Is Leptin Associated With Diabetic Retinopathy?

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OBJECTIVE — In advanced stages of diabetic retinopathy, new blood vessels are formed based on undefined mechanisms. Recently, leptin was shown to possess an angiogenic action in vitro and to induce neovascularization in vivo. The aim of the present study was to investigate the relationship between plasma leptin levels and the severity of diabetic retinopathy.

RESEARCH DESIGN AND METHODS — There were 70 patients with type 2 diabetes (age 47.9 ± 9.7 years, BMI 26.4 ± 3.3 kg/m²) who were seen in a retina outpatient clinic and assigned to subgroups according to the stage of their diabetic retinopathy. There were 66 healthy volunteer subjects matched with the diabetic patients for age, BMI, and sex who served as control subjects (age 46.0 ± 8.8 years, BMI 27.1 ± 2.3 kg/m²). Fasting plasma leptin levels were measured.

RESULTS — Plasma leptin level of the diabetic patients was not significantly different from the control subjects. In patients with proliferative diabetic retinopathy (n = 17), the mean plasma level of leptin (16.1 ± 9.2 ng/ml) was significantly higher than that in patients with nonproliferative retinopathy (n = 20) (11.5 ± 3.5 ng/ml, P = 0.039) or patients without retinopathy (n = 33) (5.8 ± 3.7 ng/ml, P = 0.001). The mean leptin level in patients with nonproliferative diabetic retinopathy was also significantly higher than that in patients without retinopathy (P = 0.002).

CONCLUSIONS — Our results show that the more advanced the diabetic retinopathy, the higher the plasma leptin levels, even after adjusting the leptin levels for BMI. The presence of such a positive correlation need not imply a causal relationship. Nevertheless, previously observed leptin-induced promotion of angiogenesis and neovascularization lends support to the possibility that leptin may play a role in the progression of human diabetic retinopathy to a proliferative phase. This possibility deserves further investigation.

Diabetes Care 23:371-376, 2000

In type 1 diabetes as well as type 2 diabetes, there is a highly significant positive correlation between metabolic dysregulation and development of microangiopathic complications, including retinopathy (1). Not all patients with poor control of diabetes over long periods of time develop retinopathy, however, particularly the advanced proliferative form. Some studies of familial clustering suggest that a genetic component is involved in the susceptibility to both diabetic nephropathy and diabetic retinopathy (DR) (2). Other cofactors are likely to be involved.

Leptin is a 167-amino acid protein transcribed from the ob gene, which was originally cloned from the adipose tissue of mice and human subjects (3). It plays an important role in the regulation of food intake, energy expenditure, and body weight. The gene is expressed in adipose tissue, gastric epithelium, and placenta (3–5). Plasma leptin levels correlate with body fat content; plasma leptin is elevated in obesity (6) and decreased in anorexia nervosa (7). A human leptin gene mutation associated with severe obesity has been reported in an English family (8). We have reported on a Turkish family with a missense mutation in the leptin gene, manifested as morbid obesity and hypogonadism, suggesting that leptin is involved in metabolic and hormonal events in addition to its effects on food intake and energy expenditure (9). Indeed, leptin has been shown to be involved in the regulation of the function of the pituitary, thyroid, adrenal cortex, and gonads (10–16).

In addition to its major involvement in metabolic and hormonal events, leptin also promotes cell proliferation. Leptin stimulates hematopoiesis in vitro (17,18). Proliferation of CD4+ T-cells is stimulated by leptin, which is associated with an increase in cytokine release (19). Pertinent to DR, recent findings show that leptin promotes vascular endothelial cell proliferation and angiogenesis in vitro (20,21). This effect of leptin can be confirmed in vivo in suitable models in the form of induction of neovascularization (20–21). These findings lead us to speculate that leptin may play a role in the progression of DR. Thus, the aim of the present study is to examine the possible relationship between the existence or stages of DR and serum leptin levels in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Study subjects and design
There were 70 patients with type 2 diabetes chosen from among patients seen in the retina division of our ophthalmology outpatient clinic. As a group, age and BMI (mean ± SD) were 47.9 ± 9.7 years and 26.4 ± 3.3 kg/m², respectively. The sex ratio was 23/47 (M/F). The duration of diabetes after diagnosis was 14.9 ± 5.9 months. All patients were being treated with sulfonylureas and were on no other medications. They were evaluated by standard physical
Leptin levels in diabetic retinopathy

Table 1—Clinical and laboratory features of the patient and control groups

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Control subjects</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>70</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>47.9 ± 9.7</td>
<td>46.0 ± 8.8</td>
<td>NS</td>
</tr>
<tr>
<td>M/F</td>
<td>23/47</td>
<td>26/40</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.4 ± 3.3</td>
<td>27.1 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130 ± 6.6</td>
<td>121 ± 7.1</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>81 ± 4.3</td>
<td>77 ± 5.5</td>
<td>NS</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>9.9 ± 6.1</td>
<td>9.5 ± 3.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

P values were determined by Student's t test.

examination, chest X-ray, baseline electrocardiogram, exercise electrocardiogram, two-dimensional echocardiography, and routine clinical laboratory tests, including liver and kidney function tests and 24-h urinary protein measurements. None of the patients had hypertension, nephropathy, depression, coronary heart disease, heart failure, or renal failure—entities that have been associated with abnormal leptin levels.

Each patient underwent ophthalmoscopic examination and fluoroangiography. The stage of retinopathy was graded in a masked fashion, in that only code numbers were made available to the graders. The patients were then stratified into subgroups according to the stage of DR. The subgroup without any DR included 33 patients (10 men and 23 women, mean age 49.6 ± 12.1 years, mean BMI 25.9 ± 3.2 kg/m²). The subgroup with nonproliferative diabetic retinopathy (NPDR) included 20 patients (7 men and 13 women, mean age 46.9 ± 7.1 years, mean BMI 26.5 ± 2.5 kg/m²). NPDR was diagnosed on the basis of one or more of the following findings: hard or soft exudates, intraretinal microvascular abnormalities, hemorrhages, microaneurysms, and venous beading in at least one eye. The subgroup with proliferative diabetic retinopathy (PDR) included 17 patients (6 men and 11 women, mean age 46.0 ± 9.7 years, mean BMI 27.2 ± 2.7 kg/m²). The diagnosis of PDR required the presence of one or more of the following abnormalities: new vessels elsewhere, fibrous proliferations elsewhere, and preretinal and/or vitreous hemorrhages in at least one eye.

There were 66 healthy volunteer subjects (18 men and 48 women) chosen to serve as the control group. They were matched with the diabetic patients according to blood pressure, age, BMI, and sex. Their age was 46.0 ± 8.8 years and BMI 27.1 ± 2.3 kg/m² (mean ± SD). The subjects underwent routine physical and laboratory evaluations to ensure that none had diabetes, hypertension, hyperlipidemia, or psychological, metabolic, hepatic, or renal disease. None of the healthy subjects had a family history of hypertension or diabetes.

All subjects gave informed consent for participating in the study. The study was approved by the local ethics committee of Gulhane School of Medicine. Subjects in both groups reported that their weight had been stable for at least 3 months preceding the entry into the study. Arterial blood pressures were measured in the right arm by mercurial sphygmomanometer three times in a resting condition in the morning, and mean values were calculated for systolic and diastolic pressures. All venous blood and urine samples were collected at 0800 after an overnight fast.

Biochemical assays
Serum levels of glucose (glucose oxidase-peroxidase calorimetric method) and creatinine (Jaffé method) were determined using a Tecnicon Dax-48 system analyzer (Miles, Tarrytown, NY). HbA₁c was measured by inhibition of latex agglutination, using a DCA 2000 analyzer (Bayer, Elkhart, IN). Microalbuminuria was detected by an immunoturbidimetric method with a Urinpak Micro Albinum immunokit (Miles, Tarrytown, NY).

Radioimmunoassay
Venous blood samples were collected in ethylenediamine tetraacetate-coated tubes. The tubes were promptly centrifuged; plasma was separated and stored at −70°C. Plasma leptin levels were measured in duplicate by radioimmunoassay (Human Leptin RIA Kit; Linco Research, St. Louis, MO). Assay sensitivity was 0.5 ng/ml. The intra-assay coefficient of variation at 4.9 ng/ml was 8.3% (n = 5) and at 25.6 ng/ml was 3.4% (n = 5). All plasma samples were run in a single assay.

Statistical analysis
All analyses were performed using version 9.0 of the computer-based statistical package of Statistical Product and Service Solutions (SPSS) (22). The data are reported as means ± SD. Student's t test was used to determine the significance of the differences between the parameters for the diabetic patients as a whole and for the healthy control subjects. Pearson's correlation test was used to analyze the relationship between variables. For comparison of the diabetic subgroups, the results were determined by one-way analysis of variance (ANOVA) and Tukey Honestly Significant Difference (HSD) test for post hoc comparison of means, automatically adjusted for unequal sample sizes by a harmonic mean. Leptin levels of the diabetic subgroups were also analyzed after adjusting for the BMI, using analysis of covariance (ANCOVA); also provided the significance of the differences between the subgroups. Receiver Operating Characteristic (ROC) curve analysis was used to estimate the cutoff value for leptin in the occurrence of DR. Differences and correlations were considered significant at P < 0.05.

RESULTS—Clinical and laboratory data of the diabetic patients and healthy control subjects are shown in Table 1. No significant differences in age, BMI, systolic and diastolic blood pressure, and plasma leptin levels were observed between the patient and control groups. As expected, female patients (12.3 ± 6.4 ng/ml) have higher plasma leptin levels than male patients (7.2 ± 6.6 ng/ml). Female control subjects (11.5 ± 6.1 ng/ml) also have higher plasma leptin levels than male control subjects (6.7 ± 3.5 ng/ml). No significant differences in plasma leptin levels were observed in both male and female groups when compared with control subjects of the same sex. Plasma leptin levels were strongly correlated with BMI in both diabetic patients (r = 0.43, P = 0.001) and healthy control subjects (r = 0.42, P = 0.001).

The age, sex, mean duration of diabetes, and levels of fasting blood glucose, HbA₁c, and serum creatinine were similar for the three patient subgroups stratified according to the stage of DR (Table 2). In these subgroups, there was no correlation between plasma leptin levels and fasting blood glucose, HbA₁c, or the duration of diabetes. Plasma leptin levels in patients with PDR (n = 17) (16.1 ± 9.2 ng/ml) were significantly higher than those in patients with NPDR (n = 20) (11.5 ± 3.5 ng/ml, P =
**Table 2— Characteristics of patients according to retinopathy grade**

<table>
<thead>
<tr>
<th></th>
<th>No DR</th>
<th>NPDR</th>
<th>PDR</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>33</td>
<td>20</td>
<td>17</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Age (years)</td>
<td>49.6 ± 12.1(^a)</td>
<td>46.9 ± 7.1(^b)</td>
<td>46.0 ± 9.7(^c)</td>
<td>0.9</td>
<td>0.410</td>
</tr>
<tr>
<td>M/F</td>
<td>10/23</td>
<td>7/13</td>
<td>6/11</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Duration of diabetes (months)</td>
<td>14.1 ± 5.1(^d)</td>
<td>16.8 ± 6.2(^e)</td>
<td>14.4 ± 7.2(^f)</td>
<td>1.4</td>
<td>0.247</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>25.9 ± 3.2(^g)</td>
<td>26.5 ± 2.5(^h)</td>
<td>27.2 ± 2.7(^i)</td>
<td>0.1</td>
<td>0.924</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>118.6 ± 8.1(^j)</td>
<td>120.1 ± 10.2(^k)</td>
<td>116.9 ± 11.1(^l)</td>
<td>0.6</td>
<td>0.532</td>
</tr>
<tr>
<td>Hemoglobin A(_1c) (%)</td>
<td>9.5 ± 2.4(^m)</td>
<td>9.4 ± 2.0(^n)</td>
<td>10.2 ± 1.6(^o)</td>
<td>0.6</td>
<td>0.525</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.9 ± 0.2(^p)</td>
<td>0.8 ± 0.1(^q)</td>
<td>0.9 ± 0.1(^r)</td>
<td>0.6</td>
<td>0.528</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>5.8 ± 3.7(^s)</td>
<td>11.5 ± 3.5(^t)</td>
<td>16.1 ± 9.2(^u)</td>
<td>20.3 &lt; 0.001*</td>
<td></td>
</tr>
<tr>
<td>Mean leptin (adjusted)</td>
<td>5.94</td>
<td>11.49</td>
<td>15.89</td>
<td>25.4* &lt; 0.001*</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) and P values were determined by one-way ANOVA. Post hoc Tukey’s HSD test: P > 0.05 for a vs. b, b vs. c, and a vs. c; P = 0.24 for d vs. e; P = 0.92 for d vs. f; P = 0.44 for e vs. f; P = 0.95 for g vs. h; P = 0.93 for g vs. i; P = 0.99 for h vs. i; P = 0.48 for j vs. k; P = 0.51 for j vs. l; P = 0.69 for k vs. l; P = 0.99 for m vs. n; P = 0.16 for m vs. o; P = 0.55 for n vs. o; P = 0.63 for p vs. r; P = 1.0 for p vs. s; P = 0.71 for r vs. s; P = 0.102 for t vs. u; P < 0.001 for u vs. v; P = 0.039 for u vs. v. Leptin mean values were adjusted for BMI by using ANCOVA. *Determined by ANCOVA.

Fasting plasma leptin levels of patients with NPDR were also significantly higher than those in patients without retinopathy (P = 0.002) (Fig. 1). There seemed to be a tendency of higher BMI levels among patients with more advanced retinopathy; however, there was no statistically significant difference in mean BMI (F = 0.1, P = 0.924). To ensure that the subgroup differences in leptin levels reported above were not skewed based on the influence of body weight on leptin levels, plasma leptin levels of DR subgroups were adjusted for BMI and determined by statistical analysis; the differences remained highly significant (F = 25.4, P < 0.001) (Table 2).

A threshold level of leptin for the existence of DR was estimated by ROC curve analysis (Fig. 3). The estimated threshold of plasma leptin for DR was 8.55 ng/ml (sensitivity 81.1% and specificity 80.8%, area under the curve = 0.875, P = 0.001).

**CONCLUSIONS** — The major finding in the present study is that fasting plasma levels of leptin are elevated in diabetic patients with retinopathy, proportionate to the severity of retinopathy. The design of the study rules out any interference by the diurnal variation in leptin levels, because all samples were collected at 0800 in the fasting state (23). There was a tendency, not reaching statistical significance, for body weight to be higher in patients with advanced retinopathy. In a previous study, overweight diabetic patients were reported to be at an increased risk of progression of DR (24). However, others have observed no such association (25,26). In our study, the significant positive relationship between the increases in leptin levels and progression of retinopathy cannot be attributed to body weight, because the relationship prevailed, after adjusting leptin levels according to BMI.

In recruiting the diabetic patients for this study, we were careful to ensure that all subgroups were similar not only in body weight, but also with respect to other factors known to contribute to the development of retinopathy, such as the level of glycosylated hemoglobin, time elapsed after diagnosis of diabetes, and coexistence of other significant health problems. Thus, the positive relationship between leptin levels and retinopathy observed in this study was not accompanied by any obvious difference among the retinopathy subgroups in factors promoting microangiopathy.

Even though there was a significant positive correlation between leptin levels and retinopathy, the mean plasma level of leptin for the entire group of diabetic patients irrespective of retinopathy was similar to the level in weight-matched nondiabetic control subjects. This finding corroborates findings in previous studies in humans (27,28) and animals (29,30). The apparent discrepancy can be explained readily on the basis of statistical scatter in a parameter regulated by multiple factors and emphasizes the value of stratifying a group in a manner suitable to test a hypothesis.

In hyperinsulinemic clamp studies, administration of insulin was shown to increase serum leptin levels (31). Such a relationship cannot explain our findings in this study, because none of the patients were being treated with insulin, and all patients, including those without retinopathy and normal leptin levels, were on a sulfonylurea drug.
The statistical cut point in the fasting plasma level of leptin for the occurrence of retinopathy of any degree was 8.55 ng/ml, a value slightly below the mean plus 1 SD (10.2 ng/ml) for the nondiabetic subjects. Thus, a predictive value cannot be assigned to leptin levels in relation to the development of DR. Very likely, such estimates would require much larger sample sizes in each diabetic subgroup.

The highest plasma levels of leptin were observed in patients with proliferative retinopathy. In this subgroup of patients, the mean plasma leptin level was 16.1 ng/ml. In the study of Bouloumie et al. (20), human umbilical venous endothelial cells were stimulated to proliferate maximally at a leptin concentration of 10 ng/ml. Endothelial proliferation is a component of PDR. Thus, the concentration of leptin reaching the vascular endothelial tissue in our patients was in an order of magnitude that can cause proliferation.

For a cell type to respond to leptin, leptin receptors must be present in the plasma membrane. Two main types of leptin receptors, also referred to as Ob receptors (Ob-R), have been identified (32). The type with a long intracellular domain (Ob-R L or Ob-R b) is the signaling form, eliciting protein phosphorylation. The type with a short intracellular domain (Ob-R S or Ob-R a) may possess a transport function, allowing leptin to traverse a capillary such as the blood-brain barrier. Both types of receptors are expressed in human endothelial cells (20,21,33). In the study of Bouloumie et al. (20) using human umbilical venous endothelial cells, leptin induced tyrosine phosphorylation of mitogenic protein kinases Erk-1 and Erk-2 at the concentration that evoked maximal cell proliferation and formation of capillary-type tubes. This phenomenon of in vitro angiogenesis was confirmed by Sierra-Honigmann et al. (21).

Bouloumie et al. (20) showed that stimulation of angiogenesis occurs also in adult endothelial cells derived from porcine aorta. Furthermore, the order of magnitude of stimulation of angiogenesis by leptin is similar to that induced by vascular endothelial growth factor (20,21). In considering the possibility that leptin may play a role in the pathogenesis of DR, one could either postulate a direct effect in terms of angiogenesis mediated by the Ob-R L receptors or an indirect effect on the surrounding retinal tissues mediated by the Ob-R S receptors. The former mechanism would be applicable to the proliferative phase of retinopathy, while the latter raises the possibility of involvement of leptin in stages of retinopathy preceding neovascularization.

The significance of leptin-induced in vitro proliferation of endothelial cells in terms of neovascularization has been documented in two different in vivo models: chick chorioallantoic membranes (20) and rat corneas (21). These effects of leptin observed by others add further credence to the possibility that the association of hyperleptinemia, which we observed in diabetic patients with retinopathy but not in patients without retinopathy, may participate in the development or progression of this complication.

**Figure 2**—Plasma leptin levels of patients with type 2 diabetes by severity of DR. Error bars indicate 95% confidence range of the subgroups.

**Figure 3**—ROC curve of plasma leptin levels for occurrence of DR. Arrow indicates threshold level. Estimated cutoff value of leptin is 8.55 ng/ml with 81.1% sensitivity and 80.8% specificity for existence of DR (area under curve = 0.875, P = 0.001).
In debating the possibility that hyperleptinemia may play a role in the evolution of DR, a question demanding an answer is “why have plasma leptin levels increased?” One may assume that this increase represents an increase in secretion rather than decreased clearance in these patients with normal kidney and liver functions. Because adipose tissue is the principal source of leptin, one may again assume that hyperleptinemia observed in association with adipose tissue may play a role in the pathogenesis of DR in humans.

Acknowledgments — We are grateful to Dr. Sumer Belbey Pek, Department of Endocrinology and Metabolism, University of Michigan, for critical review of the manuscript.

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