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Leptin and Leptin Receptor Are Expressed in the Human Endometrium and Endometrial Leptin Secretion Is Regulated by the Human Blastocyst*

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ABSTRACT

Embryonic implantation is a crucial event for the human reproductive function. Cytokines and paracrine molecules have been proposed as putative local regulators of this process. The leptin or the OB protein has been linked to the reproductive function and inflammatory response. In the present study, we describe for the first time the expression of leptin and leptin receptor (long form) in the secretory endometrium and that endometrial leptin secretion is regulated *in vitro* by the human blastocyst. Leptin and leptin receptor messenger RNA and protein were identified in secretory endometrium and in cultured endometrial epithelial cells (EECs) by RT-PCR, Western blot, and immunohistochemistry. The concentrations of immunoreactive leptin secreted by human embryos alone or cocultured with EECs were also assessed. We found that human blastocysts secrete significantly higher levels of leptin than arrested embryos. In contrast, leptin concentrations secreted by arrested embryos cocultured with EECs were significantly higher than blastocysts cocultured with EECs. These findings suggest that the human endometrium is a site for local production and a target tissue for circulating leptin. Expression of leptin and its functional receptor in the endometrium and regulation of endometrial leptin secretion by the human embryo suggests that the leptin system may be implicated in the human implantation process. (*J Clin Endocrinol Metab* **85:** 4883–4888, 2000)

LEPTIN OR THE OB gene product is a small pleiotrophic peptide of 146 amino acid residues (16 kDa) initially found to be secreted by adipose tissue, with its secretion tightly linked to food consumption and energy balance (1, 2). More recently, investigations have implicated leptin in the regulation of reproductive function (3–6). The ob/ob mutant female mouse is characterized by obesity and sterility as a result of the synthesis of a nonactive, truncated version of leptin (7). Fertility in these animals can be restored by exogenous leptin treatment but not by food restriction, suggesting that leptin is required for normal reproductive functioning (8). Moreover, impaired reproductive function of ob/ob male mice can be corrected only with leptin treatment (9).

In addition to adipose tissue, leptin has been found to be secreted by a variety of reproductive organs including placenta (10, 11). *In vitro* cultures of cytotrophoblastic cells secreted leptin, and this secretion is regulated by interleukin 1 (IL-1) and estradiol (12). Leptin protein (13) and leptin messenger RNA (mRNA) (14) have also been detected in human ovary, and follicular and serum leptin production may be influenced by the ovarian functional state (15). Leptin is coexpressed in a polarized manner with STAT3 protein in oocytes and embryos at all stages of development (16). Leptin is also expressed in mammary glands (17).

The long form of the leptin receptor (OB-R), has signaling capabilities and is primarily localized at the hypothalamus, anterior pituitary, and several other areas of the brain (18–20). OB-R mRNA has been detected in granulosa and theca cells of the ovary (13, 14), and the OB-R protein has been detected by immunofluorescence in mouse oocytes fertilized eggs and cleavage stage embryos (16). OB-R is also expressed in hepatic and adrenal cells (21, 22). A soluble form of the leptin receptor has also been reported to exist in the human (23, 24).

Although it has been suggested that leptin does not directly regulate reproduction itself, leptin could regulate the function of reproductive organs (25). However, the specific mechanism(s) whereby leptin modulates reproductive function is unknown (1, 2, 5, 6, 25). Endocrine data in *in vitro* fertilization (IVF) suggest that leptin production may be influenced by the ovarian functional state (15) and follicular fluid leptin concentrations could be a marker of assisted reproduction treatment success (26). However, a possible role for leptin in the endometrial-embryonic dialogue during implantation has not yet been described.

In the present study, we investigate the expression of leptin and its receptor (long form) by secretory endometrium and cultured endometrial epithelial cells (EECs) *in vitro* and

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analyze leptin secretion by the human blastocyst in the presence or absence of cultured EECs.

Materials and Methods

Patients and tissues

This project was approved by the institutional review board on the use of human subjects in research at the Instituto Valenciano de Infertilidad [Spanish Law of Assisted Reproductive Technologies (35/1988)]. Embryos and granulosa cells were obtained from nine patients after ovarian hyperstimulation and insemination using routine IVF procedures. Endometrial biopsies were obtained from fertile women (age, 23–39 yr) during the midsecretory phase of the menstrual cycle. Human adipose tissue, used as positive control for leptin and leptin receptor expression, was obtained from patients undergoing gynecological surgery. Human placental tissue was obtained following routine delivery.

EEC cultures

A portion of each endometrial biopsy was dated according to the Noyes's method (27). EECs were isolated and cultured until confluent in DMEM (Sigma, St. Louis, MO) and MCDB-105 (Sigma) (3:1) containing 10% heat inactivated fetal bovine serum (Life Technologies, Grand Island, NY), antibiotics, and 5 μg/mL insulin (Sigma), as described previously (28–30).

Embryo culture and coculture with EECs

A human *in vitro* model was used to study interactions between the human embryo and EECs (31, 32). Forty-eight hours after insemination, two to four cell embryos were cocultured individually on the EEC monolayer for 5 days, from day 2 until day 6 of embryonic development. Embryonic development was monitored daily, and embryos were grown in 1 mL IVF/S2 or CCM medium (1/1) (Scandinavian IVF Science, Gothenburg, Sweden) until they reached the eight-cell stage, and then were cultured in CCM medium until blastocyst stage. Conditioned media were changed every 24 h of culture, however, only the last 24 h (corresponding to 120 h at day 6 of culture) media sample was collected to measure leptin concentrations. After embryo transfer, EEC wells were classified according to embryonic status: EECs with embryos that reached the blastocyst stage and EECs with arrested embryos. In each experiment, EECs cultured under the same conditions without embryos and human embryos cultured in the absence of EECs were used as controls.

RT-PCR

Total cellular RNA was extracted, purified, and reverse transcribed from secretory phase endometrial biopsies, EECs, adipose tissue, placenta, and granulosa cells using Trizol reagent (Life Technologies, Paisley, Scotland) according to the manufacturer's instructions. First-strand cDNA was reverse-transcribed from 1 μg RNA using a MMLV Reverse transcriptase and Advantage RT-for-PCR kit (60 min/42 C) (Clontech, Palo Alto, CA). Contradictory data about leptin mRNA expression in ovary has been reported (13, 14) by RT-PCR using different primers. Therefore, we assayed two pairs of primers previously reported as successful in detecting the leptin mRNA expression in ovary (14) and placenta (11) to be more confident about the RT-PCR in the assessment of leptin mRNA expression in endometrium and EEC. Primers specific for leptin were, 5′-ATGCATTGGGAACCCTGTGCGG-3′ (forward) and 5′-TGAGGTCCAGCTGCCACAGCATG-3′ (reverse) (11), and 5′-CCAAAACCCTCATAAGAC-3′ (forward) and 5′-CACCTCTGGAGTAG-3′ (reverse) (14) were used to amplify products of 490 bp and 341 bp, respectively. Primers specific for the long form of leptin receptor were 5′-GCTATTTTGGGAAGATGT-3′ (forward) and 5′-TGCCTGGGCCTCTATCTC-3′ (reverse) (14). All primers were synthesized by Life Technologies. PCR conditions specific for each primer pair were determined following optimization with adipose tissue, placenta, and granulosa cells. The PCR amplification was performed in a final reaction volume of 25 μL consisting of 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5–3.25 mM $MgCl_2$, 0.2 mM dNTPs (Sigma), 0.2 μM forward and reverse primers, and 2.5 μL cDNA, overlayed with 50 μL mineral oil (Sigma). *Taq* DNA polymerase (0.5 U, BIOTAQ; Bioline, London, UK) was added at 94 C. PCR amplification was as 5–10 min at 94 C, followed by a 1-min cycle of denaturation at 94 C, annealing at 58–59 C, and extension at 72 C, followed by a final extension for 10 min at 72 C; repeated for 30 cycles. The negative control included in each reaction consisted of H_2O substituted for complementary DNA (cDNA). In addition, in some experiences we performed PCR using total RNA without RT treatment as additional negative controls. Also, total RNA extracted from samples was previously digested with DNAase I, and RT-PCR experiences were performed. Therefore, we were sure that genomic DNA was not present in RNA extracts. The integrity of each cDNA preparation was analyzed by RT-PCR of β-actin mRNA (33). The primers for β-actin amplification used were those reported by Ponte *et al.* (33). Concentrations of cDNA used for PCR experiments were similar and adjusted for β-actin mRNA expression. PCR reaction products were analyzed by gel electrophoresis in 2% agarose gel containing 0.5 μg/mL ethidium bromide and parallel to a molecular weight marker of 100 bp (Life Technologies). The PCR assay was repeated at least three times on each cDNA sample.

Western blot analysis

Adipose, endometrial, and placental tissue (100 mg) were disrupted by homogenization in RIPA buffer on ice and subsequently by ultrasonic treatment. Total protein was determined using the Bradford protein assay (BioRad Laboratories, Inc., Munich, Germany). Five micrograms (adipose tissue) and 10 μg (for rest of samples) proteins were loaded per lane for leptin and 10 μg proteins (all samples) for leptin receptor, and electrophoresis was performed at 125 V for 1.5 h and 2.5 h (NOVEX Electrophoresis GmbH, Frankfurt, Germany) for leptin and leptin receptor, respectively, in 8% SDS and 14% polyacrylamide gel (SDS-PAGE). Electroblotting (NOVEX apparatus) onto nitrocellulose membranes of 0.2 μm and 0.45 μm pore size (BioRad) for leptin and leptin receptor, respectively, was performed at 35 mA for 1.5 h. Membranes were blocked with a TRIS-Glycine (TBS) solution consisting of 3% BSA and 0.05% Tween 20 overnight at 4 C and washed three times with TBS plus 0.05% Tween 20 for 5 min each. The membranes were subsequently incubated at room temperature for 2 h with 0.5 μg/mL rabbit antihuman leptin antibody or 2 μg/mL goat antihuman leptin receptor antibody (Ob Y-20 and Ob R Sc-20, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TBS containing 1% BSA and 0.05% Tween 20. Following an incubation with biotinylated antirabbit (Sigma) and antigoat (DAKO Diagnosticos, S.A, Barcelona, Spain) biotinylated antibodies and peroxidase-labeled streptavidin (Sigma) for 1 h at room temperature. Positive specific antigen-antibody reactions were analyzed with a chemiluminescent assay (Amersham, Germany). Western blot experiments were also performed with human recombinant leptin at 2 μg (R&D Systems, Bühlmann Laboratories, Basel, Switzerland) and to demonstrate that the antileptin antibody specifically detects the peptide (a band about 16 kDa). To analyze nonspecific binding in the blot, PAGE-SDS gel electrophoresis and membrane electroblotting were run in parallel experiments and irrelevant rabbit or goat immunoglobulin preparations were substituted for primary specific antibodies. Afterward, nonspecific bands in the blot for leptin and leptin receptor were identified. A broad range biotinylated marker (Bio-Rad) was used to calculate protein weight.

Immunohistochemistry

Formalin-fixed endometrial tissues were sectioned and deparaffinized. Unmasking of tissue antigens was performed by heat treatment in sodium citrate buffer (pH 6, 10 mM) at 95 C for 15 min and partial digestion at 37 C by 10 min with protease (Sigma) (16). Endometrial tissue sections were blocked with PBS containing 5% BSA and 0.1% Tween 20 for 2 h at room temperature or overnight at 4 C. Samples were incubated with 0.5 μg/mL rabbit antihuman leptin antibody (Ob Y-20) or 2 mg/mL goat antihuman leptin receptor (Ob.R Sc-20) in 1% PBS-BSA/0.05% Tween 20, for 1.5 h at 37 C. Immunofluorescent labeling was performed with fluorescein isothiocianate-antirabbit or antigoat IgG for 1 h at 37 C.

Leptin measurements in conditioned media from EEC cultures

Leptin concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (DRG Diagnostic International, Inc., USA and Instruments GmbH, Germany) in conditioned media from the last 24 h (5 days) of EECs cultured alone (n = 7), with arrested embryos (n = 10) or transferred blastocyst (n = 10), as well as arrested embryos (n = 7) and transferred blastocysts (n = 3) cultured alone. Leptin was also determined in fresh IVF media (CCM) and in control samples in the kit. Samples (0.8–1.0 mL) and fresh conditioned medium were lyophilized and resuspended in 120 μL deionized water or of deionized water at basic pH, to compare total solubility of leptin after lyophilization. Recovery experiments were made by the addition of human recombinant leptin (R&D System) to fresh conditioned media, following lyophilization and resuspension of the powder. Total recovery of leptin in these samples ranged between 87% and 90%. Measured concentrations of leptin were within the dynamic range of the standard curve of the ELISA kit. Leptin concentrations were divided by the lyophilization concentration factor and expressed in pg/mL. According to the manufacturer, the performance characteristics of leptin-ELISA were as: sensitivity, 200 pg/mL; 100% specificity for human leptin; less than 0.2% cross-reactivity with rat and mouse leptin; and no detectable cross-reactivity with human insulin, human proinsulin, rat insulin, human C-peptide, glucagon, or insulin-like growth factor I. Standards, controls, and samples were assayed in duplicate. The intra-assay CV were between 0.75–5.32%.

Statistical analysis

Results are expressed as mean ± SE. Statistics were performed using SPSS computer software program (SPSS Inc., Chicago, IL). Differences in leptin protein concentrations were evaluated by ANOVA using the Scheffe test for multiple comparison; P less than 0.05 was considered to be statistically significant.

Results

Expression of leptin and leptin receptor (long form) mRNA in secretory endometrium and EEC cultures

Analysis of PCR products with the two different sets of primers demonstrated that leptin mRNA is expressed in cultured EECs (Fig. 1, A and B). In addition, leptin mRNA was also shown to be expressed in secretory endometrium, placenta, granulosa cells, and adipose tissue. Each cDNA generated a single product of the expected size, with no evidence of product generated from contaminating genomic DNA showed the same cDNA product for leptin (see Fig. 1, A and B). A PCR product of 501 bp encoding a region of mRNA for the full-length leptin receptor (OB-R) was detected in all EEC cultures and secretory endometrial biopsies (Fig. 1C). RT-PCR product specific for leptin receptor was identified in granulosa cell preparations at the same size. Analysis of the β-actin PCR product by gel electrophoresis (Fig. 1D) confirmed the initial amount of cDNA to be approximately equivalent in all samples. However, from the calculations of the yields of cDNA amplification using the ratio leptin or leptin receptor/β-actin, we were not able to demonstrate whether leptin mRNA or leptin receptor mRNA were significantly increased in EECs cocultured with blastocyst, with arrested embryos, or without embryos.

Immunohistochemical and immunoblot determination of leptin and leptin receptor

Using immunohistochemical technique, we identified a positive signal for leptin and leptin receptor in secretory endometrium. Staining for leptin receptor and leptin was found at the luminal and glandular endometrial epithelium (Fig. 2, A and B). Furthermore, these results were confirmed by Western blot analysis. Human adipose and placental tissues were used as positive controls. Figure 3 shows the Western blot results for leptin and leptin receptor. A band of approximately 16 kDa was found in all the samples and controls (Fig. 3A). Immunoblotting analysis using negative control demonstrated that leptin was present in secretory endometrium and all preparations from EEC cultured with or without embryos (Fig. 3A). The same samples, EEC cultures, and endometrium were positive for the leptin receptor (Fig. 3B). Western blotting for leptin receptor identified a major band at approximately 190 kDa (34). However, other minor but specific bands were also detected. The antileptin receptor antibody used in this investigation was raised against amino acids 1146–1165 encoding the carboxyl-terminus of the full-length OB-R. Therefore, minor bands found to be lower than 190 kDa could represent different molecular size isoforms of the leptin receptor.

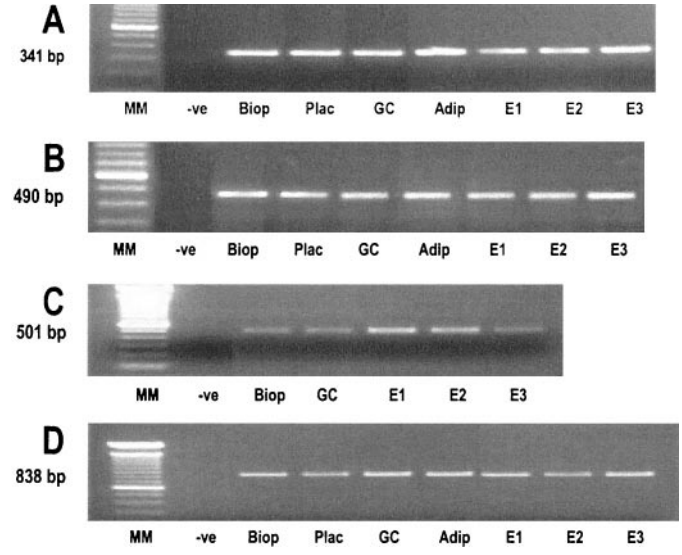

FIG. 1. Leptin ligand and receptor mRNA expression within secretory endometrial tissue and EECs cultured alone or in presence of human embryos as determined by RT-PCR. A, Leptin RT-PCR product amplified using primers reported by Senaris *et al.* (11). B, Leptin RT-PCR product amplified using primers reported by Cioffi *et al.* (14). C, RT-PCR of leptin receptor mRNA using primers reported by Cioffi *et al.* (14). D, β-Actin RT-PCR product. Note the presence of a PCR product of the appropriate size for leptin in EECs and endometrium [341 bp (A) and 490 bp (B)] and for leptin receptor (501 bp; C), which coincides with products obtained from adipose tissue (Adip), placenta (Plac), and granulosa cells (GC). Endometrial biopsies from the secretory phase of the menstrual cycle (Biop), endometrial epithelial cells cultured alone (E1), cultured with arrested embryos (E2), and cultured with competent blastocysts (E3), molecular marker (MM), negative control (-ve).

Determination of leptin in conditioned media from EEC cultures

Leptin was not detected in the lyophilized fresh culture medium by the ELISA kit. Leptin was detected in the conditioned media from both human blastocysts and endometrial epithelial cells (Fig. 4). Interestingly, two different patterns of secretion were found depending on whether

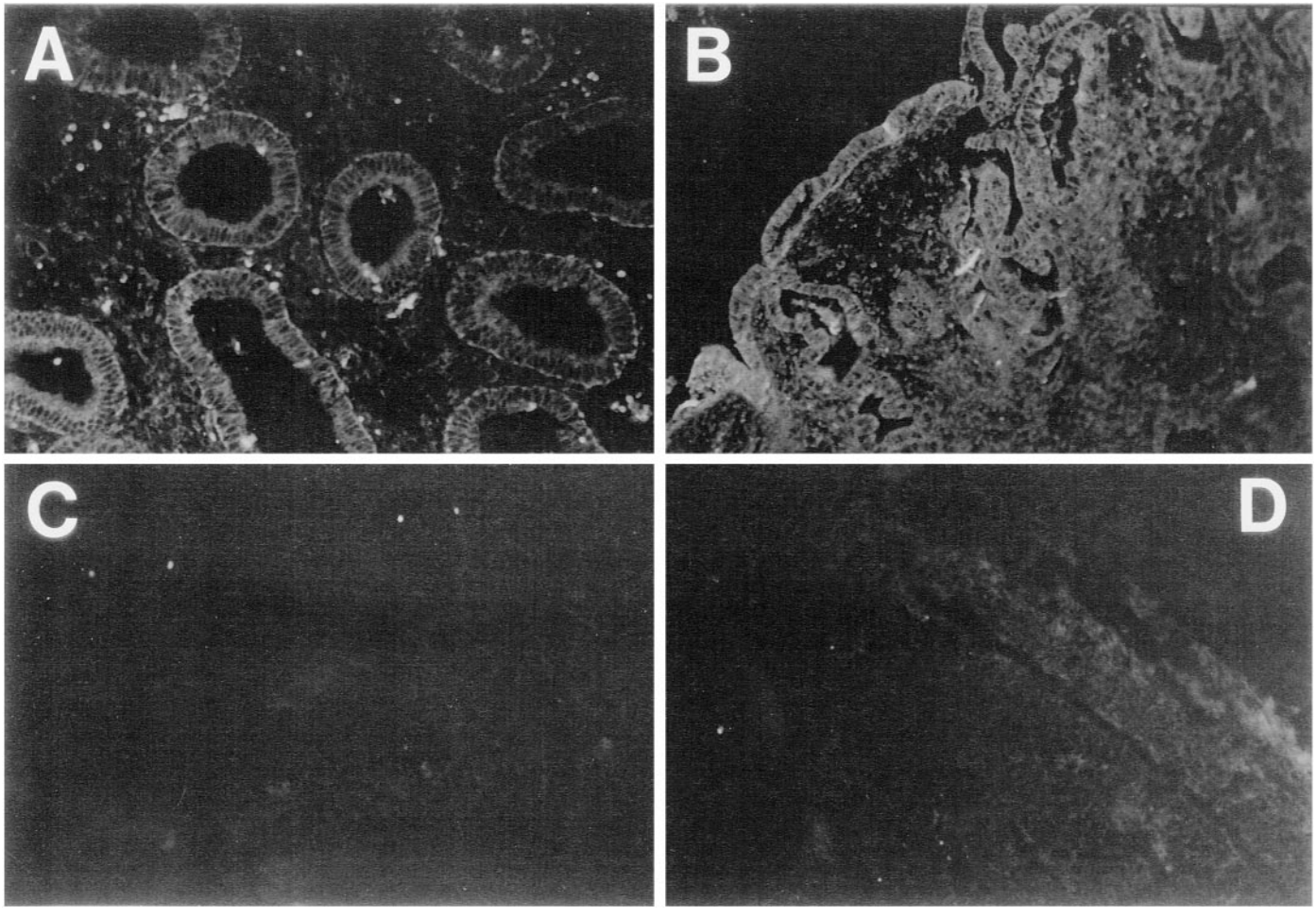

FIG. 2. Immunofluorescent detection of leptin receptor (A) and leptin (B) within secretory endometrium. Negative control for leptin receptor (C) and leptin (D).

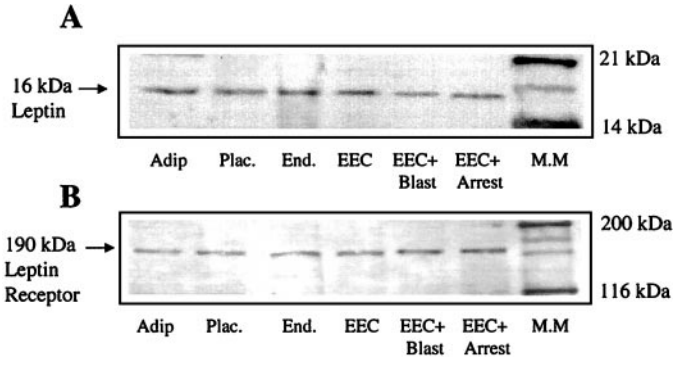

FIG. 3. Western blots of nitrocellulose membranes probed with antileptin (A) and antileptin receptor (B) antibodies loaded with secretory endometrium (End.), EEC cultures (EEC), and EECs cocultured with competent blastocysts (EEC+Blast), with arrested embryos (EEC+Arrest), adipose tissue (Adip), and placenta (Plac.). Note a major band of approximate 16 kDa corresponding to leptin (A) and of 190 kDa corresponding to leptin receptor (B). M.M, Molecular weight markers.

embryos were cultured alone or cocultured with EEC (Fig. 4). Hatched blastocysts cultured alone secreted significantly higher concentrations of leptin (453.3 ± 43.3 pg/mL) than arrested embryos alone ($P = 0.001$), blastocyst cocultured with EECs ($P = 0.001$) or EECs alone ($P = 0.003$). Nevertheless, when blastocysts were cocultured with EECs, leptin concentrations were lower than when arrested embryos were cocultured with EECs (247 ± 14.5 pg/mL *vs.* 360 ± 30 pg/mL; $P = 0.012$), suggesting a regulation in the secretion of leptin or binding to leptin receptor in the cocultures of human embryos and EECs.

Discussion

We demonstrate for the first time that the leptin system (leptin and leptin receptor) is expressed in secretory endometrium and endometrial epithelial cells cultures by RT-PCR, Western blot analysis, and immunohistochemistry. We also report that individual human blastocysts and EECs secrete leptin. Furthermore, the pattern of leptin secretion differed in blastocysts cultured alone *vs.* blastocysts cocultured with EECs.

Higher leptin secretion was found in competent blastocyst cultures compared with arrested embryos, suggesting that this molecule may be a marker of cell viability. However, when competent embryos were cocultured with EECs, leptin concentrations in conditioned media were not different from those of EECs cultured alone. This finding suggests different possibilities: leptin secreted by a competent blastocyst may

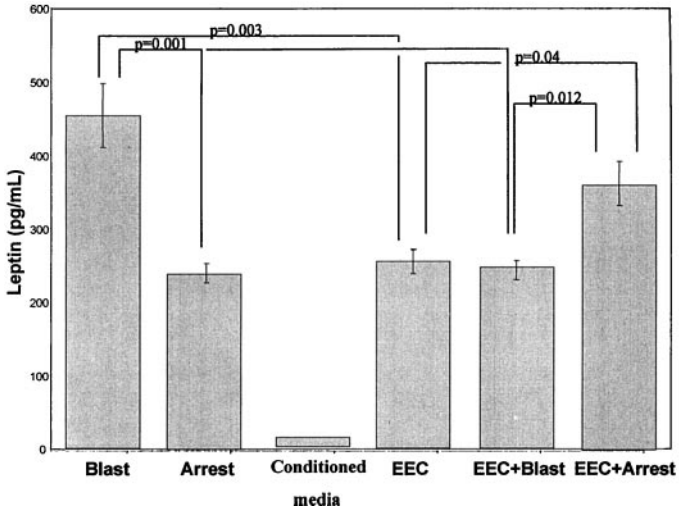

FIG. 4. Leptin concentrations in conditioned media from human EECs, human embryos cultured alone or cocultured with EECs during an IVF protocol. Conditioned media from human embryo cultures at day 6 following routine IVF were lyophilized and resuspended in deionized water, and leptin was measured by ELISA (see *Materials and Methods*). Leptin secretion from competent blastocysts (Blast; 453 ± 43.3 pg/mL; n = 3) was significantly higher than leptin secretion from arrested embryos (Arrest; 238.6 ± 13.1 pg/mL; n = 7; $P = 0.001$) and EECs cocultured with competent blastocyst (EEC+Blast; 247 ± 14.5 pg/mL; n = 10; $P = 0.001$). Leptin concentrations in cocultures of arrested embryos with EECs were higher (EEC+Arrest; 360 ± 30.0 pg/mL; n = 10) than in cocultures of competent blastocyst with EECs (EEC+Blast; 247 ± 14.5 pg/mL; n = 10; $P = 0.012$) or EECs cultured alone (EEC; 254 ± 16.3 pg/mL; n = 7; $P = 0.04$). Leptin was undetectable in lyophilized fresh culture medium.

bind to EECs or the secretion of leptin is regulated in EECs and/or in the human blastocyst. In contrast to this, leptin concentration when an arrested blastocyst is cocultured with EECs is higher than leptin secreted by an arrested blastocyst cultured alone, suggesting that EECs may increase leptin production in response to embryonic death signals or bind less leptin when is cocultured with an arrested embryo. In any case, all these findings strongly suggest that this molecule is part of the embryonic-endometrial dialogue during the adhesion phase of human embryonic implantation.

The human blastocyst expresses immunoreactive leptin in a copolarized manner with STAT3 protein (16), and leptin had also been detected in mouse oocytes, but an exogenous source (maternal) has been suggested due to the absence of leptin mRNA as determined by RT-PCR (14). However, mouse embryos express leptin receptor (16). This fact reinforces our hypothesis that blastocyst and human endometrium might communicate at the time of implantation through the leptin system.

In addition, we found the expression of leptin and leptin receptor mRNA in EECs cocultured with human embryos confirmed the observations made following measurement of leptin protein secreted during culture. Expression of leptin receptor mRNA and protein within the secretory endometrium and cultured EECs indicate potential cell targets for this system. From a functional perspective, at the time of implantation the changes observed *in vitro* in the leptin levels in the microenvironment of the EECs (that express both ligand and receptor) close to the blastocyst (that secretes leptin) might be interpreted as a molecular communication between both partners. If a similar situation occurs *in vivo*, we might think that leptin is an important factor for implantation to proceed.

Leptin produced and secreted locally by epithelial cells could act in an autocrine or paracrine manner to regulate biological functions that may mediate endometrial receptivity to facilitate the implantation process. Regulation of leptin and leptin receptor expression in EECs by the human embryo could represent a coordinate molecular event to allow implantation to occur.

Further research is needed to examine the mechanism(s) through which leptin modulates reproductive function. Based on the present investigation, we propose that leptin could be an autocrine/paracrine mediator that allows the embryo and epithelial cells to communicate during implantation.

In summary, the finding of leptin and leptin receptor mRNA expression in the endometrium and in EEC cultures and the regulation of its secretion by the human embryo suggests that leptin may be involved in the molecular mechanism of the implantation process.

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