Leptin Enhances the Recruitment of Endothelial Progenitor Cells Into Neointimal Lesions After Vascular Injury by Promoting Integrin-Mediated Adhesion

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Abstract—The adipocytokine leptin modulates vascular remodeling and neointima formation. Because endothelial progenitor cells (EPCs) participate in vascular repair, we analyzed the effects of leptin on human EPC function in vitro and in vivo. After 7 days in culture, EPCs expressed the leptin receptor and responded to leptin stimulation with increased STAT3 phosphorylation. Incubation of EPCs with leptin (at concentrations between 1 and 100 ng/mL) increased the number of EPCs adhering to vitronectin and fibronectin in a receptor-specific manner. It also enhanced the capacity of EPCs to incorporate into a monolayer of human endothelial cells and the adherence of these cells to activated platelets. Leptin upregulated αβ5 and α4 integrin expression in EPCs, and the effects of leptin on EPC function could be prevented, at least in part, by RGD peptides and function-blocking antibodies. Intravenous injection of fluorescently labeled human EPCs into athymic nude mice shortly after vascular injury revealed that preincubation of EPCs with leptin augmented their accumulation within intimal lesions, accelerating reendothelialization and decreasing neointima formation in an αβ5 and α4 integrin-dependent manner. Our findings suggest that leptin specifically modulates the adhesive properties and the homing potential of EPCs and may thus enhance their capacity to promote vascular regeneration in vivo. (Circ Res. 2008;103:536-544.)

Key Words: leptin ■ endothelial progenitor cells ■ neointima formation ■ integrins

Leptin is an adipose tissue–secreted hormone acting primarily on hypothalamic neurons to regulate body weight and energy expenditure. However, isoforms of the leptin receptor are also expressed on a number of cell types outside of the central nervous system. For example, leptin potentiates agonist-induced platelet aggregation,1 and it promotes arterial1-2 and venous3 thrombosis in vivo. Moreover, the hormone has been shown to promote the proliferation, differentiation, and functional activation of hematopoietic cells,4 and it may function as an angiogenic factor for mature endothelial cells.5,6

Accumulating evidence suggests that endothelial progenitor cells (EPCs) play an important role in maintaining vascular integrity and regulating neovascularization. For example, EPCs, which either were exogenously administered or mobilized from the bone marrow in response to stimulation with various cytokines and angiogenic growth factors, contributed to postnatal vasculogenesis associated with myocardial and skeletal muscle ischemia, wound healing, and tumor growth.7-9 In the vasculature, circulating EPCs migrate to sites of endothelial injury, and it has been suggested that they are capable of promoting reendothelialization, improving endothelium-dependent vasoreactivity, and reducing intimal hyperplasia during vascular remodeling.10,11 Importantly, the ability of EPCs to confer vascular protection appears to be related not only to their number in the circulation but also to their functional properties and particularly their ability to adhere (home) to areas of tissue damage.12,13 Based on these previous reports, and on the recent detection of the leptin receptor on human EPCs,14 we sought to determine whether leptin may affect the ability of EPCs to interact with endothelial cells and the extracellular matrix (ECM) of the vessel wall and to participate in vascular remodeling. Our results indicate that ex vivo stimulation of culture-expanded human EPCs with leptin at concentrations ranging between 10 and 100 ng/mL results in upregulation of α4 and αβ5 integrins. These effects promoted EPCs adhesion to ECM proteins, activated platelets, and mature endothelial cells in vitro, and they increased EPC recruitment to sites of vascular injury in vivo. Interestingly, although EPCs did not appear to incorporate into the neointima itself, leptin treatment enhanced reendothelialization and decreased neointima formation. Our findings

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provide novel insights into the mechanisms underlying the pleiotropic effects of leptin, and perhaps other adipocytokines, on vascular homeostasis. They may help modify existing pathophysiological concepts and improve our understanding of the links between obesity and cardiovascular disease.

**Materials and Methods**

An expanded Materials and Methods is available in the online data supplement at http://circres.ahajournals.org.

Mononuclear cells were isolated from the peripheral blood of human volunteers by density-gradient centrifugation. After 4 days in culture, nonadherent cells were removed and adherent cells were maintained in endothelial cell medium until analysis on day 7. EPCs were characterized and expression of the leptin receptor was determined using flow cytometry, immunofluorescence, RT-PCR, and Western blot analysis. The effects of leptin (1, 10, 100, or 1000 ng/mL for 24 hour) or neutralizing antibodies against leptin, leptin receptor, and α4 or αvβ5 integrins on EPC function in vitro was assessed by analyzing EPC adhesion to ECM proteins, tumor necrosis factor (TNF)-α–stimulated human umbilical vein endothelial cells (HUVECs), or collagen-activated platelets. The in vivo effects of leptin on the recruitment of EPCs to the site of vascular injury and the degree of neointima formation or reendothelialization was examined using laser fluorescence microscopy, immunofluorescence, and morphometric quantification.

**Results**

**Characterization of Endothelial Progenitor Cells**

Human mononuclear cells were isolated and cultivated under endothelial cell conditions. On day 7, numerous spindle-shaped cells were observed, and the majority of adherent cells (>95%) were found to be double-positive for uptake of DiI-labeled acetylated LDL (acLDL) and binding of *Ulex europaeus* lectin (Figure IA in the online data supplement). Flow cytometric analysis of endothelial cell surface antigens revealed that 85.9±3.5% of the adherent cells were positive for vascular endothelial growth factor receptor (VEGFR)2, 62.1±4.2% for VE-cadherin (CD144), 56.7±5.0% for CD31, and 9.5±1.4% for von Willebrand factor (vWF) (supplemental Figure IB). In addition, the adherent cells expressed surface markers consistent with leukocytic (CD45; 97.5±0.7%) and monocytic (CD14; 20.3±11.2%) cell lineage, whereas only very few cells (<2.0%) expressed the hematopoietic markers CD34 and CD133. RT-PCR analysis confirmed the expression of endothelial cell lineage markers (supplemental Figure IC), including VEGFR1 and endothelial NO synthase. Moreover, cells were capable of incorporating into a network of tubular structures provided by cocultivated mature endothelial cells (supplemental Figure ID). On the basis of these morphological and functional characteristics, and in line with published data,8,15–19 cells were characterized as peripheral blood–derived early outgrowth or culture-expanded EPCs.

**EPCs Express the Leptin Receptor**

Expression of the leptin receptor (ObR) was analyzed on EPCs cultured for 7 days. Flow cytometric analysis revealed that 24.9±3.0% of the EPCs expressed the leptin receptor (supplemental Figure II), and the majority (>90%) of the ObR-expressing cells were also found to be positive for VEGFR2 or αM integrin (Figure 1A). ObR expression was confirmed using immunofluorescence (Figure 1B) and RT-PCR (Figure 1C). Leptin resulted in upregulation of both ObR mRNA (Figure 1C) and surface (Figure 1D) expression. Moreover, Western blot analysis revealed increased STAT3 phosphorylation in response to leptin (Figure 1E), indicating that EPCs possess the long isoform of the leptin receptor.20

**Leptin Potentiates the Adherence of EPCs to ECM Proteins**

EPCs homing to the vessel wall involves the adhesion of circulating cells to ECM proteins following vascular injury. To assess whether leptin alters the adhesive properties of EPCs, cells were incubated with leptin and then allowed to attach to different ECM proteins. Preincubation of EPCs for 24 hours with leptin (1, 10, and 100 ng/mL) significantly increased the number of cells firmly adherent to vitronectin (VN) compared to PBS-treated cells (supplemental Figure IIIA), whereas stimulation with 1000 ng/mL leptin appeared to have the opposite effect. Time curve experiments (ranging from 1 to 72 hours) revealed that the effect of leptin on EPC adhesion was most pronounced after stimulation for 24 hours (data not shown). Of note, coinoculation with leptin- or leptin receptor–neutralizing antibodies both prevented the potentiating effect of leptin on EPC adhesion to VN (supplemental Figure IIIB). Stimulation with leptin also increased the number of EPCs binding to fibronectin (FN)-coated culture dishes (supplemental Figure IIIC), whereas it did not alter their ability to adhere to collagen, gelatin, or plastic (data not shown).

**Leptin Increases the Ability of EPCs to Adhere to Endothelial Cell Monolayers and Enhances Transendothelial Migration**

Apart from the interaction of EPCs with ECM proteins, their homing to the vessel wall requires adhesion and transmigration through endothelial cells. Pretreatment of EPCs with leptin (10 and 100 ng/mL) significantly enhanced their capacity to incorporate into a monolayer of TNF-α–activated HUVECs (supplemental Figure IIID). Also, preincubation with leptin, at concentrations of 10 and 100 ng/mL, increased the migration of EPCs through a HUVEC monolayer toward a chemotactic gradient induced by stromal cell–derived factor-1α (199±33% and 152±40%, respectively; *P*=0.01 for the difference between 10 ng/mL leptin and PBS-treated controls).

**Stimulation of EPCs With Leptin Upregulates the Expression of αvβ5 and α4 Integrins**

EPCs adhere to ECM proteins or cells via specific integrin receptors. For example, binding of cells to VN is mediated primarily by αvβ3 and αvβ5 integrins, whereas at least 10 different integrins are involved in mediating adhesion to FN, among which α5β1, α4β1, and αIββ3 have been studied in more detail.21 Integrins capable of mediating cell–cell contacts include α4β1 and members of the β2 family; they enable binding to vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule-1, and endothelial surface–associated fibrinogen.22 In addition, αvβ3 and αvβ5 integrins may participate in cell–cell interactions using platelet endothelial cell adhesion molecule23 and endothelial surface–bound VN24 as ligands. We analyzed the surface expression of integrin adhesion...
receptors on EPCs after 7 days in culture using flow cytometry. As summarized in Figure 2A, EPCs mostly expressed β2 (94 ± 4.4% of gated mononuclear cells), αM (89 ± 3.7%), and αL (89 ± 3.0%), and, to a lesser extent, β1 (66 ± 11%) integrins. EPCs also expressed αβ5 (26 ± 2.6%) and α4 integrins (12 ± 3.7%), whereas the expression of αβ3 on EPCs was low (7.0 ± 1.9%).

Figure 2B shows that stimulation of EPCs with 10 or 100 ng/mL leptin for 24 hours significantly increased surface expression of α4 and αβ5 integrins, as assessed by flow cytometry, compared to unstimulated controls (representative histograms shown in Figure 2C), whereas the expression of αβ3, β1, β2, and the β2-associated subunits αL and αM remained unchanged. Fluorescence microscopy confirmed that leptin increased α4 and αβ5 integrin expression (Figure 2D), and the majority of the αβ5- and α4-expressing cells were found to also express VEGFR2, αM integrin, and ObR (Figure 2E). Upregulation of α4 and αβ5 integrin expression appeared to be related, at least in part, to an increase in their mRNA levels (supplemental Figure IVA); however, this effect was not consistently observed in EPCs of all individuals studied (supplemental Figure IVB).

The Effects of Leptin on Integrin-Mediated EPC Adhesion Involve Both RGD-Dependent and RGD-Independent Mechanisms
Given the fact that the Arg-Gly-Asp (RGD) sequence is a major recognition motif for integrin-mediated cell adhesion to ECM proteins, experiments were performed to test the effect of RGD peptides on EPC adhesion with and without leptin stimulation. Our findings, which are summarized in Figure 3, show that the addition of RGD peptides (0.25 mg/mL) reduced the ability of EPCs to adhere to VN (P = 0.004; A) and mature endothelial cells (P = 0.006; Figure 3E), whereas they did not detectably affect their adhesion to FN (P = 0.944; Figure 3C). Moreover, addition of RGD peptides significantly reduced the leptin-mediated increase of EPC adhesion to VN (Figure 3A and representative findings
in Figure 3B) and to HUVECs (Figure 3E and representative findings in Figure 3F), although adhesion to HUVECs was still elevated compared to unstimulated, RGD-treated controls \( (P<0.006) \). Further experiments revealed that the effect of leptin on EPC adhesion to FN continued to be significant in the presence of RGD peptides \( (P<0.002 \) versus unstimulated controls; Figure 3C and representative findings in Figure 3D). These results suggest that both RGD-dependent (adhesion to VN and HUVECs) and -independent mechanisms (adhesion to FN and, partly, to HUVECs) are involved in mediating the effects of leptin on EPC adhesion.

Further experiments revealed that monoclonal \( \alpha \beta 5 \)-inhibiting antibodies (MAB1961; 10 \( \mu g/mL \)) significantly reduced the adhesion of both untreated and leptin-stimulated EPCs to VN (Figure 3A and representative findings in Figure 3B), whereas monoclonal \( \alpha 4 \)-inhibiting antibodies (MAB16983; 10 \( \mu g/mL \)) significantly reduced EPC adhesion to FN (Figure 3C and representative findings in Figure 3D). Adhesion of leptin-stimulated EPCs to a monolayer of TNF-\( \alpha \)-stimulated HUVECs could be completely abolished by \( \alpha 4 \)-and VCAM-function-blocking antibodies (Figure 3E and representative findings in Figure 3F), suggesting that the leptin-mediated increase of EPC adhesion involves binding via \( \alpha 4 \) integrins to VCAM, expressed on activated HUVECs.

**Leptin Enhances the Incorporation of EPCs After Vascular Injury and Reduces the Size of Neointimal Lesions In Vivo**

To determine the effects of leptin on EPCs homing to the vessel wall in vivo, vascular injury was induced at the carotid artery using the ferric chloride model.\(^1\) Fluorescent DiD-labeled EPCs, pretreated with 10 ng/mL leptin or PBS for 24 hours, were intravenously injected into athymic nude mice within 10 minutes after induction of vascular injury. Whole body fluorescence laser imaging at different time points (up to 3 weeks) revealed that DiD-labeled EPCs already began to accumulate at the injured carotid artery within the first hour. A significantly enhanced specific fluorescence intensity proj-
ected on the injured carotid artery could be observed between days 1 and 3 after injury in mice that had received leptin-treated EPCs compared to those injected with control-treated cells (representative findings shown in Figure 4A; results summarized in B). At later time points, the differences in the specific fluorescence intensity were no longer significant. Additional experiments using CM-Dil-labeled cells and fluorescence microscopy confirmed the presence of EPCs at the injured carotid artery. Fluorescent cells were detected accumulating within the thrombotic material occluding the vas-

Figure 3. The effect of leptin on EPC adhesion is integrin-mediated and partly RGD-dependent. The effects of leptin (10 ng/mL for 24 hours) on the number of EPCs adherent to VN (A), FN (C), or HUVECs (E) were quantified per high power field (HPF), also in the presence of RGD peptides, function-blocking antibodies, or isotype IgG controls, respectively. *P<0.05, **P<0.005, and ***P<0.001 vs unstimulated cells; #P<0.05 vs leptin-stimulated cells; §P<0.05 vs RGD alone. Representative photomicrographs show the adhesion of fluorescently labeled PBS- or leptin-treated EPCs to VN-coated (B) or FN-coated (D) culture plates or HUVECs (F). Magnification, ×50 (B and D) or ×200 (F).
cular lumen, and a higher cell number was detected 3 days after injection when the EPCs had been pretreated with leptin (Figure 4C). In further experiments, leptin (0.1 μg/g body weight) was injected into mice 30 minutes before induction of arterial injury followed by injection of untreated EPCs as described above. Morphometric quantification revealed that not only ex vivo leptin prestimulation but also leptin IP injection before injury was capable of significantly enhancing the accumulation of EPCs to the site of vascular injury (supplemental Figure V).

Because ferric chloride–induced injury results in the formation of platelet-rich thrombi, and platelet α-granules contain ligands for both α4 (fibronectin or thrombospondin) and αvβ5 (VN) integrins, we also assessed the adhesion of leptin- or control-stimulated EPCs to a monolayer of collagen-activated human platelets or releasates from maximally stimulated platelets in vitro. These analyses revealed that preincubation with leptin significantly enhanced the adhesion of EPCs to collagen-activated platelets (236±46.3% versus PBS-treated cells; n=5; P=0.019) or platelet releasates (182±15.6%; n=5; P<0.001). Importantly, the leptin effects were receptor-specific, because they could be inhibited by preincubation of EPCs with ObR-neutralizing antibodies (86.5±16.8%; P=0.39; and 84.3±33.2%; P=0.54, respectively).

Histological analysis of neointimal lesions 3 weeks after injury revealed that CM-DiD-labeled EPCs were located within the neointima, predominantly in the subendothelial layer (Figure 5A). Immunofluorescence labeling confirmed that these were injected human EPCs, because they colocalized with human leukocyte antigen (data not shown). Double staining with antibodies against cell type–specific antigens further showed that 46.4±5.8% of CM-DiD-labeled EPCs colocalized with CD31 and 53.2±7.7% with αM, whereas costaining with α-actin was rarely observed (Figure 5B). In this regard, binding studies using Ulex europeus lectin failed to detect the presence of human (ie, EPC-derived) endothelial cells, whereas strong staining of murine endothelial cells could be observed (Bandeiraea simplicifolia lectin; supplemental Figure VI). Finally, morphometric analysis of arterial cross sections through the neointima 3 weeks after injury revealed a significant reduction of both the neointima area and the degree of luminal stenosis in mice injected with leptin-treated EPCs (10 and 100 ng/mL) compared to those injected with PBS-treated EPCs (Figure 5C). Of note, lesions from mice injected with EPCs that had been treated with supraphysiological leptin concentrations (ie, 1000 ng/mL) did not differ from those receiving control-treated cells (data not shown). Finally, preincubation of leptin-stimulated EPCs with specific integrin function-blocking antibodies or control IgG, 30 minutes before their intravenous injection after injury, confirmed that the observed effects of leptin on the neointima formation and reendothelialization are mediated by αvβ5 or α4 integrins (Figure 5D and 5E).

Discussion

The results of the present study indicate that the adipokine leptin may enhance the ability of endothelial progenitor cells

Figure 4. Effect of leptin on the accumulation of human EPCs after carotid artery injury. A, Fluorescence laser imaging revealed a more pronounced accumulation of leptin-stimulated, DiD-labeled EPCs at the site of vascular injury in comparison to control-stimulated EPCs. Representative images 3 days after induction of vascular injury are shown. B, Quantitative analysis of 3 to 4 mice per group revealed a significant increase in the specific fluorescence intensity in mice between days 1 and 3 after injection of leptin-treated EPCs. *P<0.05 vs control-treated EPCs. C, Representative cross sections showing enhanced accumulation of CM-Dil-labeled leptin-treated (right) compared to control-treated (left) EPCs (red signal) within the thrombotic material occluding the vascular lumen 3 days after injury. Magnification, ×100.
to participate in vascular remodeling. Pretreatment of culture-expanded EPCs with leptin at concentrations ranging between 10 and 100 ng/mL promoted the adhesion of these cells to ECM proteins, activated platelets, and mature endothelial cells. In vivo, leptin stimulation increased the recruitment of EPCs to sites of vascular injury and platelet-rich thrombi, and this effect was associated with enhanced reendothelialization and decreased neointima formation. These findings unveil novel mechanisms that may contribute to the pleiotropic effects of the adipokine on vascular homeostasis.

Experimental studies have suggested beneficial effects of EPCs on intimal hyperplasia. For example, implantation of autologous circulating EPCs into balloon-injured carotid arteries of rabbits accelerated endothelialization, reduced neointima formation, and improved endothelial-dependent vasoreactivity.10,11 Similarly, spleen-derived EPCs enhanced reendothelialization and reduced neointima formation after carotid artery injury in mice.15 Importantly, homing and incorporation of EPCs into sites of vascular injury and ischemia appears to depend not only on their circulating numbers but also on their functional properties. Thus, EPCs that were culture-expanded from diabetic humans and transplanted into mice after wire-induced endothelial denudation exhibited impaired recruitment,25 whereas transgenic overexpression of vasculoprotective genes enhanced the ability of rabbit EPCs to reduce neointima hyperplasia.26

The attachment of circulating EPCs to matrix proteins and (activated) endothelial cells is a critical step in the arrest and extravasation of these cells at the site of injury and appears to be mediated, at least in part, by integrin adhesion receptors.22,27 The VN receptor, which has been shown to be upregulated in response to hypoxia28 or vascular injury,29 promotes the adhesion of cells to the plasma protein VN, released from activated platelets30 and accumulating in the vessel wall after injury.31 VN may, thus, function as an adhesive substrate guiding circulating progenitor cells to the site of tissue injury, and its role appears to
be similar to that recently ascribed to fibrin or activated platelets.\cite{32,33} Notably, it also has been suggested that VN may function as a bridging molecule between circulating cells and the endothelium itself.\cite{24} Two functionally homologous integrins (\(\alpha_v\beta_3\) and \(\alpha_v\beta_5\)) have been shown to bind VN in an RGD-dependent manner. Blocking antibodies to \(\alpha_v\beta_3\), or to VN itself, suppressed neointima formation after balloon injury in rats.\cite{24,34} and a preclinical study reported that cyclic RGD-coated stents reduced intimal hyperplasia, possibly by enhancing the recruitment of endothelial progenitor cells.\cite{36} Although \(\alpha_v\beta_5\) expression also is increased in response to balloon injury,\cite{26} its role during vascular remodeling is less well examined. In the present study, we demonstrate that EPCs express \(\alpha_v\beta_5\) integrins and that stimulation of EPCs with leptin significantly upregulated \(\alpha_v\beta_5\) surface expression resulting in enhanced, RGD-dependent EPC adhesion to VN. Importantly, in vivo studies using function-blocking antibodies suggest that \(\alpha_v\beta_5\) integrins are involved in the beneficial effect of leptin prestimulation of EPCs on neointima formation.

In addition to the \(\alpha_v\beta_5\)-VN interaction, the results of our study also suggest a critical role for \(\alpha_4\) integrins, most likely as part of the \(\alpha_4\beta_1\) or very late antigen-4 complex, in mediating the effects of leptin on EPC homing. It has been reported that EPCs, but not smooth muscle cell progenitors, express \(\alpha_v\beta_5\) and \(\alpha_4\beta_1\).\cite{37} In the present study, treatment of EPCs with leptin upregulated the expression of \(\alpha_4\) integrins, and \(\alpha_4\)-neutralizing antibodies not only prevented the binding of leptin-stimulated EPCs to FN or HUVECs but also the leptin-mediated reduction of neointima formation. Binding of leptin-stimulated EPCs to HUVECs could also be inhibited by VCAM-neutralizing antibodies, suggesting that leptin promotes adhesion of \(\alpha_4\)-expressing EPCs to VCAM present on activated endothelial cells. In this regard, ex vivo preincubation of EPCs with \(\alpha_4\)-neutralizing antibodies was shown to reduce the recovery of hindlimb blood flow and capillary density and incorporation of EPCs into ischemic tissues.\cite{38}

In accordance with the effects of leptin on integrin-mediated adhesion of EPCs to mature endothelial cells, activated platelets, and ECM proteins, our in vivo studies showed that pretreatment of human EPCs with leptin before their injection into athymic nude mice resulted in more pronounced accumulation of these cells at the site of vascular injury. This effect was associated with accelerated reendothelialization and, importantly, reduced neointima formation. Previous reports suggested that EPCs may facilitate endothelial cell repair by transdifferentiation into endothelial cells.\cite{15,39} However, in our experiments using 2 different fluorescent membrane labels, as well as indirect detection methods such as the visualization of human leukocyte antigen, we could not detect human (injected) EPCs lining the luminal surface of injured mouse arteries, although CD31- or \(\alpha_M\)-positive EPCs were found to be present within the neointimal lesions. In this regard, it has been suggested that EPCs may stimulate the recruitment and proliferation of local mature cells through paracrine mechanisms, including the secretion of angiogenic factors.\cite{40,41} Our findings confirm and extend these reports by showing that pretreatment of human EPCs with leptin enhanced reendothelialization of injured arteries with murine, ie, endogenous cells. It remains to be determined whether these endogenous cells originate from the proliferation of local endothelial cells or circulating progenitors.

We and others have previously shown that once-daily intraperitoneal administration of recombinant leptin into wild-type and leptin-deficient mice enhanced (rather than reduced) intimal hyperplasia after experimental injury.\cite{42,43} The explanation for the apparent disagreement between these earlier observations and the results of the present study is partly related to the mode of leptin application, ie, once-daily injection of the hormone into mice over several weeks, as opposed to a single ex vivo stimulation of culture-expanded, human EPCs. Also, the leptin dosages that have been used to rescue the phenotype of leptin-deficient mice\cite{42} result in transient but pronounced (and partly supraphysiological) elevations of plasma leptin concentrations.\cite{1} Notably, the EPCs used in our experiments were isolated from lean individuals, and it is possible that the effects of continuously elevated circulating leptin levels, such as those encountered in human obesity, on EPCs or other cells of the vessel wall may differ from those described in the present study.

In conclusion, we could show that the adipokine leptin enhanced the functional capacity of peripheral blood-derived human EPCs in vitro and improved their homing capacity in vivo. These findings, which were partly unexpected, point to novel mechanisms possibly contributing to the effects of leptin on vascular remodeling, and they also raise the possibility that pretreatment of progenitor cells could therapeutically modify (enhance) their regenerative properties following vascular injury. Future studies will have to examine in more detail how interaction of leptin with its receptor on endothelial (progenitor) cells upregulates specific integrins. Moreover, it needs to be clarified how obesity-associated hyperleptinemia affects the functional activity of EPCs and other vascular cells and, particularly, whether the increased cardiovascular risk associated with excess body weight is related to the hyperleptinemia itself or resistance to the possible vasoprotective and vasoregenerative effects of the hormone.

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Disclosures

None.

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Expanded Materials and Methods

In Vitro Studies

Isolation and Cultivation of Endothelial Progenitor Cells

Mononuclear cells (MNC) were isolated from the peripheral blood of healthy, normal-weight human volunteers by density-gradient centrifugation with Histopaque-1077 (Sigma-Aldrich; Munich, Germany). Immediately after isolation, total MNC (8x10^7 cells; 1.2x10^4/mm²) were plated on culture dishes coated with human fibronectin (FN; 10 µg/mL; tebu-bio; Offenbach, Germany) and maintained in Endothelial Cell Basal Medium MV (PromoCell; Heidelberg, Germany) supplemented with 1 µg/mL hydrocortisone, 0.4% (v/v) bovine brain extract, 10 ng/mL epidermal growth factor, 100 U/mL penicillin, 100 µg/mL streptomycin, and 20% (v/v) FCS. After 4 d in culture, nonadherent cells were removed by washing twice with PBS, and adherent cells were maintained in medium until analysis on d 7.

Leptin stimulation

Cells were incubated with recombinant human leptin (R&D Systems) at final concentrations of 1, 10, 100 or 1000 ng/mL for 24 h. In a subset of experiments, leptin was preincubated for 30 min at 37°C with neutralizing anti-human leptin (Ob) Ab (AF398; 1 µg/mL), anti-human leptin receptor (ObR) Ab (AF389; 5 µg/mL) or equal amounts of isotype control (both R&D Systems), before being added to the EPC culture medium.

Cell Characterization

EPC were characterized by dual staining for Dil-labeled acetylated LDL (6 µg/µL; Molecular Probes; Karlsruhe, Germany) at 37°C, and FITC-labeled Ulex europaeus lectin (10 µg/mL; Sigma-Aldrich) at RT, 60 min each. For flow cytometry analysis, EPC were gently detached using 5 mmol/L EDTA/PBS at 37°C, washed and incubated for 45 min at 4°C in PBS/0.5%
(w/v) BSA together with PE-labeled mouse antibodies (Abs) against VEGFR2 (FAB357P; R&D Systems; Wiesbaden, Germany), VE cadherin (sc-9989; SantaCruz Biotechnology; Heidelberg, Germany), CD133 (#130-090-853; Miltenyi Biotec; Bergisch Gladbach, Germany), α4 (#555 503; BD Pharmingen; Heidelberg, Germany) or ObR (FAB 867P; R&D Systems) respectively, or with FITC-labeled mouse/ rat Abs against CD34 (#555 821; BD Pharmingen), CD31 (#555 445; BD Pharmingen), αM (CD11b01; Caltag), αL (CBL 451F), αvβ5 (MAB 1961F), αvβ3 (MAB 1976F), β1 (CBL 481F) and β2 (CBL 158F; all Chemicon International; Hampshire, UK). Surface expression of vWF (A0082; DakoCytomation; Glostrup, Denmark), CD14 (#555 396; BD Pharmingen), and CD45 (#130-080-202; Miltenyi Biotec) was detected using FITC-labeled goat anti-rabbit or anti-mouse secondary Abs (Molecular Probes). Flow cytometry was performed using FACScan flow cytometry and analyzed with Cell Quest software (BD Bioscience). Each analysis included at least 10,000 events of mononuclear gated cells.

A matrigel tube formation assay was performed using the In Vitro Angiogenesis Assay Kit (Chemicon). Briefly, a mixture of Dil-acLDL-labeled EPC (3x10^4 cells/mL) and human umbilical vein endothelial cells (HUVEC; 1.2x10^5 cells/mL) were incubated for 8 h on 96-well plates precoated with matrix solution (ECMatrix™) and then analyzed using fluorescence microscopy (Zeiss Axiovert 200 and AxioVision 3.1).

**Adhesion and Transmigration Assays**

For cell adhesion experiments, EPC were gently detached with 5 mmol/L ETDA/PBS, washed and suspended in adhesion buffer (serum-free culture medium plus 0.5% (w/v) BSA, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, and 1 mmol/L MnCl₂), and labeled with DiI-acetylated LDL (Molecular Probes). EPC (1x10^5 in 500 µL adhesion buffer) were then seeded on 24-well plates, precoated with either vitronectin (VN; 0.5 ng/mm²; Promega) or fibronectin (FN; 10 ng/mm²; tebu-bio), and blocked with 1% (w/v) BSA for 30 min at RT, or on confluent
monolayers of HUVEC (PromoCell), stimulated or not with TNF-α (1 ng/mL; R&D Systems) for 6 hrs. Experiments were performed in the absence or presence of monoclonal blocking Abs against α4-integrin (clone P1H4; Chemicon) or αvβ5-integrin (clone P1F6; Chemicon), murine isotype controls (Chemicon), RGD peptides (Sigma-Aldrich), or function-blocking Abs against VCAM-1 (MAB 2144; Chemicon). After 30 min incubation at 37°C, plates were vigorously washed to remove nonadherent cells. Adherent EPC were fixed with 2% paraformaldehyde (PFA) in PBS for 10 min at RT and subsequently stained with 4’-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Experiments were performed in triplicate, and the number of adherent cells was quantified by manually counting fluorescence-labeled EPC in five randomly selected microscope fields per well.

For platelet-EPC interaction experiments, washed platelets were isolated from healthy volunteers as described.² 24-multiwell plates were coated with 4 µg/mL fibrillar type I collagen (Chronolog Corporation) for 3 hrs at RT. The wells were rinsed with PBS to remove unbound collagen and then incubated with platelets for 30 min at RT, resulting in the formation of a monolayer of activated platelets. CM-Dil-labeled EPC (3.5x10⁴ per well) were added and allowed to adhere for 30 min at 37°C. For platelets release experiments, platelets were re-suspended in modified Tyrode’s buffer (pH 7.4), containing CaCl₂ (1 mM; Sigma-Aldrich), type I collagen (2 µg/mL), thrombin (1 U/mL; Sigma-Aldrich) and calcimycin (1.3 µg/mL; Sigma-Aldrich), incubated for 10 min at RT, 5 min at 37°C and 1 min on ice, and centrifuged at 13,000 rpm for 10 min at 4°C. Then, supernatants were used to coat 24-well plates and the adhesion of CM-Dil-labeled EPC was analyzed as described above. In both assays, the number of adherent EPC was manually counted in five randomly selected microscope fields (200x) per well.

For transmigration experiments, HUVEC were seeded on gelatine-coated 6.5-mm transwell filters (8 µm pore size; BD Falcon; Le Pont De Claix, France). After 2 d, 600 µL of serum-free basal medium containing SDF-1α (100 ng/mL; R&D Systems) was added to the
lower compartment of the transwell system, and EPC (1x10^5 in 100 µL) were added to the upper compartment. After 18 h at 37°C, the filter was washed with PBS, fixed with 2% PFA and the upper side carefully cleaned with a cotton swab. For quantification, cell nuclei on the lower side were stained with DAPI and the total number of transmigrated cells per filter was counted using ImagePro Plus software (Media Cybernetics).

**Immunofluorescence**

EPC were cultivated on FN-coated culture slides (BD Falcon), fixed with 2% PFA (in PBS plus 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, and 1 mmol/L MnCl₂), permeabilized with 0.1% Triton X-100 in PBS (5 min, 37°C) and blocked with 0.5% BSA/PBS. Slides were incubated with mouse anti-human αvβ5 or α4 monoclonal Abs (both Chemicon; 1:10 dilution) or rabbit anti-human ObR polyclonal Ab, directed against an epitope within the internal domain of human leptin receptor (H-300; SantaCruz Biotechnology; 1:20 dilution) for 1 h at RT. After washing, FITC- or PE-labeled secondary Abs (1:100 dilution in PBS; Molecular Probes) were added for 30 min at RT. Slides were washed, mounted and subsequently analyzed by fluorescence microscopy.

**Reverse Transcriptase-Polymerase Chain Reaction**

Expression of endothelial cell markers and the leptin receptor (ObR) was evaluated by RT-PCR analysis. Total RNA of cultured human EPC or HUVEC was extracted using TRI Reagent (Ambion) and chloroform following the manufacturer’s instructions. 500 ng RNA was reverse transcribed using M-MLV Reverse Transcriptase (Promega). Primer pairs for VEGFR1, CD144, VEGFR2, CD31, vWF, eNOS, the common isoform of ObR, α4, β5 (5’-GTGCTCCAAAGAGGACTTCG-3’; 5’-GAAGTTGCTGGTGAGCTTCC-3’), and β-actin (5’-CCCAAGGCCAACCGCGAGAAGAT-3’; 5’-GTCCCGGCCAGCCAGGTCCAG-3’) were used. The conditions for subsequent PCR were 94°C for 2 min, followed by 36-40
cycles at 94°C for 30 seconds, 55-60°C primer-dependent annealing temperature for 30 sec and 72°C for 1 min, and ending with a 5 min-extension at 72°C. Expression of β-actin was used for semiquantification. RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and quantified under UV illumination using ChemiImager 4400, version 5.1 (Alpha Innotech Corporation; San Francisco, CA, USA).

**Immunoprecipitation, SDS-Page Electrophoresis and Western Blot Analysis**

EPC were treated with leptin (10 ng/mL) or PBS for 10 min, detached, washed with PBS, and resuspended in 100 µL lysis puffer (75 mM NaCl, 100 mM HEPES, 50 mM CHAPS, pH 7.5), sonicated and cleared by centrifugation at 13,000 rpm for 10 min at 4°C. After preclearing with protein A beads (Amersham Biosciences AB; Uppsala, Sweden), monoclonal mouse p-Tyr Ab (PY20; 2 µg; SantaCruz) was added for 2 h followed by incubation with protein A beads overnight. All incubation steps were performed under rotation at 4°C. Equal amounts of protein were loaded and fractionated by electrophoresis on a 10% SDS-polyacrylamide gel together with molecular weight standards and then transferred to nitrocellulose membranes (Protran®, Whatman; Dassel, Germany). Membranes were blocked in 3% non-fat dry milk (in TBS) prior to incubation with rabbit anti-human STAT3 or p38 Abs (dilution, 1:1000; Cell Signaling Technology; Danvers, MA) overnight at 4°C. Visualization of protein bands was achieved using a HRP-conjugated secondary donkey anti-rabbit IgG Ab (dilution, 1:3000; Amersham Biosciences) for 2 h at RT, followed by autoradiography.

**In Vivo Studies**

**Mice**

Six to eight week-old male athymic nude mice (NMRI-Foxn1nu) were obtained from Harlan Winkelmann (Borchen, Germany) and maintained at the Animal Facility of the University of Goettingen under pathogen-free conditions. All animal care and experimental procedures
were approved by the Animal Research Committee of the University of Goettingen and complied with national guidelines for the care and use of laboratory animals.

**Induction of Carotid Artery Injury**

Mice were anesthetized and subjected to carotid artery injury using 10% ferric chloride as described\(^8\). Within 10 min following induction of injury, \(1.5 \times 10^6\) CM-DiI- or DiD-labeled human EPC (non-stimulated or stimulated with recombinant human leptin for 24 h) were injected into the contralateral jugular vein. In some experiments, EPC were incubated with monoclonal blocking Abs against \(\alpha_4\)-integrin (clone P1H4; 10 \(\mu\)g/mL), \(\alpha\nu\beta_5\)-integrin (clone P1F6; 10 \(\mu\)g/mL), or murine isotype controls (Chemicon), for 30 min at 37°C prior to their intravenous injection. The wound was carefully sutured, and the mice returned to their cages. For tissue harvesting at different time points after injury, anesthetized mice were perfused with normal saline through the left ventricle. The injured carotid artery was carefully excised and immediately processed for cryopreservation, followed by preparation of frozen sections and subsequent histochemical analysis.

**Optical Imaging using eXplore Optix™**

At predefined time points after endothelial injury, mice were anaesthetized with 4% isoflurane and maintained on 1.5% isoflurane in 100% oxygen for the duration of data acquisition. CM-DiD-fluorescence-labeled cells were detected using an *in vivo* fluorescence optical imaging system (eXplore™ Optix; GE Healthcare), which allows the detection of fluorescent signals with superior tissue penetration, together with fluorescence intensity as well as lifetime determination. The device uses a pulsed laser diode with a wavelength in the near-infrared region of 670 to 700 nm, a pulse duration of approximately 100 per sec and a time-correlated single photon counting detection system. Both source and detection points are raster-scanned on the specimen and directed at the same specimen surface. Scans of whole ventral body and
regions of interest were performed, using 1 and 2 mm step size and 1.0 and 0.3 sec integration times, respectively. Laser power was calculated every time for signal intensity in regions of interest. Data were analyzed using eXplore Optix analysis workstation (GE HealthCare).

Morphometric Analysis of Neointima Formation

The neointima and the degree of luminal stenosis were quantified on 5 µm-thick, Verhoeff’s elastica-stained serial cross sections through the injured carotid artery segment (200 µm apart) using image analysis software (ImagePro Plus, version 4.1; Media Cybernetics).9

Histology

For histochemical analysis of lesions, injected human EPC were identified as CM-DiI-positive cells or by staining for HLA-ABC (BD Pharmingen). Endothelial cells were identified by binding of Ulex europeaus and Bandeiraea simplicifolia lectin (both Vector Laboratories, Peterborough, UK), or staining with Abs against vWF (Dako), CD31 (SantaCruz), αM (BD Pharmingen), or α-actin (Dako) followed by PE- or FITC-labeled secondary Abs (Molecular Probes). Cell nuclei were stained with DAPI. Reendothelialization was quantified by measuring the length of the vWF-positive endothelial layer as percentage of the total luminal circumference using image analysis software (ImagePro Plus, version 4.1).

Statistical Analysis

Data shown represent means ± SEM. Differences between means were tested by Student’s t test. Statistical significance was assumed when \( P \) was <0.05. All statistical analyses were performed using GraphPad PRISM data analysis software (version 4.01; GraphPad Software, Inc).
Reference List


Online Figure I

A

acLDL
lectin
overlay

B

VEGFR2
CD144
CD31
vWF
CD45
CD14
CD34
CD133

C

VEGFR1  VEGFR2  CD144  CD31  vWF  eNOS  β-actin
Online Figure I. Characterization of Endothelial Progenitor Cells (EPC).

A. After 7 d in culture, spindle-shaped cells were identified as EPC by uptake of Dil-labeled acLDL (red signal) and binding of FITC-labeled lectin (green signal). Double-positive cells appear yellow (overlay). Nuclei were counterstained with DAPI (blue signal). Magnification, 400x.

B. Flow cytometry revealed that cells expressed surface antigen markers characteristic for endothelial (VEGFR2, CD144, CD31, vWF), leukocytic (CD45) or monocytic (CD14) cells, whereas the hematopoietic markers (CD34, CD133) were expressed on the minority of cells. Red histograms represent specific fluorescence, translucent histograms the respective isotype controls.

C. RT-PCR analysis confirmed the expression of endothelial cell markers; β-actin mRNA expression was used as internal standard.

D. After coincubation in matrigel™, HUVEC and fluorescence-labeled EPC (red signal) developed a tubular network (left panel; magnification, 100x). High-power magnification (200x; right panel) confirmed the incorporation of EPC (red signal) into tubular structures provided by HUVEC.
Online Figure II

Online Figure II. Expression of leptin receptor mRNA and protein.
Flow cytometry was employed to quantitate ObR expression on EPC, using COS-7 cells as negative and human ObR cDNA-transfected HEK293 cells (ObR HEK293) as positive control.
Online Figure III

A

![Graph showing adhesion (% of control) vs. leptin concentration (ng/mL)]

B

![Graph showing adhesion (% of control) for different conditions: leptin, IgG, Ob-Ab, ObR-Ab]
Online Figure III. Leptin enhances adhesion of EPC to VN, FN and HUVEC.

A. EPC were preincubated with leptin (1 to 1000 ng/mL) for 24 h and their adhesion to VN-coated culture plates examined (*P<0.05; **P<0.01; ***P<0.001 vs. control).

B. The effect of 10 ng/mL leptin on EPC adhesion to VN could be blocked using leptin (Ob-Ab) or leptin receptor (ObR-Ab) neutralizing antibodies (**P<0.005 vs. control; #P<0.05 vs. leptin-treated cells). Preincubation of EPC with leptin dose-dependently enhanced their capacity to adhere to FN-coated culture plates (C) or mature endothelial cells (HUVEC; D; **P<0.01; ***P<0.001 vs. control).
Online Figure IV

A

B

Online Figure IV. Effect of leptin on α4 and β5 integrin mRNA expression in EPC. RT-PCR analysis of α4 and β5 integrin expression in EPC, untreated (black bar) and after stimulation with leptin (10 ng/mL; translucent bars). β-actin is shown as internal standard. Representative findings are shown in panel A, the quantitative analysis of n=8 experiments is shown in panel B.
Online Figure V. Accumulation of fluorescence labeled EPC within the intraluminal thrombus.

EPC (1x10^6 cells in 200 µL) were stimulated with 10 ng/mL recombinant human leptin (EPC+L10) or PBS (EPC) for 24 hours, labeled with CM-Dil and injected intravenously into nude mice within 10 min after induction of arterial injury. In addition, a subset of mice received recombinant human leptin (0.1 µg/g body weight; EPC+leptin i.v.) by intraperitoneal injection 30 min before the induction of arterial thrombosis. Injured vascular segments were removed 24 hours later and analyzed for the presence of CM-Dil-positive EPC (red signal) using fluorescence microscopy. Representative findings are shown in panel A, the quantitative analysis of findings n=3 mice per group is shown in panel B. *P<0.05; **P<0.01 vs. control.
Online Figure VI

Analysis of murine neointimal lesions (upper row) using Bandeiraea simplicifolia lectin staining (BSL, arrows) confirmed murine endothelium (arrows), whereas human endothelial cells identified by Ulex europeaus lectin staining (UEA) could not be detected. Human carotid artery lesions (lower row) were used as negative control for murine BSL and as positive control for human UEA staining. Magnification, 200x.
Online Figure VII. VCAM expression on vascular lesions.
VCAM-1 expression was examined on uninjured arteries as well as neointimal lesions 3 wks after induction of vascular injury using immunofluorescence. Uninjured arteries were found to be VCAM-1 negative, whereas VCAM-1 expression was markedly upregulated after 3 wks (arrows). Magnification, 200x (left) and 400x (right).