Role of Cytokines in the Regulation of Reproductive Function

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 1, pp. 81-85, January, 2007
Original article submitted July 14, 2005

The levels of 17 cytokines in the follicular fluid were measured by multiplex proteome analysis at the peak of stimulated ovulation during the *in vitro* fertilization cycle. In patients with ineffective folliculogenesis, the concentrations of IL-2, IL-4, IL-7, and granulocytic CSF in the follicular fluid were significantly lower than in women with greater mean number of collected oocytes. It was shown that multicomponent cytokine deficit was associated with lower production of high quality oocytes and lower efficiency of fertilization of the resultant oocytes *in vitro*. The absence of pregnancy after *in vitro* fertilization cycle is associated with lower levels of IL-2, IL-4, IL-7, granulocytic CSF, and macrophagic inflammatory protein 1b in combination with elevated contents of IL-8 and IL-13. The results attest to an important role of cytokines in the regulation of oogenesis and in preparation of the endometrium to implantation of the embryo.

Key Words: *cytokines; multiplex proteome analysis; follicular fluid; oocytes; in vitro fertilization*

Normal development and functioning of the female reproductive system are realized in close cooperation with the immune and endocrine systems. Changes during the menstrual cycle and pregnancy are regulated by the hypothalamic-pituitary system and directly result from hormone-induced remodeling of ovarian tissues and uterine mucosa. At the cellular level, the hormonal effects are realized with participation of many peptide factors, a special role is played by the lymphohemopoietic cytokines produced by immunocompetent cells [2,3,9].

The immunological aspects of the maintenance and normal course of gestation are best studied. The role of the immune system in the regulation of the earliest stages of the reproductive process (folliculogenesis, oogenesis, and embryo implantation) is less studied. Introduction of accessory reproductive technologies, for example, *in vitro* fertilization (IVF), made it possible to study the immune regulation of these processes in humans. Ovarian tissues and the endometrium contain immune cells, whose composition and count change over the course of the ovulatory cycle [4,6,8]. The follicular fluid (FF) contains cytokines, the concentrations of some of them significantly surpass those in the peripheral blood, which attests to importance of immune regulation of ovarian function [5]. Along with the regulation of ovarian function, the immune system cells and cytokines produced by them play an exceptionally important role in the preparation of the endometrium to embryo implantation [7] and pregnancy maintenance [11].

It is obvious that immunity disorders can be a serious cause of the absence of pregnancy or early spontaneous abortion, including those in IVF. Therefore, complex evaluation of cytokine levels in wo-

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men opposite by fertility levels attracts special interest. Due to the development and introduction of multiplex proteome analysis into practice it became possible to study the cytokine-mediated regulatory mechanisms at a higher level. Commercial kits for evaluation of 17 human cytokines belonging to different functionally significant groups were developed [1].

We studied the role of cytokines in the regulation of the early stages of the reproductive process in humans, including oocyte maturation, embryo implantation, and pregnancy development; to this end, we measured the content of 17 cytokines in FF from women differing by parameters of ovarian function and efficiency of IVF therapy.

MATERIALS AND METHODS

The study was carried out in 52 women aged 24-49 years (mean age 33 years) treated for infertility by the IVF method. The duration of infertility varied from 2 to 16 years (mean duration 6 years). Primary infertility was diagnosed in 32% cases, secondary in 68% cases (most often tuboperitoneal factor infertility). Control group consisted of 7 healthy fertile women aged 22-30 years with a history of normal gestation and full-term deliveries of healthy babies (these women participate in the oocyte donorship program). All clinical laboratory studies were carried out after informed consent of the examined women.

Superovulation in the IVF cycle was induced according to the standard protocols. Ovarian hyperstimulation (OHS) syndrome was diagnosed in 13.5% cases (in 7 of 52 sterile women) at the beginning of the lutein phase. The syndrome manifested by the presence of fluid in the peritoneal cavity and moderate pain. Transvaginal puncture of the folliculi was carried out under ultrasonic control. After counting of the collected oocytes and evaluation of their quality, they were cultured in IVF medium (MediCult) at 37°C in a humid atmosphere with 5% CO_2. The semen was treated by the floating method. Oocytes were fertilized 4-5 h after puncture and tested for pronuclei 18-20 h after fertilization. Division and quality of embryos were evaluated after 46-48-h culturing. The fertilization index was evaluated as a ratio of fertilized to total number of oocytes (in %). The embryos were transferred under ultrasonic control on days 3-4 of culturing. Pregnancy onset was diagnosed by ultrasonic examination by the presence of fertilized ovum in the uterine cavity 3-4 weeks after embryo transfer.

The levels of 17 cytokines (IL-1β, TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, granulocytic (G-) and granulocytic macrophageal (GM-) CSF, monocytic chemotaxic factor (MCP-1), macrophageal inflammatory protein (MIP-1β)) in FF were measured by flow fluorometry on a automated dual-beam laser analyzer (Bio-Plex Protein Assay System, Bio-Rad) using 17-Plex commercial kits (detectable dynamic range 2-32,000 pg/ml) according to manufacturer's instruction.

This method is based on specific binding of the studied cytokines to the solid phase (a suspension of 5.5-μ polystyrene granules). Granules of 17 types differing by their own fluorescent label and conjugated to respective monoclonal anticytokine antibodies were used in the analysis simultaneously. Biotin-conjugated anticytokine antibodies were added into the reaction mixture after specific binding of the granules to cytokines present in the studied samples and in calibration samples. The resultant "sandwich" structure was detected by biotin reaction with streptavidin labeled with a "reporter" fluorescent label (phycoerythrin). All reactions were carried out in a 96-well plate. The results were evaluated by flow fluorometery, the granules were automatically separated into types by their own FITC labels, while the content of bound cytokines was evaluated by summary intensity of phycoerythrin fluorescence. Using standard calibration dilutions, the cytokine concentrations in the test samples were evaluated automatically on a PC using Bio-Plex Manager software.

Cytokine values below the threshold sensitivity of the method (<2 pg/ml) were taken for 1 pg/ml in statistical data processing.

In order to evaluate possible involvement of the cytokines in the regulation of ovarian function, the relationship between FF cytokine profile parameters and the total number of maturing oocytes, percentage of high-quality oocytes among them, and fertilization index were studied. The development of iatrogenic complication of IVF was taken into consideration.

RESULTS

A wide spectrum of cytokines was identified in FF at the peak of stimulated ovulation. It included Th1/proinflammatory (IFN-γ, IL-2, TNF-α, IL-1β, IL-12, IL-17) and Th2/antiinflammatory (IL-4, IL-5, IL-10, IL-6, IL-13) cytokines, chemokines (IL-8, MIP-1, MCP), and hemoimmunopoiesis factors (G-CSF, GM-CSF, IL-7; Table 1). The groups of infertile patients and oocyte donors were comparable by the cytokine levels, but individual values considerably varied greatly. The presence of some cytokines in FF was proven [12], but the presence of

TABLE 1. Cytokine Levels in FF of Oocyte Donors and Infertile Patients (pg/ml)

Cytokine	Oocyte donors (*n*=7)			Infertile patients (*n*=52)		
	M±*m*	median	range	*M*±*m*	median	range
IFN-γ	78±36	55	1-288	65±13	59	1-604
IL-2	18±5	25	1-30	17±2	26	1-37
IL-4	8.0±1.6	9.6	1-12	7.0±0.7	8.4	1-15
IL-5	2.4±2.7	2.7	1-3	2.2±0.1	2.8	1-5.5
IL-6	1.70±3.5	13	3-29	31±10	18	1-541
IL-10	10±1	10	5-13	10.0±0.7	10	1-24
IL-13	54±16	24	20-114		26	1-225
IL-1β	25±3	23	19-41	27.0±1.6	25	9-63
TNF-α	16.5±6.0	10	8.8-52.0	19.0±3.6	10	1-156
IL-12 (p70)	12.0±0.2	12	11.0-12.3	14.0±1.5	12	1-64
IL-17	21±5	16	15-40	22.5±3.0	16	1-114
IL-7	10.7±0.5	11	8.5±11.3	8.2±0.9	11	1-38
G-CSF	132±30	166	18-203	120±12	157	1-296
GM-CSF	202±48	136	125-460	220±36	137	1-1262
IL-8	21.0±4.4	21	7.8±35.0	33.5±15.0	15	2.5±812
MIP-1	81±16	75	34-154	115±13	81	14.3-496.0
MCP-1	11±8	1	1-58	20±8	1	1-362

IL-4, IL-10, and IL-12 in FF is little studied [9]. The contents of many cytokines in FF significantly surpassed their serum levels [1], which attested to potential role of cytokines in the regulation of ovarian function. In addition to leukemia inhibitory factor, IL-8, IL-6, and G-CSF, whose selective production in FF is known, we detected higher levels of GM-CSF, IL-2, IFN-γ, MIP-1β, and IL-7.

The concentrations of IL-2, IL-4, IL-7, and G-CSF in patients, in whom the total number of collected oocytes was ≤8, were significantly higher than in the opposite group, in which the mean number of oocytes was 3-fold higher (Table 2). Presumably, these cytokines are involved in the initiation of oocyte maturation. A similar cytokine profile of FF was detected during the development of OHS syndrome, though in this case a sufficient number of oocytes were produced (median: 15 oocytes). Low concentrations of IL-2, IL-4, IL-7, and G-CSF were paralleled by a significant (by 40%) elevation of IL-1β level (from 23-25 to 34 pg/ml) and a more than 2-fold increase in the con-

TABLE 2. Cytokine Content in FF of Women Differing by the Content of Oocytes and Development of OHS Syndrome during IVF Cycle (pg/ml; median)

Parameter	Oocyte donors	Number of oocytes		
		OHS		no OHS
		<8 (*n*=21)	>8 (*n*=24)	>8 (*n*=7)
Total number of maturing oocytes	8	4**	13	15
IL-2	25	1.5**+	30.3	6.0*+
IL-4	9.6	3.5**+	11.5	2.7*+
IL-13	24	23.7	26	59**+
IL-1β	23	23.7	24.9	34*+
IL-7	11	7.3**+	11.5	5.3*+
G-CSF	166	88**+	194	46**+

Note. *p<0.05, **p<0.01 *vs.* women without OHS with 8 or less oocytes collected (according to Mann—Whitney test), +p0.05 compared to oocyte donors.

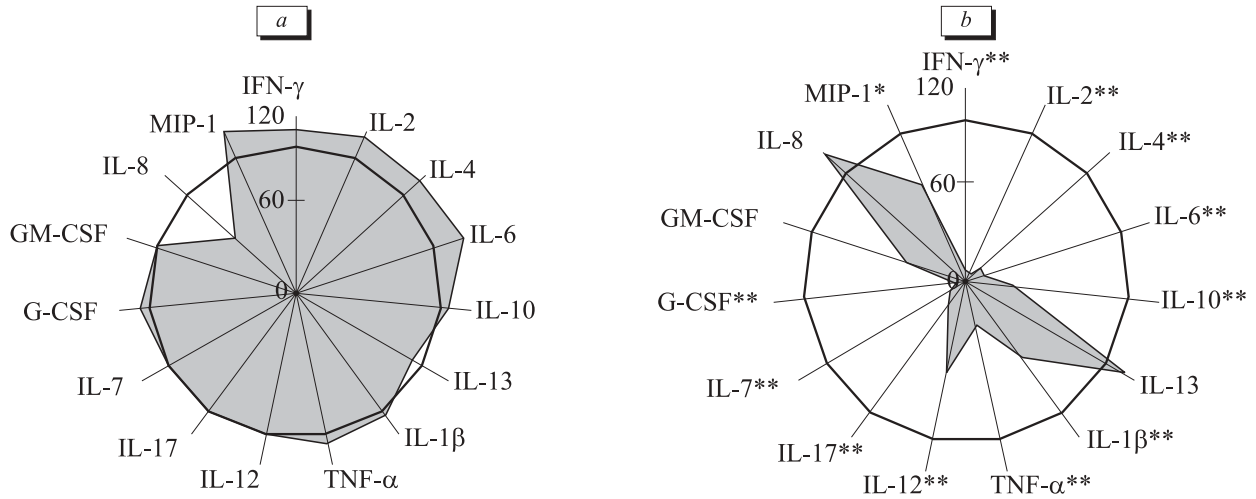

Fig. 1. Cytokine profiles of FF in women differing by quality of oocytes. *a*) women (*n*=23) with 100% high-quality cells at the peak of stimulated ovulation. *b*) women (*n*=6) with no more than 60% high-quality oocytes (median: 50%). The data are presented as the percentage of the mean level of cytokines in FF of oocyte donors (*n*=7), taken for 100%. Here and in Fig. 2: **p*<0.05, ***p*<0.01 compared to the data presented in fragment *a* (according to Mann—Whitney test).

centration of IL-13 (from 24 to 59 pg/ml). The pathogenesis of OHS is associated with excessive production of proinflammatory cytokines [10], which is seen from increased IL-1β level. The increase in the level of IL-13 can be regarded as a compensatory reaction aimed at limitation of local inflammation, which leads to a decrease in IL-2, IL-4, IL-7, and G-CSF levels.

Analysis of FF cytokine profiles in women differing by the quality of collected oocytes revealed deep multicomponent deficiency in the majority of analyzed cytokines in patients with no more than 60% high-quality oocytes (50% on average; Fig. 1). The low levels of cytokines in FF were associated with not only impaired production of high quality oocytes, but also significantly lower efficiency of their subsequent *in vitro* fertilization (fertilization index 39% *vs.* 91%, $p<0.01$). Therefore, proper maturation of oocytes requires a certain level of a system of cytokines (including IL-2, IL-4, IL-7, and G-CSF) in the follicle microenvironment. Hence, low quality of oocytes and level of their fertilization are associated with the multicomponent cytokine deficit, but not changes in just one factor.

The number of oocytes, their quality, and level of fertilization are important parameters determining the result of IVF. However, the efficiency of the initial stages of embryo development and its effective implantation are also essential for the onset of gestation. In our study, all IVF cycles eventuated in puncture of follicles and embryo transfer. A total of 24 (46%) pregnancies were diagnosed. Women in whom clinical pregnancy was attained (*n*=24) and patients in whom the treatment was inefficient (*n*=28) were comparable by the mean age, duration and forms of infertility, and drug protocols of superovulation induction (data not presented). Retrospective analysis of cytokine content in FF of women opposite by the fertility levels showed (Fig. 2) that the absence of pregnancy was associated with lower levels of IL-2, IL-4, IL-7, G-CSF, and MIP-1β in parallel with elevated levels of IL-8 and IL-13. The deficit of IL-2, IL-4, IL-7, G-CSF, and MIP-1β was associated with little count and/or poor quality of maturing oocytes. On the other hand, we detected no significant differences

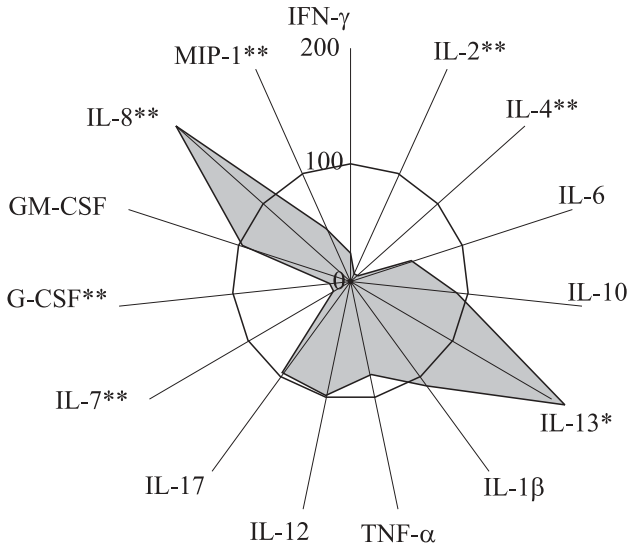

Fig. 2. Cytokine profile of FF in women with ineffective IVF cycle (*n*=28). The data are presented as the percentage of the mean levels of cytokines in FF of women (*n*=24) in whom clinical pregnancy was attained, taken for 100%.

in the levels of IL-8 and IL-13 in women differing by the number or quality of collected oocytes. Presumably, increased levels of these cytokines are associated with disorders in the early stages of implantation and maturation of the embryo.

Hence, analysis of the cytokine profiles of FF in women treated for sterility by IVF indicates an important role of the immune system in the regulation of folliculo- and oogenesis. The data demonstrate the involvement of cytokine-mediated mechanisms in oocyte maturation processes largely determining the efficiency of subsequent stages of fertilization and implantation of collected oocytes, the onset and development of pregnancy.

The practical significance of the results consists in the possibility of using some parameters of the cytokine status of women, examined at the peak of superovulation, as the early diagnostic markers of clinical efficiency of IVF. The prognosis of IVF is important for the development and introduction into practice of adjuvant cytokine therapy aimed at improvement of the efficiency of IVF, which is no higher than 30-40%. The strategy of IVF can be modified, for example, by delay of transfer of collected and frozen embryos until the period when more favorable conditions for their effective implantation and subsequent gestation development are created.

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