Leptin-Dependent and Leptin-Independent Paracrine Effects of Perivascular Adipose Tissue on Neointima Formation

Marco R. Schroeter, Norman Eschholz, Sebastian Herzberg, Isabel Jerchel, Maren Leifheit-Nestler, Frauke S. Czepluch, Georgios Chalikias, Stavros Konstantinides, Katrin Schäfer

Objective—Clinical and experimental evidence suggests that periadventitial adipose tissue may modulate vascular lesion formation. The aim of this study was to determine the role of perivascular leptin expression on neointima formation and to differentiate it from local inflammation and systemically elevated leptin levels.

Approach and Results—Increased neointima formation after carotid artery injury was observed in hyperleptinemic, diet-induced obese wild-type mice, but not in leptin-deficient ob/ob mice. High-fat diet was associated with increased leptin expression in visceral adipose tissue (VAT) as well as in perivascular adipose tissue. Perivascular leptin overexpression achieved by adenoviral vectors enhanced intimal cell proliferation and neointima formation in wild-type mice, but not in leptin receptor–deficient mice. Perivascular transplantation of VAT from high-fat diet–induced obese wild-type mice around the carotid artery of immunodeficient mice also promoted neointima formation, without affecting body weight or systemic leptin levels, and this effect was absent, if VAT from ob/ob mice was used. On the contrary, perivascular transplantation of VAT from ob/ob mice fed high-fat diet, characterized by marked immune cell accumulation, promoted neointimal hyperplasia also in the absence of leptin. In vitro, recombinant leptin and VAT-conditioned medium increased human arterial smooth muscle cell proliferation in a (partly) leptin-dependent manner.

Conclusions—Our findings suggest that locally elevated leptin levels may promote neointima formation, independent of obesity and systemic hyperleptinemia, but also underline the importance of perivascular inflammation in mediating the increased cardiovascular risk in obesity. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: adipokines ■ inflammation ■ leptin ■ neointima formation ■ obesity

Obesity is associated with an elevated risk and incidence of atherothrombotic complications, including myocardial infarction and stroke.1 In addition to comorbidities, such as diabetes mellitus or hypertension,2 cytokines, growth factors, or other mediators released from the adipose tissue have been implicated in the pathogenesis of cardiovascular disease in obesity.3 For example, circulating leptin levels are elevated in obese humans,4 and clinical studies suggest an association between the presence of hyperleptinemia and the incidence of coronary heart disease or risk of myocardial infarction.5,6 Furthermore, plasma leptin levels correlated with the intima-media thickness at the common carotid artery,7 coronary artery calcification,8 or in-stent restenosis in patients undergoing coronary revascularization.9

The leptin receptor is expressed in human and murine atherosclerotic lesions,10 and the interaction of leptin with its receptor has been shown to enhance smooth muscle cell (SMC) proliferation,11,12 angiogenesis,13 platelet aggregation,14 or immune cell activation.15 Moreover, we and others could show that mice lacking leptin (ob/ob) or its receptor (db/db) are protected from neointima formation in response to vascular injury, whereas exogenous leptin administration increased experimental lesion size in a receptor-specific manner,12,16 suggesting that leptin may represent a direct link between obesity and cardiovascular disease.

Recent clinical and experimental evidence points toward a direct, causal association between perivascular adipose tissue (PVAT) accumulation and vascular lesion formation. For example, studies in humans observed significant correlations between the amount of pericardial fat and coronary atherosclerosis or prevalent myocardial infarction, independent of conventional measures of adiposity or systemic cardiovascular risk factors.17,18 Using a mouse model of endovascular injury, Takaoka et al19 could show that removal of periadventitial adipose tissue enhances neointima formation, and that transplantation of subcutaneous adipose tissue from lean, but not diet-induced obese, mice protects against vascular remodeling. In this regard, obesity was associated with a loss of anticontractile properties of PVAT20 as well as increased inflammation21 and chemokine production.22 However, little is
known about the importance of specific adipokines expressed in the PVAT, and how they may locally affect vascular remodeling processes in obesity. Interestingly, previous studies showed markedly increased leptin levels in the PVAT of obese mice or patients with coronary atherosclerosis.

Based on these previous findings, experiments were performed in vivo and in vitro to determine the importance of perivascular leptin expression on neointimal lesion formation and to differentiate it from systemically high-leptin levels as well as perivascular inflammation in obesity.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Obesity and Systemic Hyperleptinemia Enhance Neointima Hyperplasia**

To examine the effects of obesity and hyperleptinemia on neointima formation, vascular injury was induced in wild-type (WT) mice, fed either NC or 45% high-fat diet (HFD). As shown in Table 1, body weight (P<0.0001) and circulating leptin levels (P=0.002) were significantly elevated in mice fed HFD compared with those on NC. Moreover, neointima area (Figure IA in the online-only Data Supplement), intima-to-media ratio (Figure IB in the online-only Data Supplement), and luminal stenosis (Figure IC in the online-only Data Supplement) were significantly increased in HFD-induced obese mice compared with lean mice (representative findings are shown in Figure IE in the online-only Data Supplement). On the contrary, no significant changes in neointima formation were observed in leptin-deficient obese ob/ob mice compared with lean WT mice, suggesting that leptin is involved in mediating the vascular effects of increased body weight.

**Obesity Is Associated With Elevated Perivascular Leptin Expression**

In addition to elevated visceral fat and circulating leptin levels, recent studies suggested that increased body weight is associated with enhanced perivascular leptin expression. In agreement with those previous findings, WT mice fed HFD for 8 weeks (body weight, 28±2.3 g; visceral adipose tissue [VAT] weight, 1.8±0.1 g; serum leptin levels, 29±3.9 ng/mL) exhibited significantly increased levels of leptin mRNA (Figure 1A) and protein expression (Figure 1B) compared with WT mice fed NC (body weight, 24±1.3 g; VAT weight, 0.5±0.03 g; serum leptin levels, 1.7±0.2 ng/mL), both in the VAT and PVAT. Representative findings after immunohistochemical detection of leptin in either fat depot are shown in C.

**Perivascular Overexpression of Leptin Enhances Neointima Formation in the Absence of Obesity and Elevated Circulating Leptin Levels**

To further explore the role of perivascular leptin (over)expression on neointima formation, adenoviral vector (AdV) containing leptin cDNA were applied around the carotid artery after vascular injury. Mice after perivascular application of pluronic gel containing control cDNA or an equal volume of sterile PBS or after subcutaneous injection of AdV-leptin in pluronic gel were used as negative controls. The perivascular gene expression on day 21 after vascular injury was first assessed using GFP-containing AdV (Figure IIA in the online-only Data Supplement). Of note, no specific fluorescence signal could be detected in liver or spleen (Figure IIB in the online-only Data Supplement), whereas cells coexpressing GFP and markers of SMC, macrophages or adipocytes, respectively, could be observed (Figure IIC in the online-only Data Supplement). Moreover, immunohistochemistry revealed elevated levels of (peri-)vascular leptin expression in AdV-leptin–treated carotid arteries compared with those treated with pluronic gel or AdV-null (Figure IID in the online-only Data Supplement). Importantly, serum leptin levels were similar in mice treated locally with either AdV-leptin, AdV-null, or pluronic gel alone (Table 2). On the contrary, morphometric analysis revealed a significant increase in the luminal stenosis (P<0.05) and intima-to-media ratio (P<0.05) in mice treated with AdV-leptin compared with pluronic gel alone or AdV-null (Figure 2A and 2B, representative findings shown in Figure 2C). Moreover, findings were found to be specific, because perivascular application of AdV-leptin did not enhance neointima formation in leptin receptor–deficient db/db mice (P<0.01 and P<0.05 versus AdV-leptin–treated WT mice). Further analysis revealed increased number of PCNA-immunopositive, proliferating cells in the neointima (Figure 2D and, to a lesser extent, also the media (Figure 2E) of WT mice treated with AdV-leptin compared with AdV-null (representative findings shown in Figure 2F).

**Perivascular Transplantation of Obese VAT Promotes Neointima Formation**

In addition to leptin overexpression, obesity is associated with systemic as well as local, that is, perivascular inflammation, which has been shown to affect vascular remodeling. To examine the effects of perivascular adipose tissue accumulation, independent of systemic obesity, neointima formation was quantified after induction of vascular injury followed by transplantation of VAT, derived from WT mice fed either NC (body weight, 23.5±0.3 g) or HFD for 8 weeks (body weight, 31.2±0.6 g), around the carotid artery of immunodeficient nu/nu mice. Similar to a previous study, VAT was chosen for transplantation, because the amount of endogenous perivascular fat at the carotid artery was not sufficient for heterologous transplantation. The morphology and local integration of the donor VAT into the perivascular space of the host are shown in Figure III in the online-only Data Supplement, using VAT from GFP reporter gene tg mice. Although VAT transplantation did not affect body weight or

**Table 1. Body Weight and Serum Leptin Levels at Tissue Harvest**

<table>
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<th>WT</th>
<th>WT+HFD</th>
<th>ob/ob</th>
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<tr>
<td>n</td>
<td>19</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>26±0.6</td>
<td>32±1.2</td>
<td>55±0.9</td>
</tr>
<tr>
<td>Serum leptin, ng/mL</td>
<td>5.6±1.2</td>
<td>26±6.0</td>
<td>0.0±0.0</td>
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HFD indicates high-fat diet; ob/ob, obese, leptin–deficient mice; and WT, wild-type mice.
systemic leptin levels of the hosts (Table 3); transplantation of VAT obtained from HFD-fed WT mice significantly increased luminal stenosis (Figure 3A) and intima-to-media ratio (Figure 3B) compared with *nu/nu* mice transplanted with VAT from lean WT mice (*P*<0.05) or *nu/nu* mice, injured but not being transplanted (*P*<0.05), indicating local effects of VAT accumulation (representative findings are shown in Figure 3C). Importantly, PVAT transplantation failed to enhance neointima formation if VAT from obese NC-fed, leptin-deficient ob/ob mice (body weight, 47.5±1.5 g) was used. On the contrary, mice perivascularly transplanted with VAT from ob/ob mice placed on HFD for 8 weeks to further enhance obesity (body weight, 64.6±1.4 g) showed a similar increase of neointima formation (*P*=0.05 versus VAT-Tx ob/ob NC using Student *t* test) as their HFD-fed WT counterparts, suggesting that other factors in addition to leptin are also involved in mediating the effects of VAT on intimal hyperplasia in the presence of extreme obesity.

**HFD Induces VAT Inflammation in Leptin-Deficient Mice, but Not in WT Mice**

To further investigate the mechanisms underlying the observed leptin-independent paracrine effects of VAT from HFD-fed ob/ob mice on neointima formation, the immune cell content of VAT obtained from WT and ob/ob mice fed either NC or HFD for 8 weeks was examined using flow cytometry. These analyses revealed that ob/ob+HFD mice exhibited a marked VAT inflammation, as shown by significantly elevated numbers of CD11b+ F4/80+ monocytes/macrophages (Figure 4A), Ly6C+, CD11b+ inflammatory monocytes (Figure 4B), CD11c+, F4/80+ dendritic cells (Figure 4C), and CD4+ (Figure 4D) or CD8+ (Figure 4E) lymphocytes. On the contrary, similar and low immune cell numbers were observed in the VAT of WT mice fed NC or HFD for 8 weeks or that of ob/ob mice on NC.

**VAT Promotes SMC Proliferation in a Leptin-Dependent Manner**

To further investigate the paracrine effects of VAT and the role of leptin therein, we examined the proliferation of SMCs in response to leptin or conditioned medium (CdM) of VAT obtained from WT mice or ob/ob mice fed either NC or HFD. As shown in Figure 5A, recombinant human leptin (100 ng/mL) significantly increased SMC proliferation compared with vehicle (*P*<0.01), and this effect could be abolished using leptin-neutralizing antibodies (*P*<0.001). The effect of CdM of VAT obtained from WT mice fed NC or HFD on SMC proliferation could also be reduced, at least in part, using leptin-neutralizing antibodies (*P*<0.001; Figure 5B). On the contrary, proliferation of SMC incubated with VAT-CdM from leptin-deficient ob/ob mice exhibited a reduced proliferation compared with VAT-CdM from WT mice (*P*=0.055), which was not affected by the addition of leptin-neutralizing antibodies.

**Discussion**

The main finding of this study is that perivascular overexpression of leptin, achieved by transplantation of VAT from diet-induced obese mice or leptin cDNA-containing adenoviral

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**Table 2.** Body Weight and Serum Leptin Levels at Tissue Harvest

<table>
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<tr>
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<th>WT</th>
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<th>WT</th>
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<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>28±1.0</td>
<td>26±0.6</td>
<td>26±0.8</td>
</tr>
<tr>
<td>Serum leptin, ng/mL</td>
<td>4.1±0.6</td>
<td>2.8±0.6</td>
<td>4.1±0.6</td>
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*AdV indicates adenoviral vector; and WT, wild-type mice.*
vectors, promotes neointima formation in a leptin receptor–specific manner and independent of body weight and systemic leptin levels. Moreover, leptin-mediated paracrine effects on SMC proliferation could be identified as 1 possible mechanism underlying the neointimal hyperplasia–promoting effects of obese VAT. On the contrary, our findings also underline the importance of PVAT inflammation, which was found to promote neointima lesion formation, despite the absence of leptin.

Clinical trials and observational studies indicate that obesity is associated with an increased risk and incidence of cardiovascular disease.1 Leptin is primarily expressed in adipocytes, and obesity is frequently accompanied by hyperleptinemia.4 Findings that elevated circulating leptin levels correlate with the incidence of cardiovascular events,6 or the severity of coronary atherosclerosis8 suggested that leptin may be causally involved in the development of cardiovascular complications in obesity. In this regard, it could be previously shown12 and confirmed in this study that HFD-induced obesity promotes intimal hyperplasia after vascular injury, and this effect was absent in obese, leptin–deficient ob/ob mice. Moreover, systemic administration of leptin was found to promote the formation of neointimal and atherosclerotic lesions,12,16 suggesting...
that the enhanced vascular remodeling in obesity is mediated by elevated circulating levels of this adipokine. The increase in adipose tissue mass in obesity is not limited to visceral fat, but extends to adipocyte depots surrounding blood vessels or within the pericardium. Studies in obese subjects demonstrated that pericardial or periaortal adipose tissues are enlarged in obesity and not only correlate with measures of adiposity, but also metabolic risk factors frequently associated with increased body weight.23 The association of pericardial fat thickness or volume with coronary artery disease was found not dependent on the presence of obesity or systemic risk factors,17,18 suggesting that proinflammatory and vasoactive mediators released from PVAT depots directly modulate the phenotype or function of cells within the neighboring vessel wall.

The majority of studies on the pathogenesis of atherosclerosis have so far focused on processes within the intima and media, whereas alterations in the outer vessel wall layers have been less intensively examined. It has been reported already several years ago that resection of the adventitia results in severe endothelial damage and intimal hyperplasia in rats,26 and that periaventricular myofibroblasts contribute to the neointima formation in response to endoluminal vascular injury.27 More recently, it was shown that the increase in neointima size after removal of the adventitia can be prevented by perivascular transplantation of subcutaneous adipose tissue obtained from lean mice, but not obese mice or transplantation of VAT.19 Based on these and other26 findings, it has been suggested that PVAT possesses vasculoprotective effects, which are diminished or lost in obesity, possibly because of increased inflammation, cytokine production, and oxidative stress. Importantly, the perivascular changes were found to occur independent of body weight or circulating biomarkers,28 suggesting that PVAT functions as paracrine organ transducing vasoregulatory, metabolic, and atherogenic signals to neighboring blood vessels.

Numerous vasoactive mediators are known to be secreted from adipocytes.3 Comparative analyses of different fat depots revealed an elevated perivascular expression of several proinflammatory and proatherogenic cytokines in hypercholesterolemic or diet-induced obese mice or humans,21,22,28 including interleukin-1β, interleukin 6, interleukin 8, or MCP-1, whereas the perivascular expression of adiponectin was found to be reduced.23,29 Moreover, perivascular, but not systemic, administration of adiponectin was shown to reduce the neointimal hyperplasia observed in adiponectin-deficient mice.19 Regarding the adipokine leptin, feeding WT mice HFD for 2 weeks was associated with a dramatic increase in the perivascular leptin expression,23 similar to our findings after feeding mice HFD for 8 weeks. Moreover, patients with coronary heart disease were reported to exhibit higher leptin levels within the epicardial adipose tissue compared with healthy control subjects,24 and human atherosclerosis was found to correlate with periaortic expression of leptin.30 Importantly and supporting the hypothesis that local, that is, adventitial overexpression of leptin contributes to the elevated cardiovascular risk associated with obesity, adenovirus-mediated perivascular overexpression of leptin was sufficient to enhance neointima formation, also in the absence of systemic hyperleptinemia or obesity. Previous studies have found that the coronary endothelial dysfunction induced by PVAT from pigs with metabolic syndrome could be reversed by inhibition of leptin signaling.31 Similar findings were observed after perivascular transplantation of VAT from HFD-induced obese WT mice, shown to overexpress leptin, although various other vasoactive substances may have influenced the vascular remodeling in addition to leptin. Microvascular networks connecting the PVAT with vascular lesions have been found to promote atherosclerotic plaque growth,32 and it remains to be shown whether perivascular leptin may accelerate lesion growth via enhancing local angiogenesis.33

The leptin receptor is expressed on cells present within the vessel wall, including endothelial cells, SMCs, or monocytes/macrophages.10 SMCs constitute a major component of vascular lesions, promoting lesion growth through proliferation, migration, or extracellular matrix production, and previous
studies have shown that leptin promotes SMC proliferation.\textsuperscript{11,12} In this regard, immunohistological analyses detected increased numbers of proliferating cells in neointimal lesions after AdV-mediated perivascular leptin overexpression. Moreover, studies in rats revealed that CdM from periaortic adipose tissue stimulates SMC proliferation in a leptin receptor–specific manner.\textsuperscript{34} In agreement with these findings, in vitro experiments detected paracrine effects of VAT-derived CdM on SMC proliferation, which seemed to depend, at least in part, on the presence of leptin, as shown by neutralizing antibodies or VAT-CdM obtained from leptin-deficient \textit{ob/ob} mice. On the contrary, we did not detect differences between CdM obtained from lean mice or HFD-induced obese WT mice regarding its paracrine activities on SMC proliferation, probably because of the presence of multiple factors expressed and secreted from adipose tissue. For example, others have shown visfatin, highly expressed in PVAT, to possess paracrine effects on SMC proliferation.\textsuperscript{35} Other factors expressed in PVAT and known to promote SMC proliferation include ROS, angiotensin II, or TNF-\textit{α}.

Supporting the importance of leptin in mediating the paracrine effects of obesity on the vascular wall, VAT obtained from

Figure 4. Quantification of inflammatory cells within the visceral adipose tissue (VAT) of lean and obese mice. VAT was harvested after feeding mice NC or high-fat diet (HFD) for 8 weeks, digested with collagenase, and immediately analyzed using flow cytometry. Obesity was associated with marked VAT inflammation in HFD–fed \textit{ob/ob} mice. Summarized findings after analysis of \textit{n}=5 to 8 mice per group as well as representative dot plots are shown. \textbf{A}, CD11b\textsuperscript{+}, F4/80\textsuperscript{+}; \textbf{B}, Ly6C\textsuperscript{+}, F4/80\textsuperscript{+}; \textbf{C}, CD11c\textsuperscript{+}, F4/80\textsuperscript{+}; \textbf{D}, CD4\textsuperscript{+}; and \textbf{E}, CD8\textsuperscript{+} cells; respectively. * \textit{P}<0.05 and ** \textit{P}<0.01 vs NC-fed mice; *** \textit{P}<0.01 vs Wild-type (WT) + HFD mice. Representative dot blots are shown in \textbf{F–J}. 
feeding WT mice HFD for 8 weeks was found not to be associated with inflammatory cell accumulation, despite the strong induction of leptin expression. Of note, flow cytometry analysis of VAT from WT mice fed HFD for longer time periods, that is, 5 months, resulting in adiposity resembling that of ob/ob mice, revealed a similar degree of VAT inflammation (not shown). In addition, leptin may affect the activity and function of immune cells. Among other, leptin has been shown to activate monocyte/macrophages,13 and leptin (receptor)–deficient atherosclerotic mice were found to exhibit a reduced T cell helper type 1 response and improved regulatory T cell function.40

Taken together, we identified leptin, expressed at increased levels in the PVAT during diet-induced obesity, as 1 important factor involved in mediating the paracrine effects of obesity and PVAT accumulation on the vascular response to injury. On the contrary, our findings also underline the importance of perivascular immune cell accumulation in promoting neointima formation, and these effects did not depend on the presence of leptin. Overall, our findings highlight the importance of local (ie, perivascular) alterations for the increased cardiovascular risk associated with obesity.

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Disclosures
None.

References

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Figure 5. Effects of leptin and visceral adipose tissue (VAT) conditioned medium on smooth muscle cell (SMC) proliferation. A, Addition of recombinant human leptin (100 ng/mL for 48 hours) to serum-free medium (SFM) significantly increased SMC proliferation compared with vehicle, and this effect could be abolished using leptin-neutralizing antibodies (α-Ob). **P<0.01 and ***P<0.001 vs vehicle and #/#P<0.001 vs leptin. B, The effect of conditioned medium (CdM) from VAT on SMC proliferation was reduced using α-Ob in wild-type (WT) and WT + high-fat diet (HFD), but not ob/ob mice. **P<0.01 vs CdM alone.

leptin-deficient ob/ob mice, exhibiting a similar adiposity as WT-HFD mice, failed to enhance neointimal hyperplasia. On the contrary, VAT obtained from ob/ob mice fed HFD increased neointima formation in the recipients, suggesting that leptin expression is not a prerequisite to develop vascular complications in obesity, if additional factors are present. In this regard, obesity is known to be associated with a higher expression of proinflammatory mediators9 and enhanced adipose tissue infiltration with immune cells,37 both of which may contribute to the PVAT dysfunction in obesity. Regarding the latter, a strong accumulation of immune cells was observed after feeding ob/ob mice HFD for 8 weeks, possibly explaining the observed paracrine effects of their VAT on neointima formation despite the absence of leptin. Previous studies have shown that the severity of endothelial dysfunction correlates with the perivascular macrophage and T lymphocyte infiltration.38 The perivascular overexpression of chemotactic cytokines may itself promote the recruitment of inflammatory cells, and PVAT-CdM was shown to induce the migration of peripheral blood leukocytes22 or monocyteic cells.29 The increase in adipocytokine expression was found to occur before changes in macrophage or T cell accumulation, suggesting that factors released from hypertrophied adipocytes are involved in the initiation of adipose inflammation.39 Our finding of a more pronounced VAT inflammation in HFD-fed ob/ob mice suggests that leptin is not critically involved in the recruitment of immune cells to obese adipose tissue, although our flow cytometry analyses may not have detected differences in specific immune cell subpopulations, such as NK or ancestral γδ T cells.39
A. Colombo A. Association of insulin resistance, hyperleptinemia, and impaired nitric oxide release with in-stent restenosis in patients undergoing coronary stenting. 


J Clin Endocrinol Metab. 2003;88:5163–5168.


36. Fain IN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipokine tissue matrix, and adipokines from visceral and subcutaneous abdominal adipose tissues of obese humans. 


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Materials and Methods

Experimental Animals
Male C57BL/6J wildtype (WT) mice and mice deficient for leptin (C57BL/6J-Lep\textsuperscript{ob}; \textit{ob}/\textit{ob}) or the leptin-receptor (C57BL/6J-\textit{m} \textit{+/+} Lep\textsuperscript{db}; \textit{db}/\textit{db}) were from Jackson Laboratories. Mice were fed normal rodent chow (NC) until the age of 8 wks and then either switched to high fat diet (HFD; 45% of calories from fat; D12451 Research Diets Inc.) or maintained on NC until tissue harvest.

Vascular Injury Model
Mice were anesthetized by intraperitoneal injection of 2% xylazine (CP-Pharma; 6 mg/kg body weight [BW]) and 10% ketamine hydrochloride (Pharmanovo; 100 mg/kg BW) and subjected to carotid artery injury using 10% ferric chloride (Fe\textsubscript{3}Cl\textsubscript{3}), as described.\textsuperscript{1} The carotid artery was carefully separated from the accompanying nerve and vein and any adventitial tissue, which might prevent diffusion of the ferric chloride solution, was removed. Because high fat diet-induced obesity was not associated with a significant accumulation of endogenous fat around the common carotid artery, specific preparations did not differ between hosts fed NC or HFD.

Advenoviral Vector Preparation
Adenoviral vectors (AdV) containing human leptin (AdV-leptin) or control (AdV-null) cDNA were diluted in sterile PBS, and 10 \(\mu\)L (corresponding to 1x10\textsuperscript{6} PFU per artery) were thoroughly mixed with 50 \(\mu\)L pluronic gel-F127 (Sigma; final concentration, 25%) and kept on ice until being equally applied around the injured carotid artery segment or injected subcutaneously immediately after vascular injury.
All animal experimental procedures were approved by local authorities and complied with the national and institutional guidelines for the care and use of laboratory animals.

**Perivascular Transplanatation of Visceral Adipose Tissue**

Visceral (i.e. perigonadal) adipose tissue (VAT) was harvested from WT, *ob/ob* or Green Fluorescent Protein (GFP)-transgenic (tg) mice, weighed, rinsed in sterile 0.9% normal saline and stored on ice before being carefully placed (approx. 50 mg per mouse) around the left carotid artery of immunodeficient nude mice (NMRI-Fox1^nu; nu/nu) directly after induction of vascular injury.

**Serum Analysis**

Blood was obtained at the time of tissue harvest by cardiac puncture. Serum was prepared by centrifugation at 3,000 rpm for 10 min. Supernatants were stored at -80°C pending analysis of leptin levels using enzyme-linked immunoassays (R&D Systems).

**Morphometric Quantification of Neointima Formation**

Three weeks after vascular injury, mice were anesthetized and perfusion-fixed with 4% zinc formalin through the left ventricle. The injured carotid artery together with the bifurcation were harvested and embedded in paraffin. Neointima formation was quantified on Verhoeff’s Elastica-stained (VES) serial cross sections using image analysis software (Image-Pro Plus; Media Cybernetics). Five sections equally spaced through the injured arterial segment (at 200 µm-intervals) were evaluated and results averaged for each animal.

**Immunohistochemistry**

Leptin was visualized using a polyclonal goat anti-mouse antibody (R&D Systems). Proliferating cells were detected using a biotinylated mouse anti-proliferating cell nuclear
antigen (PCNA) antibody (Zymed). The number of PCNA-positive cells was manually count
and expressed as percentage of the total cell nuclei within the neointima or media, respectively.

**Immunofluorescence**

Cryosections through the carotid artery were examined for the presence of lymphocytes, endothelial cells, macrophages, adipocytes, smooth muscle cells or fibroblasts using monoclonal rat or rabbit anti-mouse CD3 (Santa Cruz Biotechnologies), CD31 (Santa Cruz Biotechnologies), F4/80 (Serotec) or perilipin (Cell Signaling Technology) antibodies or polyclonal rabbit anti-mouse \( \alpha \)-smooth muscle actin (abcam) or FSP1 (Dako Cytomation) antibodies, respectively, followed by incubation with phycoerythrin (PE)-labeled goat anti-rat or rabbit secondary antibodies (Invitrogen) and 4',6-diamidin-2-phenylindol (DAPI; Dako) as nuclear counterstain. GFP was detected using a polyclonal goat antibody (Novus Biologicals), followed by incubation with PE-labeled secondary antibodies.

**Flow Cytometry**

Adipose tissue was minced, digested in collagenase A (from clostridium histolyticum; Roche) and then lyzed (FACS\textsuperscript{TM} Lysing Solution, BD Bioscience), washed and resuspended in PBS/0.5% bovine serum albumine (BSA). Immunolabeling was performed by incubating 100 \( \mu \)L cell suspensions together with 1 \( \mu \)L of either fluorescein isothiocyanate (FITC) or Peridinin Chlorophyll Protein Complex/Cyanin 5.5 (PerCP/Cy5.5) fluorescence-conjugated antibodies against CD4, CD8, CD11b, CD11c, F4/80, or Ly-6C (please see suppl. Table I) for 45 min at 4°C. Samples were examined by BD FACS CantoII and analyzed using BD FACS Diva software.

**RNA Isolation and Reverse Transcription Polymerase Chain Reaction**
Total adipose tissue RNA was extracted using TRI Reagent (Ambion), and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out to assess leptin mRNA expression using published primer sequences. RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and quantified by measuring the relative pixel of the target gene band and normalizing it to 18S.

**Preparation of Adipose Tissue Conditioned Medium**

Conditioned medium (CdM) was prepared by incubating VAT (500 mg) for 4 hrs at 37°C in 1 mL serum-free Dulbecco’s Modified Eagle’s medium (DMEM) containing 0.5% BSA, 50 U/mL penicillin and 50 µg/mL streptomycin. The supernatant was collected, centrifuged at 5,000 rpm for 5 min at 4°C to remove cell debris and stored at -80°C.

**Analysis of Cell Proliferation**

Human arterial smooth muscle cells (HASMCs; Lonza) were maintained in SMC growth medium (Lonza) supplemented with 5% fetal calf serum, 0.5 ng/mL Epidermal Growth Factor, 2.0 ng/mL basic Fibroblast Growth Factor, 5 g/mL insulin, and gentamicin/amphotericin B. Cells (3.2×10^4 cells/mL) were seeded in 96-well plates and serum-starved (0.2% FCS) for 72 hrs, before being incubated with either recombinant human leptin (R&D Systems) or VAT-CdM for additional 48 hrs. Specificity experiments were carried out using neutralizing antibodies against leptin (anti-Ob; 100-fold molar excess; R&D Systems) or non-specific IgG controls. Cell proliferation was evaluated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).

**Statistical Analysis**

Quantitative data are presented as mean±standard error of the mean (SEM). Normal data distribution was tested using the D’Agostino & Pearson omnibus normality test. Equality of
variances were tested using Bartlett’s or Levene’s test, depending on whether samples were normally distributed or not. When 2 groups were compared, differences were tested using Student’s t test for unpaired means, if samples were normally distributed, or Mann Whitney test, if not. When three or more groups were compared, ANOVA was employed, if samples were normally distributed, or Kruskal-Wallis test, if not. Regarding post-hoc comparisons, Bonferroni’s multiple comparison test was used, if the assumption of equality of variances was satisfied. If variances differed significantly, ANOVA was followed by Dunnet’s C post-hoc test and Kruskal-Wallis by Dunn’s post-hoc test. Statistical significance was assumed if P reached a value <0.05. Analyses were performed using GraphPad PRISM data analysis software (version 4.01; GraphPad Software Inc.) or SPSS Statistics (version 20.0; IBM Corporation).

References


**SUPPLEMENTAL MATERIAL**

**Supplemental Table I. Antibodies used for flow cytometry**

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Supp. Figure I. Effect of obesity and systemic hyperleptinemia on neointima formation.

Morphometric analysis 3 wks after induction of carotid artery injury revealed that the (A) neointima area, (B) intima-to-media ratio and (C) luminal stenosis were significantly increased in HFD-induced obese (n=9) compared to lean (n=19) WT mice (*P<0.05), whereas the intimal hyperplasia in leptin-deficient ob/ob mice (n=10) did not differ from lean WT mice and was significantly decreased compared to obese WT mice (#P<0.05). (D) Of note, the mean external elastic lamina (EEL) length was similar in all groups. (E) Representative Verhoeff’s elastica-stained cross sections through neointimal lesions are shown.
Supplemental Figure II.

A

AdV-GFP

pluronic gel only

GFP DAPI

GFP DAPI

50 µm

50 µm

AdV-GFP

AdV-GFP

α-GFP GFP DAPI

w/o 1st Ab GFP DAPI

50 µm

50 µm

B

spleen

liver

spleen

liver

50 µm

50 µm
Supplemental Figure II (continued).

C

PVAT

GFP CD31 DAPI 50 μm

PVAT

GFP SMA DAPI 50 μm

PVAT

GFP FSP1 DAPI 50 μm

PVAT

GFP Perilipin DAPI 50 μm

PVAT

GFP F4/80 DAPI 50 μm

PVAT

GFP CD3 DAPI 50 μm
Suppl. Figure II. Perivascular Application of Adenoviral Vectors. (A) Experiments using GFP cDNA-containing adenoviral vectors (AdV) were performed to visualize the gene expression within the (peri-)vascular tissue (green signal; compare left and right panels as well as inserts), both on native sections (upper row) as well as after staining with polyclonal antibodies against GFP (lower panels; red signal). (B) No specific GFP signal was detected in spleen or liver. (C) Immunofluorescence staining for CD31, SMA, FSP1, perilipin, F4/80 or CD3 (red signal) was employed to determine GFP-AdV expression (green signal) in endothelial cells, smooth muscle cells, fibroblasts, adipocytes, macrophages or lymphocytes, respectively (broken arrows indicate GFP-positive, continuous arrows double-positive cells). (D) Leptin immunohistochemistry was performed on carotid artery sections treated with either pluronic gel, AdV-null or AdV-leptin. Results after omission (w/o) of the first (1st) antibody (Ab) are also shown.
Supplemental Figure III.

Suppl. Figure III. Perivascular Transplantation of Visceral Adipose Tissue. (A) Visceral adipose tissue (VAT) obtained from Green Fluorescent Protein (GFP) reporter gene-transgenic (tg) mice was transplanted around the carotid artery of immunodeficient \( \text{nu/nu} \) mice following induction of vascular injury (upper panel). Three wks later, frozen cross sections were stained with DAPI to visualize cell nuclei and examined under the fluorescence microscope. VAT from GFP tg mice is shown as positive (left panel, lower row), VAT from a WT mouse as negative control (right panel, lower row). (B) Sections after perivascular VAT transplantation (VAT-T\( _x \)) were stained with oil red-O to confirm the presence of fat vacuoles (left panel). VAT was used as positive control (right panel).