

The role of leptin during the development of mouse preimplantation embryos

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Abstract

Leptin is known to regulate diverse reproductive functions, and recent studies have implicated involvement of leptin in the early mouse embryo development. The aim of the present study was to investigate the expression of leptin and its functional receptor (OB-Rb) in mouse oocyte and preimplantation embryo, and to examine whether leptin influenced the early embryo development. Leptin mRNA was detected in blastocyst and hatched blastocyst, and OB-Rb mRNA was detected in oocytes, 1-cell, 2-cell, morula, blastocyst and hatched blastocyst. As for the origin of leptin, leptin mRNA was identified in both the oviduct and uterus of the pregnant mouse. Furthermore, in the pregnant mouse, the levels of leptin in uterine fluid were higher than those in the non-pregnant mouse. Supplementation of culture medium with leptin promotes the development of preimplantation embryos from 2-cell stage to the blastocysts, fully expanded blastocysts and hatched blastocysts. Leptin significantly increased the total cell number of blastocysts, and the effect was preferentially observed in the trophectoderm. These findings raise the possibility that leptin regulates the development of mouse preimplantation embryo through a paracrine pathway.

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1. Introduction

Leptin is a peptide hormone mainly secreted by adipose tissue, and plays a key role to regulate body weight and energy expenditure (Zhang et al., 1994; Caro et al., 1996; Pelleymounter et al., 1995; Halaas et al., 1995; Campfield et al., 1995). In addition, leptin is known to act as a modulator of diverse reproductive functions (reviewed by Holness et al., 1999; Gonzálets et al., 2000). The leptin receptor (OB-R) is a member of the class I cytokine receptor superfamily (Tartaglia et al., 1995), and alternatively spliced into five isoforms from a single gene, i.e. OB-Ra, -Rb, -Rc, -Rd, and -Re (Lee et al., 1996). Among these splice variants, only the full-length form, OB-Rb, is capable to mediate signal

transduction through both a signal transducer and activator of transcription-3 (STAT3) and mitogen-activated protein kinase pathways (Ghilardi et al., 1996; Baumann et al., 1996; Vaisse et al., 1996; Bjørbæk et al., 1997).

In recent studies, it has been demonstrated that leptin and OB-R were present in human and mouse oocytes and preimplantation embryos detected by immunohistochemical staining (Cioffi et al., 1997; Antczak and Van Blerkom, 1997). Leptin at physiological concentrations causes tyrosine phosphorylation of STAT3 in mouse metaphase 2 stage oocytes (Matsuoka et al., 1999). These results suggest that leptin could directly exert its biological effects on mammalian oocytes and preimplantation embryos through the activation of STAT3 signal transduction pathway. In the present study, we sought to determine the biological effects of leptin on mouse preimplantation embryo development.

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2. Materials and methods

2.1. RT-PCR and nested PCR

The methods of Reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR for oocytes and preimplantation embryos at different stages (1-cell, 2-cell, 4-cell, 8-cell, morula, blastocyst and hatched blastocyst) were described previously (Kawamura et al., 2000, 2001). For positive controls, mouse adipose tissue cDNA was amplified simultaneously. For negative controls, the specimen in which water was substituted for mRNA was amplified. Due to the low expression of leptin and OB-Rb mRNAs in oocytes and preimplantation embryos, nested PCR was needed to obtain optimal results. Furthermore, total RNA was extracted from oviducts and uterus of pregnant mice on days 1 after mating, and RT-PCR for leptin was performed. To confirm identity, PCR products were subcloned and sequenced.

2.2. Enzyme-linked immunosorbent assay

Leptin concentrations in plasma, oviduct and uterine fluid were measured using a Mouse Leptin Enzyme-linked immunosorbent assay kit (Morinaga, Tokyo, Japan) with a sensitivity of 200 pg/ml, and the intra- and inter-assay coefficients of variation of the assay were less than 10%. For measurement of leptin in the oviduct and the uterine fluid, oviducts and uteri were collected from five virgin mice 4 weeks of age, non-pregnant mice 9 weeks of age at estrus and pregnant mice 9 weeks of age on days 1 and 4.5 after mating, which corresponded to the embryonic stage of 2-cell and hatched blastocyst, respectively. Each uterine and oviduct fluid was obtained by flushing the uterine and oviduct cavities with 100 μ l of 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate buffer (Sigma, St. Louis, MO) and centrifuged at $2000 \times g$ for 5 min. Each supernatant was concentrated to 10 μ l using Microcon-10 (Millipore, Bedford, MA) and the samples were stored at -20°C until assay. The plasma samples were obtained from the same mice described above and stored at -20°C until assay.

2.3. Embryo collection and culture

Female IVCS mice, aged 9 weeks, (Institute for Animal Reproduction, Ibaragi, Japan) were superovulated by 5 IU of pregnant mare serum gonadotropin (Sigma) followed at 48 h later by 5 IU of human chorionic gonadotropin (hCG) (Sigma). Two-cell stage embryos were collected from the oviducts of the mated female mice 46–47 h after hCG injection. Groups of 10–15 embryos randomly selected were placed in 30 μ l drops of HTF medium without serum (Quinn et al.,

1985) with or without recombinant leptin (Sigma) (1, 10, 100, and 1000 ng/ml) covered by mineral oil. Embryos were cultured over 72 h up to the hatched blastocyst stage at 37°C in 5% CO_2 in air. All procedures involving the care and use of animals were approved by the Animal Research Committee at Akita University School of Medicine.

2.4. Differential labeling of ICM and TE nuclei

Embryos at 2-cell stage were cultured with or without 100 ng/ml of leptin for 56 h, and the numbers of inner cell mass (ICM) and trophectoderm (TE) cells of each blastocyst were counted by the differential labeling technique using two polynucleotide-specific fluorochromes (propidium iodide and bisbenzimidazole; Hoechst 33342, Sigma) as described previously (Handyside and Hunter, 1984).

2.5. Statistical analysis

The one-way ANOVA followed Fisher's protected least significant difference (PLSD) test was utilized to evaluate the effect of leptin on the development of embryos with dose-dependent manner and the differences in leptin protein concentrations in uterine fluid. The Mann–Whitney *U*-test was carried out in the comparison of the number of total blastomeres, ICM, and TE cells.

3. Results

3.1. Expression of leptin, and OB-Rb mRNAs in mouse oocytes and preimplantation embryos

Using nested PCR, Leptin mRNA was detected in blastocyst and hatched blastocyst stage embryos as a 309-bp band, whereas OB-Rb mRNA was detected in oocytes, 1-cell, 2-cell, morula, blastocyst and hatched blastocyst stage embryos as a 137-bp band (Fig. 1a). Leptin mRNA was detected in both oviduct and uterus as a 441-bp band by RT-PCR (Fig. 1b). All PCR products were validated by sequence analysis (data not shown). Experiments in the present study were performed three times, and the results were found to be reproducible.

3.2. Detection of leptin in mouse oviduct and uterine fluids

There were no significant differences in plasma leptin concentrations among IVCS virgin mice 4 weeks of age, non-pregnant mice 9 weeks of age at estrus and pregnant mice 9 weeks of age on days 1 and 4.5 (3.88 ± 0.39 , 3.71 ± 0.24 , 3.57 ± 0.24 , 3.64 ± 0.20 ng/ml, respectively).

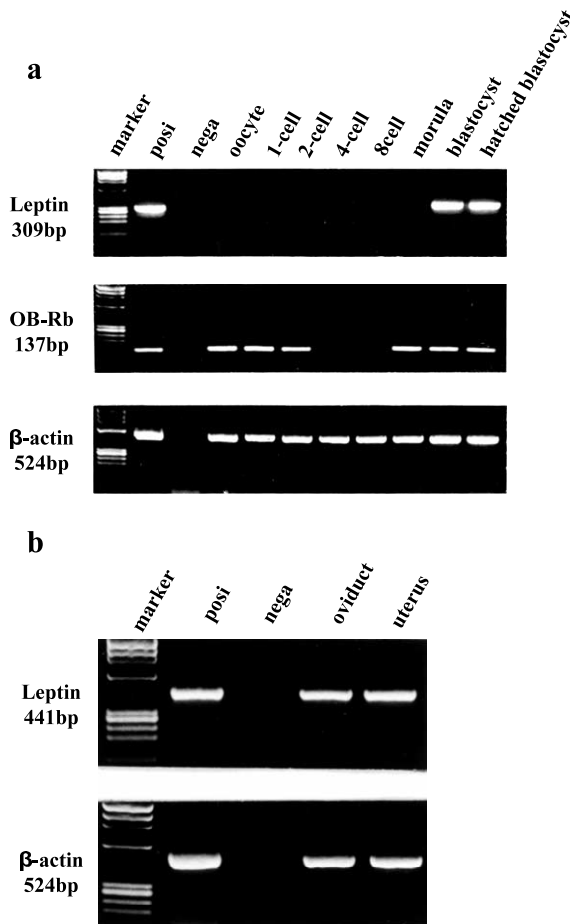


Fig. 1. RT-PCR analysis of leptin and OB-Rb mRNAs from mouse oocytes and preimplantation embryos at different stages (1-cell, 2-cell, 4-cell, 8-cell, morula, blastocyst, and hatched blastocyst). For internal control, β -actin was amplified simultaneously in each PCR reaction. Due to the low expression of leptin and OB-Rb mRNAs in oocytes and preimplantation embryos, nested PCR was needed to obtain optimal results. Marker, ϕ x 174-Hae III digest; posi, positive control for leptin; OB-Rb, and β -actin, mouse adipose tissue cDNA; neg, negative control, distilled water; bp, base pair. (From Kawamura, K., Sato, N., Fukuda, J., Kodama, H., Kumagai, J., Tanikawa, H., Nakamura, A., Tanaka, T., 2002. Leptin promotes the development of mouse preimplantation embryos in vitro. *Endocrinology* 143 (5), 1922–1931, With permission from the Endocrine Society.)

The levels of leptin in uterine fluid of pregnant mice on days 1 and 4.5 (2.16 ± 0.17 and 2.80 ± 0.13 ng/ml, respectively) were significantly higher than those of virgin mice (0.43 ± 0.50 ng/ml) ($P < 0.0001$) or non-pregnant mice (0.70 ± 0.60 ng/ml) ($P < 0.0001$). Leptin was not detected in the oviduct fluid.

3.3. Leptin promote preimplantation embryo development to the blastocyst, fully expanded blastocyst and hatched blastocyst in vitro

In each experiment, 15–26 embryos were employed in each group, consisting of exactly six observations, and the experiment was repeated five times. Finally, a total

of 100–156 embryos were examined in each group, and the results were shown in Fig. 2. The P -values of one-way ANOVA revealed the significant of dependency on leptin concentration in all stages ($P = 0.0000$, $P = 0.0001$, and $P = 0.0000$ for blastocyst, expanded blastocyst, and hatched blastocyst, respectively). Fisher's PLSD test showed that 1 ng/ml of leptin did not promote the development of all stages of embryos, whereas 10, 100, and 1000 ng/ml significantly promote the development of blastocyst, expanded blastocyst, and hatched blastocyst stage embryos. Furthermore, the most positive effect of leptin on the development of embryos was observed at expanded blastocyst stage.

3.4. Leptin increases the cell numbers of cultured mouse blastocyst

The numbers of total, TE and ICM cells of blastocyst after 56 h of culture with or without leptin are; 56.5 ± 2.7 or 45.6 ± 2.5 , 39.9 ± 1.5 or 33.0 ± 1.5 , and 16.6 ± 1.2 or 12.6 ± 1.2 , respectively. Blastocysts cultured with 100 ng/ml of leptin had a significantly higher total cell number, as compared with blastocysts cultured in HTF medium alone ($P < 0.005$). The increase in the total cell number of leptin-stimulated blastocysts was resulted from proliferation of both ICM and TE cells, and the proliferation of TE cells ($P < 0.005$) was more evidently observed as compared to that in ICM cells ($P < 0.05$).

4. Discussion

In the present study, OB-Rb mRNAs were detected in oocytes, 1-cell, 2-cell, morula, blastocyst and hatched blastocyst stage embryos, whereas leptin mRNA was absent from oocytes up to morula stage embryos. Thus, in mouse oocytes and early preimplantation embryos, an exogenously secreted leptin is considered to bind the OB-Rb and cause its biological effect. Furthermore, we have shown that leptin mRNA is expressed in mouse blastocyst and hatched blastocyst stage embryos. It has been reported that cultured human blastocysts secrete leptin, and the levels of leptin are significantly higher than that of arrested embryos (González et al., 2001). Therefore, it is likely that leptin may be produced by maternal organ(s) and/or these embryos themselves, and act in both paracrine/autocrine manner to regulate biological functions.

To confirm whether leptin was produced and secreted during early embryogenesis by the reproductive tract, (i) the presence of leptin mRNA in uterus and oviduct was examined, and (ii) the levels of leptin in plasma, oviduct and uterine fluid were measured. In human, because leptin mRNA and protein were detected in human secretory endometrium by RT-PCR, immunohistochemistry and Western blot analysis, leptin is suggested to be

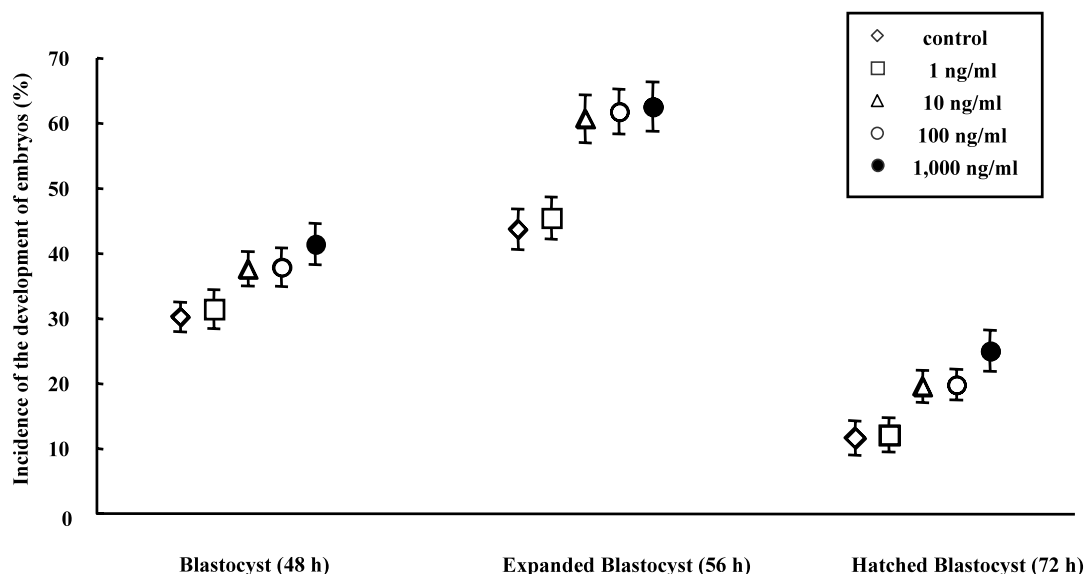


Fig. 2. The dose-dependent effects of leptin on the development of mouse preimplantation embryos. A total 100–156 embryos were examined in each group; 0 ng/ml = 156, 1 ng/ml = 150, 10 ng/ml = 100, 100 ng/ml = 110, and 1000 ng/ml = 127. Values are mean $\pm$ S.E.M. *, $P < 0.05$; **, $P < 0.01$; † $P < 0.005$; ‡, $P < 0.001$ when compared with control (0 ng/ml of leptin). (From Kawamura, K., Sato, N., Fukuda, J., Kodama, H., Kumagai, J., Tanikawa, H., Nakamura, A., Tanaka, T., 2002. Leptin promotes the development of mouse preimplantation embryos in vitro. *Endocrinology* 143 (5), 1922–1931, With permission from the Endocrine Society.)

produced by endometrium (González et al., 2001). In the present study, leptin mRNA was expressed in both oviduct and uterus of pregnant mice. Although there were no significant differences in plasma leptin concentrations among virgin mice, non-pregnant mice, and pregnant mice, the levels of leptin in uterine fluid of pregnant mice were significantly higher than those of virgin mice and non-pregnant mice. Because of very little amount of oviduct fluid, the levels of leptin in oviduct samples were below the sensitivity. These findings suggest that leptin is produced and secreted by at least pregnant endometrial epithelium, and preimplantation embryos could directly contact with leptin secreted from the reproductive tract. Further studies are needed to elucidate the mechanism(s) of up-regulation of leptin secretion in endometrium during early pregnancy.

We demonstrated co-culture of leptin promoted the development from 2-cell stage embryo to the blastocyst, fully expanded blastocyst and hatched blastocyst with dose-dependent manner. The effect of leptin was most evident at the expanded blastocyst stage after 56 h of culture, whereas it was decreased after 48 h of culture until blastocyst, or 72 h of culture until hatched blastocyst. Because the level of OB-Rb mRNA was increased from morula stage, embryos at blastocyst stage may not have enough time to respond to leptin as a ligand for OB-Rb. During longer culture period until hatched blastocyst stage, the saturation of receptors with its ligands may lead to an attenuation of the effect on preimplantation embryos.

In this study, leptin significantly increased the total cell number of blastocysts, and the effect was preferentially observed in the TE in mouse blastocysts, leptin and STAT3 were detected in mural and polar TE cells and were not detected in ICM cells (Antczak and Van Blerkom, 1997). Thus, the preferential effect of leptin on TE cells rather than ICM cells may be attributable to the different expression or sensitivity of OB-R and STAT3 in signal transduction pathway of leptin between TE and ICM cells.

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