Leptin receptor is elevated in carotid plaques from neurologically symptomatic patients and positively correlated with augmented macrophage density

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Background: Carotid artery lesions from symptomatic patients are characterized by inflammation and neovascularization. The adipokine leptin promotes angiogenesis and activates inflammatory cells, and the leptin receptor (ob gene-encoded receptor), ObR, is expressed in advanced atherosclerotic lesions. The present study quantitatively analyzed ObR messenger RNA (mRNA) expression and immunoreactivity in carotid artery plaques from symptomatic and asymptomatic persons. Plaque angiogenesis, gene expression of vascular endothelial growth factor (VEGF), and macrophage density were also analyzed.

Methods: Carotid endarterectomy specimens were collected from 26 patients undergoing surgery for hemispheric cerebrovascular symptoms (n = 13) or progressive asymptomatic internal carotid stenosis (n = 13). A representative sample, including part of the most active site, was collected from each lesion and evaluated by real-time polymerase chain reaction analysis for ObRlong and ObRcommon isoforms, VEGF165, and macrophage adhesion molecule-1 (Mac-1) mRNA, and by immunohistochemistry for ObR, von Willebrand factor (vWF), and CD68 antigen expression.

Results: All plaques exhibited advanced atherosclerosis (American Heart Association class IV through VI). Transcript levels were preferentially elevated in symptomatic plaques for ObRlong (P = .0006) and ObRcommon (P = .033), with a simultaneous upregulation of VEGF165 (P = .001) and Mac-1 mRNA expression (P = .003). Immunohistochemical analysis confirmed a significant increase of ObR antigen levels (P = .011) and CD68-positive inflammatory cells (P = .049) in symptomatic plaques, whereas neovascularization, evident in all plaques, was similar in both groups (P = .7).

Conclusion: The ObRlong and ObRcommon genes are upregulated and their protein preferentially synthesized in clinically symptomatic carotid plaques. Moreover, ObR expression is positively correlated with augmentation of gene transcripts related to macrophage density and neovascularization. These data suggest that ObRlong and ObRcommon may be linked with histologic features of carotid plaque instability, which are associated with cerebral ischemic symptoms. (J Vasc Surg 2008;48:1146–55.)

Vulnerable plaques affecting the cervical carotid artery frequently undergo rupture, leading to cerebral embolic events that present clinically as hemispheric transient ischemic attacks (TIAs) or ischemic stroke.1,2 Rupture-prone atherosclerotic plaques typically harbor a sizable necrotic core close to the lumen, with a thin fibrous cap separating the core material from the blood stream.3,4 In addition, clinically symptomatic carotid occlusive disease has been associated with angiogenesis and macrophage infiltration characteristic of complex, unstable plaques.5,6

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Leptin, the product of the ob gene, is primarily synthesized by adipocytes, and its plasma levels correlate with adipose tissue mass.7 This hormone is involved in multiple pathophysiologic processes linking obesity, insulin resistance, inflammation, hematopoiesis, and angiogenesis.8 In fact, clinical studies identified leptin as an independent risk factor for coronary heart disease.9,10 Its systemic levels correlate with those of C-reactive protein (CRP) and may be useful in predicting future coronary events.11 Moreover, hyperleptinemia has been associated with in-stent restenosis after percutaneous transluminal coronary angioplasty, suggesting involvement of the hormone in the remodeling process.12 Finally, plasma leptin levels correlate with increased carotid intima-media thickness, an independent predictor of cerebrovascular occlusive disease and future stroke,13 implying linkage with atherosclerosis.14

Leptin is assumed to exert its effects on the cardiovascular system through interaction with the leptin receptor (ObR) expressed on the surface of hematopoietic and vascular cells.15 Tissue-specific availability of ObR may therefore have a significant effect on leptin-mediated processes. Atherosclerotic plaques, and especially advanced carotid lesions, demonstrate abundance of ObR antigen. In fact, ObR immunoreactivity has been identified in macro-

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phases, endothelial cells, and to a lesser degree in vascular smooth muscle cells (SMCs).\textsuperscript{16,17} Although clinical and experimental studies have implicated ObR in atherogenesis, to our knowledge, no quantitative data on its expression in human atherosclerotic lesions have been reported so far, and its potential role in plaque progression and instability has not been explored. In the present study, we tested the hypothesis that increased ObR gene expression correlates with clinical and histologic features of plaque vulnerability.

**METHODS**

**Study population.** Atherosclerotic plaques were collected from 26 consecutive patients undergoing carotid endarterectomy for extracranial high-grade internal carotid artery (ICA) stenosis (\textgreater;70\% luminal narrowing for symptomatic patients). The study included surgical specimens from 13 symptomatic patients (defined according to the North American Symptomatic Carotid Endarterectomy Trial [NASCET] classification)\textsuperscript{18} presenting with cerebrovascular TIAs (n = 10) or stroke (n = 3) within the last 3 months. All other specimens were collected from 13 clinically asymptomatic patients exhibiting progressive ipsilateral ICA stenosis of \textgreater;80\% on serial Doppler examinations. Normal carotid arteries (n = 3) were obtained from organ donors and served as controls for polymerase chain reaction (PCR) analyses.

The degree of ICA stenosis was established in two consecutive Doppler examinations before surgery in all cases. It was additionally confirmed by computed tomography angiography (CTA) in 15 cases, including 12 patients initially evaluated at the department of neurology, and three for whom CTA was used to verify the accurate degree of ICA stenosis, to resolve ambiguous duplex results.

All patients were evaluated neurologically before and after surgery by a senior neurologist and by a member of the surgical team. Three patients with a history or clinical symptoms of stroke underwent a brain CT scan and were operated on \textlesss than or equal to 1 to 3 months after the cerebrovascular event. Ten symptomatic patients presenting with TIAs underwent surgery \textlesss than or equal to 4 weeks. The study was approved by the local ethics committee, and written informed consent was obtained from all patients.

**Tissue collection.** All plaques were assessed macroscopically for signs of luminal ulceration, intraplaque hemorrhage, and the presence of a soft or ruptured necrotic core. After the plaques were washed in normal saline solution, a representative segment was sampled from each specimen and immediately snap-frozen for future RNA analysis. Each of those representative samples included an 8- to 10-mm-long proximal plaque segment that extended from the bifurcation into the ICA. The remaining specimen, including at least 2 to 4 mm of the proximal ICA plaque, was rapidly fixed in 4\% formalin for 8 to 12 hours and then embedded in paraffin blocks. Great care was taken to prevent plaque disintegration during division of the unfixed specimens. All samples contained part of the most active site of the plaque, identified macroscopically as displaying the most advanced atherosclerotic process, frequently including part of the necrotic core.

**RNA and complimentary DNA preparation.** Plaque samples, kept frozen in liquid nitrogen, were pulverized by crushing over dry ice, followed by homogenization (Polytron PT-2100) in TRIZOL solution (Invitrogen, Carlsbad, Calif) and RNA preparation. RNA concentration was determined by spectrophotometry. Complimentary DNA (cDNA) was synthesized using random hexamer (Promega, Southampton, United Kingdom) with SUPERSCRIPT II RNase H-negative reverse transcriptase (Gibco BRL, Paisley, United Kingdom). The cDNA prepared from the total RNA was used in real-time PCR analysis for ObR\textsubscript{long}, ObR\textsubscript{common}, VEGF\textsubscript{165}, and macrophage antigen complex 1 (Mac-1) mRNA.

**Quantitative real-time PCR.** Quantitative real-time PCR was performed on cDNA prepared from total RNA isolated from vascular samples. Preordered assay-on-demand kits (Applied Biosystems, Foster City, Calif) were used according to the manufacturer’s protocol, using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Different assay kits were used for each detected gene: leptin receptor variant 1 (LEP-R or ObR\textsubscript{long}; catalog number: HS00174492), leptin receptor common (Mac-1) (HS00174497), Mac-1 (HS00167304), and VEGF\textsubscript{165} (HS009000057). A Universal PCR Master Mix (Applied Biosystems) was used. Values were normalized to the reference gene Abl (Abelson). Samples from normal donor carotid arteries were used to establish the baseline for each gene. Those were used as the internal controls to which all samples obtained from symptomatic and asymptomatic patients were compared, as well as to the external reference gene Abl.

Normalization of the results could be obtained by comparative Ct (\Delta\DeltaCt) analysis, which gave the arbitrary score of 1.0 to the control. Comparing patients’ samples with this value resulted in fold-increase or fold-decrease of the expression of each detected gene in each sample. The nominal values for each gene in the control samples, including the Abl, represented the actual transcript quantity, and were always in the range of 10,000 copies \pm 10\% (about cycle 25.5).

The fluorescent probe used was 5’-Fam-CTGGC-CCAA-CATGGGC-GGA-BHQ-3’. The primers used were forward 5’-TGGAGATAACACTCTAAGCATCAA-AGG-3’ and reverse 5’-GATGTATGCTTGGGAG-C-CCA-3’. Results were analyzed using the SDS 2.1 (Applied Biosystems) and Excel (Microsoft Corp, Redmond, Wash) computer programs.

**Histology.** All plaques were examined histologically after hematoxylin and eosin and Movat’s pentachrome staining and then classified according to the AHA criteria, as defined by Stary and modified by Virmani.\textsuperscript{5,19} The classification of advanced class IV through VI lesions relies primarily on the status of the fibrous cap according to the size and proximity of the necrotic core to the luminal surface. These criteria reliably reflect the potential of the plaque to progress and generate major vascular complica-
tions. The presence of intraluminal iron, indicative of past intraplaque hemorrhage, was visualized using Perl’s iron stain.

**Immunohistochemistry.** Immunohistochemical analyses were performed on 5-μm-thick slides prepared from paraffin blocks. All plaque specimens (except one asymptomatic) were examined by immunohistochemistry for the presence of von Willebrand factor (vWF), ObR, and CD68 antigen.

For visualization of intraplaque microvessels, a polyclonal rabbit antihuman vWF antibody (DakoCytomation, Hanburg, Germany) was used, 1:50 diluted in buffer containing background-reducing components (Dako). Fluorescein isothiocyanate (FITC)-conjugated goat antirabbit immunoglobulin (Ig) G (1:50 dilution; Zytomeded, Berlin, Germany) was used as secondary antibody. Nuclei were visualized by fluorescence using 4',6-diamidino-2-phenylindole (DAPI) counterstaining (Vectorshield; Vector Laboratories, Burlingame, Calif).

Detection of the leptin receptor antigen was performed as described previously. According to the manufacturer’s specifications, the antibody recognizes both the long and common isoforms of the leptin receptor. Macrophages were detected using a mouse monoclonal anti-human CD68 antibody (dilution 1:100; Dako), followed by a biotinylated goat antimouse secondary antibody (dilution 1:200; Zytomed) and amino-ethyl-carbazole (Zytomed) as color substrate. Sections were briefly counterstained with Gill’s hematoxylin (Thermo Shandon, Pittsburgh, Penn) and mounted in VectaMount (Vector) before inspection by Olympus BX51 microscope (Olympus, Melville, NY).

**Quantitative analysis of lesion neovascularization, macrophage density, and ObR immunoreactivity.** Blood vessels were identified by the presence of circular structures lined with cells staining positive for vWF and corresponding cell nuclei demonstrated by DAPI counterstaining. Two independent investigators, blinded to the origin of the plaque, graded the presence of blood vessels by viewing five sections per patient and at least 10 different fields per slide (×1000 original magnification). The following scoring system was used: 0, no blood vessels; 1, less than two blood vessels per microscopic field in <50% of microscopic fields; 2, more than two blood vessels per microscopic field in <50% of microscopic fields; 3, more than two blood vessels per microscopic field in at ≥50% of microscopic fields.

ObR and CD68 immunoreactivity was assessed in three randomly chosen microscope fields (×100 original magnification) per tissue section per patient and quantified by measuring the immunopositive area and the number of immunopositive signals using Image-Pro Plus 4.1 software. (Media Cybernetics, Silver Spring, Md).

**Determination of plasma parameters.** Plasma samples were collected from all patients 2 hours before carotid surgery and stored at –70°C. Samples were assayed for the biomarkers leptin, soluble leptin receptor, CRP, and interleukin (IL)-6.

Leptin was determined using radioimmunoassay kits (Human Leptin RIA kit, Linco Research Inc, St Charles, Mo). The sensitivity of the leptin assay was 0.5 μg/L, and the interassay coefficient of variation was 3% to 6%. Human soluble leptin receptor was determined using solid-phase enzyme linked immunosorbsent assay (Quantikine, Human Leptin Soluble Receptor kit, R & D Systems, Minneapolis, Minn). The sensitivity of the assay was 0.06 μg/L, and the interassay coefficient of variation was 5% to 9%, and the manufacturer’s normal range was 13.1-55.4 μg/L. CRP was determined by a standard immunological assay, using Olympus autoanalyzer (AU 2700, with a normal value of <3 mg/L). IL-6 was measured by chemiluminescent immunometric method (Immulite, Diagnostic Products Corp, Los Angeles, Calif). The sensitivity of the assay was 2 pg/mL, and the interassay coefficient of variation was 5% to 10%. Expected values for healthy individuals are <5.9 pg/mL, at the 95th percentile, as specified by the manufacturer.

**Statistical analysis.** For statistical analysis of differences in mRNA transcript levels, for ObRlong, ObRcommon, VEGF165, and Mac-1, nonparametric statistical methods were used to compare plaque samples from symptomatic and asymptomatic patients due to the small sample size. Mann-Whitney test or Wilcoxon rank test for unpaired population was applied. To correlate transcript levels for ObRlong with VEGF165 and Mac-1, the Pearson coefficient method was used. For the comparative assessment of angiogenesis in symptomatic and asymptomatic specimens, the Wilcoxon rank test was used. ObR and CD68-immunopositive signals were compared using the t test for unpaired means. To assess differences in categoric variables (eg, clinical variables in Tables I and II), the Fisher exact test was used. SAS 9.1 software (SAS Institute, Inc, NC) was used for all statistical analysis. All tests applied were two-tailed, and a value of P ≤ .05 was considered statistically significant.

**RESULTS**

Baseline demographics and clinical risk factors, including diabetes mellitus, dyslipidemia, prevalence of arterial hypertension, and elevated body mass index (>25 kg/m²) were similar in both patient groups (Table I). Although cigarette smoking appeared to be more frequent among symptomatic patients (7 of 13 vs 3 of 13), the difference was not statistically significant. Histopathologic analysis of all specimens revealed advanced or complicated atherosclerotic lesions (ie, types IV through VI) in a similar proportion of symptomatic and asymptomatic patients (Table II).

**Leptin receptor gene expression and immunoreactivity.** A significant elevation in the mean ObRlong mRNA expression was found in plaques from symptomatic compared with asymptomatic patients (90.6 ± 25.8 vs 5.12 ± 1.9 arbitrary units [AU], respectively; P = .0006; Fig 1, column 1). Similarly, analysis of mRNA expression of the ObRcommon isoform revealed significant differences between the two groups (84.49 ± 31 vs 56.9 ± 82 AU; P = .033; Fig 1, column 2). Immunohistochemical
analysis confirmed a significantly increased total ObR immunopositive area in symptomatic compared with asymptomatic lesions (1098 ± 143 × 10^3/μm² vs 591 ± 109 × 10^3/μm²; P = .011). Representative findings are shown in Fig 2, A-D. In agreement with these findings, a significant increase was observed in the number of ObR immunosignals per microscope field (540 ± 93.7 vs 873 ± 111; P = .033).

**VEGF and Mac-1 mRNA expression, neovascularization and macrophage density.** We analyzed the samples by real-time PCR for VEGF165 transcript as an indicator of active angiogenesis. Mac-1 mRNA expression was also measured as a marker of macrophage density. Both analyses demonstrated a highly significant increase in mRNA levels in the symptomatic plaques. Mean VEGF165 transcript levels were 586.2 ± 166 AU in symptomatic patients vs 36.4 ± 11 AU in asymptomatic patients (P = .001; Fig 1, column 3), and mean Mac-1 mRNA was 330.8 ± 80 AU in symptomatic vs 20.5 ± 8 AU in asymptomatic patients (P = .003; Fig 1, column 4). High correlation was found between ObRlong and VEGF165 mRNA (r = 0.98, P < .0001), and with Mac-1 mRNA (r = 0.86, P < .0001), respectively.

Morphologic analysis revealed abundant neovascularization in plaques from either symptomatic or asymptomatic patients (Fig 3). Differences in plaque vascularity at the histologic level were assessed by two independent investigators, blinded to the plaque origin. Both examiners'
scores matched perfectly in 71% of the cases, whereas 29% of specimen scores differed by one grade level. When observers’ grading differed, the average grade was used as the quantitative estimate of the presence of blood vessels in the specimens. Although symptomatic specimens scored a slightly higher average neovascular blood vessel count (1.7 vs 1.5), the differences were insignificant ($P = .7$). Neovascularization was located primarily in the subintima, interfacing the necrotic core, and at the shoulder area. There were also vessels within the necrotic core and in the adventitia (not shown).

To test for differences in the presence of intraplaque inflammatory cells, we assessed the number of CD68-positive cells (Fig 4). Inflammatory cells were most frequently found surrounding cholesterol clefts (Fig 4, B) or intrallesional microvessels (not shown). We found a significantly increased CD68-immunopositive area in symptomatic vs asymptomatic lesions ($1080 \pm 348 \times 10^3 / \mu m^2$ vs $348 \times 10^3 / \mu m^2$).

Fig 2. Immunohistochemical detection of the leptin receptor in symptomatic and asymptomatic carotid plaques. Expression of the leptin receptor was analyzed on paraffin sections through atherosclerotic lesions at the carotid bifurcation. Representative examples of findings in lesions from (A and B) 13 symptomatic patients and (C and D) 12 asymptomatic are shown. Arrows point to ObR-positive cells (red-brown signal). Inserts highlight differences in the signal intensity of ObR-positive cells lining the luminal border (compare A and C) or within the medial portion of the vessel (compare B and D). Scale bars, 25 μm. E, Quantitative analysis of the ObR-immunopositive area is presented with the standard error (error bars). The statistical significance after comparison of both groups using the t test is indicated in the graph.
Plasma levels of soluble leptin receptor, leptin, CRP, and IL-6. Mean levels of soluble leptin receptor, and of leptin, CRP, and IL-6 assessed in plasma samples from symptomatic and asymptomatic patients revealed no significant differences for any of the variables (Table III). Plasma levels of soluble leptin receptor were within the normal range. Mean CRP plasma levels were moderately and equally elevated above the normal range in both groups, and mean IL-6 levels were within the normal range. These results suggest that over-synthesis of ObR, demonstrated locally in symptomatic carotid plaques, did not correlate with systemic plasma levels of leptin soluble receptor as assessed in those patients.

DISCUSSION

The current study tested the hypothesis that ObR expression is more prevalent in carotid plaques from clinically
symptomatic patients and positively correlates with histologic features of unstable atherosclerotic plaques. We found that Ob\textsubscript{R\_long} and Ob\textsubscript{R\_common} isoforms were significantly upregulated in symptomatic carotid plaques, correlating with a simultaneous upregulation of \textit{VEGF}_{165} and \textit{Mac-1} mRNA levels. Moreover, symptomatic lesions contained significantly higher amounts of ObR protein and CD68-positive macrophages, suggesting a link between ObR expression and features of plaque inflammatory components.

In corroboration with previous reports\textsuperscript{2,20}, histopathologic analysis of specimens from symptomatic and asymptomatic patients revealed similar proportions of advanced or complicated atherosclerotic lesions. Our findings suggest that modulation of ObR gene expression is related to specific circumstances selectively affecting symptomatic plaques.

Quantitative analysis demonstrated that ObR was significantly more abundant in symptomatic atherosclerotic plaques, both at the mRNA and the protein level available.
Recent clinical studies demonstrated abundant mature ObR protein in advanced atherosclerotic lesions.\textsuperscript{16,17} Elevated levels of ObR protein observed in symptomatic plaques could be due to increased density of cells that normally express the receptor. No statistically valid correlation was demonstrated between CD68 and ObR signal quantitation (data not shown), and unfortunately, we could not colocalize both antigens because fixation conditions would not allow costaining. Therefore, our findings suggest that increased macrophage density could not be the sole source of the elevated ObR. As demonstrated in Fig 2 and in a recent publication,\textsuperscript{17} ObR antigen is present in both endothelial cells and SMCs, in addition to macrophages, in advanced carotid plaques. Regardless of the origin of ObR, it seems reasonable to assume that local availability and direct contact between leptin and its receptor may play a role in plaque progression.

Limitations of clinical and imaging (duplex, magnetic resonance imaging) criteria as guides to decisions in carotid disease lend importance to the search for diagnostic modalities and biomarkers of the vulnerable carotid plaque.\textsuperscript{28,29} Such biomarkers are expected to reflect a systemic inflammatory state. This is based on the observation that multiple vulnerable plaques frequently coincide, thereby underlying the current concept of “the vulnerable patient.”\textsuperscript{30} In accordance with previous clinical reports,\textsuperscript{31} however, we found that neither plasma CRP nor IL-6 nor plasma leptin or soluble leptin receptor levels indicated a state of systemic vulnerability in clinically symptomatic carotid patients. Overexpression of ObR in the plaque may therefore represent a local indicator of elevated risk, independent of systemic plasma biomarkers.

Experimental data in mice have shown that recombinant leptin promotes arterial thrombosis\textsuperscript{52} and atherosclerosis progression.\textsuperscript{33} Exogenous leptin administration aggravated the response to experimental vascular injury and resulted in enhanced neointimal formation and lumen narrowing.\textsuperscript{22} However, no such effect has been recorded in vessels from leptin receptor-deficient mice. Furthermore, it has been demonstrated that defective leptin/leptin receptor pathway signaling protects mice from atherosclerosis through regulation of the immune response.\textsuperscript{34} Collectively, these data imply that although leptin is readily available systemically, its thrombogenic and proatherogenic properties can be expressed in vivo only in the presence of intact leptin receptor at the target organ. Furthermore, we have performed preliminary analyses of leptin protein in a new series of carotid plaques that reveal substantial antigen levels in the range of 0.1- to 2.0-ng/mg protein for specimens from both symptomatic and asymptomatic patients (unpublished data). These results corroborate available data regarding leptin protein in coronary artery advanced atherosclerotic plaques,\textsuperscript{35} thus supporting the assumption that leptin is involved in carotid atherogenesis.

**CONCLUSIONS**

We have demonstrated that $\text{ObR}_{\text{long}}$ and $\text{ObR}_{\text{common}}$ transcripts are significantly upregulated in carotid plaques...
of symptomatic patients. The simultaneous increase in VEGF	extsubscript{165} and Mac-1 gene expression within the plaque suggests a link between elevated leptin receptor levels and increased inflammation and stimulus for angiogenesis. Abundant ObR antigen and increased macrophage density could contribute to plaque instability and may eventually translate into clinical symptoms. Large-scale simultaneous genetic and clinical studies are warranted to further substantiate the potential linkage between leptin receptor, enhanced atherosclerosis, and plaque vulnerability in the carotid artery and other vascular beds. Further investigation of ObR in conjunction with imaging techniques may support the concept of the receptor as a novel local biomarker for plaque and patient vulnerability.

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**AUTHOR CONTRIBUTIONS**

Conception and design: JS, KS
Analysis and interpretation: JS, AS, MS, MF, SK, KS
Data collection: JS, AS, KS
Writing the article: JS, KS
Critical revision of the article: JS, AS, MS, MF, SK, KS
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