

Leptin modulates extracellular matrix molecules and metalloproteinases: possible implications for trophoblast invasion

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Leptin is a circulating hormone which plays an important role in the regulation of energy balance, haemopoiesis and reproduction. Leptin and its receptor (leptin-R) are localized in human placental tissue but their function is not known. In this study we have investigated the expression of leptin and leptin-R in the human placenta with particular attention to extravillous cytotrophoblastic cell islands and cell columns which play a pivotal role in trophoblast invasion and placental growth. We demonstrate that leptin-R immunoreactivity shows a strong expression in the distal extravillous cytotrophoblastic cells of cell columns invading the basal plate, whereas leptin expression is homogeneously expressed in all the cellular components of cell columns. Since the invasive ability of the distally located extravillous cytotrophoblast of cell columns is known to be regulated by a variety of proteases and some extracellular matrix molecules, we tested the influence of leptin on the in-vitro production of matrix metalloproteinase (MMP)-2, MMP-9 and fetal fibronectin (fFN) by cytotrophoblastic cells. We demonstrate that leptin increases, in a dose-dependent manner, the secretion of immunoreactive MMP-2 and fFN and enhances the activity of MMP-9 in cultured cytotrophoblastic cells. Our results suggest that leptin and leptin-R could have a role in the invasive processes of the extravillous cytotrophoblastic cells by modulating the expression of MMPs. In addition, these results provide a foundation for studying pathological conditions characterized by insufficient or excessive trophoblast invasion.

Key words: fibronectin/leptin/leptin-R/metalloproteases/placenta

Introduction

Leptin, the obese gene product, is a hormone that regulates food intake and energy expenditure (Zhang *et al.*, 1994; Ahima *et al.*, 1996). Leptin is expressed in adipose tissue (Cinti *et al.*, 1997), human placenta (Masuzaki *et al.*, 1997; Senaris *et al.*, 1997), gastric fundus (Bado *et al.*, 1998) and mammary gland (Smith-Kirwin *et al.*, 1998). The leptin receptor (leptin-R) has been cloned and shown to be a member of the class I cytokine receptor family (Tartaglia *et al.*, 1995; Tartaglia, 1997). In addition, the *leptin-R* gene has been demonstrated to have at least five splice variants encoding proteins with identical extracellular domains, but different lengths of intracellular domains (Chen *et al.*, 1996; Lee *et al.*, 1996). Relevant changes in plasma leptin concentrations are observed in pregnant women (Butte *et al.*, 1997) and leptin is also detected in amniotic fluid and in arterial and venous cord blood (Matsuda *et al.*, 1997; Schubring *et al.*, 1997) as well as in the villous trophoblast (Masuzaki *et al.*, 1997). Bodner *et al.* (1999) have shown that leptin-R protein is localized at the apical membrane of the syncytium and its mRNA is present in the cytoplasm of the syncytiotrophoblast in human term placenta. These data suggest that leptin and leptin-R may have a role in the regulation of placental physiology. The human placenta is able to synthesize and secrete a variety of hormones and molecules

that regulate the fine balance between proliferation, differentiation and invasion of trophoblastic cells. The trophoblast includes the villous trophoblast (syncytiotrophoblast and cytotrophoblast), which covers the placental villi, and the extravillous cytotrophoblast of cell islands and cell columns. In the latter, important proliferative, differentiative and invasive processes of the placenta occur (Bulmer *et al.*, 1988; Castellucci *et al.*, 1991; Mühlhauser *et al.*, 1993, 1995; Benirschke and Kaufmann, 1995; Kaufmann and Castellucci, 1997).

The extravillous cytotrophoblastic cells of cell columns located proximally to the villous stroma have been identified as proliferating cells, whereas extravillous cytotrophoblastic cells located distally to the villous stroma show invasive characteristics and are no longer proliferative (Bulmer *et al.*, 1988; Castellucci *et al.*, 1991; Mühlhauser *et al.*, 1993). The invasive ability of these extravillous cytotrophoblastic cells has been demonstrated to be, at least in part, regulated by a variety of proteinases, their activators and inhibitors (Fisher *et al.*, 1985, 1989; Graham and Lala, 1991; Bischof and Martelli, 1992; Huppertz *et al.*, 1998; Liu *et al.*, 1998; Hu *et al.*, 1999; Feng *et al.*, 2000) and it is promoted by some extracellular matrix molecules, e.g. fetal fibronectin (fFN) (Feinberg *et al.*, 1991). Different families of matrix degrading proteases have been found to be involved in this process. Matrix metalloproteinases (MMPs) are a group of degradative

enzymes which are secreted as inactive zymogens and must be cleaved to become active. Huppertz *et al.* (1998) have shown strong immunostaining for MMP-2 and MMP-9 in the distal, invasive part of cell columns.

As one of the most important features of the placenta is the tightly regulated invasive behaviour and leptin has been identified in some of the main components of this organ (Masuzaki *et al.*, 1997; Senaris *et al.*, 1997), we postulated that leptin and its receptor could influence and modulate the invasive behaviour of the trophoblast during its invasiveness. In this study, we used primary cultures of cytotrophoblastic cells to test the influence of human leptin on MMP-2, MMP-9 and fFN production as these are molecules which regulate the invasive ability of the cytotrophoblastic cells. In addition, we investigated the immunolocalization of leptin and its receptor in the extravillous cytotrophoblastic cells of cell columns because no such data are available for these structures which play a pivotal role in trophoblast invasion.

Materials and methods

Tissues

In all, 19 human placentae at 7 ($n = 3$), 8 ($n = 2$), 10 ($n = 1$), 12 ($n = 5$), 38 ($n = 5$) and 40 ($n = 3$) weeks post-menstruation and umbilical cords (from 38–40 week placentae) were collected from clinically normal pregnancies interrupted by curettage (aspiration technique) for psychosocial or medical reasons that were unlikely to affect placental structure and function, or terminated by Caesarian sections or normal vaginal deliveries. Placentae were cut into small blocks. Specimens of white adipose tissue were collected from the abdominal wall of five patients undergoing Caesarian section at 38 weeks gestation. Ovarian tissue was obtained from young women undergoing laparotomy for reasons unrelated to ovarian pathology.

Tissue preparation for immunohistochemistry

Placental specimens ($n = 19$) were rapidly washed with 0.1 mol/l phosphate buffer at pH 7.4 and fixed for 4–12 h in 4% buffered formalin at 4°C. The specimens were then dehydrated in ethanol and embedded in paraffin wax. Serial sections (3 µm) were used for light microscopy and immunohistochemistry.

Samples for reverse transcription-polymerase chain reaction (RT-PCR) and Northern blotting

Placental specimens ($n = 19$), umbilical cords ($n = 8$), human white adipose tissue ($n = 5$) and human ovarian tissue ($n = 5$) were collected and immediately frozen and stored in liquid nitrogen until being used for RNA extraction. Human white adipose tissue was used as a positive control.

Immunohistochemistry

Primary antibodies

The following primary antibodies were used: (i) mouse monoclonal antibody anti-human pan-cytokeratin (Dako, Glostrup, Denmark; diluted 1:500 v/v); (ii) rabbit polyclonal antibody (A-20) raised against a peptide corresponding to amino acids 137–156 mapping at the carboxy terminus of human leptin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:100 v/v); (iii) affinity-purified goat polyclonal antibody (N-20) recognizing the amino terminus of all the alternatively spliced forms of the leptin receptor, i.e. ObR_S + ObR_L (amino acids 32–51 of the human leptin-R, Santa Cruz Biotechnology, diluted 1:200 v/v); (iv) affinity-purified goat polyclonal antibody (C-20) directed against the peptide corresponding to amino

acids 1146–1165 at the carboxy terminus of the human leptin-R, thus recognizing only the long form of the leptin receptor, ObR_L (Santa Cruz Biotechnology; diluted from 1:100 to 1:200 v/v). The specificity of the antibodies for the leptin receptor has been tested on human tissues and cells by the manufacturer (Santa Cruz Biotechnology) and by previous studies (Glasow *et al.*, 1998; Morton *et al.*, 1998) using Western blotting and immunohistochemistry.

Immunostaining

Immunohistochemical demonstration of leptin, leptin-R and pan-cytokeratin was performed on serial sections using avidin-biotin peroxidase (ABC) (Hsu *et al.*, 1981). Briefly, dewaxed sections (3 µm) were processed through the following incubation steps: (i) hydrogen peroxide 0.3% in methanol for 30 min to block endogenous peroxidase; (ii) normal rabbit serum (for leptin-R), normal goat serum (for leptin), or normal horse serum (for pan-cytokeratin), diluted 1:75 v/v, for 20 min to reduce non-specific background staining; (iii) primary antibody, overnight at 4°C; (iv) biotinylated secondary antibody: rabbit anti-goat immunoglobulin G (IgG) (for leptin-R), goat anti-rabbit IgG (for leptin), or horse anti-mouse IgG (for pan-cytokeratin) diluted 1:200 v/v, for 30 min (Vector Laboratories, Burlingame, CA, USA); (v) ABC complex for 1 h (Vectastain ABC kit; Vector Laboratories); (vi) histochemical visualization of peroxidase using 0.075% 3',3'-diaminobenzidine hydrochloride as chromogen (Sigma, St Louis, MO, USA) and hydrogen peroxide 0.02% in Tris buffer 0.05 mol/l, pH 7.6, for 5 min in a dark room. Sections were then rinsed in tap water, counterstained with haematoxylin, dehydrated and mounted with Eukitt (Kindler GmbH & Co, Freiburg, Germany).

Specificity tests were performed: (i) by omitting the primary antibody in the immunostaining procedure; (ii) by incubating sections with the antiserum saturated with homologous antigen (for this procedure, the antibody was incubated with a 10-fold excess of homologous peptide (15 µg/ml), for 48 h); or (iii) by replacing the primary antibody with the pre-immune serum. These controls showed negative results.

RNA extraction and analysis

Total RNA was extracted after homogenization of tissues considered above in the presence of 4 mol/l guanidinium isothiocyanate by ultracentrifugation through 5.7 mol/l caesium chloride gradient. cDNA synthesis was obtained from 5 µg of total RNA in 50 mmol/l Tris-HCl pH 8.3, 75 mmol/l KCl, 3 mmol/l MgCl₂, 10 mmol/l dithiothreitol, 1 mol/l of each dNTP, 250 ng of oligo(dT)₁₅, 250 ng random hexamers, 100 ng/µl nuclease-free bovine serum albumin, 20 IU rRNasin (Promega, Madison, WI, USA) and 500 IU of recombinant Molony murine leukemia virus reverse transcriptase (Gibco BRL, Milano, Italy). RNA and primers were kept at 70°C for 5 min and then cDNA synthesis was allowed to proceed at 37°C for 1 h.

Oligonucleotide primers sequences

Leptin primers:

L+ (5'-GATGACACCAAAACCCTCATC-3')

L- (5'-GGCCACCACCTCTGTGGAGTA-3')

Leptin receptor primers for the short form (ObR_S) of the receptor (exon 2):

LR2+ (5'-GGCTAAAAGGACACTTAAAT-3')

LR2- (5'-TTTCATTCAGATTGAAAAAGT-3')

Leptin receptor primers for the long form (ObR_L) of the receptor (exon 5):

LR5+ (TAGAATCTCTTCATCTGTTAAGA-3')

LR5- (5'-AATCCTTCTGAGAATGTG-3').

PCR conditions

Of the cDNA synthesized from 5 µg of total RNA 20% was used for PCR amplifications in 10 mmol/l Tris-HCl pH 8.3, 50 mmol/l

KCl, 5 mmol/l MgCl₂, 0.2 mmol/l of each dNTP, primers at a concentration of 0.5 µmol/l and 4 IU of recombinant *Taq* DNA Polymerase in a final volume of 50 µl. After denaturation at 96°C for 5 min, samples were subjected to 40 cycles (50 s at 94°C, 50 s at 60°C and 1 min at 72°C) using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). All cDNAs were also amplified with β-actin-primers to test their quality.

Cloning of PCR amplified cDNAs into phagemid vectors

The amplified leptin fragments were sequenced (using the same primers as those used for PCR amplification) with a PRISM™ sequencing kit (Perkin-Elmer) to confirm their identity, then cloned into plasmids (pCRII – TA Cloning, Invitrogen, Grohingen, The Netherlands) and used as probes after labelling with α-32P [dCTP] by random primers method (Megaprime Labelling Kit; Amersham, Buckinghamshire, UK).

Northern Blot analysis

RNA was quantified as follows: 10 µg of total RNA was electrophoresed in denaturing conditions (formaldehyde–agarose gel) and capillary-blotted with 20× sodium chloride/sodium citrate (SSC; 1× SSC = 150 mmol/l NaCl, 15 mmol/l sodium citrate, pH 7.0) overnight onto a nylon membrane (Hybond-N, Amersham), then UV cross-linked and baked. RNA blots were hybridized overnight at 68°C in hybridization buffer containing 5× SSPE (20× SSPE = 3 mol/l NaCl, 0.2 mol/l NaH₂PO₄, 25 mmol/l EDTA, pH 7.4), 2% sodium dodecyl sulphate (SDS), 10% dextran sulphate and 1.5 µg denaturated herring sperm DNA. Post-hybridization washing was performed twice in a solution of 2× SSC, 0.1% SDS and 1× SSC, 0.1% SDS at 56°C for 10 min each time. RNA blots were then exposed to Kodak X-Omat film at –70°C. Hybridization with the β-actin probe was performed to verify the RNA integrity and loading efficiency.

Preparation of cytotrophoblastic cells (CTB)

CTB were isolated, purified, characterized and cultured as previously described (Bischof *et al.*, 1991). Briefly, trophoblastic tissue obtained from legal abortions (at 7–12 weeks gestation) was digested by trypsin and the CTB were separated from blood cells and syncytiotrophoblast on a discontinuous Percoll gradient with the contaminating leukocytes being removed by immunopurification with an antibody to CD45 (Dako) coupled to magnetic particles (Dyna Beads; Dynal, Geneva, Switzerland). CTB were counted in a Neubauer cell in the presence of Trypan Blue (Sigma) and diluted to 10⁶ cells/ml. Cells were cultured overnight in Dulbecco's modified Eagle's medium (DMEM) containing 2 mmol/l L-glutamine, 4.2 mmol/l magnesium sulphate, 2.5 mmol/l HEPES, 1% gentamycin, 1% amphopterin B, 100 µg/ml streptomycin and 100 µg/ml penicillin in presence of 10% fetal bovine serum. The next morning (day 0), the medium was changed to serum-free DMEM and cells were incubated in the presence or absence of recombinant human leptin (rhLept, 0.001–1 µg/ml; R&D Systems, Bühlmann AG, Basel, Switzerland). Incubation was performed under a 5% CO₂ and 95% air atmosphere in a humid incubator at 37°C. The medium was changed on days 2 and 4 and the culture was stopped on day 4. The supernatants were divided into aliquots and stored at –20°C until assayed. The cells were lysed with 200 µl Triton X-100 (25% in water) and stored at –20°C for total cell protein measurements.

MMP-9 and MMP-2 enzyme-linked immunosorbent assays (ELISA)

MMP-2 ELISA

Microplates (96-well) were coated overnight at 4°C with 100 µl of sheep anti-human MMP-2 IgG (The Binding Site, Anawa, Wangen, Switzerland; 30 µg/ml in sodium carbonate buffer, 50 mmol/l, pH 9.6).

Unbound sites were blocked for 2 h at room temperature with 250 µl of 10% Blotto (non-fat dry milk; BioRad, Hercules, CA, USA) in phosphate-buffered saline (PBS) containing 0.02% NaN₃. Plates were then washed twice with PBS containing 0.1% Tween 20 (PBST; 250 µl/well) and once with PBST + 10% Blotto (PBSTB). Samples and standards were diluted in PBS containing 10% Blotto, applied in duplicate (100 µl/well) and incubated overnight at room temperature. After incubation, the plates were washed as described above, and incubated with biotinylated anti-MMP2 IgGs (100 µl/well) for 2 h at room temperature on a rotating platform. Plates were washed three times for 15 min with PBST, and once with PBSTB and re-incubated for 30 min at 20°C with avidin peroxidase (1/4000 in PBSTB, 100 µl/well). After washing four times with PBST, the plates were incubated in the dark for 10 min with *o*-phenylenediamine (1.2 mg/ml, 100 µl/well) and H₂O₂. The reaction was stopped by the addition of H₂SO₄ (2M, 50 µl/well) and the absorbance was measured at 492 nm in an ELISA plate reader (Lasysystem Multiscan; BioConcept, Allschwill, Switzerland).

Biotinylation of antibodies

Sheep anti-human MMP-2 IgG (500 µl, 13 mg/ml) was diluted 1:1 with bicarbonate buffer (0.1 mol/l, pH 8.4) and dialysed against this buffer for 48 h at 4°C. Activated biotin, at a concentration of 10 mg/ml in DMSO, was added (110 µl) and incubated for 2 h at room temperature. The preparation was then extensively dialysed against PBS containing 0.02% NaN₃, and stored at 4°C.

Purification of MMP-9

The purification procedure was performed using a human histiocytic lymphoma cell line (U937), according to a previously described protocol (Ward *et al.*, 1991). Pooled conditioned medium from U937 cells (4.8 l) to which 48 ml of 1 mol/l Tris, pH 7.6 was added, was applied on a Gelatin–Sephacrose column (5×2.5 cm), equilibrated in Tris 10 mmol/l, NaCl 1 mmol/l, CaCl₂ 10 mmol/l, 0.04% Brij, pH 7.6 (buffer A). The column was thoroughly washed with buffer A and eluted with 10% dimethylsulphoxide (DMSO) in buffer A. The presence of MMP-9 in the fractions was tested by gelatin zymography (see below). Fractions containing MMP-9 activity were pooled, dialysed against buffer A and applied to a Concanavalin-A–Sephacrose column (2.5×9 cm), equilibrated in buffer A. After washing, the column was eluted with 0.5 mol/l Methyl-α-D-manno-pyranoside. MMP-9-containing fractions were concentrated on a small Gelatin–Sephacrose column (0.5×10 cm). The pooled MMP-9 fractions were dialysed against Tris 0.01 mol/l, NaCl 0.1 mol/l, CaCl₂ 10 mmol/l, Brij 0.04%, divided into aliquots and stored at –20°C.

Production of anti-MMP-9 polyclonal antibodies

Purified MMP-9 was dialysed against PBS and 40 µg/rabbit was injected s.c. in different sites in two rabbits. A second 20 µg injection was carried out 5 weeks later, and a third was performed 4 weeks after the second one. Rabbits were bled 7 days after the third injection. Titration was monitored by Ouchterlony double-diffusion. Sera with a titre of ≥1/32 were pooled. An IgG preparation was obtained by ammonium sulphate precipitation of these pooled rabbit sera. The IgG concentration was estimated by measuring the optical density at 280 nm and found to be 9.6 mg/ml.

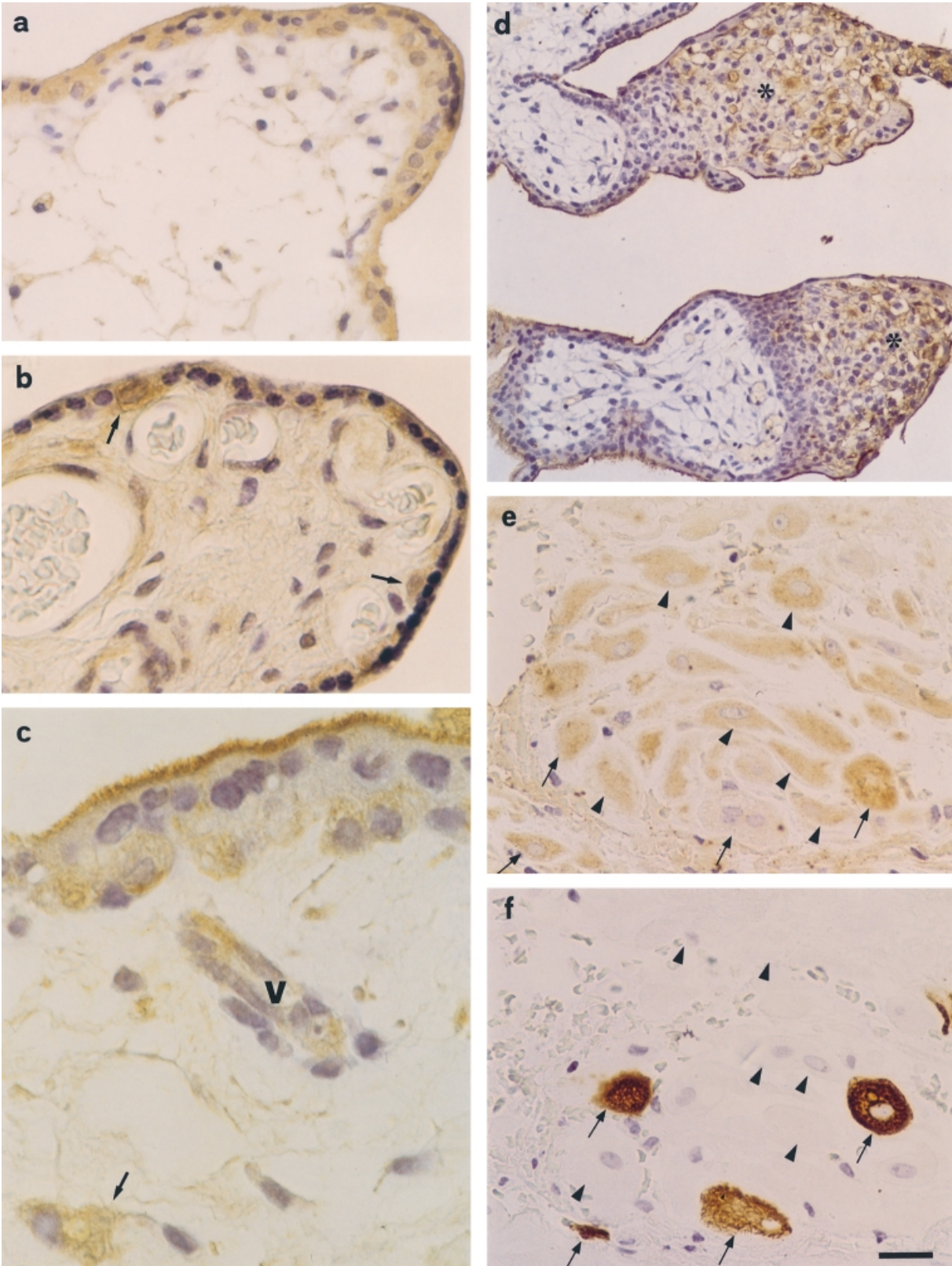
MMP-9 ELISA

Washing and incubation procedures were essentially the same as for the MMP-2 ELISA. Our rabbit anti-human MMP-9 IgG preparation was used for coating the plates (48 µg/ml). The second antibody was the commercially available sheep anti-MMP-9 IgG (The Binding Site, Anawa, Wangen, Switzerland), diluted 1:2000 in PBSTB. Peroxidase-labelled rabbit anti-sheep antibodies (100 µl/well) were then used in an incubation for 1 h at room temperature. Detection was performed as for MMP-2 ELISA.

Measurement of MMP-2 and MMP-9

The concentration of immunoreactive MMP-2 and MMP-9 were calculated by comparison to the standard curves (calibrated supernatants from gingival fibroblasts or U937 cells respectively), calcu-

lated as the optical density versus the log concentration of the MMPs and the results were expressed as ng/ml. These calculations were performed on a Macintosh computer (Power PC) using regression analysis from the StatView Programme (Abacus).



Gelatinolytic assays

Zymography was performed as previously described (Bischof *et al.*, 1991; Martelli *et al.*, 1993). Zymograms were scanned in an 'Apple Onescanner' and the surface of the digestion bands measured by the NIH Image 1.60 program with a Power Macintosh 7100/66 computer. All zymograms were evaluated using the same preset standards and the activities of MMP-2 and MMP-9 were expressed in arbitrary units/ml.

Hormone and protein assays

fFN was measured by a commercially available enzyme immunoassay with a sensitivity of 50 ng/ml and a coefficient of variation of 7.5% (Adeza Biochemical, Sunnyvale, CA, USA). Total cell proteins were measured in the lysed cells using the Bio-Rad protein assay according to the manufacture's instructions and using bovine serum albumin as the standard (Bio-Rad, München, Germany).

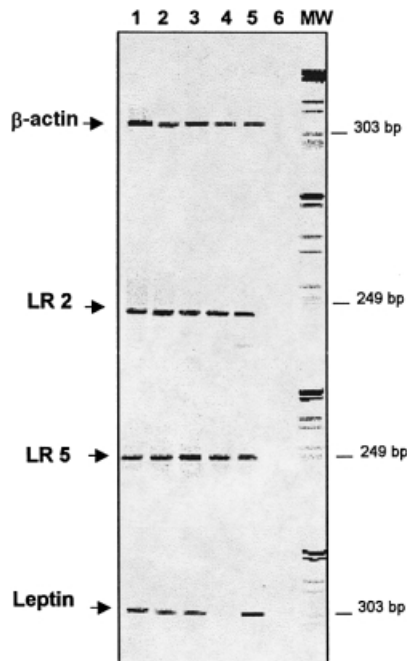


Figure 2. Reverse transcription–polymerase chain reaction (RT–PCR) expression analysis of Leptin Receptors (LR) splice variants and leptin in human placenta. Lane 1 = first trimester placenta; lane 2 = third trimester placenta; lane 3 = umbilical cord; lane 4 = human ovary; lane 5 = human white adipose tissue; lane 6 = control reaction (without DNA); MW = molecular markers (low range, BIO-RAD). β -actin, LR2 (the short splice variant of leptin receptor), LR5 (the long splice variant of leptin receptor), and leptin are shown. All tissues were extracted and reverse transcribed at the same time.

Statistical analysis

To evaluate the effects of rhLept on the different trophoblastic parameters, the individual values were transformed into values per mg cell proteins and per day [(conc.day2 + conc.day4)/4] and expressed as a percentage of the respective controls (CTB in absence of rhLept). All experiments were run in duplicates and repeated with three different preparations of CTB. Statistical analyses were performed by one-way analysis of variance, and analyses of correlation using the Statview 4.5 program on a Power Macintosh 7100/66 computer.

Results

Since the material from first trimester placentae only occasionally included substantial parts of the basal or chorionic plate our results concern the villous trees, cell islands and cell columns. However, the tissue probes from third trimester placentae covered representative parts of the whole placenta from the chorionic to the basal plate.

Immunohistochemical analysis of leptin and leptin receptor

Identical leptin receptor immunostaining patterns were observed with the antibodies recognizing the short and long form of leptin-R, suggesting that these forms are similarly expressed in the human placenta. The immunostaining patterns reported below were consistent across all samples.

Chorionic villi

Leptin immunoreactivity was present in the cytoplasm of the villous trophoblast (syncytiotrophoblast and cytotrophoblast), at all stages of pregnancy (Figures 1a,b). All the other villous components were negative for leptin. Leptin-R immuno-

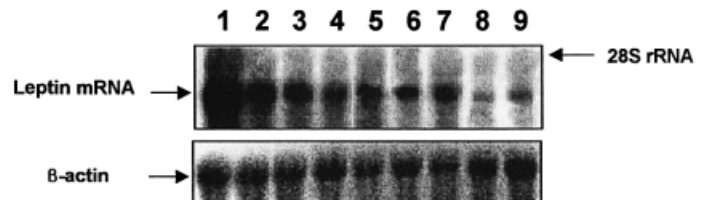


Figure 3. Northern blotting for evaluating the expression of *leptin* mRNA in human placenta. Lane 1 = sample from human white adipose tissue; lanes 2–4 = samples from first trimester human placentas; lanes 5–7 = samples from third trimester human placentas and lanes 8 and 9 = samples from human umbilical cords. β -actin probe hybridization of the same blot is shown at the bottom.

Figure 1. Immunolocalization of leptin and leptin-R in human placenta of first and third trimester of gestation. (a) Immunohistochemical localization of leptin. The immunostaining is mainly located in the cytoplasm of villous trophoblast (syncytiotrophoblast and cytotrophoblast, ninth week of gestation). (b) Leptin immunostaining pattern during the 38th week of gestation. Syncytiotrophoblast and villous cytotrophoblastic cells (arrows) are positive. (c) Immunostaining for leptin-R at the eighth week of gestation. The positive reaction product is on the apical plasma membrane of the syncytiotrophoblast. Villous cytotrophoblastic cells are also stained. Note the immunostaining of endothelial cells of the fetal vessel (V) and of a Hofbauer cell (arrow). (d) Placental villi which are continuous with cell columns. In the cell columns the positive reaction product for leptin-R is in the extravillous cytotrophoblastic cells distally located (*) from the villous stroma (eighth week of gestation). (e) Basal plate. Immunostaining for leptin-R. This section is parallel to the one showing cytokeratin immunostaining depicted in part (f). Extravillous cytotrophoblastic cells (arrows) are positive for leptin-R. Note the decidual cells (arrowheads) which are positive for leptin-R and negative for cytokeratin in (f). (f) Basal plate at the 38th week of gestation. Immunostaining for cytokeratin. Section parallel to the one depicted in (e) and immunostained for leptin-R. Extravillous cytotrophoblastic cells (arrows) are positive for cytokeratin. Note the negative immunostaining of decidual cells (arrowheads). (a, e, f) scale bar = 23 μ m; (b) scale bar = 14 μ m. (c) scale bar = 9 μ m; (d) scale bar = 90 μ m.

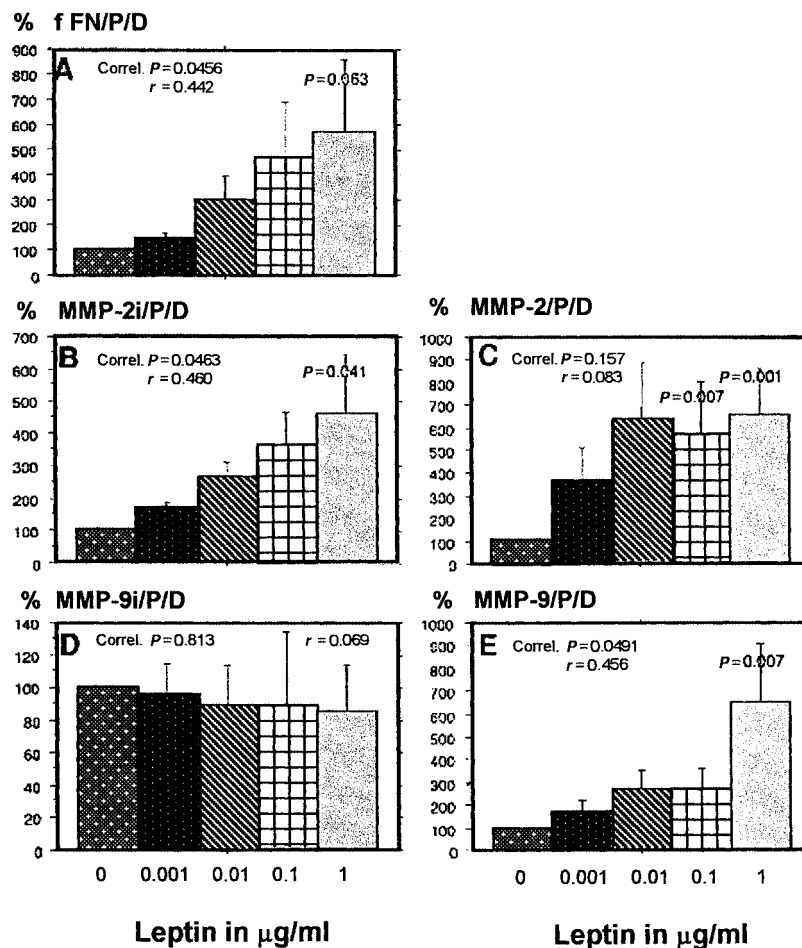


Figure 4. Effects of recombinant human leptin (rhLept) on the trophoblastic secretion of (A) fetal fibronectin (fFN), (B) of immunoreactive matrix metalloproteinase (MMP)-2 (D) and MMP-9, (C) of the gelatinolytic activity of MMP-2 and (E) MMP-9. Results are presented as mean $\pm$ SEM per mg of cell protein and per day and expressed as percentage of control values (cell without leptin). Culture conditions are described in Materials and methods. % fFN/P/D = percentage fFN/mg cell protein per day; % MMP-2i/P/D = percentage immunoreactive MMP-2 per mg cell protein per day; % MMP-9i/P/D = percentage immunoreactive MMP-9 per mg cell protein and per day; % MMP-2/P/D = percentage of zymographic activity of MMP-2 per mg cell protein per day; % MMP-9/P/D = percentage of zymographic activity of MMP-9 per mg cell protein per day.

reactivity was expressed in villous trophoblast at all stages of pregnancy (Figure 1c). The apical plasma membrane of the syncytiotrophoblast was intensely labelled (Figure 1c). In the first trimester of gestation the endothelial cells of various fetal vessels were positive for leptin-R (Figure 1c) whereas at term few blood vessels were stained for this antigen. Hofbauer cells also showed leptin-R immunostaining (Figure 1c).

Cell columns and cell islands

Leptin immunostaining was present in all extravillous cytotrophoblastic cells of cell columns and cell islands. Interestingly, leptin-R immunoreactivity was strongly expressed in the extravillous cytotrophoblastic cells distally located from the adherent villous (Figure 1d).

Chorionic plate and basal plate

In the third trimester of gestation, leptin immunostaining was found in both the extravillous cytotrophoblastic cells of the chorionic plate and in amniotic epithelial cells. Both cellular components were also positive for leptin-R (data not shown). Leptin and leptin-R (Figure 1e) were detectable in the majority of the extravillous trophoblastic cells (positive for cytokeratin;

Figure 1f) of the basal plate. However, some of these cytotrophoblastic cells were weakly stained and/or negative for this antigen. Decidual cells (negative for cytokeratin; Figure 1f), were positive for leptin and leptin-R (Figure 1e).

Leptin and leptin receptor mRNA expression

RT-PCR yielded bands of the expected sizes for ObR_L (LR5) and ObR_S (LR2) forms in all samples considered (Figure 2). *Leptin* mRNA was detected in first and third trimester placentae, umbilical and white adipose tissue. Human ovary was used as the negative control for leptin expression (Karlsson *et al.*, 1997). The identities of the amplified fragments were confirmed by restriction enzyme cleavage. Northern blot analysis revealed *leptin* mRNA in all samples considered, in line with the RT-PCR data (Figure 3) and with a previous study (Senaris *et al.*, 1997).

Effects of leptin on cytotrophoblastic cells

Positive immunostaining for leptin-R in both the invading extravillous cytotrophoblastic cells of cell islands and cell

columns and RT-PCR-positive results prompted us to investigate the influence of leptin on the enzymes responsible for trophoblast invasion. As shown in Figure 4, rhLept increased in a dose-dependent manner ($P = 0.0456$), the trophoblastic secretion of fFN (Figure 4A) and the concentration of immunoreactive MMP-2 ($P = 0.0463$, Figure 4B), but not of MMP-9 (Figure 4D). However, the MMP-9 activity was increased in a dose-dependent manner ($P = 0.0491$, Figure 4E). Although rhLept did not stimulate MMP-2 activity in a dose-dependent manner ($P = 0.157$), the higher concentrations of rhLept (0.1 and 1 $\mu\text{g/ml}$) significantly increased MMP-2 activity (Figure 4C).

Discussion

The functional roles of leptin and leptin-R in the human placenta have not yet been clarified. In this study we demonstrate for the first time that leptin and leptin-R are expressed in the extravillous cytotrophoblastic cells of cell columns which are structures of the placental villous tree that play a pivotal role in placental growth and in trophoblast invasion (Mühlhauser *et al.*, 1995; Kaufmann and Castellucci, 1997). Immunostaining for leptin-R was peculiar in these structures, showing a clear gradient, i.e. weak expression in the more proximal (proliferating) elements and strong expression in the distal extravillous trophoblastic cells involved in the invasive processes. The functional significance of leptin and leptin-R expression in these cells could thus be related to the invasive processes, possibly involving an enhancement of the activity and/or synthesis of the molecules regulating trophoblastic invasion. Trophoblast invasion requires degradation of extracellular matrix molecules by the activity of specific proteases (Bischof and Martelli, 1992; Graham and Lala, 1992) as well as synthesis of fFN, a molecule that facilitates the migration of the extravillous cytotrophoblastic cells into the uterine wall (Bischof and Martelli, 1992; Graham and Lala, 1992). It has been previously demonstrated that MMP-2, MMP-9 and fFN (Feinberg *et al.*, 1991; Castellucci *et al.*, 1993; Huppertz *et al.*, 1998) are expressed in the distal part of the extravillous cytotrophoblastic cells of cell columns where we detect a strong expression of leptin-R (this study). These data led us to consider leptin as a potential specific regulator of the production of these molecules. Our in-vitro studies support this hypothesis because leptin regulates MMP-2 and MMP-9, as well as the extracellular matrix molecule, fFN. We showed that leptin increases in a dose-dependent manner the secretion of immunoreactive MMP-2 and fFN and enhances the activity of MMP-9 in cytotrophoblastic cells. Interestingly, leptin does not affect the cytotrophoblastic secretion of immunoreactive MMP-9, although it significantly enhances MMP-9 activity. This enzyme is known to be essential for in-vitro and in-vivo invasion (Librach *et al.*, 1991; Bischof *et al.*, 1995a; Huppertz *et al.*, 1998). This observation might indicate that leptin does not influence the production of MMP-9, but does stimulate its activation. It is tempting to speculate that leptin could regulate the synthesis of potential activators of MMP-9 (Ogata *et al.*, 1992).

In contrast to other regulators of trophoblast invasion, e.g.

leukaemia inhibitory factor (LIF) (Bischof *et al.*, 1995b) and tumour necrosis factor α (Meisser *et al.*, 1999), leptin stimulates fFN and MMP-2 secretion indicating that this hormone probably acts by a different mechanism. In the present study, leptin-stimulated matrix deposition (measured as fFN) as well as the proteolytic activity of cytotrophoblastic cells, thereby acting similarly to interleukin (IL)-1 α (Meisser *et al.*, 1999), a known stimulator of MMPs (Rawdanowicz *et al.*, 1994).

Extending previous observations (Masuzaki *et al.*, 1997; Senaris *et al.*, 1997; Mise *et al.*, 1998), we show that leptin-R (long and short forms) is expressed at the apical surface of the syncytiotrophoblast of the first and third trimester of gestation suggesting that plasma leptin may have an autocrine and/or paracrine role at this level. The function of leptin and its receptor in villous trophoblast might be related to the secretion of human chorionic gonadotrophin (HCG) since rhLept induces a dose-dependent stimulation of HCG secretion by primary cultures of cytotrophoblastic cells *in vitro* (Chatdonnens *et al.*, 1999). Thus, the role of leptin-R may be considered similar to that of other molecules, e.g. *c-erbB-2*, which are expressed both on the syncytiotrophoblast and on extravillous cytotrophoblastic cells, but are considered to play different roles in these two locations (Mühlhauser *et al.*, 1993).

Recently, it has been demonstrated that leptin promotes angiogenesis in cultured human vein endothelial cells and in porcine aortic endothelial cells (Bouloumié *et al.*, 1998; Sierra-Honigsmann *et al.*, 1998). Moreover, leptin exhibits a potent neovascularization effect in corneal assays conducted with normal rats (Sierra-Honigsmann *et al.*, 1998). Our findings showed leptin-R positivity in endothelial cells of various fetal blood vessels of chorionic villi during the first trimester of gestation. Leptin might therefore contribute to the development of placental vessels together with other growth factors such as vascular endothelial growth factor (Shiraishi *et al.*, 1996), basic fibroblast growth factor (Mühlhauser *et al.*, 1996) and platelet derived growth factor B (Holmgren *et al.*, 1991).

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