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Leptin-directed embryo implantation: Leptin regulates adhesion and outgrowth of mouse blastocysts and receptivity of endometrial epithelial cells

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Abstract

Leptin is a 16-kDa multifunctional protein. Recent reports indicate that leptin is an important molecule during implantation and placentation, implicated in embryonic-maternal cross-talk and cytotrophoblast invasiveness, however, the role of leptin playing in the process of normal blastocyst implantation has not been well characterized. In the present study, the possible mechanisms of leptin playing in mouse blastocyst implantation were investigated. Leptin and receptor isoforms mRNAs were detected in whole mouse uteri during estrous cycle and peri-implantation periods. Immunofluorescent analysis further confirmed Ob-R protein was present in mouse uterus. The differential amounts of leptin and Ob-R isoforms suggested a role for leptin in such endometrial issues as blastocyst implantation. In vitro culture model for studying embryo implantation, leptin promoted mouse blastocyst adhesion and blastocyst outgrowth on fibronectin. Blastocysts treated with 300 ng/ml leptin had the greatest adhesion rate of $76.58 \pm 6.41\%$ ($P = 0.046$), and blastocysts treated with 30 ng/ml leptin had

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the greatest outgrowth rate of 78.64 ± 8.48% ($P = 0.005$). In isolated endometrial epithelial cells, leptin upregulated amounts of alpha v and beta 3 integrin, and promoted cell adhesion to such extracellular matrix proteins as fibronectin, laminin and type IV collagen, showing a dose- and time-dependent cell-adhesive capacity. Collectively, the information from the present study may partly account for leptin-induced mouse blatocyst implantation.

Keywords: Mice; Leptin; Implantation; Adhesion; Outgrowth; Integrin

1. Introduction

Leptin, the 16-kDa protein product of the *ob* gene, is a multifunctional hormone produced primarily in adipose tissue (Zhang et al., 1994). The biological actions of leptin on target tissues are carried out through interaction with its specific receptors, Ob-R. Ob-R is a member of the gp130 family of cytokine receptors which occurs in several receptor variants (Ob-Ra through Ob-Rf) that are generated by alternative splicing of the *db* leptin receptor gene (Tartaglia et al., 1995; Lee et al., 1996). In addition to regulating energy homeostasis, neuroendocrine, immune functions, etc. leptin was also found to be essential for normal embryo implantation (Zhang et al., 1994; Pelleymounter et al., 1995; Masuzaki et al., 1997; Schwartz et al., 1999; Ahima and Flier, 2000; Chehab et al., 1996; Ramos et al., 2005).

Implantation is an important event in the establishment of successful pregnancy. It requires delicate interactions between the embryos and the maternal uterine milieu. The adhesion of the trophoblasts to the endometrium and its subsequent invasion into the maternal tissue is highly regulated, by a pool of cell adhesive molecules and endocrine, paracrine, autocrine signals. Integrins are a family of transmembrane, heterodimeric adhesion receptors comprised of alpha and beta subunits. The combination of different subunits produces distinct integrin molecules that mediate cell adhesion to a variety of adhesive proteins of extracellular matrix such as fibronectin, vitronectin, laminin, and collagen. The interaction of integrin with matrix proteins regulates cell adhesion and cell migration responding to hormones and growth factors. Gonzalez et al. have shown that in human, leptin upregulates expression of beta 3 integrin subunit in endometrial epithelial cells, expression of alpha 2, alpha 5, alpha 6 integrin subunits, and activity of metalloproteinase-9 in cytotrophoblats (Gonzalez et al., 1999, 2001; Gonzalez and Leavis, 2001). Disruption of leptin signaling results in a significant reduction in amounts of beta 3 integrin in the mouse uterus (Ramos et al., 2005). The underlying mechanisms of leptin on mouse blastocyst implantation, however, have not been fully elucidated. In the present study, we characterized a differential gene expression of leptin and Ob-R in the mouse uteri during estrous cycle and early pregnancy. Moreover, leptin was found to enhance adhesion and outgrowth of mouse blastocysts on fibronectin. We also demonstrated that the increased capacity of leptin-treated cells to adhere to matrix proteins was paralleled by the change in the expression of alpha v and beta 3 integrins.

2. Materials and methods

2.1. Animals

Adult (virginal; 22–25 g) mice of the outbred Kunming white strain were purchased from the Experimental Animal Center, Institute of Zoology, Chinese Academy of Sciences, and maintained under a controlled photoperiod of 12-h light:12-h dark with food and water supplied ad libitum. The Guidelines for the Care and Use of Animals in Research enforced by Beijing Municipal Science and Technology Commission were followed. Virgin female mice were mated with fertile males of the same strain overnight. The following morning of finding a vaginal plug was designated as day 1 of pregnancy. Estrous cycle determination was conducted using vaginal smear and animals were classified according to the type of cells present in the smear.

2.2. Embryo collection and culture

The mice were induced to ovulate by an intra-peritoneal injection of a single dose of 5 IU of equine chorionic gonadotrophin (eCG) followed by a single injection of human chorionic gonadotrophin (hCG) 48 h later. Blastocysts flushed from the uterus on day 4 of pregnancy were transferred into droplets of preheated Ham's F-12 containing 2.20 mmol/L calcium lactate, 2.05 mmol/L glutamine, 12.5 mmol/L $NaHCO_3$, 0.4% bovine serum albumin, 3.67×10^{-7} M estrogen, and 400 IU/ml gentamicin sulfate, and incubated at 37 °C, 5% atmospheric CO_2 in a humidified chamber. To study effects of leptin on blastocyst attachment and outgrowth, groups of 30–50 blastocysts that were randomly selected were placed in 100 μl droplets of Ham's F-12 culture medium containing indicated concentration recombinant mouse leptin (R&D Systems, Abingdon, UK) covered by mineral oil. Criteria for attachment and outgrowth of blastocysts were followed as described by Liu et al. (2002).

2.3. Isolation and culture of endometrial epithelial cells

Endometrial epithelial cells were isolated by a modification of the method established by Grant and Wira (2003). Briefly, mouse uterine horns were cut to expose the endometrial luminal surface, and minced into small pieces of 2–3 mm. The tissue fragments were incubated in DMEM/F-12 medium containing 1 mg/ml type IV collagenase (Invitrogen, San Diego, CA) and 6 mg/ml dispase (Invitrogen) for 1 h at 4 °C followed by 20 min at 37 °C. After tissue sample was gently pipetted for 20 times in a centrifugation tube, the supernatant was passed through 100 μm and 40 μm nylon mesh (Small Parts Inc., Miami Lakes, FL), the epithelial sheets was recovered from the retained fragments on the 40 μm nylon mesh. The cells were incubated in culture medium consisting of DMEM/F-12 medium plus 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. After isolated cells had plated for 1 h, debris and unattached cells were removed by washing for three times with culture medium.

2.4. Indirect immunofluorescence

For immunofluorescent staining, as previously described (Cai et al., 2000), frozen sections (10 μm) and epithelial cells mounted onto poly-lysine-coated slides were fixed for

10 min in 4% paraformaldehyde solution at room temperature. The sections and cells were then washed in PBS and permeabilized for 15 min in 0.1% Triton X-100 solution. After one additional washing with PBS, the cells were incubated in 5% bovine serum albumin (BSA) for 1 h, and subsequently incubated with the primary antibodies against Ob-R (Abcam Ltd., Cambridge, UK), alpha v integrin (Sigma, St. Louis, MO), vimentin (Sigma), or pan-cytokeratin (Sigma) overnight at 4 °C. After four times of washing with PBS, cells were treated with anti-rabbit IgG or anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC) for 1 h at 37 °C. Amount of Ob-R and alpha v integrin proteins was assessed by confocal laser microscopy.

2.5. cDNA synthesis and PCR amplification

Total RNA was extracted using Trizol Reagent (Gibco-BRL, Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed in 25 μl of reaction mixture containing 30 U avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI). The PCR was conducted in a total volume of 50 μl containing 1.25 U Taq DNA polymerase (Tianwei Times Biotechnology Co. Ltd., Beijing) for 30 cycles of 45 s at 94 °C, 45 s at 57 °C, and 45 s at 72 °C. The specific primers of alpha v integrin, beta 3 integrin (Kim et al., 2003), beta 1 integrin, E-cadherin (Oka et al., 2002), leptin, Ob-Rt (a common extracellular domain of the Ob-R), Ob-Rb (the functional leptin receptor), Ob-Ra, Ob-Rc, Ob-Rd and Ob-Re (Morton et al., 1998) were used. Beta-actin mRNA (primer: forward 5′-GGC CCA GAG CAA GAG AGG TAT CC-3′ and reverse 5′-ACG CAC GAT TTC CCT CTC AGC-3′, 460 bp) was measured as an internal standard in all RNA samples.

2.6. Cell adhesion assay

The cell adhesion assay was modified according to the method described by Guo et al. (2000). In brief, 96-well tissue culture plates were coated with 20 μg/ml fibronectin (Sigma), 20 μg/ml type IV collagen (Sigma), 20 μg/ml laminin (Sigma), 100 μg/ml poly-lysine, or 1% BSA in PBS. A 100 μl solution was added to the wells and incubated for 2 h at 37 °C. Wells were then washed twice with PBS, and non-specific binding sites were blocked by incubation with 1% BSA in PBS for 1 h at 37 °C. After blocking, wells were washed twice with PBS and stored at 4 °C before use. Cells were treated with DMEM medium in the absence or presence of leptin at the indicated concentrations for various times, then, collected and suspended in complete medium without fetal calf serum. Subsequently, 2×10^4 cells in 100 μl were added per well and allowed to adhere for 1 h at 37 °C. Adhesion assays were terminated by the addition of 100 μl per well of 4% formaldehyde in PBS to the cell culture medium. Cells were fixed for 10 min at ambient temperature, washed three times with water, and stained for 1 h with 0.2% crystal violet in PBS. Cells were then washed five times with PBS and incubated for 30 min with 100 μl dimethyl sulfoxide (DMSO). The number of cells in each well was evaluated by measuring the absorbance (Abs) at 595 nm. The amount of cell adhesion was expressed as percent of cells adhering to fibronectin (FN), type IV collagen (COL) or laminin (LM) in comparison with that to poly-lysine (LY). Cell

adhesion (%) was calculated with the following formula:

$$\frac{\text{Abs(FN, COL, LM, LY-coated wells)} - \text{Abs(BSA-coated wells)}}{\text{Abs(LY-coated wells)} - \text{Abs(BSA-coated wells)}} \times 100$$

Each experiment was repeated four times, with three replicates.

2.7. Flow cytometric analysis

Endometrial epithelial cells were prepared for flow cytometry using previously described methods (Ahmed et al., 2002). Briefly, endometrial epithelial cells were washed with PBS, and incubated in the presence of primary antibodies as alpha v integrin, beta 1 integrin (Sigma) or E-cadherin (Sigma) for 45 min. After three washings with PBS, FITC-conjugated anti-mouse IgG or anti-rat IgG antibodies were added and incubated for 45 min. After an additional three washings, the fluorescence intensity of 10,000 cells was measured by an EPICS XL-MCL flow cytometer (Coulter, Miami Lake, FL). A negative control consisting of untreated cells was used to set the gate in each experiment.

2.8. Statistical analysis

All data are presented as mean ± S.E. Statistical analyses were performed using least squares regression analysis and one-way ANOVA with the Bonferroni–Dunnett's T3 post hoc multiple comparisons to examine for differences between every two groups. The relationships between concentration of leptin and the effects on the blastocysts and epithelial cells were determined by analysis of correlation. Only $P < 0.05$ was accepted as being statistically different ($^{*}P < 0.05$; $^{**}P < 0.01$). Each experiment was repeated at least three times.

3. Results

3.1. Amount of leptin and Ob-R in the mouse uteri

To examine whether uterus express leptin and receptor, mouse uteri were collected on different days of estrous cycle and early pregnancy. Total RNA was isolated from at least three mouse uteri each day and repeated for three times. The results depicted in Fig. 1A revealed that leptin and all receptor isoforms of Ob-Rt, Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re evaluated were present in the uterus during the estrous cycle. The amount of leptin and Ob-Rd mRNA appeared to be greater in estrus and diestrus phases as compared with metestrus and proestrus phases. The amount of Ob-Rc mRNA was greatest in the diestrus phase, whereas there were no significant changes in amounts of mRNA for Ob-Rt, Ob-Ra, Ob-Rb and Ob-Re mRNA during the estrous cycle.

The changes in amount of mRNA for leptin and Ob-R during the peri-implantation period are depicted in Fig. 1A. There were small amounts of Ob-Rt and Ob-Rc mRNA on days 1–3 of pregnancy with an increase on day 4. There were small amounts of Ob-Ra mRNA on day 1 pregnancy. There were no differences in amounts of Ob-Rd, Ob-Re, and Ob-Rb during the

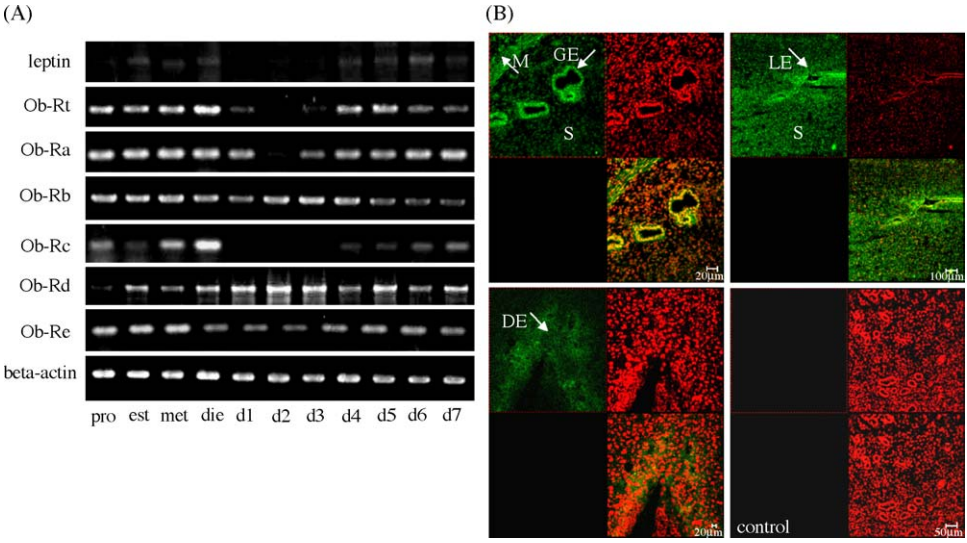

Fig. 1. Gene expression leptin and Ob-R on the mouse uterus. (A) Leptin and Ob-R mRNAs in mouse uterus during estrous cycle and peri-implantation. Ethidium bromide-stained gel showed products from RT-PCR of total RNA from whole uterus during peri-implantation period using primers specific for leptin and receptor isoforms. (B) Immunohistochemical localization of Ob-R in mouse uteri. pro, proestrus; est, estrus; met, metestrus; die, diestrus; LE, luminal epithelium; GE, glandular epithelium; S, stroma; M, myometrium, DE, decidua.

periods when this was assessed. To confirm the presence of Ob-R, immunohistochemistry experiments were performed on the mouse uterus. The Ob-R immunostaining was observed in both epithelial- and stromal cell-compartments, and the staining was more intense in the former than in the latter cell type (Fig. 1B). The Ob-R immunostaining was also observed in the myometrium, and decidua on day 7 of pregnancy. The differential amounts of leptin and Ob-R isoforms suggested a role for leptin in endometrial tissues during the peri-implantation period.

3.2. Effect of leptin on blastocyst attachment and outgrowth on fibronectin

Blastocysts began to adhere to fibronectin after 6 h and spread onto the substrate after 12 h. The numbers of attaching and outgrowing blastocysts were collected at 12 and 24 h, respectively. There was a positive correlation between blastocyst adhesion rate and concentration of leptin ($r = 0.516$, $P = 0.001$), and a positive correlation between blastocyst outgrowth rate and concentration of leptin ($r = 0.562$, $P = 0.001$). As depicted in Fig. 2, leptin promoted blastocyst attachment and outgrowth when compared with the control group. Blastocysts treated with 300 ng/ml leptin had a greater adhesion rate, with $76.58 \pm 6.41\%$ as compared with $50.53 \pm 6.35\%$ for the control group ($P = 0.046$, Fig. 2B), and blastocysts treated with 30 ng/ml leptin had the greatest outgrowth rate ($78.64 \pm 8.48\%$ compared with $36.44 \pm 7.08\%$ for the control group ($P = 0.005$, Fig. 2C).

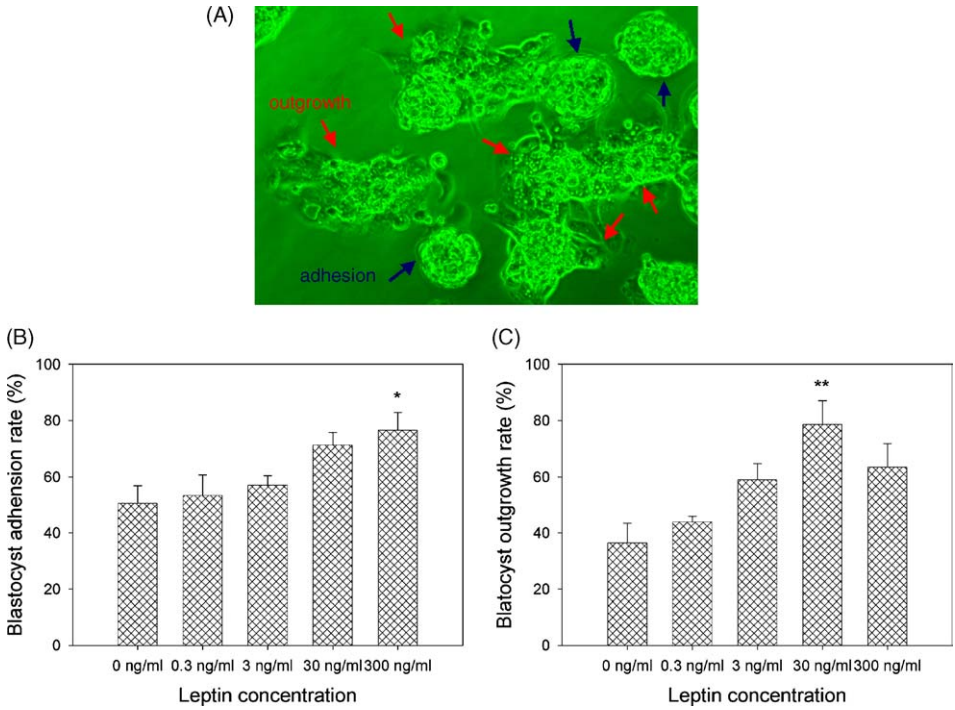

Fig. 2. Effect of leptin on attachment and outgrowth of blastocysts on fibronectin. Blastocyst attachment and outgrowth rates were determined by comparing the total number of blastocysts with the total numbers of attaching or outgrowing blastocysts. (A) The representative illustration of blastocyst attachment and blastocyst outgrowth. (B) The effect of leptin on blastocyst adhesion to fibronectin. The number of adhering blastocysts was collected after treatment with leptin for 12 h. (C) The effect of leptin on blastocyst outgrowth on fibronectin. The number of outgrowing blastocysts was collected after treatment with leptin for 24 h.

3.3. Leptin promoted epithelial cells adhesion to extracellular matrix

Primary endometrial epithelial cells were isolated (Fig. 3A), and the purity of the epithelial cells, checked by immunofluorescent staining of pan-cytokeratin (Fig. 3B) and vimentin (Fig. 3C), was over 90%. The cells were subjected to immunocytochemical analysis with Ob-R antibody, and immunoreactive staining was detected in the membrane and cytoplasm (Fig. 3E), while no fluorescence was observed in cells in the presence of a control rabbit IgG antibody (Fig. 3F).

There were positive correlations between adhesion rate of epithelial cells and concentration of leptin ($r = 0.866$, $P = 0.003$ in all cases). As depicted in Fig. 4A, leptin promoted a dose-dependent adhesion of endometrial epithelial cells on all matrix proteins. Endometrial epithelial cells were treated with leptin at a range of 0–30 ng/ml for 24 h, with a maximal number of cells adhering to the matrix proteins at a concentration of 30 ng/ml ($P < 0.001$ in all cases). Moreover, there were positive correlations between adhesion rate of epithelial cells and time of treatment (FN: $r = 0.596$, $P = 0.014$; COL: $r = 0.745$, $P = 0.005$; LM:

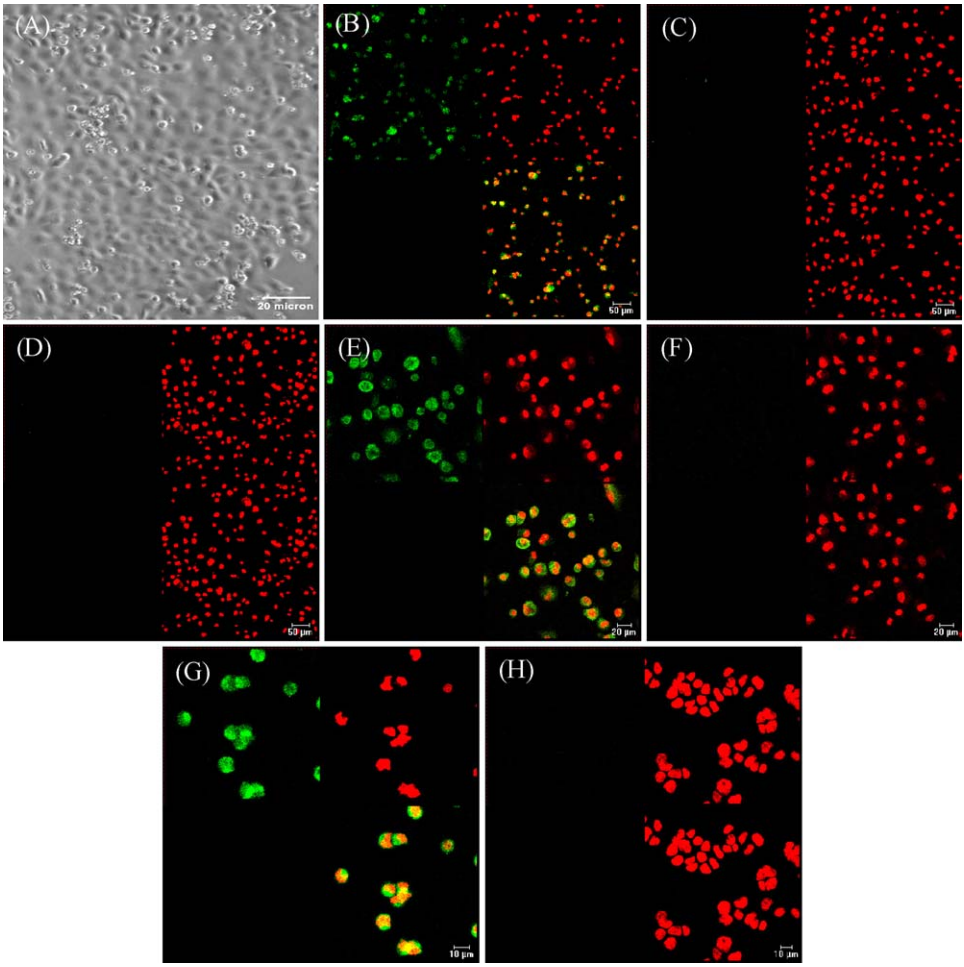

Fig. 3. Immunocytochemical staining for Ob-R in the cultured mouse endometrial epithelial cells. Endometrial epithelial cells were cultured on sterile coverslips for 3 h, then, prepared for immunocytochemistry. (A) Morphology of isolated primary endometrial epithelial cells. The purity of the cells, checked by immunofluorescent staining of pan-cytokeratin (B) and vimentin (C), was over 90%. The cells showed positively staining for Ob-R (E). (D) and (F) served as the controls. RL95-2 cell line stained with Ob-R (G) was used as a positive control, and cells incubated with rabbit IgG control antibody (H) was as negative control.

$r = 0.447$, $P = 0.023$). Leptin had a time-dependent cell-adhesive capacity, with a maximal effect observed at 24 h. (FN, $P = 0.023$; COL, $P = 0.008$; LM, $P = 0.038$; Fig. 4B).

3.4. Alpha v and beta 3 integrins in mouse endometrial epithelial cells

Mouse endometrial epithelial cells were also isolated for studying the regulation of integrins expression by leptin. Alpha v integrin was clearly detected in endometrial epithelial

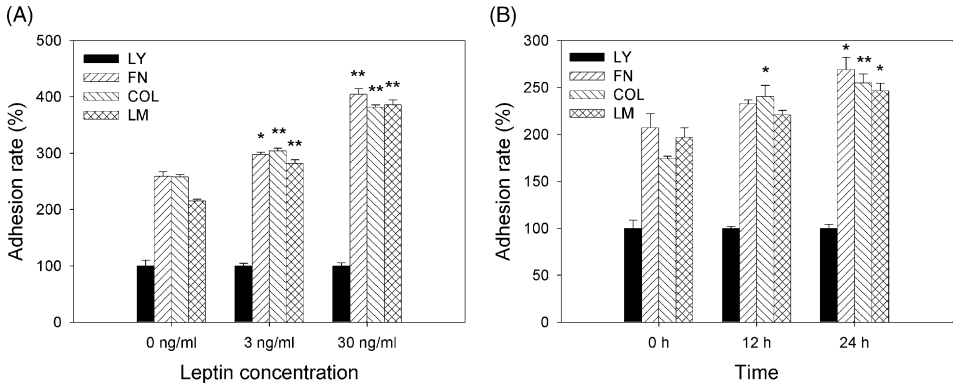

Fig. 4. Effect of leptin on adhesion of mouse endometrial epithelial cells. Cells were treated with DMEM medium in the absence or presence of leptin at the indicated concentrations for various times. Cell adhesion assay was performed as described in Section 2. Each data represents the mean ± S.E. ($n = 4$ experiments with three replicates). (A) Epithelial cells were treated with various concentration of leptin for 24 h. (B) Epithelial cells were treated with 3 ng/ml leptin for 0, 12 and 24 h. FN, fibronectin; COL, type IV collagen; LM, laminin; LY, poly-lysine.

cells (Fig. 5A). As depicted in Fig. 5B, leptin increased the amount of mRNA for alpha v integrin at concentrations in the range of 0.3–300 ng/ml, but not of beta 1 integrin or E-cadherin. Leptin also upregulated the amount of beta 3 integrin mRNA. We next examined the effect of leptin on alpha v integrin protein expression by flow cytometric analysis, a amount of alpha v integrin protein was stimulated enhanced by leptin as shown depicted in Fig. 5C and D.

4. Discussion

Leptin is structurally related to cytokines and exerts its action through Ob-R, its receptor that belongs to the cytokine-receptor superfamily. Previous studies have demonstrated that alternative splicing of Ob-R results in the production of multiple isoforms with an identical extracellular domain and a variety of cytoplasmic domains (Tartaglia et al., 1995; Lee et al., 1996). In this study, we have analysed gene expression of leptin and Ob-R isoforms in mouse uteri and observed a variation in the isoforms of Ob-R present in different days of early pregnancy. The temporal expression profile of leptin and Ob-R isoforms throughout early pregnancy suggested that leptin could be a modulator in the regulation of endometrial issues such as embryo implantation.

Embryo implantation is a complex process that involves interactions between the embryo and endometrium. During the “implantation window” period, appropriate development and attachment of the embryo is the initial event for establishment of a successful pregnancy. Many key molecules such as hormone, growth factors and cell adhesion molecules function in concert to stimulate embryos to develop and attach to the endomentrium. Leptin, functioning through a receptor present in the embryo from the oocyte to the hatched blastocyst stage, has been shown to stimulate development of embryos (Fedorcsak and Storeng, 2003;

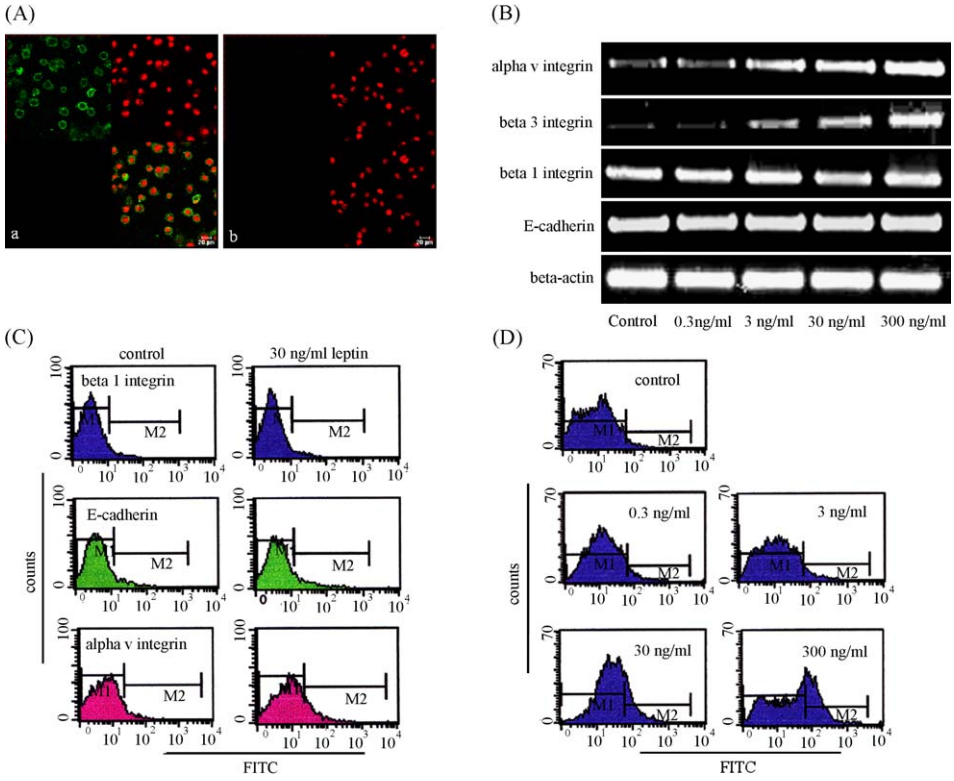

Fig. 5. Effect of leptin on alpha v and beta 3 integrins in mouse endometrial epithelial cells. (A) Immunocytochemical staining for alpha v integrin in the cultured mouse endometrial epithelial cells. Endometrial epithelial cells were cultured on sterile coverslips for 3 h, then, prepared for immunocytochemistry: (a) cells incubated with alpha v integrin antibody; (b) cells incubated with mouse IgG control antibody. (B) Effect of leptin on alpha v integrin mRNA expression. RNA was prepared from cells treated with leptin at a concentration of 0.3–300 ng/ml for 24 h. The mRNA for alpha v, beta 1, beta 3, and E-cadherin were analyzed. (C) Effect of leptin on beta 1 integrin, E-cadherin and alpha v integrin protein expression. The flow cytometric analysis revealed that no significant changes were observed for E-cadherin and beta 1 integrin. In contrast, alpha v integrin was upregulated. (D) Expression of alpha v integrin protein was significantly increased by leptin.

Kawamura et al., 2002, 2003; Cervero et al., 2004; Swain et al., 2004). Furthermore, leptin has been shown to upregulate gene expression for the matrix metalloproteinases (MMPs), and enzymes involved in trophoblast invasion in cultured human and mouse trophoblast cells (Gonzalez et al., 2001; Schulz and Widmaier, 2004). Our results showed that leptin could promote adhesion and outgrowth of mouse blastocysts on fibronectin. This suggested that leptin might play a physiological role in adhesion and invasion of the trophoblast cells to maternal endometrium in vivo.

Synchronized development of the embryo to the blastocyst stage, and differentiation of the uterus to the receptive state are essential to the embryonic implantation process (Ma et al., 2003). The endometrial epithelium is a specialized structure that displays a receptive state for embryo during the brief “implantation window”. Integrins, the glycoproteins

serving as receptors for extracellular matrix ligands and acting as modulators of cellular processes, are one of the most thoroughly characterized of the immunohistochemical markers of uterine receptivity function (Hynes, 1992; Damsky et al., 1993). Integrin-mediated interactions of cells with components of the extracellular matrix regulate cell adhesion and cell migration (Troussard et al., 1999). Leptin upregulates the expression of beta 3 integrin in human endometrial epithelial cell, gene expression for the alpha 2, alpha 5, alpha 6 integrin subunits, and activity of metalloproteinase-9 in the human cytotrophoblast (Gonzalez et al., 1999, 2001; Gonzalez and Leavis, 2001). The most likely maternal integrins to be involved in blastocyst attachment are those of the alpha v family (Aplin, 1997). Results from the present study indicate leptin to be a strong stimulator of the gene expression of the alpha v and beta 3 integrin subunits in endometrial epithelium. Moreover, leptin promoted endometrial epithelial cells adhering to fibronectin, collagen and laminin. An attachment mechanism involving alpha v and beta 3 integrins may require a bifunctional bridging ligand to span between receptors on the embryonic and maternal cell surfaces (Aplin, 1997). Fibronectin, type IV collagen and laminin are the potential bridging ligands (Kimber and Spanswick, 2000). Based on these studies as well as the present data, we propose that leptin-increased gene expression for alpha v and beta 3 integrins stimulated endometrial epithelial cell adhesion, with the functional consequence being enhanced endometrial epithelial cell receptivity to embryonic implantation.

In conclusion, this current study for the first time revealed differential expression of leptin and its multiple Ob-R isoforms in mouse uterus throughout estrous cycle and early pregnancy. In vitro model of embryo implantation, leptin enhanced blastocyst attachment and outgrowth. Moreover, leptin promoted adhesion of primary endometrial epithelial cells onto fibronectin, collagen and laminin probably by up-regulating the expression of alpha v and beta 3 integrins. These data supported that leptin is required for blastocyst implantation in mouse.

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