Elevation of Vitreous Leptin in Diabetic Retinopathy and Retinal Detachment

Ray F. Gariano, Anjali K. Nath, Donald J. D’Amico, Thomas Lee, and M. Rocio Sierra–Honigmann

PURPOSE. Leptin is a cytokine that regulates energy metabolism and is linked to diabetes mellitus through its metabolic actions. Leptin is angiogenic and promotes wound healing, and therefore this investigation was conducted to determine whether leptin is associated with neovascular and fibrotic complications of diabetes and other retinopathies.

METHODS. Serum and vitreous samples were collected from patients classified by the presence and type of diabetic retinopathy or other ocular diseases. Leptin was measured in serum and vitreous by radioimmunoassay, and leptin and leptin receptor were localized in epiretinal membranes immunohistochemically.

RESULTS. Leptin levels in serum and vitreous were higher in patients with diabetes than in those without, and vitreous leptin concentrations were especially elevated in patients with proliferative diabetic retinopathy or retinal detachment. Leptin and leptin receptor were detected in fibrovascular epiretinal membrane of patients with diabetes.

CONCLUSIONS. Leptin in human vitreous is elevated in proliferative diabetic retinopathy, and retinal detachment and is present in fibrovascular epiretinal tissue. These data suggest an involvement of leptin in retinal disease. (Invest Ophthalmol Vis Sci. 2000;41:3576–3581)

Leptin is a pleiotropic cytokine with circulating serum concentrations that are directly proportional to the size of the subcutaneous adipose mass. Leptin synthesis was initially localized within adipose tissue, where it is produced by adipocytes. It has since been detected in other tissues, including placenta, ovaries, mammary gland, gastric mucosa, and hepatic stellate cells. Since the discovery that the defective gene in certain genetically obese mice encodes leptin or its receptor, leptin has been proposed to function as an adipose-derived endocrine signal that acts through hypothalamic receptors to induce satiety and enhance lipid metabolism and energy expenditure.

Mice homozygous for defects in leptin or leptin receptor expression exhibit hyperglycemia, insulin resistance, and morbid obesity. In some patients with diabetes mellitus, serum levels of leptin are elevated, and leptin resistance has been implicated in the pathogenesis and treatment of this disease. Leptin modulates insulin production by human pancreatic cells, and serum insulin levels correlate with those of leptin in patients with diabetes and experimental animals. Leptin has also been proposed to play a role in insulin resistance.

Leptin has not, however, been previously associated with long-term complications of diabetes—in particular, diabetic retinopathy. In advanced diabetic retinopathy, retinal angiogenesis and growth of fibrotic tissue may result in vitreous hemorrhage and traction retinal detachment, respectively, which are the principal causes of severe vision loss in patients with diabetes.

We and others have reported that leptin is an angiogenic factor and that leptin is associated with wound healing. These findings led us to speculate that leptin participates in fibrovascular complications of diabetic retinopathy by direct proangiogenic effects within the eye. As an initial step to evaluate this hypothesis, this study assessed the presence of leptin in the human eye and whether ocular leptin levels correlate with angiogenic and fibrotic complications of diabetic and other retinopathies.

METHODS

All research involving human subjects adhered to the Declaration of Helsinki. Institutional review and approval were obtained, and informed consent was obtained from all patients enrolled in the study. Patients were characterized by age, gender, presence and type of diabetes, body mass index, and ocular diagnosis (Table 1). Those with type 1 diabetes had onset of diabetes before the age of 30 and were insulin dependent, and those with type 2 diabetes had onset after 30 and were initially insulin independent. The body mass index was calculated as the weight in kilograms divided by the square of the height in meters; body mass indexes of 18.5 to 24.9 are normal, and 25.0 to 29.9 and higher than 29.9 indicate overweight and obesity, respectively.

Based on ocular diagnoses, patients were subdivided into several categories. The proliferative retinopathy (PR) group included patients with proliferative diabetic retinopathy (i.e., retinal neovascular growth due to pathologic angiogenesis); the nonproliferative diabetic retinopathy group (NPDR) con-
### TABLE 1. Description of Patients

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<td>22.0</td>
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**Retinal Detachment**

- 26 | | 27 | F | 25.8 | UVE |
- 27 | | 37 | F | 16.5 | CMV |
- 28 | | 52 | M | 27.8 | TRAU |
- 29 | | 40 | M | 23.0 | ROP |
- 30 | | 49 | M | 25.0 | ROP |
- 31 | | 56 | M | NA | |
- 32 | | 34 | M | 28.8 | TRAU |
- 33 | | 49 | F | 21.0 | |
- 34 | | 14 | F | 19.6 | |
- 35 | | 83 | F | 25.0 | TRAU |
- 36 | | 74 | M | 27.2 | |
- 37 | | 25 | F | 22.5 | UVE |
- 38 | | 66 | M | 26.5 | |
- 39 | | 65 | M | 25.1 | |
- 40 | | 45 | M | 28.8 | |
- 41 | | 27 | M | 37.7 | |
- 42 | | 24 | M | 31.0 | GRT |
- 43 | | 67 | M | NA | |

**Proliferative Retinopathy**

- 44 | 1 | 49 | M | 33.1 | |
- 45 | 1 | 25 | M | 54.7 | |
- 46 | 1 | 57 | M | 22.9 | |
- 47 | 1 | 58 | F | 49.0 | |
- 48 | 1 | NA | M | NA | |
- 49 | 1 | 57 | M | 27.3 | |
- 50 | 1 | 57 | M | 28.0 | |
- 51 | 1 | 67 | M | 31.8 | |
- 52 | 1 | 53 | F | 27.1 | |
- 53 | 1 | 30 | F | 23.8 | |
- 54 | 1 | 47 | M | 27.5 | |
- 55 | 1 | 30 | F | 30.8 | |
- 56 | 1 | 57 | M | 23.0 | |
- 57 | 1 | 27 | M | 26.0 | |
- 58 | 1 | NA | M | NA | |
- 59 | 1 | 41 | M | 25.3 | |
- 60 | 2 | 45 | M | 31.1 | |
- 61 | 2 | 51 | M | 29.0 | |
- 62 | 2 | 65 | M | NA | |
- 63 | 2 | 75 | M | 22.2 | |
- 64 | 2 | 65 | M | 21.8 | |
- 65 | 2 | 41 | F | 40.1 | |
- 66 | 2 | 61 | M | 30.0 | |
- 67 | 2 | 58 | M | 30.7 | |
- 68 | 2 | 66 | F | 42.0 | |
- 69 | 2 | 61 | F | 32.0 | |
- 70 | 2 | 63 | F | 41.1 | |
- 71 | 2 | 70 | M | 25.2 | |
- 72 | | 62 | M | 29.2 | CRVO* |
- 73 | | 31 | M | 23.3 | PSR* |

BMI, body mass index; CH, choroidal hemorrhage; CMV, cytomegalovirus retinitis; CNV, choroidal neovascularization; CSME, clinically significant macular edema; DL, dislocated lens; ENDP, bacterial endophthalmitis; ERM, epiretinal membrane; GRT, giant retinal tear; MH, macular hole; RD, retinal detachment; ROP, retinopathy of prematurity; TRAU, trauma; UVE, uveitis; VH, vitreous hemorrhage.

* Nondiabetic proliferative retinopathy due to central retinal vein occlusions (CRVO) or proliferative sickle retinopathy (PSR).

Vitreous samples were obtained at the time of vitrectom via vitrectomy surgery by using a syringe attached to an automated vitrector, immediately placed on ice, and maintained in a freezer at −80°C until analysis. Blood samples were also obtained at the time of surgery, and the serum was separated by centrifuge and frozen at −80°C. Samples were stored less than 3 months before testing. In selected patients, preretinal or subretinal fibrovascular membranes were removed from the eye, fixed for 24 hours in formalin, embedded in paraffin, and sectioned at 4 μm.

Leptin concentrations in serum and vitreous were measured using a commercially available radioimmunoassay kit (Linco Research, St. Louis, MO), with a sensitivity range for human leptin from 0.05 ng/ml to 10 ng/ml. Serial dilutions were made in duplicate at 4, 8, and 16-fold for serum samples, so that one or more values were within the standard curve. Vitreous samples were tested undiluted and with 2-fold dilution.

Preretinal and subretinal fibrovascular proliferative tissue was obtained in three patients during vitrectomy surgery; these membranes were fixed in buffered formalin (Sigma) for 24 hours, paraffin embedded, and sectioned at 4 μm. Slides were treated with 1.5% hydrogen peroxide in phosphate-buffered saline (PBS; Gibco, Rockville, MD) to inactivate endogenous peroxidase and incubated with 10 mM sodium citrate (pH 6.0) at 95°C for 15 minutes. Sections were then incubated with either polyclonal goat anti-human leptin (OB) antibody at 1 μg/ml (R&D, Minneapolis, MN) or rabbit polyclonal antibodies directed against synthetic peptides based on the sequence of human transmembrane long-form leptin receptor, followed by incubation with a biotin-conjugated secondary antibody. The reaction product was developed with a streptavidin-horse radish peroxidase system (Vector, Burlingame, CA). Specificity of anti-leptin antibodies was assessed by preincubation of the primary antisera with 10 mM sodium citrate (pH 6.0) at 95°C. Immunolabeling was performed on computer (10Xtra statistical software; Minitab, State College, PA). Results are expressed as means ± SEM.

### RESULTS

**Description of the Patient Population**

Seventy-three patients were included in this study; details of patient subpopulations are given in Tables 1 and 2. In the PR group (n = 28), patients had proliferative retinopathy due to diabetes. In the NPDR group (n = 11) patients were operated on for primary or recurrent retinal detachment (n = 2), macular hole (n = 2), epiretinal membrane (n = 2), displaced crystalline lens (n = 2), macular edema (n = 2), and vitreous hemorrhage.
hemorrhage due to choroidal neovascular membrane (n = 1).

In the NPR group (n = 32), patients were operated on for primary or recurrent retinal detachment (n = 16), vitreous hemorrhage (n = 3), endophthalmitis (n = 3), dislocated crystalline lens (n = 2), macular hole (n = 2), submacular choroidal neovascular membrane (n = 2), epiretinal membrane (n = 2), and trauma (n = 2).

The body mass index was significantly higher in patients with type 2 diabetes (30.0 ± 1.7 kg/m²; n = 20) than in those without diabetes (25.1 ± 0.8; n = 34, P < 0.05) and was nonsignificantly higher in patients with type 2 disease than in those with type 1 (29.1 ± 1.1, n = 19).

### Leptin Concentrations in Human Serum and Vitreous

Serum leptin levels positively correlated with the body mass index in patients with type 2 diabetes (r = 0.6, P < 0.05, n = 20) and were higher in females than in males (females: 32.8 ± 6.8 ng/ml, n = 24; males: 11.8 ± 1.9, n = 49; P < 0.05), as described previously.28 Serum leptin levels were not related to age (r = −0.02, age range, 6–83 years, n = 73).

Serum leptin levels were highest in the PR group (25.2 ± 6.9 ng/ml), intermediate in the NPDR group (14.6 ± 4.1 ng/ml), and lowest in the NPR group (12.1 ± 1.3 ng/ml; PR versus NPR: P < 0.05; PR versus NPDR: P = 0.09; Table 2). Serum leptin levels higher than 60 ng/ml were seen only in the PR group. Mean serum leptin concentration was 11.1 ± 1.2 ng/ml in those without diabetes, 20.1 ± 4.9 ng/ml in patients with type 1 diabetes, and 25.6 ± 9.5 ng/ml in those with type 2 diabetes (P < 0.05, diabetic versus non-diabetic; P > 0.05, type 1 versus type 2 diabetes).

Vitreous leptin levels also were higher in the PR group than in the NPDR and NPR groups (PR: 5.7 ± 1.8 ng/ml; NPDR: 0.7 ± 0.3; NPR: 1.2 ± 0.3, P < 0.05; Table 2). Because vitreous leptin levels may in part reflect those in the serum, we calculated the ratio of vitreous to serum leptin concentration in each patient. The ratio was approximately twice as high in the PR group as in the NPDR and NPR groups (PR: 0.197, NPDR: 0.095, NPR: 0.125). Vitreous leptin concentrations were highest in patients with type 2 diabetes (6.2 ± 2.8 ng/ml), intermediate in those with type 1 (3.2 ± 0.8 ng/ml), and lowest in those without diabetes (1.4 ± 0.3 ng/ml; P < 0.05). Vitreous leptin levels were higher in females (5.7 ± 2.2 ng/ml) than males (1.9 ± 0.6 ng/ml); however, the ratios of vitreous to serum leptin concentration was similar in females (0.18) and males (0.16).

Two patients without diabetes who had proliferative retinopathy due to central retinal vein occlusion or proliferative sickle retinopathy exhibited a mean serum leptin (13.6 ng/ml) and vitreous leptin (6.1 ng/ml) concentrations comparable with those of patients in the PR group.

To assess the effect of retinal detachment on vitreous leptin level, we divided patients into a control group of all patients with neither proliferative retinopathy nor retinal detachment (n = 25) and a group of patients with retinal detachment but without proliferative diabetic retinopathy (n = 18; Table 2). In 15 of 18 eyes with retinal detachment, epiretinal fibrotic proliferation (proliferative vitreoretinopathy) was clinically evident. The retinal detachment subgroup had a significantly higher level of leptin in the vitreous than the control subgroup, whereas serum leptin levels were similar in the two groups (retinal detachment serum and vitreous leptin levels, respectively: 10.5 ± 1.6 ng/ml, 2.2 ± 0.5 ng/ml; control groups: 13.2 ± 2.2 ng/ml, 0.5 ± 0.01 ng/ml; Fig. 1).

Concentrations of leptin in vitreous and in serum correlated positively, for all patient subgroups (PR: r = 0.74; NPDR: r = 0.65; NPR: r = 0.34; retinal detachment: r = 0.74; control: r = 0.46; P < 0.05). As seen in Figure 2, the slope of the correlations was greater in the PR and detachment groups than in the control group, perhaps suggesting greater influx of leptin into the eye from serum in these patients.

To further evaluate the possibility that intravitreal leptin arises from the bloodstream, we subdivided patients with PR into those with vitreous hemorrhage (n = 19, including two patients without diabetes who had vitreous hemorrhage) and those without intraocular bleeding (n = 11; Table 2). Both serum and vitreous leptin levels were higher in the group with vitreous hemorrhage (serum: 28.5 ng/ml versus 14.9 ng/ml; vitreous: 6.5 ng/ml versus 3.6 ng/ml). However, these differences were not statistically significant (P > 0.2), and the ratio of vitreous to serum leptin concentration was comparable in patients with (0.23) and without (0.17) vitreous hemorrhage.

**Table 2. Description of Patient Subpopulations**

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<th>Vitreous*</th>
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BMI, body mass index; NPR, no proliferative retinopathy; NPDR, nonproliferative diabetic retinopathy; PR, proliferative retinopathy; CTL, non-proliferative retinopathy without retinal detachment; RD, non-proliferative retinopathy with retinal detachment; ND, non-diabetic; T1D, Type 1 diabetes; T2D, Type 2 diabetes; VH+, proliferative retinopathy and vitreous hemorrhage; VH−, proliferative retinopathy without vitreous hemorrhage.

* Micrograms per milliliter.
Proliferative fibrovascular tissue was removed during surgery from the preretinal \((n = 4)\) or subretinal space \((n = 1)\) in patients with proliferative diabetic retinopathy and traction retinal detachment. All preretinal tissues appeared clinically as fibrovascular membranes, and the subretinal specimen appeared fibrotic only. Antisera to leptin specifically bound to each of these specimens. Figure 3 shows the localization of leptin immunoreactivity in a subretinal (Fig. 3A) and a preretinal (Fig. 3B) membrane. Leptin was associated with both stromal and cellular components of the membranes, in a patchy distribution. Figure 3C shows absence of immunoreactivity in a negative control section, when anti-leptin antibodies were preincubated with an excess of exogenous recombinant leptin. Leptin receptor was also detected in these specimens, where it was primarily associated with cell bodies. The distribution of leptin receptor labeling was patchy (Figs. 3D, 3E, 3F).

**DISCUSSION**

Several endogenous growth factors and cytokines appear to participate in development of epiretinal angiogenesis and fibrosis, including vascular endothelial growth factor, fibroblast growth factors, and insulin-like growth factors.\(^{29-31}\) Criteria to implicate these factors in retinal disease have included their presence or upregulation in vitreous humor from patients with proliferative retinopathies and their detection in pathologic ocular tissue from these patients.\(^{29-33}\) After discoveries that leptin is angiogenic, we speculated that leptin contributes to the angiogenic complications of proliferative diabetic retinopathy.

Leptin was detected in human vitreous humor. Leptin concentrations in vitreous humor correlated with those in serum, suggesting that intravitreal leptin at least in part derives from the systemic circulation. The increase in vitreous leptin compared with the increase in serum leptin was greater in patients with proliferative retinopathy or retinal detachment, consistent with enhanced access of serum proteins to the vitreous cavity in these conditions, due to disruption of blood–ocular barriers.\(^{21,34}\) It is unclear whether leptin enters the eye by passive transfer or by an active permissive mechanism. The latter transport system is thought to facilitate passage of leptin from the bloodstream into the central nervous system, through the choroid plexus.\(^{35,36}\)

Alternatively, leptin may also be produced locally in the eye as a manifestation of retinal disease. Five observations support this suggestion. First, the ratio of vitreous to serum leptin concentrations was higher in patients with proliferative retinopathy than in control patients, even though control patients had ocular conditions (e.g., trauma, diabetic macular edema, uveitis) associated with enhanced permeability of ocular blood vessels.\(^{34,37}\) Second, vitreous leptin was higher in patients with retinal detachment than in those without detachment, even though serum leptin levels were comparable in both groups. Third, the presence of blood in the vitreous cavity did not result in a higher ratio of vitreous-to-serum leptin concentrations, as would be expected if the bloodstream were the sole source of intraocular leptin. Fourth, leptin and leptin receptor were present in epiretinal fibrovascular and fibrotic tissue from patients with diabetic retinopathy. Finally, human leptin gene expression appears to be enhanced by hypoxia,\(^{38}\) and retinal hypoxia is a consistent feature of proliferative retinopathies and retinal detachment.\(^{39}\)

Leptin receptor is expressed by vascular endothelial cells, and binding of leptin to the receptor stimulates tyrosine phosphorylation.\(^{23}\) Leptin enhances endothelial cell migration and promotes assembly of endothelial cells into tubes and capillary-vessel networks.
Therefore, intraocular leptin receptors and leptin (whether produced locally or arising from systemic circulation) may participate directly in neovascular ocular disease. The mean intravitreal concentration of leptin in PR eyes (5.7 ng/ml) is comparable to levels effective in promoting angiogenesis in in vitro assays. Furthermore, leptin concentrations within the retina or at the vitreoretinal interface may be greater if leptin is produced locally or diffuses into the vitreous chamber from the retinal or choroidal vascular beds. It is also possible that leptin potentiates other mediators of angiogenesis in vivo.

Elevated vitreous leptin concentrations in patients with retinal detachment may be explained by the fact that many of these patients had prior ocular surgery that may disturb the blood-ocular barriers. However, because the ratio of vitreous to serum leptin levels was elevated in patients with retinal detachment and because detached outer retina is ischemic, leptin may be produced in association with retinal hypoxia. In addition, because leptin promotes wound healing and is upregulated at sites of scar production, leptin may relate to preretinal scar tissue formation after retinal detachment. Thus, the presence of leptin and its receptor in fibrovascular membranes may reflect participation of leptin in angiogenic or fibrogenic processes or both. Preliminary studies have detected leptin in nondiabetic epiretinal scar tissue.

Serum leptin levels were 70% (nonsignificantly) higher in patients with diabetes who had proliferative retinopathy than in those without angiogenesis, and all four patients with diabetes with leptin levels higher than 60 ng/ml exhibited proliferative retinopathy. These findings, if confirmed in a larger
population, suggest serum leptin concentrations may be useful to designate patients with diabetes who have, or are at risk to develop, severe retinopathy.

In conclusion, leptin was present in vitreous humor, leptin levels were elevated in eyes with vascular and fibrotic proliferation, and leptin and leptin receptor were localized within diabetic epiretinal proliferative tissue. These results expand the list of diseases associated with leptin, to include microvascular and proliferative complications of diabetes mellitus and other conditions.

Acknowledgments
The authors thank Dante Pieramici, John Berreen, and Nancy Miller-Rivero for collection of specimens.

References