

Leptin Promotes the Development of Mouse Preimplantation Embryos *in Vitro*

KAZUHIRO KAWAMURA, NAOKI SATO, JUN FUKUDA, HIDEYA KODAMA, JIN KUMAGAI, HIDEO TANIKAWA, AKIRA NAKAMURA, AND TOSHINOBU TANAKA

Department of Obstetrics and Gynecology (K.K., N.S., J.F., J.K., H.T., T.T.), Department of Medical Information Science (A.N.), Akita University School of Medicine, and Akita University College of Allied Medical Science (H.K.), Akita 010-0041, Japan

Leptin acts as a modulator of diverse reproductive functions, and recent studies have implicated involvement of leptin in the early embryo development in mammal. The aim of this study was to investigate the expression of leptin and its receptor (OB-R) 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 two splice variants of OB-R (OB-Ra and OB-Rb) mRNAs were detected in oocytes, 1-cell, 2-cell, morula, blastocyst, and hatched blastocyst. As for the origin of leptin, both leptin mRNA and protein were identified in the oviduct epithelium and endometrium of

pregnant mouse. In the pregnant mouse, the levels of leptin in uterine fluid were higher than those in nonpregnant mouse. Addition of leptin to embryo culture media promotes the development from 2-cell stage embryos to the blastocysts, fully expanded blastocysts and hatched blastocysts. This effect was neutralized by an antibody against the extracellular domain of OB-R. Leptin significantly increased the total cell number of blastocysts, and the effect was preferentially observed in the trophectoderm. These findings raise the possibility of a paracrine/autocrine leptin signaling system regulating the development of mouse preimplantation embryo. (Endocrinology 143: 1922–1931, 2002)

LEPTIN, THE PRODUCT of *obese (ob)* gene, is a 16-kDa nonglycosylated peptide hormone mainly secreted by adipose tissue, and it plays a key role to regulate body weight and energy expenditure (1–5). In addition, accumulating evidence indicates that leptin acts as a modulator of reproductive function (reviewed in Refs. 6 and 7). The *ob/ob* mutant mice, which exhibit a congenital deficiency in leptin, are sterile (8). This sterility is recovered by exogenous leptin administration but not by restriction of food intake (9, 10). Treatment of *ob/ob* female mice with leptin causes an elevation of the level of plasma LH and increases in ovarian and uterine weight (10). The concomitant injection of leptin to LH could inhibit ovulation of immature rats (11). The prepubertal leptin surge may trigger the onset of puberty (12–15), and administration of leptin could accelerate onset of puberty in female mice (16, 17). During the menstrual cycle, serum leptin concentrations were lowest in the early follicular phase and increased during the luteal phase with a close correlation with serum progesterone levels (18, 19). Furthermore, abnormal regulation of the leptin system may be associated with human menstrual abnormalities (20), including polycystic ovary syndrome (21, 22).

The leptin receptor (OB-R), the product of *diabetes (db)* gene, is a member of the class I cytokine receptor superfamily (23). Mouse OB-R is alternatively spliced into five isoforms from a single gene (24). The full-length form, referred to as OB-Rb, has an intracellular domain that mediates signal transduction through both a signal transducer and activator of transcription-3 (STAT3) and MAPK pathways (25–28).

Abbreviations: ICM, Inner cell mass; OB-R, leptin receptor; PLSD, protected least significant difference; STAT3, signal transducer and activator of transcription-3; TE, trophectoderm.

OB-Rb is highly expressed in the hypothalamus and plays a key role in the control of energy balance (24, 25). In several peripheral tissues, including the reproductive tissues, adipose tissue, hematopoietic tissues, endothelial cells, pancreatic β cells, brain, and lung, OB-Rb is also expressed and may contribute to a variety of biological effects (6, 7, 29–38). Two other types of OB-R isoforms have been reported; short (OB-Ra, -Rc, and -Rd) and soluble (OB-Re) isoforms lacking the intracellular domain (25). Among these isoforms, OB-Ra is most abundantly expressed in central and peripheral tissues and is considered to mediate leptin transport from peripheral blood to hypothalamus across the blood-brain barrier (39, 40). On the other hand, OB-Re may modulate leptin's biological activity by controlling the amount of unbound leptin in circulation (41).

Several reports demonstrated that leptin protein was detected in human and mouse oocytes, and preimplantation embryos by immunohistochemical staining (42, 43). Recently, Antczak and Van Blerkom (43) reported that leptin from maternal origin and STAT3 were immunolocalized in human and mouse oocytes in a polarized manner, and differentially allocated between blastomeres in mouse preimplantation embryos. Presence of Ob-R protein and mRNA were demonstrated in mouse oocytes (44), and presence of Ob-R protein was shown in preimplantation embryos (43). Leptin at physiological concentrations caused tyrosine phosphorylation of STAT3 in mouse metaphase 2 stage oocytes (44). 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 1) the temporal

expression of leptin, OB-Ra, and Ob-Rb mRNAs in mouse oocytes and preimplantation embryos up to the hatched blastocyst stage by RT-PCR; 2) whether leptin is secreted by the reproductive tracts; and 3) the biological effects of leptin on mouse preimplantation embryo development.

Materials and Methods

RT-PCR and nested PCR

The methods of RT-PCR and nested PCR for oocytes and preimplantation embryos were described previously (45, 46). Briefly, poly (A)⁺ mRNA was isolated from 15 mouse oocytes or preimplantation embryos of each stage (1-cell, 2-cell, 4-cell, 8-cell, morula, blastocyst, and hatched blastocyst stage), and each mRNA was reverse transcribed into cDNA. Exogenous rabbit α -globin mRNA (Life Technologies, Inc., Rockville, MD) was added to each sample before mRNA extraction to evaluate the efficiency of mRNA extraction and the RT procedure. The amount of cDNA subjected to each PCR reaction was equivalent to the number of genomes (*e.g.* one 2-cell stage embryo or one quarter of an 8-cell stage embryo), so that each PCR product was derived from the same number of transcribing genomes.

The primers for leptin, OB-Ra, and OB-Rb were made based on published sequences (1, 25) as shown in Table 1. The PCR was performed according to the programs described in the legend of Table 1. 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, OB-Ra 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 d 1 after mating, and RT-PCR for leptin was performed according to the programs described in legend of Table 1.

The PCR products were separated by 2% agarose gel electrophoresis (Agarose-LE, Nacalai Tesque, Inc., Kyoto, Japan) in the presence of ethidium bromide solution (Sigma, St. Louis, MO), and visualized with an UV transilluminator (Funakoshi, Tokyo, Japan). To confirm identity, PCR products were sequenced on an ABI 100 DNA sequencer (PE Applied Biosystems, Tokyo, Japan).

Embryo collection and culture

Female IVCS mice, aged 9 wk (Institute for Animal Reproduction, Ibaragi, Japan), were superovulated with ip injections of 5 IU of PMSG (Sigma) followed at 48 h later by 5 IU of human CG (Sigma). Two-cell stage embryos were collected from the oviducts of the mated female mice 46–47 h after human CG injection. The embryos were washed three times with M2 medium (Sigma). Groups of 10–15 embryos randomly selected were placed in 30- μ l drops of HTF medium (47) with or without recombinant leptin (Sigma) (1, 10, 100, and 1,000 ng/ml) covered by

mineral oil. Embryos were cultured over 72 h up to the hatched blastocyst stage at 37 C in 5% CO₂ in air.

To examine whether the effects of leptin on preimplantation embryos were mediated through OB-R, embryos were cultured in HTF medium containing 0.1 μ g/ml of goat anti-OB-R antibody raised against the extracellular domain of the receptor (K-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with or without 1,000 ng/ml of leptin. For controls, embryos were cultured in HTF medium containing the same concentration of goat IgG and goat IgG with 1,000 ng/ml of leptin. Embryonic development was monitored daily by phase-contrast microscopy (Olympus Corp., Tokyo, Japan) and the rate of development of embryos was assessed under various culture conditions.

All procedures involving the care and use of animals were approved by the Animal Research Committee at Akita University School of Medicine.

Immunohistochemistry

Oviducts and uteri were obtained from virgin mice 4 wk of age, nonpregnant mice 9 wk of age at estrus, and pregnant mice 9 wk of age on d 1 and 4.5 after mating, and fixed with 4% paraformaldehyde in PBS for 6 h at 4 C. Fixed frozen tissue sections were blocked with 10% normal goat serum (DAKO Corp., Kyoto, Japan) for 30 min at room temperature. Samples were incubated with 0.2 μ g/ml of rabbit antimouse leptin antibody (Sigma) in 1% PBS-BSA/0.1% Triton X-100 (Sigma), overnight at 4 C. After three washes in cold PBS, samples were incubated with 1.0 μ g/ml of goat antirabbit Cy3 fluorescein antibody (Chemicon, Temecula, CA) in 1% PBS-BSA/0.1% Triton X-100, for 1 h at room temperature in the dark. After three washes in cold PBS, slides were covered in a drop of anti-fade mounting medium (DAKO Corp.) and analyzed under an epifluorescence microscope (Olympus Corp.). For negative controls, control sections were subjected to the same method, except that the primary antibodies were replaced by the same concentrations of rabbit IgG (DAKO Corp.) and by the primary antibodies that were preabsorbed by purified leptin (Sigma) at 50 μ g/ml.

ELISA

Leptin concentrations in plasma, oviduct and uterine fluid were measured using a mouse leptin ELISA kit (Morinaga, Tokyo, Japan) with a sensitivity of 200 pg/ml, and the intraassay and interassay 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 at 4 wk of age, nonpregnant mice 9 wk of age at estrus, and pregnant mice 9 wk of age on d 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) and centrifuged at 2,000 \times g for 5 min. Each supernatant was concentrated to

TABLE 1. Primers used for RT-PCR and nested PCR, PCR cycles and temperatures for amplification of the different cDNA

Transcript	PCR round		Primer sequence (5'-3')	Size of product (bp)
Leptin	1st	Sense	GTGCCTATCCAGAAAGTCCAG	441
		Antisense	TCAGCATT CAGGGCTAACATC	
	2nd	Sense	TTACACACGCAGTCGGTAT	309
		Antisense	CTCAAAGCCACCACCTCTGT	
OB-Ra	1st	Sense	ACACTGTTAATTTACACCAGAG	237
		Antisense	AGTCATTCAAACCATAGTTTAGG	
	2nd	Sense	TGGGCACAAGGACTGAATTT	165
		Antisense	AGTCATTCAAACCATAGTTTAGG	
OB-Rb	1st	Sense	TGGCCACAATGTAGGTTTGA	428
		Antisense	TGGGATCGGATTTTATTACG	
	2nd	Sense	TAATGGTGTGACGGTTGCTG	137
		Antisense	CCCTGCAGTTTGTATGTGGA	
β -Actin		Sense	GGACCTCACTGACTACCTCATGAA	524
		Antisense	GGTGAAGGTGGTCAACACCTAG	
α -Globin		Sense	GCAGCCACGGTGGCGAGTAT	257
		Antisense	GTGGGACAGGAGCTTGAAT	

PCR cycles: denaturation at 94 C for 30 sec, annealing 55 C for 30 sec, extension at 72 C for 30 sec, total 35 cycles were performed.

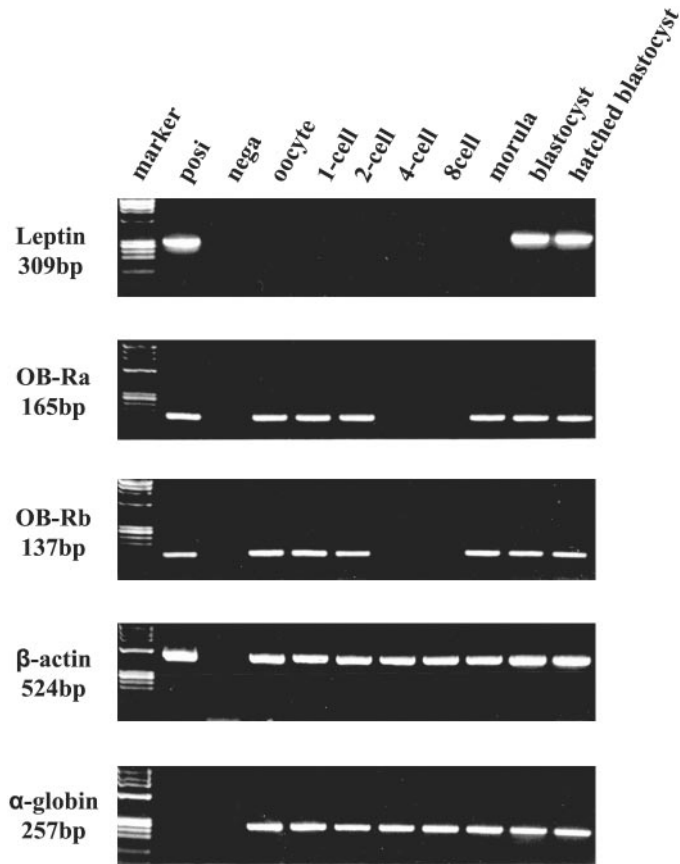


FIG. 1. RT-PCR detection of leptin, OB-Ra, and OB-Rb mRNAs from mouse oocytes and preimplantation embryos. Each of 15 oocytes and embryos at different stages (1-cell, 2-cell, 4-cell, 8-cell, morula, blastocyst, and hatched blastocyst) was used to mRNA extraction. To compare amounts of each PCR product from the same number of actively transcribing genomes, the amount of cDNA for each PCR reaction was corrected by the genome copies. Exogenous α -globin mRNA was added to each sample before mRNA extraction to evaluate the efficiencies of mRNA extraction and RT. For internal control, β -actin was amplified simultaneously in each PCR reaction. Due to the low expression of leptin, OB-Ra and OB-Rb mRNAs in oocytes and preimplantation embryos, nested PCR was needed to obtain optimal results. The expected 309-bp leptin product is present at blastocyst and hatched blastocyst stage embryos, and both 165-bp OB-Ra and 137-bp OB-Rb products are present at oocytes, 1-cell, 2-cell, morula, blastocyst, and hatched blastocyst stage embryos. No significant differences are observed in the band intensities of α -globin and β -actin amplification products among oocytes and embryos at different stages. Marker: ϕ x 174-*Hae*III digest; posi, positive control for leptin, OB-Ra, OB-Rb, and β -actin = mouse adipose tissue cDNA; neg, negative control = distilled water.

10 μ l using Microcon-10 (Millipore Corp., 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.

Differential labeling of inner cell mass (ICM) and trophoblast (TE) nuclei

Embryos at 2-cell stage were cultured with or without 100 ng/ml of leptin for 56 h, and the numbers of ICM and 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 (48). Briefly, the zona pellucida were removed in acid Tyrode's solution (Sigma), incubated in antimouse serum (Sigma) for 10 min at 37 C, and exposed to a 1:5

guinea-pig complement solution (sigma) containing 10 μ g/ml of propidium iodide for 5 min at 37 C. Then, after washing PBS, they were transferred to absolute ethanol with 25 μ g/ml of bisbenzimidazole, and left overnight at 4 C. After staining, the blastocysts were mounted on a glass slide, and a number of total, TE and ICM cells in each blastocyst was counted under an epifluorescence microscope (Olympus Corp.). The nuclei stained in pink were scored as TE cells, whereas blue nuclei were scored as ICM cells.

Statistical analysis

Two types of statistical analysis were employed: 1) To analyze the effect of leptin on the development of embryos with dose-dependent manner, one-way ANOVA followed Fisher's protected least significant difference (PLSD) test as well as trend analysis (49–51) were carried out. 2) To analyze the effects of anti-OB-R antibody on the development of embryos, ordinal unpaired comparison, *t* test, were performed on all of the possible pairs.

The one-way ANOVA was used to evaluate differences in leptin protein concentrations in uterine fluid, and the Mann-Whitney *U* test was carried out in the comparison of the number of total blastomeres, ICM, and TE cells.

Results

The temporal expression of leptin, OB-Ra, and OB-Rb mRNAs in mouse oocytes and preimplantation embryos

RT-PCR and nested PCR were performed to detect mRNAs for leptin and two splice variant forms of the leptin receptor (OB-Ra and OB-Rb) in the mouse oocytes and embryos at different stages (1-cell, 2-cell, 4-cell, 8-cell, morula, blastocyst and hatched blastocyst). Leptin mRNA was detected in blastocyst and hatched blastocyst stage embryos as a 309-bp band (Fig. 1). Both OB-Ra and OB-Rb mRNAs were detected in oocytes, 1-cell, 2-cell, morula, blastocyst, and hatched blastocyst stage embryos as 165-bp and 137-bp bands, respectively (Fig. 1). All PCR products were validated by sequence analysis (data not shown). The expression of β -actin and α -globin mRNAs was detected in the first PCR amplification, and no significant differences were observed in the band intensities of α -globin and β -actin amplification products among oocytes and preimplantation embryos at different stages. Experiments in the present study were performed three times on five separate pools of 15 oocytes and

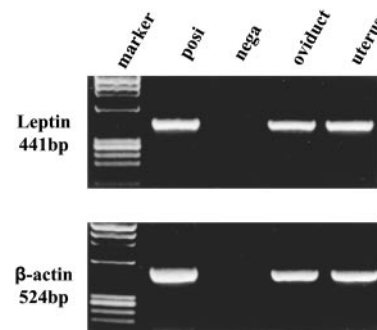


FIG. 2. RT-PCR detection of leptin mRNA from oviducts and uterus of pregnant mice on d 1. Total RNA was extracted from oviducts and uterus of pregnant mice on d 1, and RT-PCR was performed. For internal control, β -actin was amplified simultaneously in each PCR reaction. The expected 441-bp leptin product is present at both oviducts and uterus. Marker: ϕ x 174-*Hae*III digest; posi, positive control for leptin and β -actin = mouse adipose tissue cDNA; neg, negative control = distilled water.

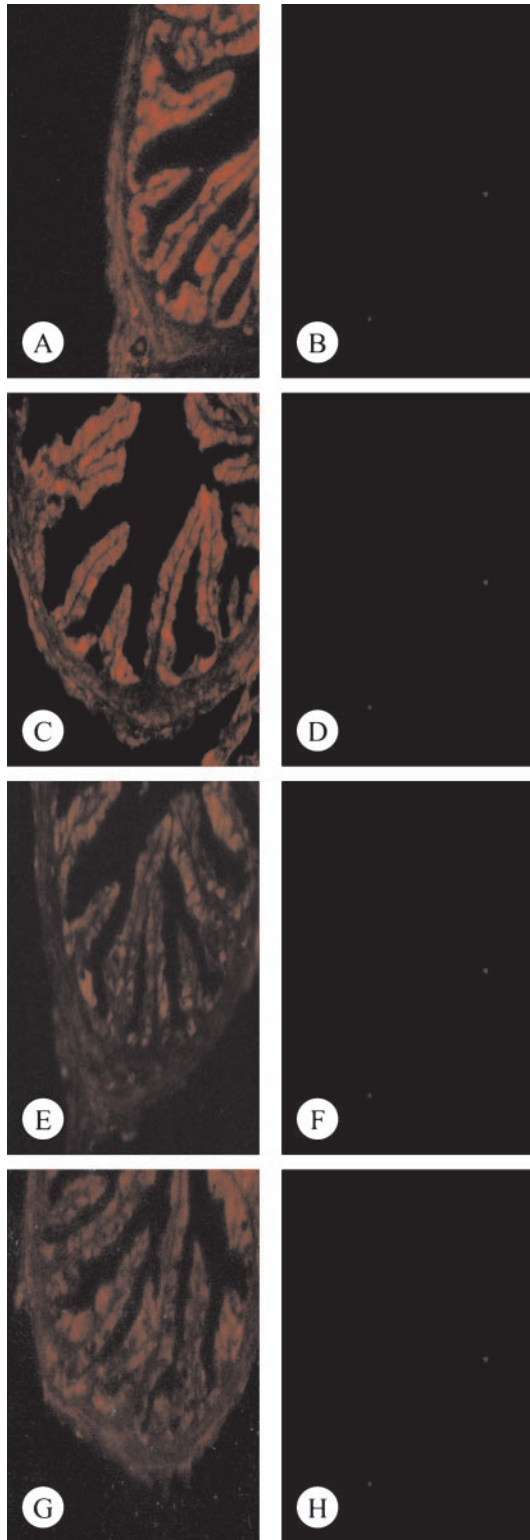


FIG. 3. Immunofluorescence staining of leptin in mouse oviduct. Shown are epifluorescence images of optical sections of the following. A and B, Pregnant mouse 9 wk of age on d 4.5; C and D, pregnant mouse 9 wk of age on d 1; E and F, virgin mouse 4 wk of age; G and H, nonpregnant mice 9 wk of age at estrus. Samples were fixed in 4% paraformaldehyde and stained using 0.2 $\mu\text{g}/\text{ml}$ of polyclonal antileptin antibody as primary antibodies and 1.0 $\mu\text{g}/\text{ml}$ of goat antirabbit Cy3 fluorescein antibody as secondary antibodies. Strong immuno-

embryos at each stage, and the results were found to be reproducible.

Detection of leptin mRNA and protein in mouse oviduct and endometrium

RT-PCR was performed to detect leptin mRNA in the mouse oviduct and uterus. Leptin mRNA was detected in both oviduct and uterus as a 441-bp band (Fig. 2). All PCR products were validated by sequence analysis (data not shown). Experiments in the present study were performed three times on five individual samples, and the results were found to be reproducible. Immunohistochemical staining was performed to detect leptin protein in the mouse oviduct and endometrium. In pregnant mice 9 wk of age on d 1 and 4.5 after mating, the luminal epitheliums of the oviduct were strongly stained, whereas in virgin mice and nonpregnant mice 9 wk of age were weakly stained (Fig. 3). Furthermore, the endometrium of both pregnant mice showed a strong staining at the luminal and glandular epitheliums, whereas virgin and nonpregnant mice showed a weak staining (Fig. 4). Consistent staining was observed in three experiments in which a total of 10 mouse oviduct and endometrium were surveyed, and no staining was found in the negative controls containing the nonimmunized IgG (data not shown) and preabsorbed primary antibodies for leptin (Figs. 3 and 4).

Detection of leptin in mouse oviduct and uterine fluids

To examine whether leptin is secreted by the oviduct and endometrium, the levels of leptin in plasma, oviduct and uterine fluid were measured using the leptin ELISA. There were no significant differences in plasma leptin concentrations among IVCS virgin mice 4 wk of age, nonpregnant mice 9 wk of age at estrus and pregnant mice 9 wk of age on d 1 and 4.5 (3.88 ± 0.39 , 3.71 ± 0.24 , 3.57 ± 0.24 , 3.64 ± 0.20 ng/ml, respectively) (Fig. 5A). The levels of leptin in uterine fluid of pregnant mice on d 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 nonpregnant mice (0.70 ± 0.60 ng/ml) ($P < 0.0001$) (Fig. 5B). Leptin was not detected in the oviduct fluid.

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

To determine the effects of leptin on the *in vitro* development of mouse preimplantation embryo, 2-cell stage embryos were cultured in the presence of 1, 10, 100, and 1,000 ng/ml mouse recombinant leptin. 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

reactivity is detected in the luminal epithelium of pregnant mouse (A, C). The luminal epithelium of virgin mouse (E) and nonpregnant mice (G) is weakly stained. Absorption with 50 $\mu\text{g}/\text{ml}$ of purified leptin before immunostaining abolished all positive staining (B, D, F, H). Original magnification, $\times 200$. Similar results were obtained in the other samples, and representative results were shown.

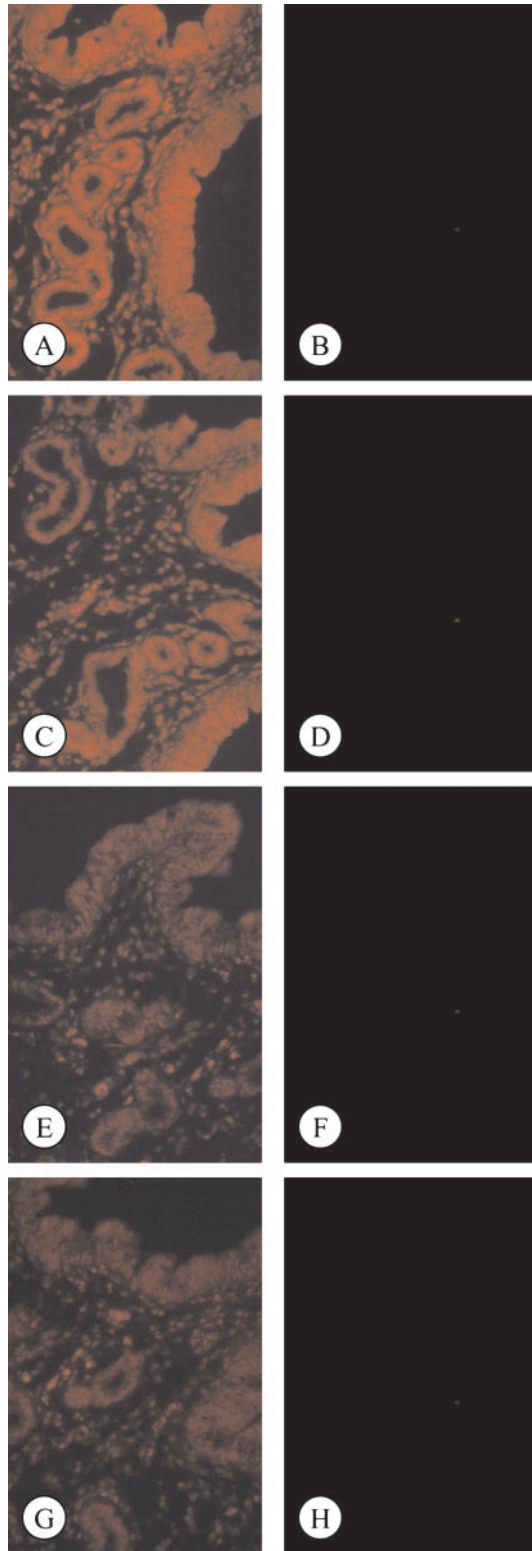


FIG. 4. Immunofluorescence staining of leptin in mouse uterus. Shown are epifluorescence images of optical sections of the following. A and B, Pregnant mouse 9 wk of age on d 4.5; C and D, pregnant mouse 9 wk of age on d 1; E and F, virgin mouse 4 wk of age; G and H, nonpregnant mice 9 wk of age at estrus. Strong immunoreactivity with polyclonal antileptin antibody is detected in the luminal and glandular epitheliums of pregnant mouse (A, C). The luminal epithelium of virgin mouse (E) and nonpregnant mice (G) is weakly

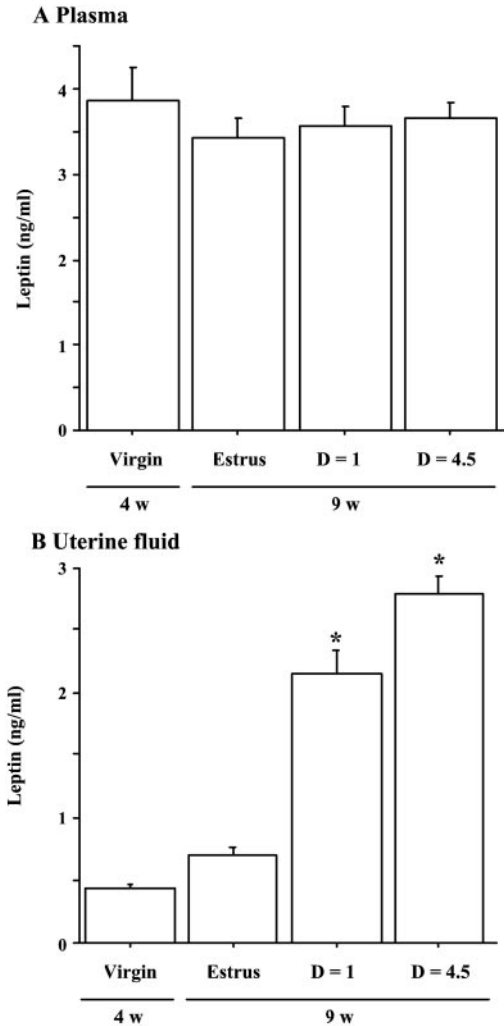


FIG. 5. The levels of leptin in mouse (A) plasma and (B) uterine fluid during early pregnancy. Bars represent mean \pm SEM ($n = 5$). Virgin, Virgin mouse 4 wk of age; Estrus, nonpregnant mouse 9 wk of age at estrus; D = 1, pregnant mouse 9 wk of age on d 1; D = 4.5, pregnant mouse 9 wk of age on d 4.5. 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 and centrifuged at $2,000 \times g$ for 5 min. Each supernatant was concentrated to 10 μ l. The plasma samples were obtained simultaneously. Leptin concentration was determined by ELISA. Data were analyzed one-way ANOVA. *, $P < 0.0001$.

group, and the results were summarized in Tables 2 and 3. 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 (0 ng/ml vs. 1 ng/ml; $P = 0.7622$, $P = 0.8993$, and $P = 0.7243$ for blastocyst, expanded blastocyst, and hatched blastocyst, respectively). Further-

stained. Absorption with 50 μ g/ml of purified leptin before immunostaining abolished all positive staining (B, D, F, H). Original magnification, $\times 200$. Similar results were obtained in the other samples, and representative results were shown.

TABLE 2. The dose-dependent effects of leptin on the development of mouse preimplantation embryos

Leptin concentration (ng/ml)	The embryonic development after each period of culture		
	48 h Blastocyst	56 h Expanded blastocyst	72 h Hatched blastocyst
0 (n=156)	0.301 ± 0.023 (−0.853 ± 0.110)	0.436 ± 0.031 (−0.263 ± 0.127)	0.115 ± 0.026 (−2.141 ± 0.227)
1 (n=150)	0.313 ± 0.030 (−0.802 ± 0.139)	0.453 ± 0.032 (−0.191 ± 0.131)	0.120 ± 0.021 (−2.061 ± 0.188)
10 (n=100)	0.375 ± 0.026 (−0.520 ± 0.117)	0.606 ± 0.037 (0.440 ± 0.155)	0.194 ± 0.025 (−1.458 ± 0.156)
100 (n=110)	0.378 ± 0.030 (−0.509 ± 0.128)	0.617 ± 0.034 (0.488 ± 0.148)	0.197 ± 0.023 (−1.436 ± 0.145)
1,000 (n=127)	0.413 ± 0.032 (−0.360 ± 0.134)	0.624 ± 0.038 (0.525 ± 0.166)	0.249 ± 0.032 (−1.140 ± 0.174)

In each experiment, 15–26 embryos were employed in each group, consisting of exactly six observations, and the experiment was repeated five times. Values are mean ± SEM, and *in parentheses* are the incidence transformed by logit function. n = Total number of cultured two-cell stage embryos.

TABLE 3. Results of statistical analysis of the dose-dependent effect of leptin on the *in vitro* development of mouse preimplantation embryos

Type of analysis	48 h Blastocyst	56 h Expanded blastocyst	72 h Hatched blastocyst
One-way ANOVA ^a (<i>F</i> test)	0.0000 (27.1164)	0.0001 (9.1513)	0.0000 (92.9223)
Fisher's PLSD ^b			
Leptin 1 ng/ml	0.7662	0.7243	0.8993
10 ng/ml	0.0778	0.0019	0.0380
100 ng/ml	0.0679	0.0011	0.0323
1,000 ng/ml	0.0100	0.0007	0.0010
Trend analysis ^c			
Linear trend (<i>F</i> test)	0.7046 (0.1479)	0.5924 (0.2961)	0.5400 (0.3887)
Non-Linear trend (<i>F</i> test)	0.0010 (9.9260)	0.0011 (9.8520)	0.0011 (9.8056)

Values are *P* values and *in parentheses* are *F* values.

^a Five sets of experiments, each of which consisted of six observations; Degree of freedom (*df*) = 4 (between group), *df* = 25 (within groups); ^b Fisher's protected least significant difference as a *post hoc* test, 0 ng/ml of leptin *vs.* each concentration of leptin; ^c the loading coefficients are [−3, −1, +1, and +3] for [1 ng/ml, 10 ng/ml, 100 ng/ml, and 1,000 ng/ml, respectively], four sets of experiments, each of which consisted of six observations, *df* = 3 (between group), *df* = 20 (within groups), *df* = 1 (linear), *df* = 2 (nonlinear).

more, the most positive effect of leptin on the development of embryos was observed at expanded blastocyst stage. The logarithms of observed four leptin concentrations are ordered from 0 (log [1_{ng/ml}]) to 3 (log (1,000_{ng/ml})) with equal interval, 1. Thus, multiple comparisons of a series of observations at four different concentrations can be considered as those of ordered groups. In other words, further information about the nature of dependency induced by logarithmic concentration of leptin can be elucidated by using trend analysis. Considering both linear (*df* = 1) and nonlinear (*df* = 2) factors between four groups (*df* = 3), trend analyses were carried out, and the results were summarized in Table 3. Though statistical test dose not give any information about the mathematical formula of nonlinear trend, observed *P* values revealed that any developmental stages were not significant in linearity (*P* > 0.42 for all stages of linear trend) but were significant in nonlinearity (*P* < 0.0002 for all stages of nonlinear trend).

Furthermore, to confirm the ligand-receptor mediated effect of leptin on the preimplantation embryos, the inhibitory effect of polyclonal anti-OB-R antibody raised against extracellular domain was examined by additional four sets of experiments. 2-cell stage embryos were cultured with 1) leptin and anti-OB-R antibody, 2) leptin and goat IgG, 3) anti-OB-R antibody, and 4) goat IgG. In each experiment, 20–26

embryos were employed in each group, consisting of exactly three observations, and the experiment was repeated twice. Finally, a total of 70–78 embryos were tested in each group; their development were summarized in Table 4 and Fig. 6B. The rates of formation of expanded blastocyst (after 56 h) and hatched blastocyst (after 72 h) were significantly inhibited when embryos were cultured with leptin and anti-OB-R antibody (*P* < 0.01, and *P* < 0.05, *vs.* leptin only, respectively), and such influence was not observed in a control group in which anti-OB-R antibody was substituted for goat IgG. Anti-OB-R antibody, by itself, slightly inhibited the rates of blastocyst, expanded blastocyst and hatched blastocyst formation, but the effects did not reach significant levels.

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 summarized in Fig. 7. Blastocysts cultured with 100 ng/ml of leptin had a significantly higher total cell number, as compared with blastocysts cultured in HTF medium alone. The increase in the total cell number of leptin-stimulated blastocysts was resulted from proliferation of both ICM and TE cells, and the

TABLE 4. The effects of anti-OB-R antibody on the development of mouse preimplantation embryos

	The embryonic development after each period of culture		
	48 h Blastocyst	56 h Expanded blastocyst	72 h Hatched blastocyst
Leptin ^a (n = 73)	0.426 ± 0.026 (−0.299 ± 0.106)	0.634 ± 0.044 (0.559 ± 0.190)	0.251 ± 0.030 (−1.104 ± 0.162)
Leptin ^a + anti-OB-R ^b (n = 78)	0.359 ± 0.026 (−0.584 ± 0.114)	0.436 ± 0.034 (−0.260 ± 0.138)	0.128 ± 0.026 (−1.965 ± 0.260)
Leptin ^a + Goat IgG ^b (n = 70)	0.397 ± 0.026 (−0.422 ± 0.109)	0.623 ± 0.038 (0.510 ± 0.163)	0.227 ± 0.027 (−1.239 ± 0.147)
None (n = 78)	0.295 ± 0.026 (−0.878 ± 0.121)	0.436 ± 0.034 (−0.260 ± 0.138)	0.115 ± 0.022 (−2.076 ± 0.226)
Anti-OB-R ^b (n = 73)	0.286 ± 0.034 (−0.930 ± 0.176)	0.396 ± 0.042 (−0.428 ± 0.175)	0.109 ± 0.026 (−2.151 ± 0.248)
Goat IgG ^b (n = 70)	0.297 ± 0.033 (−0.873 ± 0.155)	0.423 ± 0.038 (−0.313 ± 0.160)	0.113 ± 0.024 (−2.099 ± 0.232)

In each experiment, 20–26 embryos were employed in each group, consisting of exactly three observations, and the experiment was repeated two times. Values are mean ± SEM, and *in parentheses* are the incidence transformed by logit function. n = Total number of cultured two-cell stage embryos. None, HTF medium only.

^a, 1,000 ng/ml; ^b, 0.1 μg/ml.

proliferation of TE cells ($P < 0.005$) was more evidently observed compared with that in ICM cells ($P < 0.05$).

Discussion

In the present study, we have shown that leptin mRNA was expressed in blastocyst and hatched blastocyst stage embryos. Both OB-Ra and OB-Rb mRNAs were detected in oocytes and then rapidly decreased at the 2-cell stage but increased in morula stage embryos. During mouse embryo development, embryonic genome activation, which is an event of translation from maternally inherited RNA to newly translated embryonic ones, is known to take place between the early and the late 2-cell stages (52). Thus, transcripts from embryonic genes are detectable at the late 2-cell stage. In this experiment, leptin mRNA was detected after the blastocyst stage, suggesting that this transcript may be originating from an embryonic gene. Both OB-Ra and OB-Rb mRNAs detected at morula, blastocyst, and hatched blastocyst stage, are also newly translated embryonic mRNA, whereas both OB-Ra and OB-Rb mRNAs detected at oocytes, 1-cell, and 2-cell stage may be maternally derived RNA because it was detected at the stages before embryonic genome activation.

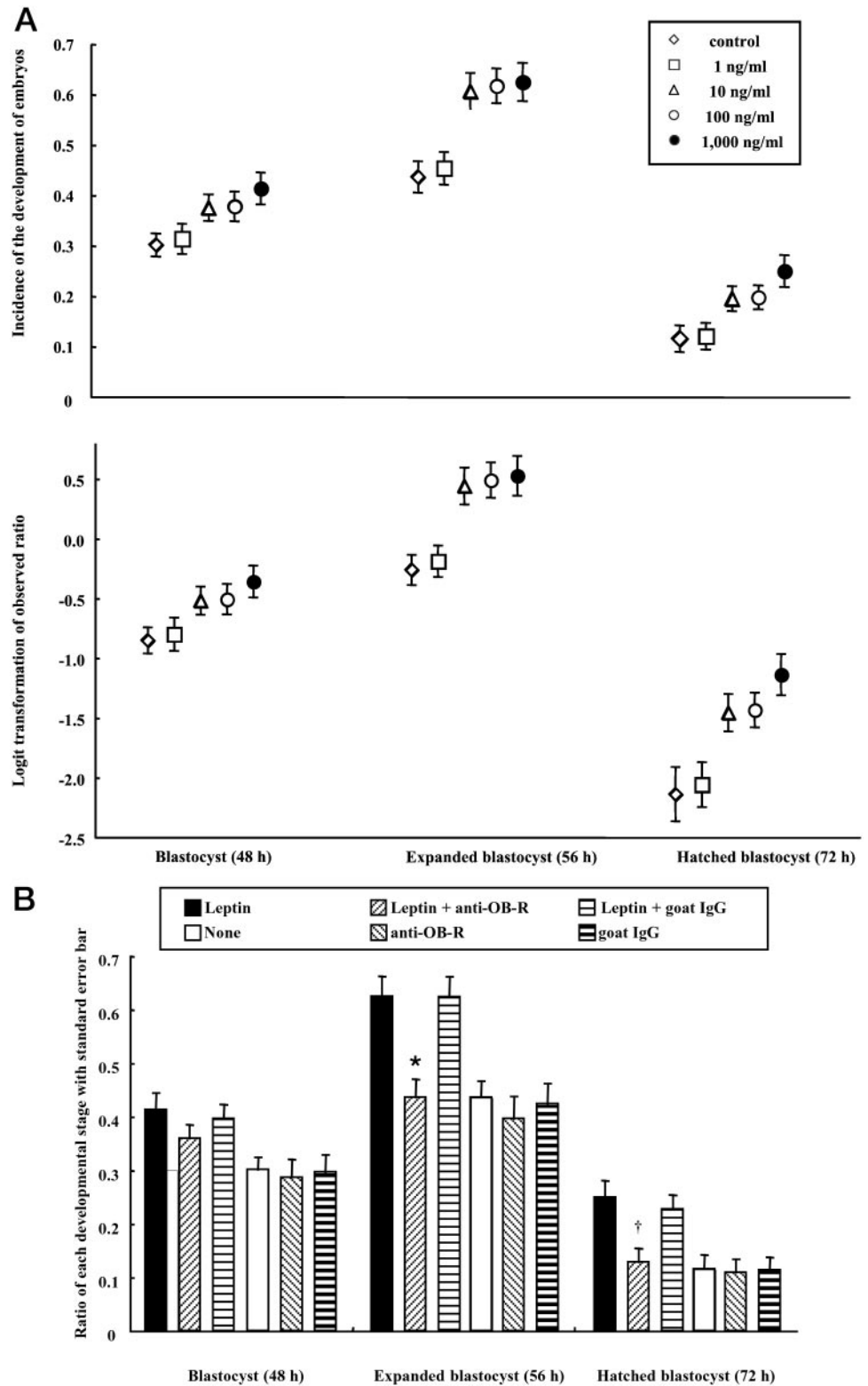
It has been reported that both OB-R mRNA and protein were present in mouse oocytes, but leptin was detected only at the protein level. Because of the lack of leptin mRNA, it is presumed that an exogenous source of leptin would be transported into the cytoplasm through its specific receptor (42–44). In mouse preimplantation embryos, it has not been elucidated whether leptin and the functional receptor, OB-Rb, mRNAs are expressed. Here, we were able to demonstrate the temporal expression of leptin, OB-Ra, and OB-Rb mRNAs. Both OB-Ra and 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, it is possible that an exogenously secreted leptin causes ligand receptor-mediated effect through the OB-Rb. 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 (53). 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. However, the role of the splice variants, OB-Ra and OB-Rb in mouse oocytes and preimplantation embryos is unknown.

We further confirmed that leptin was secreted during early embryogenesis by the reproductive tract of mouse. It has been shown that leptin mRNA and protein are detected in human secretory endometrium by RT-PCR, immunohistochemistry, and Western blot analysis (53). In the present study, leptin mRNA was expressed in both oviduct and uterus of pregnant mice. Leptin protein was more strongly expressed in glandular and luminal endometrial epithelium of pregnant mice on d 1 and 4.5, which corresponded to 2-cell and hatched blastocysts stage, than in those tissues of virgin mice 4 wk of age and nonpregnant mice 9 wk of age at estrus as shown by immunohistochemistry. Leptin protein was also strongly expressed in the oviduct epithelium of pregnant mice. Thus, it is strongly suggested that leptin is produced by oviduct and endometrial epithelium during early pregnancy. Using the leptin ELISA, it was reported that leptin was detected in the conditioned media from cultured endometrial epithelial cells (53). In this experiment, although there were no significant differences in plasma leptin concentrations among virgin mice, nonpregnant mice, and pregnant mice, the levels of leptin in uterine fluid of pregnant mice were significantly higher than those of virgin mice and nonpregnant mice. Unfortunately, owing to very little amount of oviduct fluid, the levels of leptin in oviduct samples were below the sensitivity. These findings suggest that leptin is secreted by at least pregnant endometrial epithelium, and preimplantation embryos could directly contact with leptin secreted from the reproductive tract.

The development of the mammalian preimplantation em-

FIG. 6. A, The dose-dependent effects of leptin on the *in vitro* development of mouse preimplantation embryos. In each experiment, 15–26 embryos were employed in each group, consisting of exactly 6 observations, and the experiment was repeated 5 times. A total of 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 1,000 ng/ml = 127. The incidences observed at blastocyst stage (after 48 h), expanded blastocyst stage (after 56 h), and hatched blastocyst stage (after 72 h), were transformed by logit function. Values are mean \pm SEM. The data transformed by logit function were analyzed by one-way ANOVA and trend analysis in the multiple comparison tests, and nontransformed data were analyzed by Fisher's PLSD as a *post hoc* test of one-way ANOVA. B, The inhibitory effects of anti-OB-R antibody on the *in vitro* development of embryos with or without leptin. In the additional four experiments, embryos were cultured three times in each of different HTF media of 1) leptin + anti-OB-R antibody; 2) leptin and goat IgG; 3) anti-OB-R antibody; and 4) goat IgG. In each experiment, 20–26 embryos were employed in each group, consisting of exactly three observations, and the experiment was repeated twice. A total of 70–78 embryos were tested in each group; 1) 78; 2) 70; 3) 73; and 4) 70. None, HTF medium only; Leptin, 1,000 ng/ml; anti-OB-R, 0.1 μ g/ml; goat IgG, 0.1 μ g/ml. Values are mean \pm SEM. Data were analyzed by unpaired *t* test. *, *P* < 0.01; †, *P* < 0.05, significantly different from corresponding control.



bryo *in vitro* occurs more slowly and less successfully compared with development in the reproductive tract. *In vitro* conditions for the culture of preimplantation embryos are generally considered to be suboptimal and usually associated with high rates of implantation failure especially in

human *in vitro* fertilization programs. Accumulated evidence indicates that a number of growth factors and cytokines contribute in a paracrine and/or autocrine fashion to preimplantation development (reviewed in Ref. 54). The OB-R is similar to other cytokine receptors such as IL-6 (24). Leptin

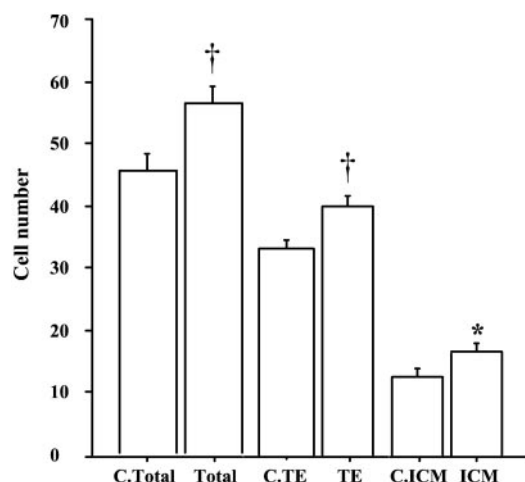


FIG. 7. The effect of leptin on the number of total cells (Total), ICM cells, and TE cells in 56-h cultured blastocysts. Values are mean \pm SEM of blastocysts cultured in 100 ng/ml of leptin ($n = 30$) and blastocysts cultured in HTF medium alone (c. Total, c. ICM, and c. TE) ($n = 30$). Data were analyzed by Mann-Whitney *U* test. *, $P < 0.05$; †, $P < 0.005$, significantly different from corresponding control.

appears to modulate cell function through the activation of the STAT system (26–29). Leptin stimulates the proliferation of diverse cells through the activation of STAT system and/or MAPK (32–38, 55–57). These findings prompted us to investigate the effect of leptin on the development of mouse preimplantation embryos. The data obtained from the present study have shown that addition of leptin to mouse embryo culture media can promote mouse preimplantation embryo development from 2-cell stage embryo to the blastocyst, fully expanded blastocyst, and hatched blastocyst *in vitro* in a 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 leptin as a ligand for OB-Rb. A decreasing effect in longer culture period until hatched blastocyst stage was observed also in the experiment of leukemia inhibitory factor on human embryos in a complex serum-free culture media, in which leukemia inhibitory factor promoted blastocyst development, but do not allow hatching of the blastocysts (58).

The promoting effect of leptin on embryo development was significant at concentrations of ≥ 10 ng/ml of leptin concentration, but not clear at concentration of 1 ng/ml. The concentration of leptin in uterine fluid was determined to be 2.16 ± 0.17 to 2.80 ± 0.13 ng/ml, whose effect on embryos was considered to be between the significant and nonsignificant levels. Thus, further studies would be necessary to elucidate whether the physiological level of leptin actually promotes the development of preimplantation embryos *in vivo*. Because embryos were shown to promote secretion of leptin from endometrium *in vitro* (53), concentrations of leptin around embryos in the uterine cavity before implantation *in vivo* may be higher than those in the uterine fluid.

The influence on mouse preimplantation embryos cultured in the medium containing leptin was neutralized by

anti-OB-R antibody. Thus, leptin can promote embryonic development through its specific receptors *in vitro*. In blastocyst stage embryos, which presumably secrete leptin, anti-OB-R antibody alone slightly inhibited the development of preimplantation embryos. This result suggests that anti-OB-R antibody alone may block autocrine mechanism within mouse preimplantation embryos.

In this study, leptin increased the total cell number of blastocysts. The increase in the total cell number was due to proliferation of both ICM and TE cells, and a significantly high number of cells developed on TE compared with ICM. The TE cells are necessary for implantation and form the placenta and extra-embryonic membranes. The ICM cells form all three germ layers and all tissues of the embryo, as well as providing complementary contributions to formation of extra-embryonic membranes. Thus, cell numbers in the TE, in the ICM or in both cell populations of blastocyst are the indicators of embryo growth and viability (59). Although access of leptin to the ICM cells may be restricted, leptin may have selective effects on the different cell populations of the blastocyst. In mouse blastocysts, leptin and STAT3 were detected in mural and polar TE cells and were not detected in ICM cells (43). 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 leptin signal transduction between TE and ICM cells.

In conclusion, we demonstrate the temporal expression of leptin, OB-Ra, and OB-Rb mRNAs in mouse oocytes and preimplantation embryos. Both OB-Ra and OB-Rb mRNAs were expressed in mouse oocytes and preimplantation embryos. Leptin mRNA was detected after blastocyst stage embryos. Oviduct and endometrial epithelium may produce and secrete leptin to the reproductive tract. Leptin can promote the development of mouse preimplantation embryos through OB-R.

Acknowledgments

Received October 22, 2001. Accepted January 31, 2002.

Address all correspondence and requests for reprints to: Kazuhiro Kawamura, Department of Obstetrics and Gynecology, Akita University School of Medicine, Hondo 1-1-1, Akita 010-8543, Japan. E-mail: kawamurak@obgyn.med.akita-u.ac.jp.

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