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Role of Reproductive Hormones in Control of Apoptosis of T-Lymphocytes

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Abstract—Effects of chorionic gonadotropin (CG), estradiol, progesterone, and their physiological combinations on apoptosis of human peripheral blood T-lymphocytes were studied. Neither the hormones separately nor their combinations affected the spontaneous apoptosis of T-cells. On stimulation with mitogens, a high dose of CG (100 IU/ml) significantly increased apoptosis of T-lymphocytes, but its combination with steroid hormones specific for trimester I of pregnancy decreased this parameter. Apoptosis of T-lymphocytes induced by neutrophils in mixed culture was also inhibited by the hormone combination corresponding to trimester I. In greater detail, this hormonal combination was shown to display differential effects on different T-cell subpopulations: it stimulated apoptosis of $CD8^+$-lymphocytes (which seemed to be provided by CG) and inhibited apoptosis of $CD4^+$-cells. Apoptosis of T-lymphocytes induced by anti-CD95 was suppressed by a high dose of progesterone (100 ng/ml) and also by its combination with CG and estradiol specific for trimester III of pregnancy. Thus, the reproductive hormones studied effectively regulated apoptosis of peripheral blood T-lymphocytes. The effect of the hormones depended on the cell type and their activation and seemed to be an important mechanism of hormonal control of immune reactions in pregnancy.

Key words: apoptosis, chorionic gonadotropin, estradiol, progesterone, T-lymphocytes, neutrophils, cAMP, pregnancy

Apoptosis is an active form of cell death which underlies many biological processes. For T-lymphocytes, apoptosis is an important mechanism which controls their development, number, and also the intensity and duration of the immune response, which is associated with the clonal expansion of antigen-specific lymphocytes and needs the activated cells to be eliminated in time to prevent development of tumors and autoimmune processes [1]. The T-lymphocyte population is heterogeneous and includes both effector (cytotoxic T-lymphocytes (CTL)) and regulatory (T-helpers, Th) cells; therefore, the selective elimination of certain subpopulations by apoptosis may also be considered a factor determining the strategy of the immune response.

The membrane molecule Fas (CD95) is an important mediator of apoptosis of T-lymphocytes and of other cells of the immune system. This molecule binds to the appropriate ligand (FasL) and initiates an apoptotic signal [2]. Fas is constitutively expressed on the T-cell membrane, whereas FasL is synthesized and secreted by lymphocytes only on activation and causes the death of $CD95^+$-cells acting as a paracrine or autocrine factor [3]. Just the FasL/Fas-dependent apoptosis is the main control mechanism of expansion of activated T-lymphocytes during the immune response.

Changes in the endocrine background in pregnancy are accompanied by significant disorders in T-cell reactions of the body [4]. These changes are mainly determined by chorionic gonadotropin (CG) and placental sex steroids which not only control the gestation processes but also markedly modify immunity [4, 5]. The level of apoptosis of T-lymphocytes is also modified in pregnancy: the Fas-dependent apoptosis of these cells is suppressed [6], the expression of Fas [6, 7] and also of the antiapoptotic molecule bcl-2 [6] are changed. Because apoptosis is an early event in the activation of T-cells and its disorders can provoke subsequent functional changes in these cells, studies on the role of reproductive hormones in the regulation of this process are important for

Abbreviations: CD) leukocyte membrane molecules; FITC) fluorescein isothiocyanate; IL-2) interleukin 2; MAb) monoclonal antibodies; MHC) major histocompatibility complex; Th) T-helpers; CG) chorionic gonadotropin; CTL) cytotoxic T-lymphocytes.

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comprehension of the control mechanism of the functional activity of T-lymphocytes and rearrangement of the immune system in pregnancy.

The purpose of the present work was to study the effects of CG, estradiol, progesterone, and of their physiological combinations on apoptosis of human peripheral blood T-lymphocytes and also to assess the intracellular mechanisms of the hormonal effects using inhibitors of adenylate cyclase (H-89), calcium (verapamil), and phosphoinositide (lithium oxybutyrate) signals. The first signal mediates the effects of CG in the traditional targets, luteocytes [8], and also in T-lymphocytes [9], whereas two other signals are involved in the non-genomic effects of steroid hormones [10, 11]. Considering the heterogeneity of T-lymphocyte population and different roles of individual T-cell subpopulations in the immune response, we analyzed the hormone-dependent regulation of apoptosis separately in $CD4^+$- and $CD8^+$-cells. Moreover, taking into account the ability of neutrophils to effectively regulate apoptosis of T-cells [12] and their sensitivity to reproductive hormones [13], the roles of autologous and allogenic neutrophils were studied in the hormone-dependent control of apoptosis of T-lymphocytes.

MATERIALS AND METHODS

The hormones were used in concentrations corresponding to their levels in peripheral blood during the first and third trimesters of pregnancy: CG (Profasi, Italy), 100 and 10 IU/ml [14]; estradiol (Sigma, USA), 1.0 and 10 ng/ml [15]; progesterone (Sigma), 20 and 100 ng/ml [16]. Doses of the hormones were combined according to their presence in pregnancy, i.e., the high dose of CG was introduced into the culture together with a low dose of steroids and vice versa.

Intracellular mechanisms of the hormonal effects were analyzed using the following reagents: the protein kinase A inhibitor H-89 (ICN, USA) (1.0 μg/ml) [17], the inhibitor of Ca^{2+}-channels verapamil (Knoll, AG, Germany) (0.025 mg/ml) [18], the inhibitor of inositol-1-monophosphatase lithium oxybutyrate (Tallinn Chemical Pharmaceutical Plant, Estonia), 1.5 mg/ml [19].

Peripheral blood leukocytes were fractionated as follows: venous blood treated with heparin (25 IU/ml) was centrifuged in a double density gradient of Ficoll–Verographin (Serva, Germany; Spofa, Czechia). Densities of the upper and lower gradients were 1.077 and 1.112 g/ml, respectively [20]. From the upper and lower interphase mononuclear cells of peripheral blood and neutrophils were collected, respectively (according to histological evaluation, the isolation purity was 98%, the cell viability by the test with 0.1% Trypan Blue was 99%).

Then T-lymphocytes were isolated by rosette-formation with sheep erythrocytes labeled with the rosette stabilizer 2-aminoethylisothiouronium bromide (the procedure was repeated twice). This method was described in detail earlier [21]. The isolation purity assessed by expression of the T-specific membrane marker CD3 was 95% and the cell viability was 97%.

T-Lymphocytes at the concentration of $2 \cdot 10^6$ cells/ml were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Serva), L-glutamine (300 μg/ml; Serva), 0.01 M HEPES (Sigma), and gentamicin (100 μg/ml; Pharmacia, Sweden) at 37°C in the presence of 5% CO_2 for 24 h. As stimulators we used phytohemagglutinin (PHA; Sigma) at the concentration of 10 μg/ml, the recombinant interleukin 2 (IL-2; Sigma) at the concentration of 10 ng/ml [22], and apoptosis-inducing monoclonal antibodies (MAb) to the CD95 molecule (Fas) which were kindly presented by Doctor of Medicine A. V. Filatov (State Research Center - Institute of Immunology, Ministry of Health of Russia, Moscow). T-Lymphocytes were incubated with the hormones for 1 h, and then an appropriate stimulator was added into the culture. In experiments with the inhibitors, the cells were preincubated with them for 1 h, supplemented with the corresponding hormone, repeatedly incubated for 1 h, and washed before the addition of the stimulator.

In the mixed cultures T-lymphocytes ($0.5 \cdot 10^6$ cells/ml) and autologous or allogenic neutrophils ($1.5 \cdot 10^6$ cells/ml) were taken at the ratio 1 : 3, which corresponded to that in the peripheral blood. The cells were introduced into the sample directly or were cultured in a transwell system preventing their immediate contact: in two dishes incompletely inserted one into the other (Flow, USA) supplemented with a separating membrane impermeable for cells but not for proteins and other molecules.

Apoptosis of leukocytes was determined by cytofluorimetry by the level of hypoploid cells in the sample fixed with 70% ethanol and stained with propidium iodide (50 μg/ml; Sigma). The cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, USA).

Expression of CD95 was determined by immunofluorescence with the FACSCalibur cytometer using MAb LT95 labeled with fluorescein isothiocyanate (FITC; Sorbent, Russia).

The level of apoptosis in $CD4^+$- and $CD8^+$-subpopulations of T-lymphocytes was determined by two-color immunofluorescence using appropriate Mab—LT4-FITC and LT8-FITC (Sorbent). Apoptosis of T-cells in the mixed culture with neutrophils was also assessed by two-color immunofluorescence using MAb LT3-FITC (Sorbent) to the T-specific membrane molecule CD3.

Data were processed using Student's *t*-test and Pearson's coefficient (*r*).

RESULTS AND DISCUSSION

The spontaneous apoptosis of T-lymphocytes was insignificant and was not influenced by reproductive hor-

Table 1. Role of reproductive hormones in regulation of apoptosis of human peripheral blood T-lymphocytes

Group number	Experimental conditions	Percent of apoptotic cells (M ± m)		
		spontaneously (n = 10)	PHA + IL-2 (n = 5)	PHA + IL-2 + anti-CD95 MAb (n = 6)
1	control	6.79 ± 1.01	13.6 ± 1.64*	60.8 ± 4.19*
2	CG, 10 IU/ml	9.15 ± 0.708	12.1 ± 1.83	58.9 ± 4.33
3	CG, 100 IU/ml	8.35 ± 0.922	18.7 ± 2.82 $p(3-1) < 0.05$	56.3 ± 4.19
4	estradiol, 1.0 ng/ml	7.13 ± 0.808	15.2 ± 2.32	57.2 ± 3.39
5	estradiol, 10 ng/ml	7.71 ± 1.24	15.1 ± 1.49	61.4 ± 2.07
6	progesterone, 20 ng/ml	6.89 ± 1.34	12.6 ± 1.94	66.1 ± 2.89
7	progesterone, 100 ng/ml	7.11 ± 0.539	13.5 ± 2.41	51.3 ± 5.26 $p(7-1) < 0.05$
8	CG (100 IU/ml) + estradiol (1.0 ng/ml) + progesterone (20 ng/ml) (trimester I of pregnancy)	8.44 ± 0.916	9.48 ± 0.376 $p(8-1) < 0.05$ $p(8-3) < 0.05$	56.7 ± 6.14
9	CG (10 IU/ml) + estradiol (10 ng/ml) + progesterone (100 ng/ml) (trimester III of pregnancy)	7.09 ± 0.687	11.2 ± 1.14	49.7 ± 6.04 $p(9-1) < 0.05$ $p(9-2) < 0.05$

Table 2. Role of reproductive hormones in regulation of apoptosis of $CD4^+/CD8^+$ T-lymphocytes

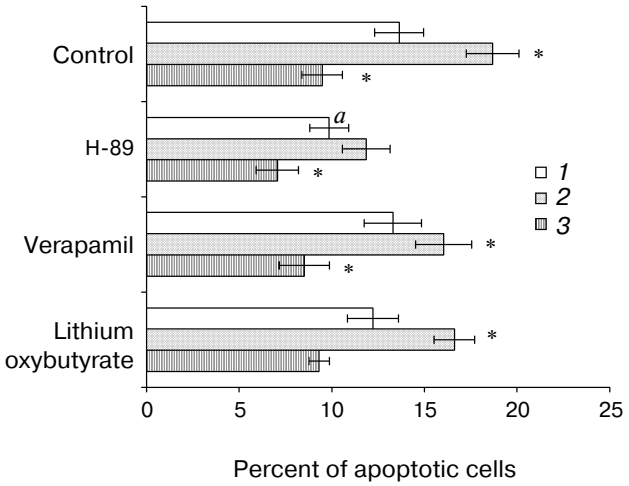
Group number	Experimental conditions	Percent of apoptotic cells in subpopulation (M ± m)			
		spontaneously (n = 5)		PHA + IL-2 (n = 5)	
		$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
1	control	6.94 ± 0.552	8.23 ± 0.727	18.3 ± 0.701	10.1 ± 1.70
2	CG, 10 IU/ml	7.28 ± 0.746	6.92 ± 0.849	15.2 ± 1.24	11.2 ± 1.91
3	CG, 100 IU/ml	7.69 ± 0.937	8.01 ± 0.804	15.3 ± 0.808	16.3 ± 2.73 $p(3-1) < 0.01$
4	estradiol, 1.0 ng/ml	7.07 ± 0.828	8.02 ± 0.668	17.2 ± 1.52	11.6 ± 1.54
5	estradiol, 10 ng/ml	7.36 ± 0.811	7.92 ± 0.777	15.8 ± 2.31	11.8 ± 1.79
6	progesterone, 20 ng/ml	6.38 ± 0.823	7.87 ± 0.857	17.8 ± 3.31	14.0 ± 1.76
7	progesterone, 100 ng/ml	7.27 ± 0.866	7.99 ± 0.947	17.4 ± 3.16	14.9 ± 2.11
8	CG (100 IU/ml) + estradiol (1.0 ng/ml) + progesterone (20 ng/ml) (trimester I of pregnancy)	7.95 ± 0.829	8.44 ± 0.843	8.16 ± 0.908 $p(8-1) < 0.01$ $p(8-3) < 0.01$ $p(8-4) < 0.01$ $p(8-6) < 0.05$	15.9 ± 1.46 $p(8-1) < 0.01$ $p(8-4) < 0.05$
9	CG (10 IU/ml) + estradiol (10 ng/ml) + progesterone (100 ng/ml) (trimester III of pregnancy)	7.93 ± 0.818	8.84 ± 0.716	13.4 ± 1.41	13.0 ± 1.05

mones or their physiological combinations (Table 1). The level of apoptosis in individual subpopulations of intact T-cells ($CD4^+/CD8^+$) also was not changed under the influence of the hormones (Table 2).

Stimulation of the cells with PHA combined with IL-2 significantly increased apoptosis of T-cells (Table 1). And the high physiological dose of CG (100 IU/ml) specific for trimester I of pregnancy increased apoptosis, whereas its appropriate combination with estradiol or progesterone suppressed the stimulated apoptosis (Table 1). As to different T-cell subpopulations, CG stimulated apoptosis only of $CD8^+$-cells, whereas the appropriate hormonal combination inhibited apoptosis only of the subpopulation of $CD4^+$-T-lymphocytes (Table 2).

The CG-dependent stimulation of apoptosis was prevented by the protein kinase A inhibitor H-89, and this suggested that cAMP should be involved in proapoptotic effects of CG (figure). However, the inhibitory effect of the appropriate combination of CG with steroid hormones was not changed in the presence of inhibitors of adenylate cyclase (H-89), calcium (verapamil), or phosphoinositide (lithium oxybutyrate) metabolism (figure).

Expression of the membrane molecule CD95 by activated T-lymphocytes had similar features: the level of $CD95^+$-cells significantly increased in the presence of the high dose of CG and decreased at its appropriate combination with estradiol and progesterone (Table 3). A significant correlation between the expression of CD95 and apoptosis in the presence of the high dose of CG (r = 0.941, $p < 0.05$) suggests a key role of this molecule in mediation of the CG-dependent regulation of apoptosis.



Mechanisms of the hormone-dependent regulation of apoptosis of T-lymphocytes stimulated with PHA + IL-2. *1*) Hormone-free control; *2*) CG, 100 IU/ml; *3*) CG (100 IU/ml) + estradiol (1.0 ng/ml) + progesterone (20 ng/ml) (trimester III of pregnancy); * $p < 0.05$ (compared to the hormone-free control); *a*) $p < 0.05$ (compared to the inhibitor-free control)

On stimulation of T-lymphocytes with apoptosis-inducing MAb to CD95 only progesterone at the high dose of 100 ng/ml and also its combination with CG and estradiol specific for trimester III of pregnancy suppressed the apoptosis induced (Table 1). Because neither CG nor estradiol alone displayed a modifying effect in

Table 3. Effects of reproductive hormones on the expression of CD95 by peripheral blood T-lymphocytes activated with PHA + IL-2

Group number	Experimental conditions	Percent of $CD95^+$-cells (M ± m) (n = 5)
1	control	62.0 ± 4.11
2	CG, 10 IU/ml	65.2 ± 5.19
3	CG, 100 IU/ml	76.4 ± 3.19 $p(3-1) < 0.05$
4	estradiol, 1.0 ng/ml	64.1 ± 6.36
5	estradiol, 10 ng/ml	67.8 ± 4.28
6	progesterone, 20 ng/ml	66.6 ± 5.03
7	progesterone, 100 ng/ml	67.2 ± 5.73
8	CG (100 IU/ml) + estradiol (1.0 ng/ml) + progesterone (20 ng/ml) (trimester I of pregnancy)	53.1 ± 1.17 $p(8-1) < 0.05$
9	CG (10 IU/ml) + estradiol (10 ng/ml) + progesterone (100 ng/ml) (trimester III of pregnancy)	66.3 ± 4.13

Table 4. Role of neutrophils in hormone-dependent regulation of apoptosis of T-lymphocytes

Group number ($n = 5$)	Experimental conditions	Percent of apoptotic cells (M ± m)		
		control	hormones (trimester I)	hormones (trimester III)
1	T-lymphocytes	7.17 ± 0.703	9.34 ± 0.761	7.71 ± 0.823
2	T-lymphocytes + autologous neutrophils	18.5 ± 2.39 $p(2–1) < 0.05$	8.29 ± 0.868*	11.9 ± 1.57
3	T-lymphocytes + autologous neutrophils (trans-well system)	7.90 ± 0.856	7.56 ± 0.861	9.58 ± 1.64
4	T-lymphocytes + allogenic neutrophils	15.4 ± 1.75 $p(4–1) < 0.05$	6.99 ± 1.43*	11.8 ± 1.48
5	T-lymphocytes + anti-CD95 MAb	60.8 ± 4.19 $p(5–1) < 0.05$	56.7 ± 6.14	49.7 ± 6.04*
6	T-lymphocytes + autologous neutrophils + anti-CD95 MAb	52.1 ± 2.10	50.0 ± 6.56	45.9 ± 2.67

* $p < 0.05$ as compared to the control.

this system (Table 1), the inhibitory effect of the hormonal combination should be assigned to progesterone.

In the presence of neutrophils the level of apoptosis of intact T-cells was significantly increased (Table 4). And both autologous and allogenic neutrophils had the same stimulating effect. The apoptosis-inducing effect of neutrophils seemed to be not restricted to molecules of the major histocompatibility complex (MHC). The lack of this effect in the trans-well system (Table 4) suggested that the cells should interact directly to provide apoptosis of T-cells by neutrophils. As to the effect of reproductive hormones in the mixed culture, the neutrophil-induced apoptosis of T-cells was significantly suppressed by the hormonal combination corresponding to trimester I of pregnancy (Table 4). However, on stimulation of the cells with anti-CD95 MAb the inhibitory effect of the hormonal combination (trimester III of pregnancy) found in the T-lymphocyte monoculture was not reproduced in the presence of neutrophils (Table 4).

Thus, of the reproductive hormones studied only CG at the high physiological dose corresponding to trimester I of pregnancy could alone modify apoptosis of T-lymphocytes stimulated with PHA/IL-2. The CG-dependent activation of apoptosis was limited to $CD8^+$-cells, mediated by cAMP, and associated with increase in the expression of the CD95 molecule on the membrane. Note that although cAMP is traditionally believed to be a proapoptotic factor for T-cells, the proapoptotic effect has been shown only on thymocytes [23] and intact T-lymphocytes [24], whereas this secondary messenger usually inhibits apoptosis in antigen-stimulated cells [25]. The cAMP-dependent increase in the activated apoptosis found in our work has several explanations: first, this effect has been detected only on the minor population of $CD8^+$-cells, and activating signals are different in different T-cell subpopulations [26]; second, signal mechanisms of the mitogen-dependent activation of cells are unlike antigenic mechanisms; third, the stimulating effect of CG is mediated by increase in the CD95 expression on the membrane and can be unassociated with regulation of apoptotic signals in the cell.

The high dose of CG combined with steroid hormones also increased the activated apoptosis of $CD8^+$-T-lymphocytes (that obviously was due to CG) but suppressed apoptosis of $CD4^+$-cells. The differential regulation of apoptosis in different T-cell subpopulations has physiological significance: the elimination of activated CTL ($CD8^+$) can suppress immune rejection, whereas the promotion of Th-clones ($CD4^+$) results in increase in production of cytokines which have trophic effect on the placenta and fetus [27]. It should be emphasized that these hormonal effects displayed only at the combination appropriate for trimester I of pregnancy and were likely to be a mechanism preventing spontaneous abortions of immune nature during this period. Moreover, the differently directed hormonal regulation of apoptosis of $CD4^+/CD8^+$-cells seems to cause changes in the immune resistance of the body in pregnancy associated with suppression of cell-mediated immune reactions concurrently with increase in the humoral immunity [28].

As to the decrease in the level of T-cell apoptosis found in pregnancy [6], it seems that the hormones studied contribute to this decrease by suppressing apoptosis of $CD4^+$-cells.

Overall, the ability of reproductive hormones to regulate apoptosis of T-cells shown in this work seems to be the most important control factor of immune reactions in pregnancy, and the changes in the functional activity of T-cells are essentially provided by regulation of their apoptotic activity.

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