

Leptin, the Product of Ob Gene, Promotes Angiogenesis

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Abstract—The adipocyte-derived cytokine leptin is thought to play a key role in the control of satiety and energy expenditure. Because adipogenesis and angiogenesis are tightly correlated during the fat mass development, we tested the hypothesis that leptin is able to modulate the growth of the vasculature. Experiments were performed using cultured human umbilical venous endothelial cells (HUVECs) and porcine aortic endothelial cells. The presence of 170-kDa endothelial leptin receptor (Ob-R) was assessed in HUVECs by Western blot analysis. Reverse transcriptase–polymerase chain reaction analysis using specific oligonucleotides for the short and long Ob-R forms further revealed the expression of both Ob-R transcripts in endothelial cells. Moreover, leptin evoked a time-dependent tyrosine phosphorylation of a number of endothelial proteins, the most prominent of which were the mitogen-activated protein kinases Erk1/2. Treatment of HUVECs with leptin led to a concentration-dependent increase in cell number that was maximal at 10 ng/mL leptin and equivalent to that elicited by vascular endothelial growth factor. This effect was associated with an enhanced formation of capillary-like tubes in an in vitro angiogenesis assay and neovascularization in an in vivo model of angiogenesis. These results indicate that leptin, via activation of the endothelial Ob-R, generates a growth signal involving a tyrosine kinase-dependent intracellular pathway and promotes angiogenic processes. We speculate that this leptin-mediated stimulation of angiogenesis might represent not only a key event in the settlement of obesity but also may contribute to the modulation of growth under physiological and pathophysiological conditions in other tissues. (*Circ Res.* 1998;83:1059-1066.)

Key Words: obesity ■ adipocyte ■ growth ■ cytokine

Angiogenesis, the formation of new blood vessels by capillary sprouting from preexisting vessels, is a major physiological event that occurs for example in the female reproductive system throughout the menstrual cycle and in pregnancy, as well as during wound healing.¹ The angiogenic process is under the control of proangiogenic factors, including vascular permeability factor/vascular endothelial growth factor (VEGF), fibroblast growth factor, and antiangiogenic factors such as endostatin and angiostatin.²

Obesity is characterized by an excess of fat mass as a consequence of adipocyte hypertrophy and hyperplasia.³ The excessive growth of adipose tissue requires the formation of new capillaries for proper function. Because the development of the vascular bed in adipose tissue is tightly connected to both number and size of adipocytes and adipose tissue serves as an important conduit for growing blood vessels, it is conceivable that adipocytes may modulate the growth of the vasculature in a paracrine manner. Indeed, adipose tissue has regularly been used in clinical applications to facilitate revascularization and healing of compromised or ischemic organs and tissues.⁴ Moreover, immortalized preadipocyte cell lines promote the formation of highly vascularized fat pads after injection into

nude mice,⁵ suggesting that adipocytes per se possess proangiogenic activity. Several recent reports have shown that adipocytes are not only sites of energy storage but also are important sources of cytokines such as tumor necrosis factor- α , growth factors including VEGF,⁶ and lipid derivatives such as 1-butyryl glycerol⁷; some of these are potentially involved in the regulation of angiogenesis. Recently, leptin, the product of the ob gene, was identified as an adipocyte-secreted protein.^{8–10} Leptin appears to play a key role in the regulation of body weight and more specifically on the control of food consumption, sympathetic nervous system activation, and thermogenesis.¹¹ Additional roles have been proposed in the control of the reproduction,^{12,13} hematopoiesis,¹⁴ and proinflammatory immune responses.¹⁵ The expression and the plasma concentration of leptin were found to be markedly increased in human obesity and positively correlated to body fat mass.¹¹ Because leptin is secreted into the plasma, endothelial cells could be exposed to much higher concentrations of this cytokine than other cell types. We therefore assessed whether leptin interacts with endothelial cells via specific receptors and thereby modulates cell growth and angiogenic processes.

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Materials and Methods

Materials

Chemicals were obtained from either Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany). Human recombinant leptin was provided by Biomol (Hamburg, Germany) and human recombinant VEGF₁₆₅ was provided by Chiron Corp (Emeryville, Calif). The murine monoclonal phosphotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY), the murine monoclonal activated mitogen-activated protein kinase (clone 12D4) from nanoTools (Biomol, Hamburg, Germany), the goat polyclonal leptin receptor (Ob-R) C-20 and Ob-R N-20 from Santa Cruz Biotechnology, the agarose-conjugated antiphosphotyrosine antibody from Oncogene Science (P-Tyr [Ab-1]-A), and the prestained protein marker from New England Biolabs. The [α^{32}]dCTP was purchased from Hartmann Analytic (Braunschweig, Germany).

Cell Culture

HUVECs and porcine aortic endothelial cells (PAECs), isolated as previously described,¹⁶ were seeded at a density of 45 000 cells/cm² in culture dishes containing M-199 medium (Life Technologies) and 10% FCS (Biochrom, Berlin, Germany) supplemented with penicillin (50 U/mL) and streptomycin (50 μ g/mL). All experiments were performed on quiescent cells from the first passage.

Western Blot Analysis and In-Gel Protein Kinase Assays

Cells were lysed with 1% Triton X-100 and 0.1% SDS in 20 mmol/L Tris-HCl, pH 7.4, containing (in mmol/L) NaCl 50, NaF 50, EDTA 5, sodium pyrophosphate 20, Na₂VO₃ 1, and protease inhibitors (100 μ g/mL phenylmethylsulfonyl, 1 μ g/mL aprotinin, and 1 μ g/mL leupeptin). The protein content of the crude preparations was measured by the method of Bradford, using serum albumin as standard. Thirty-microgram proteins were subjected to SDS/PAGE gel, either directly or after immunoprecipitation using agarose-conjugated phosphotyrosine antibody, and transferred to nitrocellulose membranes (Schleicher and Schuel), as previously described.¹⁷ Ponceau staining was performed to verify the quality of the transfer and the equal amount of protein in each lane. Proteins were detected using specific antibodies as described (see Results) and were visualized by enhanced chemiluminescence using a commercially available kit (Amersham). The autoradiographs were analyzed by scanning densitometry using as software Image master 1D (Pharmacia). For in-gel protein kinase assays, 30- μ g proteins were separated by SDS-PAGE. Myelin basic protein (0.4 mg/mL) was used as substrate that was polymerized in the gel. After protein renaturation, the kinase reactions were performed in the gel as previously described.¹⁸

Analysis of the Expression of Ob-R by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted according to the method of Chomczynski and Sacchi.¹⁹ For RT analysis, 2 μ g total RNA was incubated with 200 U reverse transcriptase (Gibco), dNTP (125 μ mol/L), oligo(dT) (200 ng), and reaction buffer in a final volume of 20 μ L at 37°C for 60 minutes. In some reaction mixtures, reverse transcriptase or total RNA was omitted to determine the amplification of contaminating genomic DNA or cDNA. After a final denaturation at 94°C for 7 minutes, 10 μ L cDNA was subjected to PCR consisting of a denaturation at 94°C for 1 minute, followed by 90 seconds of annealing at 55°C and 90 seconds of elongation at 72°C for 32 cycles. The last cycle ended with 7 minutes of elongation at 72°C. The primers used to amplify the long and short forms of the leptin receptors were chosen as previously described¹⁴: a common forward primer (5'-GAAGGAGTGGGAAAACCAAG-3') used in combination with a specific reverse primer either for the long form (5'-CATAGGTTACCTCAGTACCCTC-3'), allowing the amplification of 433 bp, or the short form (5'-CCACCATATGTAACTCTCAG-3'), allowing the amplification of 365 bp. The PCR

contained 0.4 μ mol/L of each primer, dNTP (200 μ mol/L), MgCl₂ (1 mmol/L) reaction buffer, and 2.5 U Taq polymerase (Promega) in a final volume of 50 μ L. The amplified cDNAs were size-fractionated by agarose gel electrophoresis, visualized under UV with an ethidium bromide staining, transferred to a nylon membrane (porablot NY amp, Macherey-Nagel) and hybridized with ³²P-end-labeled oligonucleotides specific for the long form (5'-CTGTGGTCTCTCTACTTTCAAC-3') or the short form (5'-CTAATCATGATCACTACAGATG-3'). Autoradiographs were then exposed for 4 to 8 hours.

Viability Assays

Cell viability was assessed by the use of an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide) assay allowing the quantification of viable cells. HUVECs were seeded in 96-well plates and incubated the next day with serum-deprived medium supplemented with 0.1% BSA (Gibco Life Technologies, Eggenstein-Leopoldshafen, Germany) for the next 72 hours, in the presence of increasing concentrations of leptin or VEGF₁₆₅ (0.1 to 100 ng/mL). Twenty microliters of MTT (5 mg/mL) was then added to 200 μ L medium culture and incubated for 3 hours. At the end of the incubation period, the medium was removed and the converted dye solubilized with acidic isopropanol. Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630 nm.

Proliferation Assays

The total cell number was determined in HUVECs seeded in 24 wells and incubated the next day with serum-deprived medium supplemented with 0.1% BSA or with 1% FCS for the next 72 hours in the presence or absence of leptin. At the time indicated, the cells were counted after trypsinization using a cell counter (CASY1, Schärfe System).

Apoptosis Assays

HUVECs were cultured in 12-well plates and incubated the next day with serum-deprived medium supplemented with 0.1% BSA for 24,

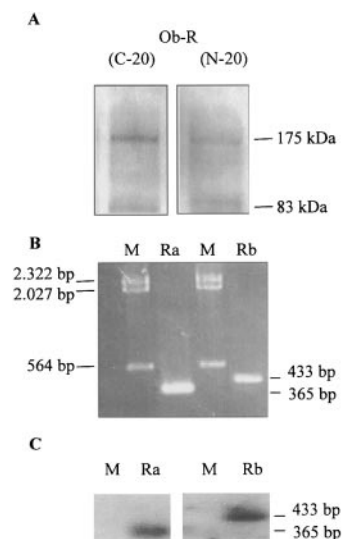


Figure 1. Characterization of Ob-R expressed in HUVECs. A, Western blot performed with 30 μ g total cellular extracts from HUVECs using a goat polyclonal antibody directed against the carboxy (C-20) or amino (N-20) terminus of human Ob-R. B, Ethidium bromide staining of RT-PCR products separated by electrophoresis performed in an agarose gel. Lane M, DNA marker; lane Ra, RT-PCR performed with selective Ob-Ra oligonucleotides; and lane Rb, RT-PCR performed with selective Ob-Rb oligonucleotides. C, Southern autoradiography. Left, Hybridization performed with Ob-Ra-specific [³²P]-end-labeled oligonucleotide. Right, Hybridization performed with Ob-Rb-specific [³²P]-end-labeled oligonucleotide.

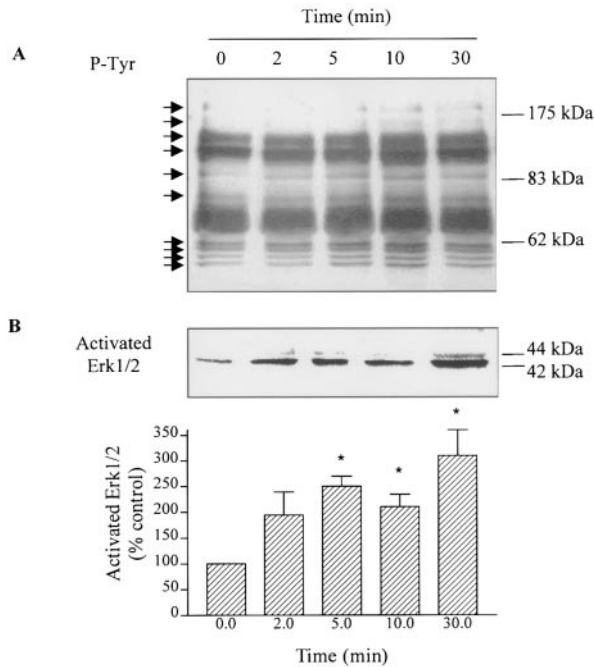


Figure 2. Effect of leptin on tyrosine kinase-dependent pathways. HUVECs, maintained in serum-deprived medium for 24 hours, were treated with 10 ng/mL leptin for the time indicated (2, 5, 10, and 30 minutes) and 30 μ g total proteins were subjected to SDS/PAGE electrophoresis. A, Western blot performed with a phosphotyrosine antibody; the results presented are representative of data obtained in 3 independent experiments. Arrows indicate tyrosine-phosphorylated endothelial proteins (P-Tyr) increased under leptin treatment. B, Time course of the activation of the mitogen-activated protein kinase Erk1/2. Top, Western blot using an antibody directed against activated mitogen-activated protein kinase Erk1/2. Bottom, Densitometric analysis is mean \pm SEM from experiments performed on 3 different cell batches. * $P < 0.05$ vs control (time 0).

48, or 72 hours, in the presence of increasing concentrations of leptin (1 to 100 ng/mL). The plates were centrifuged (2000g, 10 minutes). Thereafter, the medium was removed, cells were fixed in 4% formaldehyde, and stained with DAPI (4',6-diamino-2-phenylindole; 0.2 μ g/mL in 10 mmol/L Tris-HCl [pH 7], 10 mmol/L EDTA, and 100 mmol/L NaCl) for 30 minutes. Nuclei were analyzed by fluorescence microscopy, and apoptotic cells were counted in a double-blind manner.

In Vitro Angiogenesis Assays

The microcarrier-based fibrin gel angiogenesis assay was performed as previously described.²⁰ Trypsinized PAECs were allowed to attach onto cytodex-2 microcarrier beads for 4 hours at 37°C (Sigma) in M-199 supplemented with 10% FCS and subsequently were grown to confluence for 48 hours. Porcine fibrinogen (Sigma) was dialyzed overnight against PBS (pH 7.0; 10 mmol/L phosphate, 140 mmol/L sodium chloride) and then diluted to 1 mg/mL in PBS, pH 7.0. Packed microcarrier beads (40 μ L) were pipetted into 2 mL of fibrinogen solution in 35-mm Petri dishes, and gels were allowed to polymerize for 30 minutes after addition of 0.625 U/mL thrombin. The fibrin gels were then equilibrated in M-199 supplemented with 1 mg/L insulin, 1 mg/mL transferrin, 1 μ g/L selenium, 50 U/mL penicillin, and 50 μ g/mL streptomycin for 60 minutes at 37°C, and fresh medium was subsequently added on top of the gels. The number of positive beads for angiogenesis was determined under the microscope, by observers who were blind to the experimental conditions, in 3 randomly chosen fields by counting the number of capillary-like tubes of a length >150 μ m. For angiogenesis assays with HUVECs, trypsinized cells were plated directly on the surface

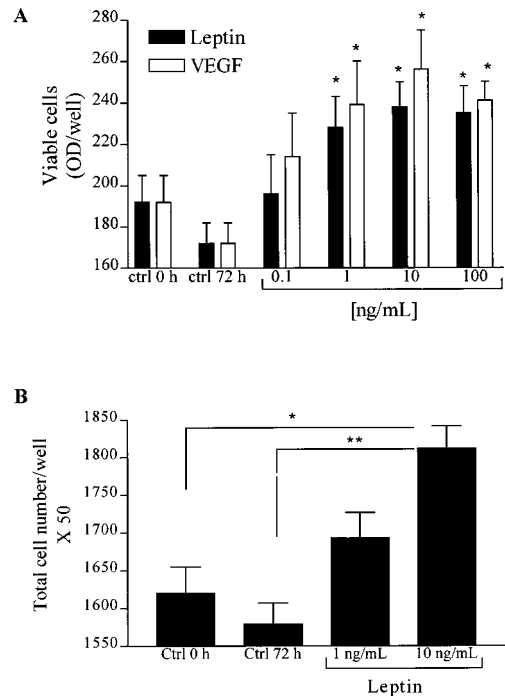


Figure 3. Effects of leptin on cell viability and proliferation. A, HUVECs were cultured in the presence of increasing concentrations of human recombinant leptin or VEGF₁₆₅ (0.1 to 100 ng/mL) for 72 hours in a serum-deprived medium supplemented with 0.1% BSA. Before the beginning of the treatment (ctrl 0 hours) and after 72 hours, the number of viable cells was assessed as described in Materials and Methods. Results presented are mean \pm SEM from 4 wells. * $P < 0.05$ vs control (ctrl 72 hours). Two independent experiments gave identical results. B, HUVECs were cultured in presence of human recombinant leptin (1 and 10 ng/mL) for 72 hours in a medium supplemented with 1% FCS. Before the beginning of the treatment (ctrl 0 hours) and after 72 hours, total number of cells was determined as described in methods. Results presented are means \pm SEM from 4 wells. * $P < 0.05$, ** $P < 0.01$ vs control. Two independent experiments gave identical results.

of the fibrin gels and cultured in M-199 supplemented with 0.1% BSA. Where appropriate, leptin was added to both the fibrinogen solution prior to polymerization and the medium bathing the cells.

Chick Chorioallantoic Membrane (CAM) Assays

All experiments with chick embryos ($n=28$) were carried out in ovo. On 3-day old embryos, a window of 7 to 10 mm in diameter was cut into the eggshell, resealed with parafilm, and further incubated until day 9. For the CAM assay, 70 μ L of a sterile 1% methylcellulose solution were mixed with 70 μ L of a leptin solution in sterile water. Seven-microliter aliquots of this mixture, each containing a total amount of either 200 ng, 1 μ g, or 3 μ g leptin were pipetted onto bacteriological-grade Petri dishes, air-dried for 2 hours, and the resulting discs placed onto 9 day-old CAMs for 2 days. Two to 3 discs were placed onto each CAM about 10 mm apart. Control discs contained sterile water only. Evaluation of the CAMs was performed on 11 day-old CAMs. To better visualize the vascular system of the CAM, 20% Luconyl Blue in PBS (BASF, Ludwigshafen) was injected into a vitelline vein using glass capillaries. Photographs were taken using a Zeiss SV6 stereomicroscope with camera adapter. The angiogenic response was assessed as positive after appearance of a significantly increased vascularization under or in the vicinity of the disc.

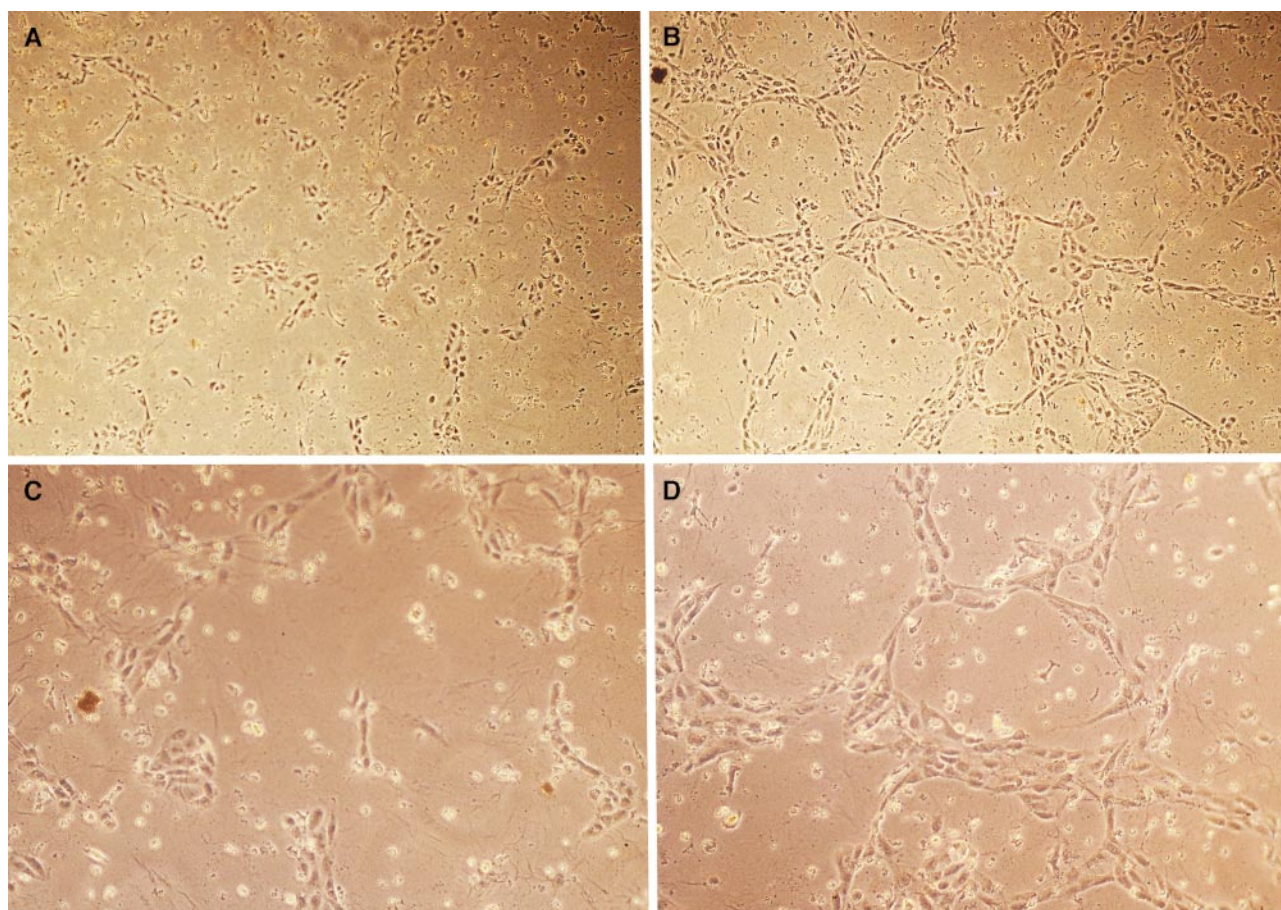


Figure 4. Effect of leptin on the formation of capillary-like structures in cultured HUVECs in fibrin gel. HUVECs plated on fibrin matrix and cultured for 5 days in serum-deprived medium in the absence (A and C) or in the presence of 100 ng/mL human recombinant leptin (B and D). Original magnification $\times 50$ (A and B) and $\times 100$ (C and D).

Statistics

Data are expressed as mean \pm SEM. Statistical analyses were performed by 1-way ANOVA followed by Bonferroni *t* test. Values of $P < 0.05$ were considered statistically significant.

Results

To determine whether endothelial cells express leptin receptors, Western blot and RT-PCR analysis were performed on whole protein and RNA extracts from cultured HUVECs. Antibodies directed against the carboxyl or the amino terminus of the human leptin receptor recognized a 170-kDa protein (Figure 1A), the size of which is consistent with the human leptin receptor.²¹ Using specific primers for Ob-Rb and Ob-Ra, cDNAs of the expected length were amplified from reverse-transcriptase reaction performed with HUVEC RNA (Figure 1B). These cDNA fragments were specifically recognized by the respective Ob-Rb and Ob-Ra radiolabeled oligonucleotides (Figure 1C). Sequence analysis confirmed the identities of the cDNA fragments as Ob-Rb and Ob-Ra.¹³ These results indicate that cultured HUVECs express at least 2 forms of the leptin receptor, Ob-Ra and Ob-Rb.

To further characterize the functionality of Ob-R, Western blot analysis was performed on whole protein extracts from HUVECs (30 μ g) treated with human recombinant leptin (10 ng/mL) at different time points (2, 5, 10, or 30 minutes). Leptin enhanced, in a time-dependent manner, the tyrosine

phosphorylation of endothelial proteins, the most prominent of which exhibited molecular weights of 180, 170, 130, 91, 86, 76, 60, 56, 52, 44, and 42 kDa (Figure 2A). Immunoprecipitation using a phosphotyrosine antibody, followed by Western blot analysis, confirmed this finding (data not shown). Western blot analysis using antibody directed against the activated form of extracellular signal-regulated kinases (Erk1 and Erk2) showed that both isoforms are time-dependently activated in leptin-treated HUVECs (10 ng/mL) (Figure 2B). This stimulatory effect of leptin on Erk1/2 was confirmed by in-gel kinase assays using myelin basic protein as substrate (data not shown). These results demonstrate that endothelial Ob-R is functionally active and linked to activation of tyrosine kinase-dependent pathways. Because activation of mitogen-activated protein kinases, and more specifically, the Erk1 and Erk2 isoforms, are involved in the control of proliferation and/or differentiation in various cell types, we studied the effect of leptin on endothelial cell growth and viability.

The leptin effect on cell viability was assessed on endothelial cells cultured with increasing concentrations of human recombinant leptin (0.1 to 100 ng/mL) for 72 hours in a serum-deprived medium. The number of viable cells per well in the presence of leptin was compared with control and VEGF₁₆₅-treated cells. As shown in Figure 3A, the number of

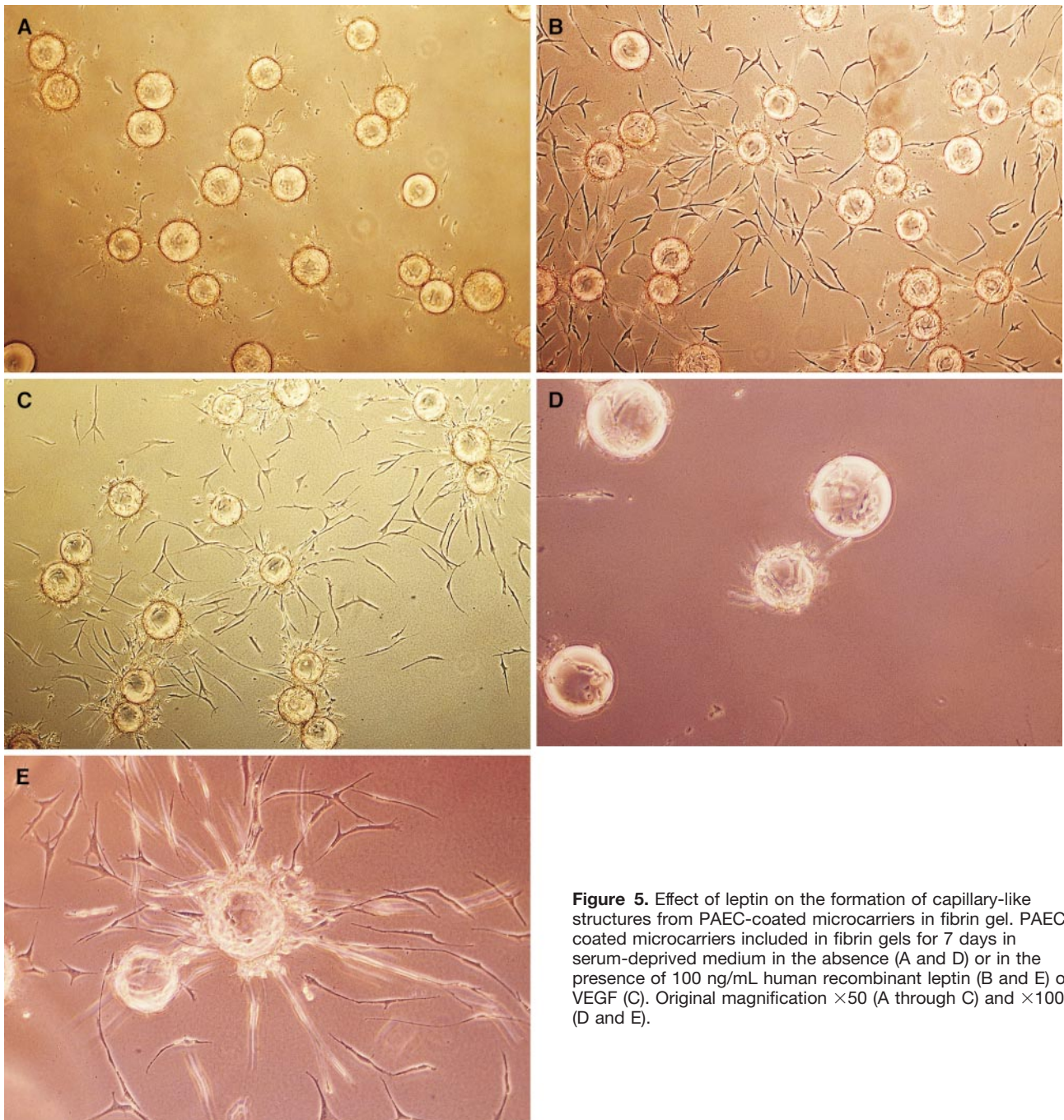


Figure 5. Effect of leptin on the formation of capillary-like structures from PAEC-coated microcarriers in fibrin gel. PAEC-coated microcarriers included in fibrin gels for 7 days in serum-deprived medium in the absence (A and D) or in the presence of 100 ng/mL human recombinant leptin (B and E) or VEGF (C). Original magnification $\times 50$ (A through C) and $\times 100$ (D and E).

viable cells maintained in serum-deprived medium for 72 hours decreased slightly compared with initial cell number, although this decrease did not reach significance. Leptin dose-dependently increased the number of viable cells. The maximal effect observed with 10 ng/mL leptin was not significantly different from that elicited by 10 ng/mL VEGF₁₆₅ and was associated with a significant increase in the initial cell number ($P < 0.05$, $n = 3$). This effect was also observed when cells were cultured in medium supplemented with 1% FCS (Figure 3B). The total number of cells was significantly increased with 10 ng/mL leptin compared with the initial cell number or after the 72-hour incubation period. To determine whether this survival/proliferative effect could be linked to an antiapoptotic effect of

leptin, the apoptotic rate was determined by visual analysis of DAPI-stained cells. The apoptotic rate in untreated cells was low and not affected by the length of the incubation period in serum-deprived medium ($1.9 \pm 0.3\%$ for 24 hours, $2.4 \pm 0.7\%$ for 48 hours, and $2.2 \pm 0.8\%$ for 72-hour incubation period). Leptin treatment did not modify the percentage of apoptotic nuclei detected (2.2 ± 0.3 in 10 ng/mL leptin versus 2.2 ± 0.8 in control cells, $n = 3$).

The effect of leptin was studied using in vitro (3D fibrin gels) and in vivo (CAM) angiogenesis assays. As shown in Figure 4, in HUVECs cultured on fibrin gels, addition of leptin (100 ng/mL) induced marked changes in HUVEC morphology (Figure 4B and 4D) compared with control

Effect of Leptin on Angiogenesis in CAM

Leptin Concentration, μg	Total Number of Eggs	Positive Discs, %
Control	4	0
0.2	8	5
1	8	53
3	8	92

Angiogenesis was induced by methylcellulose discs in the absence (control) and presence of human recombinant leptin (0.2, 1, and 3 μg) and placed on the CAM of 9-day-old chick embryos. Angiogenesis was monitored by photomicroscopy after 48 hours. Positive discs were determined as described in Materials and Methods.

(Figure 4A and 4C), with structural rearrangements leading to the formation of capillary-like networks. In a second approach to determine whether the leptin-promoted angiogenesis was also evident on “adult” macrovascular endothelial cells, experiments were performed on microcarrier beads coated with PAECs and included in a fibrin gel. As shown in Figure 5, 7 days after fibrin polymerization, addition of leptin (100 ng/mL) in both the medium and the gel increased the number as well as the length of the capillary-like structures derived from the cell-coated microcarriers (Figure 5B and 5E). A similar angiogenic effect was observed with 10 ng/mL leptin (data not shown). A 4- to 5-fold increase in the number of microcarriers that developed capillary-like tubes of $>150\text{ }\mu\text{m}$ in length was observed with 100 ng/mL leptin.

VEGF₁₆₅ (100 ng/mL) elicited a comparable angiogenic effect (Figure 5C).

Experiments were then performed *in vivo* on CAMs using methylcellulose discs containing 0.2, 1, and 3 μg leptin. After 48 hours of contact with the CAM, the discs containing 1 and 3 μg leptin were associated with a significant and marked stimulation of neovascularization (Table). A representative area of the CAM with a control disc and a disc loaded with 3 μg leptin is shown in Figure 6.

Discussion

In the present study, we have demonstrated the presence of functionally active leptin receptors on cultured HUVECs, linked to tyrosine kinase-dependent intracellular pathways. The stimulation of endothelial cells by leptin led to an increase in cell proliferation and/or survival and elicited a marked enhancement of angiogenesis.

Several forms of Ob-R, which differ in their cytoplasmic domains, have been described on the basis of mRNA analysis. The receptor with the longest cytoplasmic domain (Ob-Rb) shares sequence similarity with the corresponding regions of the leukemia inhibitory factor (LIF) receptor α -chain and gp130, whereas the short form (Ob-Ra) contains the conserved box 1-motif of the hematopoietin receptor family.²¹ Ob-Rb receptors, originally described in the central area, are far less abundant in peripheral sites than the short Ob-Ra form.²¹ It was recently reported that endothelial cells from the human brain capillaries exhibited specific and saturable

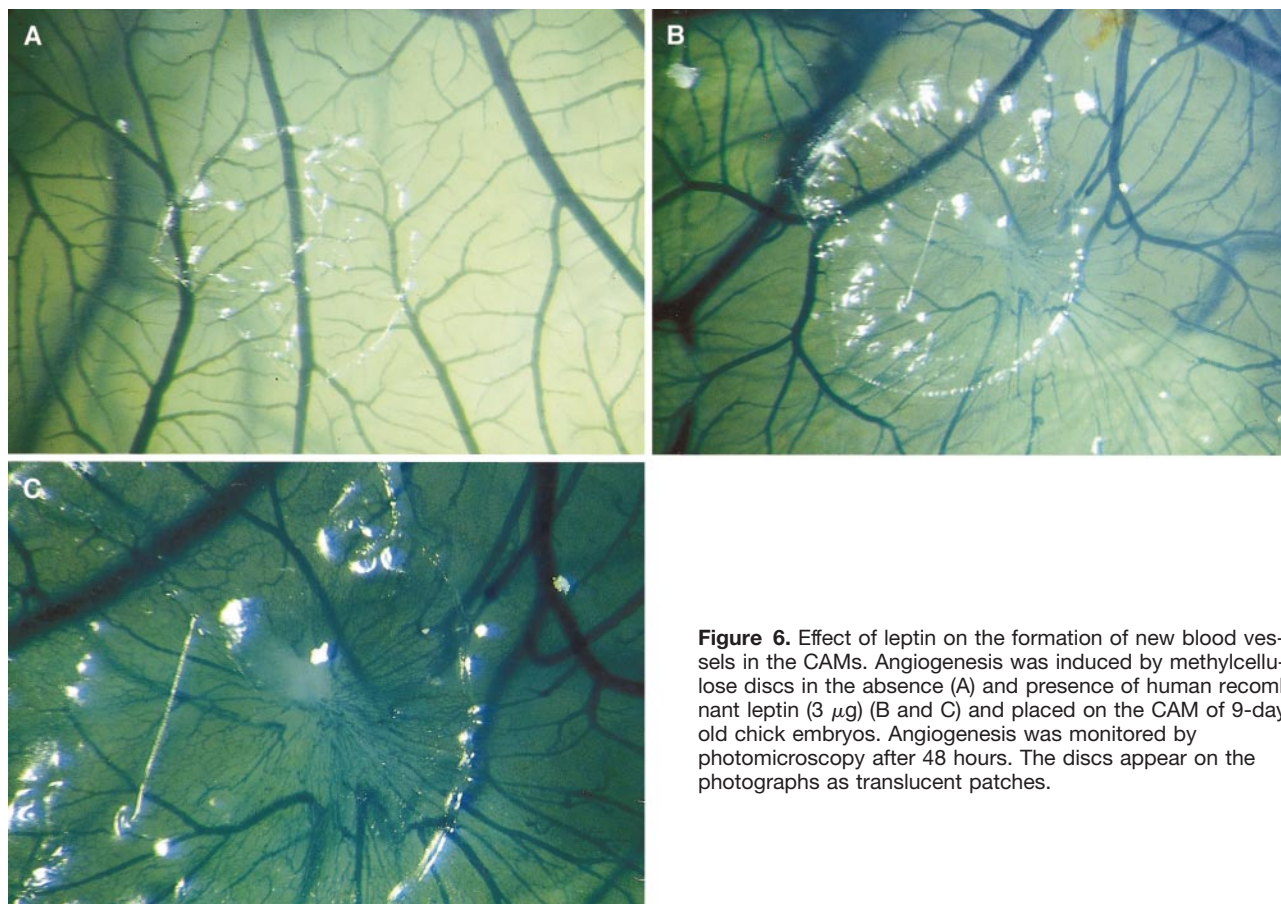


Figure 6. Effect of leptin on the formation of new blood vessels in the CAMs. Angiogenesis was induced by methylcellulose discs in the absence (A) and presence of human recombinant leptin (3 μg) (B and C) and placed on the CAM of 9-day-old chick embryos. Angiogenesis was monitored by photomicroscopy after 48 hours. The discs appear on the photographs as translucent patches.

binding sites to leptin, which were thought to play a role in the leptin endocytosis at the blood-brain barrier.²² The present study extends this first observation to human macrovascular endothelial cells and delivers by the use of Western blot and RT-PCR analysis a characterization of the type of Ob-R expressed on endothelial cells: Ob-Ra and Ob-Rb. Ob-Rb receptors have been suggested to play a role in the leptin-mediated control of cell differentiation, whereas Ob-Ra receptors may be involved in the leptin endocytosis.²¹ It is therefore conceivable that endothelial cells, in addition to their ability to internalize leptin, probably through Ob-Ra, may also be a target for the modulating effect of leptin on proliferation and differentiation through the Ob-Rb form. In particular, HUVECs might be affected by leptin, because leptin was shown to be produced in the human placenta²³ and found in the venous cord blood.²⁴

The functionality of endothelial Ob-R was demonstrated by the leptin-induced increase in the tyrosine phosphorylation of several endothelial proteins. The molecular weights from some of them were consistent with those of proteins already described to be activated under leptin treatment in other target cell types, such as members of the Janus family (JAK1 and JAK2), the STAT family (STAT1 and STAT3), and the mitogen-activated protein kinase family Erk1/2.²⁵ The finding of a leptin-induced activation of Erk1/2, as assessed with specific antibody directed against the activated Erk1/2 and in-gel kinase activity, led to the hypothesis that leptin may stimulate endothelial cell growth. Our results clearly indicate that leptin in serum-deprived medium produced a marked increase in the number of viable endothelial cells, whereas the number of cells undergoing apoptosis was unaffected. Moreover, in the presence of serum, a significant increase in total cell number was observed. Thus, leptin treatment of endothelial cells is associated with proliferation and/or survival. The maximal effect was elicited by a physiological plasma concentration of leptin (10 ng/mL) and was equivalent to that evoked by VEGF₁₆₅ in the same concentration range. Given that VEGF₁₆₅ is considered to be a major proangiogenic factor, this observation underlines the significance of leptin as an endothelial growth factor. Proliferative effects of leptin have already been reported on hematopoietic or embryonic fibroblast cell lines,^{14,25,26} but this is the first report on vascular cells. Proliferation of endothelial cells constitutes one key event in the complex angiogenic process.² Angiogenesis starts by cell-mediated degradation of the basement membrane, followed by the migration and the proliferation of endothelial cells. The morphogenesis of the cells into capillary tubes finishes this process. Using 2 different in vitro models of angiogenesis (ie, endothelial cell-coated microcarrier-induced and monolayer-induced formation of capillary-like tubes in fibrin gels), we demonstrated that the leptin-induced proliferation and/or survival is associated with the enhanced formation of capillary-like tubes in a 3D matrix in a similar manner to that elicited by VEGF₁₆₅. Moreover, in vivo angiogenic assay using CAMs showed clearly that leptin enhanced the formation of new blood vessels in vivo. This positive angiogenic effect was observed using higher concentrations of leptin than those that were effective in vitro. This is probably a result of leptin diffusion being hindered by

methylcellulose. However, our observation is in accordance with a study published during the preparation of this article that describes an angiogenic effect of leptin in the rat corneas.²⁷

Thus, our results demonstrate that leptin could be a potent modulator of the angiogenic process. This peripheral action of leptin, observed in concentrations usually found in plasma or in adipose tissues, opens new perspectives toward links between this adipocyte-secreted cytokine and angiogenesis. It may be argued that this effect of leptin is not of great physiological relevance, because animals with a defect in the leptin system (Ob and Db mice)¹¹ seem to exhibit no manifest alteration in angiogenesis. However, the use of the genetically manipulated mouse as animal model has clearly evidenced that those mice quite often develop compensatory mechanisms to overcome the genetic defect. It is therefore likely that the defect in the leptin system in the Ob or Db mice might be compensated by other angiogenic factors. Experimental models in which angiogenesis is provoked, eg, unilateral ligation of femoral artery, will help to elucidate the modulatory role of leptin in the control of angiogenic processes.

Studies of fetal adipose tissue have indicated a remarkable spatial and temporal relationship between adipogenesis and vasculogenesis.²⁸ In situations of continuing adipose tissue growth (in the adult and during the settlement of obesity), it is documented that angiogenesis is present.²⁸ The factors influencing fat pad development, at both the adipocyte and endothelial cell levels, are at an early stage of characterization. Because obesity in humans is associated with an elevation of leptin in the plasma as well as adipose tissue, it is tempting to speculate that the leptin-mediated cross talk between adipocytes and endothelial cells promotes angiogenesis, which in turn participates in the additional increment of the adipose mass and ultimately to a progressive aggravation of the obese state. The present study opens a promising perspective concerning future investigations of leptin-dependent modulation of angiogenesis more specifically in adipose tissue during the settlement of obesity but also in other tissues under physiological and pathophysiological conditions.

Acknowledgments

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