Boar seminal immunosuppressive fraction attenuates the leptin concentration and restores the thymus mass during pregnancy in mice

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Abstract

The immunosuppressive fraction (ISF) of boar seminal vesicle fluid was recently demonstrated to inhibit production of T helper (Th)1 cytokines and enhance production of Th2 cytokines. The present study shows the effect of the ISF on leptin concentrations in blood plasma and adipose tissue in mice during pregnancy. The ISF effect on thymus weight during pregnancy is also demonstrated. The leptin concentration in blood plasma and adipose tissue increased and remained high in the latter half of pregnancy. ISF treatment at the beginning of pregnancy significantly lowered the leptin concentration both in blood plasma and adipose tissue of pregnant mice. Thymus involution has been described previously in pregnant mice. ISF treatment compensated for the loss of thymus mass during the whole pregnancy in the ISF-treated mice. The treatment of pregnant mice with ISF did not affect pregnancy and litter size.

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Introduction

Leptin is essential for fertility in rodents (Zhang *et al.* 1994, Chehab *et al.* 1996). However, Mounzich *et al.* (1998) found that after conception, ob/ob mice could successfully establish and maintain pregnancy in the absence of leptin. Recently, Malik *et al.* (2001) demonstrated that exogenous leptin is necessary for successful implantation, but not for the maintenance of pregnancy beyond 7 days in mice. Leptin may promote vascular permeability (Cao *et al.* 2001), a key event in the process of decidualization and implantation (Rabbani & Rogers 2001), and directly influence the development of the embryo.

It is commonly accepted that maternal leptin concentrations are elevated during most of the pregnancy period in rats and mice and decline just prior to parturition (Kawai *et al.* 1997, Tomimatsu *et al.* 1997, Seeber *et al.* 2002). This is supported by the observations of Garcia *et al.* (2000) who found increased leptin mRNA concentrations both in placenta and maternal adipose tissues throughout gestation in the rat; it is also supported by Amico *et al.* (1998) who reported that placental leptin mRNA increased over the final third of rat pregnancy. However, Kawai *et al.* (1997) did not find expression of leptin mRNA in the rat placenta, but reported expression of leptin receptor mRNA in late gestation (Kawai *et al.* 1999). In mouse, leptin transcript

was abundant in maternal adipose tissue, placenta and in numerous fetal tissues (Hoggard *et al.* 2000), but leptin did not seem to be directly associated with conceptus growth or development.

Boar seminal vesicle fluid contains immunosuppressive fraction (ISF, Dostál *et al.* 1995). We previously demonstrated that ISF had an immunosuppressive effect on the lymphocytes (Dostál *et al.* 1997). ISF inhibited mitogen-stimulated proliferation of lymphocytes and attenuated production of T helper (Th)1 cytokines but increased production of Th2 cytokines, shifting the Th1/Th2 ratio in favor of Th2 (Veselský *et al.* 2003). In rats with adjuvant arthritis, the ISF treatment attenuated hind-paw edema and thymus involution and inhibited both IgG production and expression of interleukin-6 (IL-6) in peritoneal macrophages (Veselský *et al.* 2001).

The present study used pregnant mice as a model of the elevated leptin concentration. The study was undertaken to evaluate the effect of ISF on the concentration of leptin in the blood and adipose tissue of mice during pregnancy. It has been documented that pregnancy substantially lowers the weight of thymus (Endo & Kanayama 1998), which is linked to the expression of progesterone receptor in thymic stroma cells in pregnancy. Therefore, we followed the ISF influence on thymus weight during pregnancy.

Materials and Methods

Animals

BALB/c female mice (12 weeks old) were housed under controlled lighting (12 h light: 12 h darkness) at an ambient temperature of 22–23°C and humidity of 35–50%, with free access to food and water. They were mated overnight and the morning when the vaginal plug was found was designated as day 1 of pregnancy. The animals were bred in animal facilities of the Academy of Sciences of the Czech Republic in Prague and were treated in accordance with National Law 167/1993 on the use of laboratory animals.

Isolation of ISF from seminal vesicle fluid

The usual purification procedure was followed (Veselský *et al.* 2000). Briefly, 30 ml seminal vesicle fluid (containing about 6 g of protein) were precipitated in 8% ethanol at pH 7.2 and –2.5°C for 1 h. The precipitate was centrifuged (3000 *g*, 30 min, 4°C), dissolved in 100 ml water, lyophilized and dissolved in 16 ml water. Then 8 ml of solution were chromatographed on Sephacryl S-200 column (2.4 × 63 cm) and eluted with PBS at 16.8 ml/h. Fractions with immunosuppressive activity were pooled, desalted, lyophilized and dissolved in 4 ml water. Two milliliters of the solution were applied to the Sephadex G 75 column (1.4 × 94 cm) and eluted with PBS at a flow rate of 4.5 ml/h. Fractions with immunosuppressive activity were lyophilized and subjected to the Biocompatible Quaternary Gradient system of HPLC (Waters, Milford, MA, USA). RP HPLC was performed using a 218 TP 54 Vydac C₁₈ column (4.6 × 250 mm, 5 µm particle size). A sample of 1 mg in 1 ml of 0.05% (v/v) trifluoroacetic acid was applied and proteins were eluted by the linear gradient of 20–50% v/v acetonitrile at 1 ml/min for 60 min. Fractions with immunosuppressive activity were collected and lyophilized.

Sample collection and ISF treatment

Four experimental groups (ten mice in each time point) were established. Blood plasma, parametrial adipose tissue and thymus were obtained from decapitated animals.

Group 1 were naive virgin mice, group 2 were virgin mice that received intraperitoneally 150 µg ISF in 0.1 ml PBS for 3 days. Samples from decapitated animals were taken on days 1, 7 and 15 after the first injection of ISF.

Groups 3 and 4 were pregnant mice. Group 3 remained untreated, group 4 was injected intraperitoneally with 150 µg ISF in 0.1 ml PBS on days 1, 2 and 3 of pregnancy. Samples from decapitated animals were obtained on days 7, 10, 13, 15, 17 and 19 of pregnancy and day 23 (postpartum). Blood plasma and adipose tissue were frozen and stored at –20°C until use.

Adipose tissue extraction

For determination of the leptin concentration in adipose tissue, parametrial adipose tissue was collected from pregnant animals and virgin mice. The adipose tissue was homogenized in 100 mM NH₄HCO₃, 10 mM EGTA, 10 mM EDTA, pH 9.3 for 1 min. The homogenate was centrifuged at 5000 *g* for 20 min and the supernatant was used for the test. The leptin concentration in adipose tissue was expressed in nanograms per gram of wet tissue.

Leptin ELISA

Leptin concentrations in blood plasma and adipose tissue extract were determined by the ELISA kit using the procedure described by the manufacturer (Crystal Chem. Inc., Chicago, IL, USA).

Statistical analysis

Values represent the means ± s.d.

Data were analyzed for homogeneity of variance by ANOVA test. Subsequent significance of the differences between experimental and control groups was analyzed using Student's *t*-test.

Results

Leptin levels in blood plasma and adipose tissue of pregnant and virgin mice and the effect of ISF on leptin concentration

From day 10 of pregnancy to day 23 (postpartum), the blood plasma leptin level was significantly higher than that in virgin mice ($P < 0.01$). On day 15, the leptin level in pregnant mice (64.7 ± 11.1 ng/ml) was approximately 7 times higher than that in virgin mice (8.4 ± 2.4 ng/ml) or ISF-treated virgin mice (9.7 ± 2.4 ng/ml). After day 19 of pregnancy, the blood plasma leptin level declined sharply (Fig. 1).

ISF applied for the first 3 days of pregnancy significantly lowered the leptin concentration in blood plasma (from 64.7 ± 11.1 ng/ml in pregnant mice to 12.6 ± 7 ng/ml in pregnant mice treated with ISF as found out on day 15 of pregnancy). After that the leptin concentration in ISF-treated mice increased rapidly and exceeded that of pregnant untreated mice (Fig. 1).

The leptin concentration in adipose tissue of pregnant animals increased after day 13 of pregnancy and peaked on day 15 (41.3 ± 8.4 ng/g wet tissue), when it was about 4 times higher than that of naive virgin or ISF-treated virgin mice (9 ± 3.3 and 10.4 ± 2.8 ng/g wet tissue respectively). After that, it decreased linearly and on day 23 (postpartum) reached the values of naive virgin or ISF-treated virgin mice (Fig. 2).

ISF applied in the first 3 days of pregnancy significantly decreased the leptin level in adipose tissue (from 41.3 ± 8.4 ng/g wet tissue in pregnant mice to 14 ± 6 ng/g wet tissue in pregnant mice treated with ISF,

Figure 1 ISF maintained a low level of plasma leptin until 15 day of pregnancy. Blood was collected from pregnant mice (■) and from pregnant mice treated with ISF (◆) on indicated days and the plasma leptin concentration was assessed by sandwich ELISA. Leptin plasma concentrations of virgin mice were 8.5 ± 2.8 and 8.4 ± 2.4 ng/ml on days 7 and 15 respectively. Leptin plasma concentrations of ISF-treated virgin mice were 10.8 ± 1.8 and 9.7 ± 2.4 ng/ml on days 7 and 15 respectively. Values represent the means $\pm$ S.D. * $P < 0.01$ versus pregnant mice not treated with ISF.

as found out on day 15 of pregnancy). After that the leptin concentration increased and reached that of pregnant untreated mice on day 19 of pregnancy (Fig. 2).

In virgin mice, ISF did not significantly affect the leptin level in blood plasma and adipose tissue.

Thymus weight of pregnant and virgin mice and the effect of ISF on thymus weight during pregnancy

The weight of thymus of virgin mice was not significantly influenced by ISF treatment (40.7 ± 10.1 and

Figure 2 ISF maintained a low concentration of leptin in adipose tissue during pregnancy. Tissues were collected from pregnant mice (■) and from pregnant mice treated with ISF (◆) on indicated days, homogenized in extraction buffer and leptin concentration determined by sandwich ELISA. Leptin concentrations in adipose tissue of virgin mice were 9.5 ± 3.1 and 9.0 ± 3.3 ng/g wet tissue on days 7 and 15 respectively. Leptin concentrations in adipose tissue of ISF-treated virgin mice were 9.3 ± 3.0 and 10.4 ± 2.8 ng/g wet tissue on days 7 and 15 respectively. Values represent the means $\pm$ S.D. * $P < 0.01$ versus pregnant mice not treated with ISF.

35.7 ± 7.0 mg respectively on day 7 after the beginning of the treatment). The thymus weight was substantially lowered by pregnancy: from day 7 of pregnancy it decreased linearly and after delivery reached a minimum value of 10 ± 2.5 mg on day 23 (postpartum). The loss of thymus mass in pregnant mice was totally prevented by the ISF treatment until day 17 of pregnancy; then it was prevented only partially, but significantly (26.4 ± 5.8 mg in ISF-treated and 10 ± 2.5 mg in untreated postpartum mice; Fig. 3).

Effect of ISF treatment on litter size

ISF decreased leptin levels in both plasma and adipose tissue and totally prevented thymus involution until day 17 of pregnancy. In addition, it significantly decreased the loss of thymus mass even after delivery. Regardless of all these ISF effects, we did not find any deviations from normal pregnancy, including litter size (Table 1).

Discussion

In this study, we found a substantial increase in leptin concentration both in blood plasma and adipose tissue in the latter half of mouse pregnancy; these results corresponded with previous studies in mice (Tomimatsu *et al.* 1997) and in rats (Kawai *et al.* 1997). During mouse pregnancy, OB-R_e, a soluble form of leptin receptor is secreted into the peripheral circulation, where it has been suggested that it binds leptin and prevents it from binding to a signaling form of the receptor, thereby potentiating leptin resistance (Gavrilova *et al.* 1997, Mounzich *et al.* 1998). Leptin resistance in pregnancy is believed to ensure the demands of rapid fetal growth are met in the latter period of pregnancy (Henson & Castracane 2000).

Figure 3 The loss of thymus mass observed in pregnant mice was significantly recovered by treatment with ISF. In pregnant mice (■) and in pregnant mice treated with ISF (◆) the weight of thymus was assessed on the days indicated. Thymus weights of virgin mice were 36.2 ± 11.7 and 40.7 ± 10.1 mg on days 7 and 15 respectively. Thymus weights of virgin mice treated with ISF were 33.4 ± 5.7 and 36.0 ± 7.0 mg on days 7 and 15 respectively. Values represent the means $\pm$ S.D. * $P < 0.01$ versus pregnant mice not treated with ISF.

Table 1 Effect of decreased leptin level and increased thymus weight on litter size in mice. Values represent the means $\pm$ s.d. for ten mice decapitated in one time interval.

Days of pregnancy	Pregnant + ISF	Pregnant
10	5.2 $\pm$ 1.5	4.9 $\pm$ 1.5
13	5.9 $\pm$ 1.6	5.6 $\pm$ 1.6
15	5.2 $\pm$ 1.4	6.5 $\pm$ 1.5
17	5.1 $\pm$ 1.9	5.1 $\pm$ 1.4
19	6.2 $\pm$ 0.8	6.0 $\pm$ 2.0
23 (postpartum)	5.4 $\pm$ 1.7	5.0 $\pm$ 2.0

ISF, immunosuppressive fraction of boar seminal plasma.

Although ISF caused a decrease in leptin concentration and attenuation of thymus involution until day 17 of pregnancy, it did not hamper implantation and development of the fetuses. Our previous results indicated that ISF adsorbed on the zona pellucida of porcine or murine oocytes had no effect on the embryo and fetus development *in vitro* or *in vivo* (Veselský *et al.* 1991). This was confirmed by the fact that monoclonal antibodies to ISF did not affect the ability of boar spermatozoa to penetrate porcine zona pellucida (Veselský *et al.* 1993).

We found that a short-term treatment with ISF after immunization led only to a temporary suppression of immune response but not to a permanent tolerance to antigens (Dostál *et al.* 1997). We observed that ISF persisted in circulation for 15 days (Dostál *et al.* 1995). This could explain the fact that in this study, after day 15 of pregnancy and after clearing of ISF from the circulation, leptin concentration increased.

We suppose that the ISF-induced decrease in leptin concentration is responsible for the inhibition of thymus involution. In normal pregnancy, the Th2 immune response with preferential production of IL-4, IL-5 and IL-10 cytokines occurs while Th1 cytokines such as interferon (IFN)- γ and IL-2 or tumor necrosis factor (TNF)- α are down-regulated. If the cytokine pattern of the normal pregnancy was shifted, pregnancy failed both in rodents and humans (Tangri & Raghupathy 1993, Hill *et al.* 1995, Raghupathy 2001). However, new data demonstrated that neither maternal nor fetal production of Th2 cytokines IL-4 and IL-10 was crucial for the successful completion of pregnancy in mice (Svenson *et al.* 2001). The effect of cytokines on leptin production may be more complicated. It has been shown that TNF- α significantly inhibited leptin production by cultured adipocytes and pre-adipocytes differentiated *in vitro* (Fawcett *et al.* 2000). *In vivo* TNF- α and IL-1 α increased serum leptin concentration in humans (Janik *et al.* 1997, Zumbach *et al.* 1997). *In vivo* cytokine-induced release of leptin into serum may not result from a direct effect of the cytokines on the adipose tissue and we do not suppose that the decrease of leptin concentration in this study is caused by an ISF effect on the Th1/Th2 pattern. Leptin secretion both *in vivo* and *in vitro* is potently stimulated by glucocorticoids (Fried *et al.* 2000) and 11 β -hydroxysteroid dehydrogenase has been recognized

as a major modulator of local glucocorticoid concentration in adipose tissue (Sandeep & Walker 2001). Local glucocorticoid concentrations may therefore play an important role in the regulation of leptin synthesis and release. Recently we documented that high concentrations of corticosterone in arthritic rats were suppressed by ISF treatment (Veselský *et al.* 2001). We suggest that the ISF-induced inhibition of glucocorticoid synthesis also inhibits leptin release from adipose tissue.

We did not record any effect of the ISF-blocked leptin secretion, both in plasma and adipose tissue, on fetal weight, survival of neonatal descendants or the number of neonatals. The weight of fetuses depended on the number of fetuses implanted in the uterus but not on the leptin concentration: the higher the number of fetuses in the uterus, the lower their individual weights. We did not find any malformation or deviation from normal physiological development of descendants caused by ISF-induced leptin reduction.

In this study, ISF lowered the concentration of leptin in blood plasma and adipose tissue in the first period of pregnancy and prevented thymus involution during pregnancy. ISF did not affect pregnancy, delivery or litter size, confirming that leptin is not essential to the maintenance of pregnancy in mice.

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