The Effect of Leptin on Mouse Trophoblast Cell Invasion

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ABSTRACT

The hormone leptin is produced by adipose tissue and can function as a signal of nutritional status to the reproductive system. The expression of leptin receptor and, in some species, leptin, in the placenta suggests a role for leptin in placental development, but this role has not been elucidated. Leptin is required at the time of embryo implantation in the leptin-deficient ob/ob mouse and has been shown to upregulate expression of matrix metalloproteinases (MMPs), enzymes involved in trophoblast invasion, in cultured human trophoblast cells. This led us to the hypothesis that leptin promotes the invasiveness of trophoblast cells crucial to placental development. We found that leptin stimulated mouse trophoblast cell invasion through a matrigel-coated insert on Day 10, but not Day 18 of pregnancy. Optimal stimulation occurred at a concentration of 50 ng/ml leptin, similar to the peak plasma leptin concentration during pregnancy. Leptin treatment did not stimulate proliferation of mouse trophoblast cells in primary culture. Leptin stimulation of invasion was prevented by 25 μM GM6001, an inhibitor of MMP activity. Our results suggest that leptin may play a role in the establishment of the placenta during early pregnancy and that this function is dependent on MMP activity. This effect of leptin may represent one mechanism by which body condition affects placental development.

implantation, invasion, leptin, matrix metalloproteinase, MMP, pregnancy, placenta, trophoblast

INTRODUCTION

Normal development of the placenta is essential for human health. The placenta is responsible for the exchange of oxygen, nutrients, waste products, and regulatory factors between the fetal and maternal circulations. Consequently, alterations in the placenta alter the fetal environment and can affect fetal development. For example, higher rates of adult mortality due to heart disease have been reported in children born with abnormally large and small ratios of placental weight to neonatal weight [1, 2]. It is known that altered nutrition can affect placental development [3], but the mechanism of this effect is poorly understood.

Leptin is a 16-kDa peptide hormone secreted by adipose tissue that participates in the regulation of energy homeostasis [4]. In addition, leptin is a known mediator of nutritional status to the reproductive system [5] and has been shown to play a role in many reproductive processes, including ovulation, lactation, and puberty [6]. In ob/ob mice, which lack functional leptin, leptin treatment is required through the time of embryo implantation and placental formation for pregnancy to proceed [7]. Leptin is synthesized by the placenta in some species, including humans [8], baboons [9], little brown bats [10], and rats [11, 12]. Receptors for leptin are expressed during pregnancy in the placentas of all mammalian species reported thus far and have been localized to the trophoblast and endometrium [8, 9, 12–16]. These data suggest that leptin plays a functional role in placental physiology. For example, leptin has been shown to increase secretion of human chorionic gonadotropin by cultured human cytotrophoblast cells and placental explants [17, 18] and to inhibit cytotrophoblast cell secretion of vascular endothelial growth factor (VEGF) [19] and progesterone [20].

Leptin also promotes the expression of matrix metalloproteinase-2 (MMP-2) and the activity of MMP-9 in cultured human cytotrophoblast cells [21]. MMP-2 and -9, also known as gelatinases A and B, are members of a large family of extracellular matrix-degrading enzymes. During embryo implantation, embryonic trophoblast cells must penetrate the maternal endometrium, eventually allowing exchange between maternal and fetal blood. MMPs are expressed by both maternal endometrium and invading trophoblast cells during the time of embryo implantation in mice [22]. MMP activity, particularly that of MMP-9, is required for human and mouse cytotrophoblasts to invade extracellular matrix in vitro [23, 24]. Thus, leptin may be involved in trophoblast invasion. The potential association between leptin and trophoblast invasion is also suggested by the observation of abnormal leptin levels in preeclampsia, a complication of pregnancy associated with reduced trophoblast invasion [25].

Here we test the hypothesis that leptin promotes trophoblast invasion, a key step in the formation of the placenta. We have established a primary mouse trophoblast cell culture system and have used it to test the ability of leptin to stimulate invasion through a matrigel-coated membrane. Trophoblast cells were also cultured in the presence of GM6001, an inhibitor of MMP activity, to determine whether leptin stimulation of trophoblast cell invasion is dependent on MMP activity.

MATERIALS AND METHODS

Reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

Animals

All animal procedures were approved by the Boston University Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Timed-bred, Swiss-Webster mice were obtained from Taconic (Germantown, NY). On Day 10 or Day 18 of pregnancy, mice were killed by CO₂ inhalation, and tissues were collected for cell culture. Day 10 was chosen because it is a time of active invasion, the latest day of pregnancy at which MMP-9 is expressed [22], and the earliest stage of

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pregnancy at which placentas could be readily dissected from the uterus. Day 18 was chosen to represent late pregnancy.

Trophoblast Cell Isolation

Trophoblast cells were cultured according to a previously described method [26], with modification [27]. Briefly, placentas were separated from the underlying endometrium using dissecting forceps in a sterile dish containing wash medium (medium 199, 20 mM HEPES, 10 mM NaHCO₃, penicillin/streptomycin). The placentas were then incubated in dissociation medium (wash medium containing 1 mg/ml collagenase, 20 μg/ml DNase) for 1 h at 37°C, with periodic pipetting to separate cells. Cells were washed to remove dissociation medium, then collected and filtered to remove undigested tissue. The cells were separated on an isotonic 40% Percoll gradient. The trophoblast cell layer was collected and plated in NCTC-135 medium, with 20 mM HEPES, NaHCO₃, 1.65 mM cysteine, 10% fetal calf serum, and penicillin/streptomycin.

Immunocytochemistry

An antibody to cytokeratin was used to verify the cell type of isolated trophoblast cells. The cells were plated on uncoated inserts in a 24-well plate in NCTC-135 medium with 10% fetal calf serum for 24 h. Cells were washed in 0.1 M PBS and then fixed in 1.5% paraformaldehyde, 0.1% Triton X-100 for 20 min. Cells were incubated in methanol containing 0.3% H₂O₂ for 10 min and rinsed three times in PBS. After 20 min blocking in 5% normal serum, cells were incubated with mouse monoclonal anti-pan-cytokeratin at a 1:100 dilution for 1 h. Cells were rinsed in PBS, then incubated with sheep-anti-mouse IgG conjugated to horseradish peroxidase for 1 h. Diaminobenzadine was used as a peroxidase substrate for visualization of antibody binding. Cells were counterstained in hematoxylin (Fisher Scientific, Pittsburgh, PA) and coverslipped in a glycerol mounting medium. The slides were coded and controls were incubated with secondary antibody only.

Matrigel Invasion Assay

On Pregnancy Day 10, isolated trophoblast cells from the placentas of 2–8 mice were pooled and divided into matrigel invasion chambers at a density of 5 × 10⁴ cells per insert in a 24-well plate (BD Biosciences, Bedford, MA). The cells were cultured overnight in NCTC-135 containing 10% fetal calf serum (FCS) at 37°C, in an air/5% CO₂ atmosphere. Medium was replaced the following morning with serum-free NCTC-135, containing 0.25, 50, 100, or 250 ng/ml recombinant mouse leptin. Duplicate wells were used for each treatment. Each of the eight experiments included a control group (medium only) and leptin treatment groups derived from the same pool of trophoblast cells.

After a 24-h incubation, cells remaining above the insert membrane were removed by gentle scraping with a swab. Cells that had invaded through the matrigel to the bottom of the insert, were fixed in 1.5% paraformaldehyde, 0.1% TritonX-100 for 1 h. After washing with 0.1 M PBS, the cells were stained with hematoxylin-eosin. The insert was then washed in 70% ethanol and briefly air dried, then mounted on a glass slide and coverslipped in a glycerol mounting medium. The slides were coded to prevent counting bias, and the number of invaded cells on a representative section of each membrane were counted under a light microscope. The number of invaded cells for each experimental sample (n = 1) represents the average of duplicate wells.

The effect of leptin treatment on the number of invaded cells was analyzed by two-way ANOVA, with leptin treatment and trophoblast pool as effects. The trophoblast pool was included to account for the potential effect of isolating and plating different numbers of cells in each experiment. Tukey test was used for post hoc comparisons. A P-value < 0.05 was considered significant.

The same procedure was used to test the effect of leptin on trophoblast cells from Day 18 of pregnancy. All placentas from 1–2 pregnant mice were pooled for each experiment. Only the control (serum-free medium) and 50 ng/ml leptin treatments were tested. A paired t-test was used to compare the number of invaded cells.

Bromodeoxyuridine Assays

Cell proliferation assays were performed to determine whether changes in the number of invaded cells in the matrigel assays were due to changes in cell invasiveness or to changes in total cell number. Bromodeoxyuridine (BrdU), an analog to thymidine, was used to incorporate BrdU into dividing cells in the place of thymidine. The amount of incorporated BrdU was then measured using specific antibody. Isolated trophoblast cells from the placentas of 1–2 mice on Pregnancy Day 10 were pooled and 5 × 10⁴ cells were plated in each well of a 96-well plate. For each experiment (n = 1), trophoblast cells from a common pool were divided into control and leptin-treated wells. After an overnight incubation in NCTC-135 medium containing 10% FCS, cells were treated with BrdU in serum-free medium or in serum-free medium containing 50 ng/ml leptin, which is the treatment that resulted in the highest number of invaded cells in the matrigel invasion assays. Twenty-four hours later, BrdU incorporation was measured using a BrdU Cell Proliferation Assay (Oncogene Research Products, San Diego, CA) according to the manufacturer’s instructions. Briefly, the medium was removed and cells were washed in PBS, then exposed to fixative/denaturation solution for 30 min. Cells were then incubated with an anti-BrdU antibody for 60 min. After washing, cells were incubated with a peroxidase-conjugated secondary antibody for 60 min. After washing, the cells were exposed to a peroxidase substrate. The peroxidase reaction was quantified by measuring absorbance at 450/540 nm in a microplate reader. Background absorbance (from cells cultured without BrdU) was subtracted from the absorbance in sample wells to produce a relative measure of cell proliferation. Absorptions in the control (medium alone) and leptin-treated cells were compared by paired t-test. A P-value < 0.05 was considered significant.

Matrix Metalloproteinase Inhibitor

Matrigel invasion assays were also conducted in the presence of an MMP inhibitor, GM6001, to determine whether the effect of leptin on trophoblast invasion is MMP dependent. Trophoblast cells were isolated and cultured from mice on Pregnancy Day 10, as described above. After overnight incubation, cells were treated with 0 or 50 ng/ml leptin in combination with 25 μM GM6001 (Chemicon, Temecula, CA) in serum-free medium. The GM6001 concentration was chosen from the effective range used to inhibit matrigel invasion in a previous study [24]. Cells were then fixed and counted as described previously. Invasion in each of the GM6001-treated groups was compared with invasion in paired controls (0 GM6001, 0 leptin) taken from the same pool of isolated trophoblast cells. A P-value < 0.05 was considered significant in the paired t-tests.

RESULTS

Immunocytochemistry

The identity of trophoblast cells was confirmed by immunostaining for cytokeratin, an epithelial cell marker. Nearly all cells showed positive staining for cytokeratin (Fig. 1), indicating that the cell cultures did not contain large numbers of contaminating fibroblast or endothelial cells.

Trophoblast Invasion

On Pregnancy Day 10, leptin treatment significantly increased the number of trophoblast cells that invaded through matrigel-coated inserts (P = 0.006) (Fig. 2). The number of invaded cells was higher than the number in control wells at all of the leptin concentrations tested. The 50 ng/ml treatment, which is similar to peak plasma leptin concentrations during late pregnancy in the mouse, had the greatest effect on invasion. Leptin concentrations greater than 50 ng/ml did not cause additional cell invasion.

In trophoblast cells isolated from mice during late pregnancy (Day 18), there was no significant effect of leptin treatment on the number of invaded cells (P = 0.13, n = 14; not shown). Although a larger number of cells invaded in cultures treated with 50 ng/ml leptin, (443 ± 107 vs. control, 374 ± 90), the difference was small and highly variable.

Cell Proliferation

The level of cell proliferation, as determined by BrdU incorporation, was low in both control and leptin-treated cultures of Day 10 trophoblast cells. Leptin caused a small, but significant, decrease in cell proliferation (Fig. 3). Thus,
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FIG. 1. Immunocytochemistry. A) Cultured placental cells were plated on uncoated inserts and immunostained with an antibody to cytokeratin to confirm their identity as trophoblast cells. Immunostaining appears brown (arrows). Cells were counterstained with hematoxylin (purple). B) As a control, cells were incubated with secondary antibody only. No immunostaining was observed in control cells. The dark circles are pores in the insert (arrowheads).

FIG. 2. Effect of leptin on trophoblast cell invasion. Trophoblast cells were isolated and cultured in matrigel invasion chambers in serum-free medium containing 0, 25, 50, 100, or 250 ng/ml leptin for 24 h, and the number of cells that invaded through the matrigel were counted. Leptin treatment had a significant effect on the number of invaded cells (P < 0.006 ANOVA). Points with different letters are significantly different (Tukey test). The number of replicates for each treatment is indicated within the data points. Bars indicate standard error.

FIG. 3. Effect of leptin on cell proliferation. Trophoblast cells were cultured for 24 h in serum-free media (control) or serum-free media containing 50 ng/ml leptin (leptin) in the presence of BrdU. Absorbance at 450-540 nm reflects BrdU immunoreactivity in the treated cells. The * indicates a significant difference by paired t-test. The number of replicates is indicated within each column. Bars indicate standard error.

the leptin-induced increase in the number of invaded cells (above) was due to increased invasiveness by the trophoblast cells present in culture, not to an increase in cell number.

Role of Matrix Metalloproteinases

GM6001, an inhibitor of MMP activity, was used to determine whether the leptin stimulation of trophoblast invasion in Day 10 trophoblast cells was dependent on MMP activity (Fig. 4). At the concentration tested, GM6001 had no effect on the basal level of trophoblast invasion. There was no significant difference in trophoblast invasion between control cells (0 leptin, 0 GM6001) and cells treated with 25 μM GM6001. However, in the presence of GM6001, leptin treatment did not stimulate invasion. In cells treated with GM6001 and 50 ng/ml leptin, trophoblast invasion was similar to invasion in paired controls (0 leptin, 0 GM6001).

DISCUSSION

The present study demonstrates that leptin significantly increased invasion by Day 10 mouse trophoblast cells in vitro and that this effect was dependent on matrix metalloproteinase activity. This is the first report to establish a potential link between the effects of leptin on MMP activity [21] and the effects of MMPs on trophoblast cell invasion [22–24].

Leptin had the greatest effect on trophoblast cell invasion at the 50 ng/ml dose, which roughly corresponds to the highest plasma leptin levels that occur during pregnancy in the mouse [28]. Higher concentrations did not cause any additional stimulation. This suggests that there is an optimal range of leptin concentrations, and therefore body fat, for trophoblast invasion.

Leptin did not stimulate cell proliferation, but rather caused a small decrease in proliferation, during the 24-h culture period used in this study. Thus, the increased invasion associated with leptin treatment was not due to an increase in cell proliferation. The BrdU results contrast with
column. Bars indicate standard error. Data from columns indicated with

different from controls. The number of replicates is indicated within each
column. Bars indicate standard error. Data from columns indicated with
†are also included in the averages shown in Figure 2.

two previous studies, in which leptin stimulated cell prolif-
eration in transformed human trophoblast cells, BeWo and JAR cells [29, 30]. The discrepancy could be due to a
species difference between mice and human trophoblast

cells, but is more likely the result of differences between
primary and transformed cells. Unrestrained proliferation is
a fundamental characteristic of transformed cells, and thus,
hormones may affect proliferation very differently in pri-
mary and transformed cells. Studies in primary human tro-
phoblast cells are needed to resolve this discrepancy.

We found that the ability of leptin to stimulate trophob-
blast invasion was completely blocked in the presence of an
inhibitor of MMP activity. Further study is needed to
fully elucidate the mechanism by which leptin induces tro-
phoblast invasion. However, we can conclude that MMP
activity is a part of this mechanism and that it is required for
leptin to induce trophoblast invasion. This is consistent
with previous findings that leptin increases MMP expres-
sion in trophoblast [21] and endothelial cells [31], but dif-
fers from the finding that leptin had no effect on MMPs in
cultured human endometrial epithelial or stromal cells [32].
This discrepancy suggests that leptin’s effect on MMPs and
invasion may be cell-type specific. Alternatively, leptin ef-
fects on MMPs and invasion may depend on reproductive
status or stage, an issue we address with respect to the
trophoblast below. For example, leptin receptor expression
varies during the menstrual cycle in human endometrium,
with peak levels occurring at the late secretory phase and
high expression visible during the implantation period [16].
The cultured endometrial cells studied by Quinton et al.
[32] did express leptin receptors, but were taken from bi-
opies of nonpregnant women. It is not known whether lep-
tin would have a similar effect on the endometrium during
pregnancy.

The expression of leptin receptors in the placenta ap-
ppears to be universal. Leptin receptor has been found in the
placenta in mice [11], rats [15], humans [8, 16], baboons
[9], bats [14], and sheep [13]. This suggests that leptin has
a conserved physiological function in the placenta, and our
results suggest that trophoblast invasion may be one such
function. We reported previously that total leptin receptor
expression increases in the mouse placenta between early
and late pregnancy [14], and a similar increase has been
reported in the rat [15], although no change in expression
was detected during baboon pregnancy [9]. Although leptin
receptor expression peaks in late pregnancy, trophoblast inva-
sion occurs primarily in early pregnancy. Thus, we com-
pared the effect of leptin on trophoblast cells from early
and late pregnancy. Leptin treatment was not as effective
at stimulating invasion in Day 18 trophoblast cells as in
stimulating cells from Pregnancy Day 10. This suggests that
leptin stimulates trophoblast invasion during early pregnan-
cy but has a different function(s) in trophoblast cells during
late pregnancy. Further study is needed to identify physi-
ological functions of leptin during late pregnancy and to
identify the cellular changes that underlie the diminished
stimulation of invasion that occurs in late pregnancy. Stud-
ies in the rat found that placental expression of the b iso-
form of the receptor, associated with JAK-STAT signaling,
remains constant, while expression of the short a isoform
increases from early to late pregnancy [15, 33]. It is pos-
sible that leptin stimulation of invasion occurs through the
ObRb isofrom, whereas leptin function in the placenta of
late pregnancy occurs through ObRa.

Nutrition is an important regulator of placental devel-
velopment. In humans, leptin levels are proportional to total
body fat, and thus, reflect nutritional status [34]. Although
the placenta is a significant source of leptin in later preg-
nancy in humans, maternal fat is still the major source of
leptin production at the time of embryo implantation and
placental formation. Our finding that leptin promotes in-
vasion of trophoblast cells, an initial step in placental de-
velopment, reveals one mechanism by which nutrition may
affect placental development.

In addition, our findings may shed light on the associa-
tion between leptin and preeclampsia. Preeclampsia is a
condition in which high resistance at the maternal-fetal in-
terface causes maternal hypertension, and it is associated
with impaired placental development due to reduced tro-
phoblast invasion [25]. Cytotrophoblasts from normal hu-
man pregnancies showed reduced MMP-2 activity when co-
cultured with decidual endothelial cells from preeclamptic,
but not normal, pregnancies [35]. Elevated plasma leptin
concentrations and placental leptin content have been found
in women with preeclampsia [36–38]. This increase in pla-
cental leptin expression in patients with preeclampsia has
been correlated with umbilical artery resistance [38]. The
association between high leptin levels and a condition of
reduced trophoblast invasion could imply that leptin inhib-
its trophoblast invasion. However, our data show that, in
vitro at least, leptin stimulates trophoblast invasion. Thus,
elevated leptin is not likely to be the cause of impaired
trophoblast invasion in preeclampsia. Placentas from pre-
eclamptic pregnancies may instead be leptin resistant, or a
failure of proper trophoblast invasion may affect normal
negative feedback loops that regulate placental leptin pro-
duction. Further studies to determine downstream effectors
of leptin in trophoblast invasion or to examine leptin resis-
tance in trophoblast cells may provide additional insight
into preeclampsia.

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