSM capable of inducing extrathymic differentiation during pregnancy [2]. It was established that hormones produced no significant effect on this parameter, but in the presence of OSM, the expression of RAG-1 in Treg increases under the influence of both E_3 concentrations and CG in the high concentration corresponding to the first trimester of pregnancy (Table 1; Fig. 2).

We also evaluated the regulatory potential of hormones and OSM as well as the hormone-cytokine combinations on Th17 induction and expression of RAG-1 in this subpopulation. It was found that the percentage of CD4+RORC+ T lymphocytes (Th17) decreased under the influence of E_3 in the concentration

TABLE 1. Effect of E_3, CG, OSM, and Their Combinations on the Percentage of CD4+FoxP3+ Cells and RAG-1 Expression in These Cells (Me (LQ; UQ))

Experimental conditions	CD4+FoxP3+ cells, %	RAG-1 expression in CD4+FoxP3+cells, %
Control	0.72 (0.55; 0.92)	5.35 (4.02;8.10)
E_3, 2 ng/ml	0.96 (0.88; 1.44)*	4.82 (4.03; 8.65)
E_3, 20 ng/ml	0.79 (0.50; 1.16)	4.97 (3.75; 16.05)
CG, 100 IU/ml	0.87 (0.72; 1.15)	5.28 (3.37; 13.68)
CG, 10 IU/ml	0.86 (0.60; 1.08)	7.86 (4.92; 15.77)
OSM, 2 ng/ml	1.02 (0.49; 1.19)	5.21 (3.37; 7.78)
E_3, 2 ng/ml+OSM, 2 ng/ml	0.87 (0.81; 0.99)	17.15 (6.91; 28.63)*
E_3, 20 ng/ml+OSM, 2 ng/ml	0.96 (0.74; 1.57)*	12.18 (4.12; 22.86)*
CG, 100 IU/ml+OSM, 2 ng/ml	1.03 (0.87; 1.42)*	13.19 (5.29; 28.05)*
CG, 10 IU/ml+OSM, 2 ng/ml	0.80 (0.61; 1.64)	6.23 (4.93; 18.43)

Note. Here and in Table 2: $*p<0.05$ in comparison with the control.

TABLE 2. Effect of E_3, CG, OSM, and Their Combinations on the Percentage of CD4+RORC+ Cells and RAG-1 Expression in These Cells (Me (LQ; UQ))

Experimental conditions	CD4+RORC+ cells, %	RAG-1 expression in CD4+RORC+ cells, %
Control	0.91 (0.67; 1.39)	2.79 (2.31; 6.57)
E_3, 2 ng/ml	0.51 (0.40; 0.78)*	2.60 (2.36; 3.76)
E_3, 20 ng/ml	1.08 (0.29; 1.88)	3.84 (2.55; 4.26)
CG, 100 IU/ml	0.67 (0.44; 0.82)	4.04 (2.80; 7.39)
CG, 10 IU/ml	0.77 (0.54; 1.36)	3.52 (2.41; 6.22)
OSM, 2 ng/ml	0.87 (0.69; 1.10)	2.42 (1.72; 5.04)
E_3, 2 ng/ml+OSM, 2 ng/ml	0.56 (0.35; 0.74)	4.57 (3.27; 7.37)
E_3, 20 ng/ml+OSM, 2 ng/ml	0.54 (0.44; 0.73)	4.85 (3.23; 6.24)
CG, 100 IU/ml+OSM, 2 ng/ml	0.59 (0.48; 1.71)	3.22 (2.34; 3.86)
CG, 10 IU/ml+OSM, 2 ng/ml	0.68 (0.56; 1.26)	3.86 (2.77; 4.66)

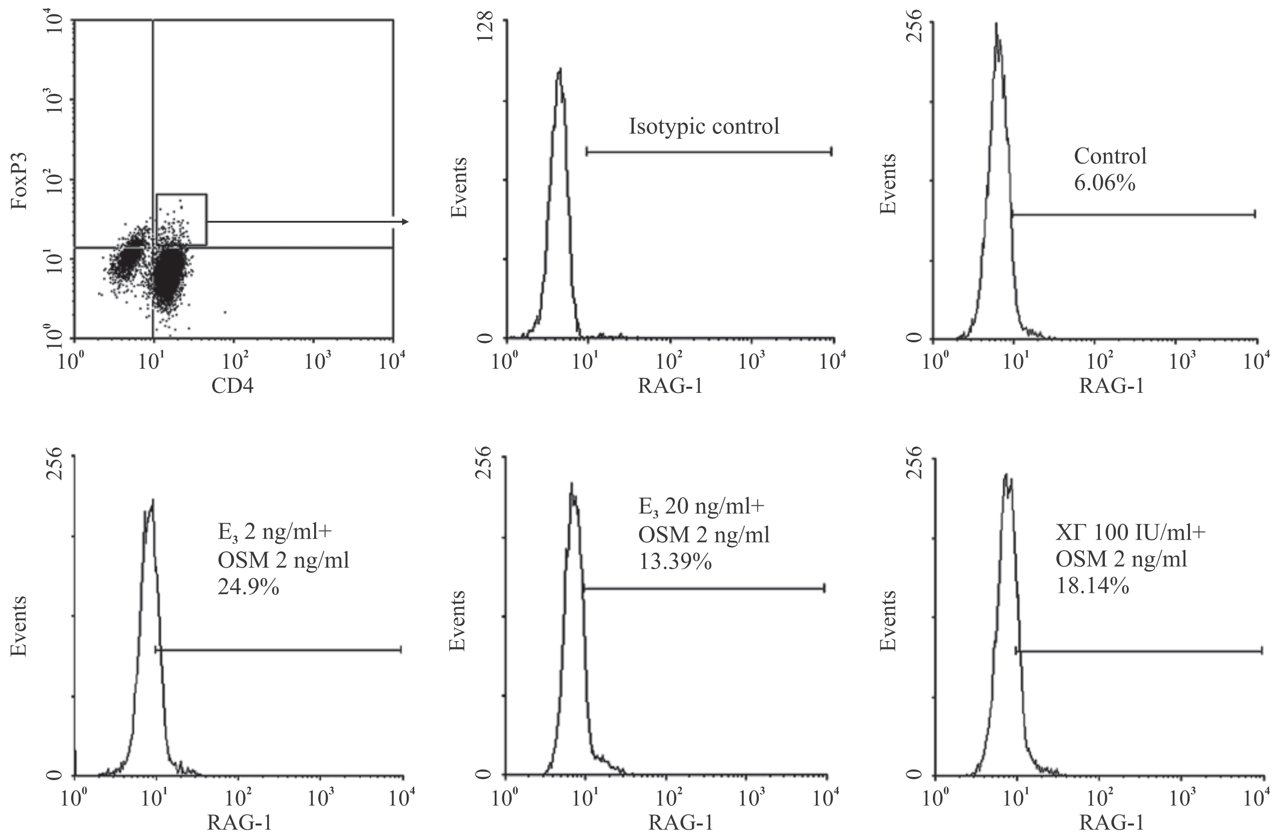

Fig. 2. Histograms demonstrating the effect of OSM combinations with E_3 and CG on RAG-1 expression in $CD4^+FoxP3^+$ cells (results of a representative experiment).

corresponding to the first trimester, *i.e.* the concentration effective for Treg induction. It is known that the risk of immune-mediated abortion in higher during the first and second trimesters [2]; therefore, realization of physiological tolerogenic potential of E_3 is expedient during this period. Under the influence of CG and OSM and their combinations as well as combinations of OSM with E_3, no significant regulatory effect on induction of $CD4^+T$ cells into Th17 and on the expression of RAG-1 in this subpopulation was observed (Table 2).

Thus, E_3 without additional stimuli can enhance iTreg generation and inhibit cell differentiation towards Th17 during the first trimester of pregnancy. In this case, both CG and E_3 in combination with OSM increase RAG-1 expression in $CD4^+FoxP3^+$ cells. According to published reports, OSM activates CG production by the chorion [12]. Conversely, activated or resting peripheral blood mononuclear cells can secrete OSM under the influence of high concentrations of CG [11]. This mutual stimulation probably promotes direct participation of these bioactive compounds in the formation of immune tolerance during the first trimester of pregnancy, the most critical in terms of induction of spontaneous abortions. During the third trimester, E_3 also promotes Treg αβTCR rearrangement, but only in the presence of OSM. Apparently, E_3 production increasing by this term in combination with OSM supports the necessary level of immunosuppression stimulating both induction of Treg and revision of their receptor.

In general, the studied hormones in combination with OSM stimulate the formation of iTreg and expression of RAG-1 throughout pregnancy, thereby promoting tolerance to fetal antigens. The mutual influence of hormones and OSM corroborates the concept of revision of αβTCR of mature T cells in the area of placental products action, which probably provides the basis for the development of a local network $CD4^+T$ cells that recognize fetal antigens as their own due to correction of their antigen-recognition repertoire. This can either enhance the suppressor functions of Treg or promote neglect of fetal structures as antigens.

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