Leptin as an Immunocorrecting Agent during Normal Pregnancy

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Experiments were performed with leptin in doses observed during pregnancy. We studied the effect of leptin on expression of membrane molecules and cytokine production by peripheral blood mononuclear cells from women. Leptin increased the expression of HLA-DR on T lymphocytes, stimulated the production of TNF-α and IL-6, and did not affect secretion of IL-2, IL-4, and IL-10 by mononuclear leukocytes. Leptin in a dose comparable to that during the 1st trimester of pregnancy increased the percentage of NK cells with membrane CD16 and CD56, stimulated the production of IFN-α by mononuclear leukocytes, and did not modulate the number of $CD16^+56^+$NKT cells. Treatment with leptin in a dose for the second-third trimesters of pregnancy was followed by a decrease in the percentage of $CD16^+56^+$NKT cells and increase in the number of NKT cells expressing CD16 and CD56. Our results indicate that leptin play an important role in the regulation of membrane molecule expression and cytokine production by mononuclear leukocytes.

Key Words: *leptin; NK cells; NKT cells; cytokines; pregnancy*

Peptide hormone leptin is mainly synthesized by adipocytes [6,7]. The major biological role of leptin is regulation of energy balance in the organism and prevention of excessive accumulation of fat [6,7]. Leptin concentration increases significantly during pregnancy (particularly in the second and third trimesters) [9]. This hormone is essential for successful implantation of the fertilized ovum. Strong production of leptin is observed in the placenta [9]. Pregnancy is accompanied by significant changes in the maternal immune response, which determines viability of the semiallogeneic embryo. The maternal immune system can recognize fetal antigens, which is followed by the local and systemic response. Under normal conditions, these reactions do not cause fetal rejection [1,2]. Tolerance of the maternal immune system to genetically foreign fetus is an urgent problem of reproductive immunology. Studying the role of hormones in immune regulation during pregnancy is of considerable importance.

The role of leptin in the regulation of immune reactions is associated with its direct effect on immune cells that express specific membrane receptors for the hormone, including $CD4^+$ and $CD8^+$ T lymphocytes, NK cells, and T cells with function of natural killer cells (NKT cells) [5-7,14,15]. Leptin is a proinflammatory hormone, which contributes to the predominance of cellular immune reactions due to an increase in cytokine production by type 1 T helper cells (Th1) [6-8]. Pregnancy is accompanied by a shift of immune reactions toward the Th2-mediated immune mechanism [1,2]. It is interesting to study the regulatory effect of leptin on functional activity of mononuclear leukocytes during pregnancy is of significance.

Here we studied the effect of leptin in doses that were comparable to hormone concentration during the first and second-third trimesters of pregnancy on the expression of functionally important membrane molecules and cytokine production by mononuclear leukocytes *in vitro*.

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MATERIALS AND METHODS

Experiments were performed with the fractionated suspension of venous blood mononuclear cells from nonpregnant women of reproductive age (23-32 years). Blood samples were collected during the follicular phase of the menstrual cycle (day 5-11). Mononuclear cells were obtained by centrifugation in a Ficoll-Verografin density gradient (1.077 g/cm^3). The cell suspension was washed 2 times with Hanks solution. Mononuclear leukocytes (10^5-10^6 cells/ml) were incubated in complete nutrient medium (medium 199 with 10 mM HEPES, 2 mM L-glutamine, 100 μg/ml gentamicin, and 10% fetal bovine serum) with leptin (Sigma) at 37°C and 5% CO_2 for 24 h. The hormone in doses of 10-35 ng/ml was added to cultured cells. These doses of leptin correspond to hormone concentration during the first and second-third trimesters of pregnancy, respectively [9]. The supernatants were collected after incubation with leptin. The cells were washed with phosphate-buffered saline (0.01 M Na_2HPO_4 and 0.145 M NaCl, pH 7.2). The phenotype of lymphocytes was estimated by flow cytometry. Cell viability (95-98%) after 1-day incubation with this hormone was evaluated in the eosin test.

Mononuclear leukocytes were stained with monoclonal antibodies to the corresponding surface antigens according to the manufacturer's recommendations (Beckman Coulter). The expression of pairs of membrane molecules was studied with two-parameter reagents labeled with fluorochromes of contrast colors, fluorescein isothiocyanate (FITC) and phycoerythrin (PE). The results were recorded on an EPICS XL flow cytofluorometer (Beckman Coulter). The lymphocyte gate was set on the basis of combination of forward and side light scatter and cell size. At least 10,000 cells were counted. The corresponding isotypic controls were used to control nonspecific binding and to distinguish a fluorescence-negative lymphocyte gate.

The following subpopulations of T lymphocytes were assessed: T cells $CD3^+CD19^-$ (CD3-FITC/CD19-PE), Th cells $CD3^+CD4^+$ (CD3-FITC/CD4-PE), cytotoxic T lymphocytes $CD3^+CD8^+$(CD3-FITC/CD8-PE), activated T cells expressing the major histocompatibility complex class II molecule (HLA-DR) $CD3^+$HLA-DR^+ (CD3-FITC/HLA-DR-PE), NK cells with the $CD3^-CD16^+56^+$ phenotype (CD3-FITC/CD16,56-PE), and NKT cells with the $CD3^+CD16^+56^+$ phenotype (CD3-FITC/CD16,56-PE).

The concentrations of cytokines TNF-α, IL-6, IFN-α, IL-2, IL-4, and IL-10 in supernatants of cultured mononuclear cells were measured after 1-day incubation with the hormone by means of enzyme immunoassay with commercial kits (Tsitokin Company) according to manufacturer's recommendations. Control samples contained a similar amount of complete nutrient medium instead of the hormone. The results were analyzed by paired Student's *t* test and Spearman correlation test.

RESULTS

Addition of leptin in doses comparable to hormone concentration in various trimesters of pregnancy was followed by an increase in the expression of activation molecule HLA-DR on the membrane of T lymphocytes. However, leptin had no effect on the expression of coreceptor membrane molecules CD4 and CD8 on T cells and total number of T lymphocytes (Table 1). Previous studies showed that the increase in the number of activated T lymphocytes expressing HLA-DR correlates with the reaction of immune rejection [1,2]. It cannot be excluded that leptin contributes to activation of T lymphocytes and stimulates the cytotoxic response during pregnancy. The increased expression of HLA-DR on T lymphocytes can be due to direct interaction of leptin with the cell. These changes may be also associated with the effect of proinflammatory cytokines [6-8], whose production increases under the influence of leptin (Table 2).

Leptin in these doses increased the production of proinflammatory cytokines TNF-α and IL-6, but had no effect on the secretion of IL-2, IL-4, and IL-10 by mononuclear leukocytes. Moreover, leptin in a dose comparable to hormone concentration during the first trimester of pregnancy was shown to increase the production of IFN-α. Hence, leptin serves as a proinflammatory agent that stimulates the secretion of Th1 cytokines during pregnancy. Overproduction of inflammatory cytokines induces a cytotoxic reaction of the maternal immune system, which is directed against allogeneic fetal cells [1,2]. During normal pregnancy, Th1 cytokines are essential for successful implantation, contribute to invasive growth of the syncytiotrophoblast, and play a role in tissue remodeling [1,2]. Leptin probably maintains the balance between proinflammatory and antiinflammatory cytokines by stimulating the secretion of Th1 cytokines. This fact is of considerable importance at the early stages of gestation, which explains the necessity of leptin for successful implantation.

We studied the modulatory effect of leptin on functional activity of NK cells during pregnancy. The hormone in a concentration comparable to that during the first trimester of pregnancy increased the percentage of $CD16^+56^+$NK cells (Table 1). These changes correlated with the increased production of IFN-α by mononuclear leukocytes (R=0.76, p<0.05). However, leptin in a dose for the second-third trimesters of pregnancy was shown to decrease the number of NK cells with the $CD16^+56^+$ phenotype. It should be emphasized

TABLE 1. *In Vitro* Modulatory Effect of Leptin on the Expression of Membrane Molecules on Peripheral Blood Lymphocytes from Women (*M*±*m*, %)

Parameter	Control	Leptin, 10 ng/ml	Leptin, 35 ng/ml
T lymphocytes ($CD3^+CD19^-$)	72.22±9.24	71.12±7.40	70.07±9.13
T helper cells ($CD3^+CD4^+$)	46.87±5.03	47.20±5.07	46.52±6.13
Cytotoxic T lymphocytes ($CD3^+CD8^+$)	21.40±5.79	20.81±6.41	20.69±5.66
Activated T lymphocytes ($CD3^+HLA\text{-}DR^+$)	10.44±2.22	15.09±3.12*	14.31±3.17*
NK cells ($CD3^-CD16^+56^+$)	7.27±1.55	9.03±0.90*	5.42±1.34*+
NKT cells ($CD3^+CD16^+56^+$)	2.12±0.94	2.19±1.08	2.83±0.53*

Note. Here and in Table 2: $p<0.05$: *compared to the control; +compared to leptin in a concentration of 10 ng/ml (paired Student's *t* test).

TABLE 2. *In Vitro* Modulatory Effect of Leptin on Cytokine Production in Cultures of Peripheral Blood Mononuclear Cells from Women (*M*±*m*, pg/ml)

Parameter	Control	Leptin, 10 ng/ml	Leptin, 35 ng/ml
IL-2	6.98±0.18	8.77±1.37	6.53±0.14
TNF-α	1.33±0.07	3.77±0.94*	4.38±0.93*
IFN-α	3.58±0.55	6.07±0.59*	4.45±0.57
IL-4	2.52±0.11	4.02±0.78	4.68±1.89
IL-6	66.55±9.46	4773.01±1344.01*	1463.59±364.67*+
IL-10	5.33±0.09	5.88±0.43	5.43±0.13

that the subpopulation of peripheral blood NK cells is presented by $CD16^+56^+(CD16^+56^{dim})$ and $CD16^-56^+(CD16^-56^{bright})$ NK cells. $CD16^+56^+$ NK cells are most mature cells exhibiting maximum cytolytic activity [11,12]. Leptin probably has a modulatory effect on the expression of CD16 molecule on $CD56^+$ NK cells, which is associated with the regulation of their cytotoxic potential. The modulatory effect of leptin is probably realized via activation of IFN-α production by mononuclear phagocytes. Published data show that this hormone can directly increase the basal and induced cytotoxicity of NK cells, which is associated with stimulation of phosphatidylcholine-specific phospholipase C during ligation of the leptin receptor. This conclusion is derived from the fact that enzyme activation contributes to the transport of the CD16 molecule from cytoplasmic granules of the Golgi complex to the membrane of NK cells [4,15]. Therefore, leptin regulates functional activity of NK cells by modulating their cytolytic activity during pregnancy. The effects of leptin are realized in a direct contact with the cell or stimulation of IFN-α production. The leptin-induced increase in the count of $CD16^+56^+$ NK cells during the first trimester of pregnancy probably protects the maternal organism and fetus from microbial attack. These changes are of particular importance at the early stages of pregnancy [1,2]. Moreover, they can be associated with the necessity of increasing the pool of peripheral blood $CD16^+56^+$ NK cells due to further migration to the decidual membrane [11]. The inhibitory effect of leptin on $CD16^+56^+$ NK cells during the late stages of pregnancy probably contributes to fetal protection from cytotoxic NK cells.

Addition of leptin in a dose comparable to that during the first trimester of pregnancy had no effect on functional activity of $CD16^+56^+$ NKT cells. By contrast, leptin in a dose comparable to hormone concentration during the second-third trimesters was shown to increase the percentage of NKT cells expressing CD16 and CD56 (Table 1). A positive correlation was found between the increase in the number of $CD16^+56^+$ NKT cells and elevation of Th1 cytokines TNF-α (R=0.857, $p<0.05$) and IFN-α (R=0.96, $p<0.05$). These data suggest that the effects of leptin on NKT cells are due to induction of cytokine synthesis. The leptin-induced increase in the percentage of $CD16^+56^+$ NKT cells at the late stages of gestation probably reflects the induction of phenotypic maturation of NKT cells. Published data show that the formation of this phenotype occurs at the final stage of NKT cell activation and is associated with acquisition of the highest cytotoxic activity [10,13]. It should be emphasized that NKT cells constitute a subpopulation of regulatory T lymphocytes. Activation of these cells is followed by secretion of considerable amounts of IFN-γ and IL-4. In addition to dendritic cells, these cells play the major role in Th1/Th2 deviation and development of peripheral tolerance [3,10,12]. Peripheral blood NKT cells are important for implantation. They induce the Th2 cytokine microenvironment, which contains IL-4 in high concentration

[3]. The observed effects of leptin on $CD16^{+}56^{+}$ NKT cells probably contribute to the role of this hormone in the maintenance of cytokine balance and peripheral tolerance during pregnancy.

We conclude that leptin has a direct effect on target cell during pregnancy. The influence of leptin is realized at the posttranslational level or results from increased production of Th1 cytokines. Leptin activates T lymphocytes and contributes to phenotypic maturation of NK cells and NKT cells, which is associated with the increase in cytotoxic activity of these cells. Leptin serves as an immunoregulatory agent during pregnancy. Leptin regulates the expression of membrane molecules and production of cytokines by mononuclear leukocytes.

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