Development of a sensitive ELISA for human leptin, using monoclonal antibodies

Keiichi Imagawa,1 Yayoi Matsumoto,1 Yoshito Numata,1* Atsushi Morita,1 Shino Kikuoka,1 Mikio Tamaki,1 Chie Higashikubo,1 Tetsuo Tsuji,1 Kazuyuki Sasakura,1 Hiroshi Teraoka,1 Hiroaki Masuzaki,2 Kiminori Hosoda,2 Yoshihiro Ogawa,2 and Kazuwa Nakao2

A new, sensitive ELISA for human leptin in plasma and cerebrospinal fluid (CSF) was developed, using monoclonal antibodies. The lower limit of detection of this ELISA was 0.78 pg/assay. Both intra- and interassay imprecision values were <7%. The dilution curves of plasma and CSF showed good linearity, and the recovery was 83.2–95.6%. There was good correlation between plasma leptin concentrations by the ELISA and a commercially available RIA (r = 0.99). Our ELISA is advantageous because it does not require radioisotopes, it produces results in hours rather than days, and more importantly, it improves on the detection limit and plasma interference of the RIA kit. The new ELISA enables measurement of low concentrations of leptin, as are seen in CSF and in plasma of patients with anorexia nervosa.

The discovery of leptin has caused a breakthrough in the fields of endocrinology, nutrition, and metabolism (1, 2). This 16-kDa protein is produced in adipose tissue, is secreted into the bloodstream, and is thought to act as an afferent satiety signal in a feedback loop affecting the satiety center of the brain (3–10). To understand the physiological role of leptin, sensitive and precise measurement of leptin concentrations in body fluids [for example, blood, cerebrospinal fluid (CSF),3 amniotic fluid, and urine] was needed. Maffei et al. (11), using an immunoprecipitation/Western blotting technique, found that plasma leptin concentrations were highly correlated with body mass index (BMI); however, this method was tedious and semiquantitative. Thereafter, several groups developed RIAs with polyclonal antibodies raised against recombinant human leptin; they observed a close correlation of leptin concentrations with the percentage of body fat (12–14). A single meal does not substantially alter leptin concentrations (13); however, short-term fasting and overeating lead to a rapid decrease and increase, respectively, in leptin synthesis, which precedes weight alteration (15–18). Pregnant women also secrete a considerable amount of leptin from the placenta into the maternal circulation when compared with nonpregnant, obese women (19).

At present, the commercially available RIA kit developed by Ma et al. (20) is the one used most widely to measure leptin. This RIA kit allows accurate analysis; however, it has some disadvantages: (a) it uses a radioisotope; (b) it is time-consuming; and (c) it has an insufficient detection limit (0.5 μg/L) to measure concentrations in CSF.

To overcome these problems, we developed a new ELISA that uses monoclonal antibodies. Here, we describe its performance and advantages.

Materials and Methods

Antigens

Recombinant human leptin was expressed in Escherichia coli and purified in a soluble form as described previously.

1 R & D and Manufacturing Department for Diagnostics, Diagnostic Science Division, Shionogi Co., Ltd., 2-5-1 Mishima, Settsu-shi, Osaka 566-0022, Japan.
2 Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.
*Author for correspondence. Fax 81-6-319-4109; e-mail yoshito.numata@shionogi.co.jp.
Received April 24, 1998; revision accepted July 14, 1998.

3 Nonstandard abbreviations: CSF, cerebrospinal fluid; BMI, body mass index; PBS, 10 mmol/L phosphate-buffered saline; pH 7.4; and AN, anorexia nervosa.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified leptin showed a single band of 16 kDa. The purity was 98% as determined by HPLC analysis.

MONOCLONAL ANTIBODIES
Seven Balb/c mice were immunized with intraperitoneal injections of leptin-bovine thyroglobulin conjugate in Freund's adjuvant (Sigma Chemical Co.) over a period of 3 months at 3-week intervals. Fusion of spleen cells from the immunized mouse with mouse myeloma cells, P3U1, was performed in a ratio of 5:1, using 500 g/L polyethylene glycol 1500 (Boehringer Mannheim GmbH) (22). The cell supernatants were screened by the method described below, and the positive cells were cloned by the limiting dilution technique and expanded intraperitoneally in Balb/c mice. Monoclonal antibodies were purified from ascitic fluids using a Protein G-Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech). Isotype determination was carried out using an the enzyme immunoassay method with an isotyping kit (PharMingen).

ANTIBODY SCREENING
Recombinant human leptin was radioiodinated by the chloramine-T method (23). The plasma samples from immunized mice or culture fluid were incubated with recombinant human leptin and 125I-leptin (30 000 cpm) in 300 μL of assay buffer for 18 h at 4 °C. The antibody-antigen complexes were precipitated by adding 1.0 mL of 16.3 g/L polyethylene glycol 6000 in 10 mmol/L phosphate-buffered saline, pH 7.4 (PBS). Unbound antigen was removed by centrifugation, followed by aspiration. The radioactivity remaining in the pellet was counted with an ARC-600 gamma counter (Aloca Co., Ltd.).

MICROPLATES COATED WITH ANTI-LEPTIN MONOCLONAL ANTIBODIES
Each well of a microplate (module plate F8; Nunc A/S) was filled with a solution (200 μL) of anti-leptin antibody, mAb1, (2.5 mg/L in PBS), and incubated overnight at 4 °C. After removal of the antibody solution, the wells were washed three times with 0.5 mL/L Tween 20 in PBS (250 μL) and aspirating it out. PBS (300 μL) containing 100 g/L sucrose and 10 g/L bovine serum albumin was added to each well of the microplates, which were then incubated at room temperature for 1 h. After aspiration, the microplates were dried in a desiccator under vacuum overnight and stored at 4 °C.

PREPARATION OF ANTI-LEPTIN MONOCLONAL ANTIBODY-ENZYME CONJUGATE
One monoclonal antibody (mAb2) was conjugated with horseradish peroxidase (EC 1.11.1.7; Boehringer) as described by Kato et al. (24).

REAGENTS
Buffers and solutions used in the ELISA were as follows: assay buffer of 0.05 mol/L phosphate buffer (pH 7.2) containing 1 g/L bovine serum albumin, 0.1 mol/L NaCl, 1 mmol/L EDTA, 0.2 mmol/L l-cystine, and 1 mL/L Kathon CG (Rohm and Haas Co.); washing solution of 0.5 mL/L Tween 20 in PBS; substrate solution of TMB plus (Dako A/S); and stop solution of 0.5 mol/L H2SO4.

ELISA FOR DETERMINATION OF HUMAN LEPTIN
In the typical assay procedure, all incubations were performed at 30 °C. Plasma and CSF samples were diluted at least three times and two times, respectively, with assay buffer. Aliquots of recombinant human leptin or samples (100 μL) were added to the wells of the antibody-coated microplates and incubated for 2 h (first reaction). After the wells were washed three times with the washing solution (250 μL), Fab'-enzyme conjugate (25 ng) in assay buffer (100 μL) was added. The samples were left standing for 1 h (second reaction). The wells were aspirated and washed again, and then substrate solution (100 μL) was added to each well. After 15 min of incubation (enzyme reaction), stop solution (50 μL) was added, and the absorbance at 450 nm was measured with ImmunoReader NJ-2000 (Nippon InterMed K.K.). The experiment was performed in duplicate except where noted otherwise. Every component for ELISA was stable for at least 3 months at 4 °C.

RIA FOR DETERMINATION OF HUMAN LEPTIN
RIA kits were purchased from Linco Research, Inc. The assay was performed according to the standard assay procedure of the RIA kits (20).

PREPARATION OF LEPTIN-FREE PLASMA
Human plasma samples (1.0 mL) were combined with 25 mg of charcoal. After incubation with continuous mixing for 24 h at 4 °C, the charcoal was removed by centrifugation.

SAMPLES
The plasma and CSF samples of anorexia nervosa (AN) patients were obtained from Kyoto University Graduate School of Medicine. Blood was withdrawn from the antecubital vein and immediately transferred to glass tubes containing Na3EDTA (1 g/L) and centrifuged at 4 °C. CSF samples were obtained by lumbar puncture from patients at Kyoto University Hospital who had been subjected to various diagnoses and were at different stages of the severity of illness. Plasma and CSF were kept frozen at −40 °C until analysis. Informed consent was obtained from the patients, and the study was approved by the ethical committee on human research of Kyoto University.

Plasma samples were also obtained from healthy volunteers in accordance with the policies and procedures of
the Institutional Review Board for use of human subjects in research at the Diagnostic Science Department, Shionogi & Co., Ltd.

C57BL/6j ob/ob mice were maintained in Shionogi Research Laboratories.

**Results**

**PREPARATION AND CHARACTERIZATION OF ANTI-LEPTIN MONOCLONAL ANTIBODIES**

As the result of cell fusion, four monoclonal antibodies (mAb1-mAb4) were established. Among the preliminary sandwich assays involving combinations of the obtained antibodies, the assay involving mAb1 (IgG2a,κ) as the immobilized antibody and mAb2 (IgG1,κ) as the labeled antibody showed a dose-dependent response to leptin with great sensitivity. The association constants of mAb1 and mAb2 were $1.2 \times 10^{10}$ and $7.2 \times 10^{8}$ L/mol, respectively, by Scatchard plot analysis. We used these monoclonal antibodies to develop the ELISA.

**ASSAY CHARACTERISTICS**

**Calibration curve.** A representative calibration curve based on leptin calibrators of 0.78–50 pg/assay is shown in Fig. 1. The absorbance at 450 nm against the amount of calibrator exhibited a linear relation.

**Intraassay precision.** The analysis of five determinations of the same plasma or CSF samples gave a CV ≤4.0% at all leptin concentrations tested. The mean ± SD concentrations measured (and CV) were as follows: plasma 1, 83.6 ± 3.4 ng/L (4.0%); plasma 2, 308.2 ± 4.1 ng/L (1.3%); plasma 3, 2476.8 ± 49.0 ng/L (2.0%); plasma 4, 11 730 ± 175 ng/L (1.5%); CSF 1, 90.7 ± 1.8 ng/L (1.9%); and CSF 2, 114.4 ± 2.9 ng/L (2.5%).

**Interassay precision.** The analysis of 10 determinations of the same plasma or 5 determinations of the same CSF samples gave a CV ≤6.6%: plasma 1, 83.6 ± 2.2 ng/L (2.7%); plasma 2, 295.4 ± 13.6 ng/L (4.6%); plasma 3, 2560.7 ± 89.6 ng/L (3.5%); plasma 4, 12 220 ± 599 ng/L (4.9%); CSF 1, 87.0 ± 4.1 ng/L (4.8%); and CSF 2, 116.2 ± 7.7 ng/L (6.6%).

**Analytical recovery and linearity.** Recoveries of exogenously added leptin from plasma and CSF samples ranged from 86.1% to 95.6% and from 83.2% to 92.2%, respectively (Table 1). Dilution curves of plasma and CSF samples showed good linearity (Table 2).

**Interferences and specificity.** Hemoglobin (5 g/L), bilirubin (200 mg/L), and total lipids (10 g/L) had no effect on the present ELISA. The cross-reactivity with mouse leptin was <0.0002%.

**Stability.** Leptin concentrations in whole blood remained mostly unchanged for 48 h at 25 °C. Use of EDTA plasma

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**Table 1. Analytical recovery of calibrator added to plasma and CSF samples.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Endogenous leptin, ng/L</th>
<th>Added, ng/L</th>
<th>Found,a ng/L</th>
<th>Recovery, %</th>
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</thead>
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<tr>
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<td>160</td>
<td>187</td>
<td>161</td>
<td>86.1</td>
</tr>
<tr>
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<td>160</td>
<td>375</td>
<td>341</td>
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</tr>
<tr>
<td>Plasma 5</td>
<td>160</td>
<td>750</td>
<td>655</td>
<td>87.3</td>
</tr>
<tr>
<td>Plasma 6</td>
<td>236</td>
<td>344</td>
<td>329</td>
<td>95.6</td>
</tr>
<tr>
<td>Plasma 6</td>
<td>236</td>
<td>688</td>
<td>657</td>
<td>95.5</td>
</tr>
<tr>
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<td>1375</td>
<td>1281</td>
<td>93.2</td>
</tr>
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<td>688</td>
<td>640</td>
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<tr>
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<td>1375</td>
<td>1273</td>
<td>92.6</td>
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<tr>
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<td>2400</td>
<td>87.3</td>
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<td>221</td>
<td>88.4</td>
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<tr>
<td>CSF 3</td>
<td>49</td>
<td>500</td>
<td>461</td>
<td>92.2</td>
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</table>

*a Increase over endogenous leptin.

**Table 2. Serial dilution linearity of ELISA.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution factor</th>
<th>Observed, ng/L</th>
<th>Expected, ng/L</th>
<th>% of expecteda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 8</td>
<td>3</td>
<td>218</td>
<td>218</td>
<td>100</td>
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<tr>
<td>Plasma 8</td>
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<td>252</td>
<td>218</td>
<td>116</td>
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<tr>
<td>Plasma 8</td>
<td>12</td>
<td>231</td>
<td>218</td>
<td>106</td>
</tr>
<tr>
<td>Plasma 9</td>
<td>3</td>
<td>655</td>
<td>655</td>
<td>100</td>
</tr>
<tr>
<td>Plasma 9</td>
<td>6</td>
<td>675</td>
<td>655</td>
<td>103</td>
</tr>
<tr>
<td>Plasma 9</td>
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<td>653</td>
<td>655</td>
<td>100</td>
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<tr>
<td>Plasma 10</td>
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<td>951</td>
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<td>92</td>
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<tr>
<td>CSF 4</td>
<td>8</td>
<td>100</td>
<td>113</td>
<td>88</td>
</tr>
</tbody>
</table>

*a Calculated as (observed/expected) ×100.
or serum gave equivalent results. Leptin in EDTA plasma, serum, or CSF was stable at least 1 month at 4°C. Ten freeze/thaw cycles had little effect on plasma leptin (data not shown).

**Comparison with RIA using a commercially available kit**

**Correlation.** The correlation between the values obtained by the newly developed ELISA (y) and an RIA using a commercially available kit (x) could be expressed by the Passing–Bablok regression equation (25), \( y = 1.37x - 1.42 \) (μg/L), for which the correlation coefficient was 0.99 (n = 57), as shown in Fig. 2. At a leptin concentration <3 μg/L, \( r \) was 0.75.

**Imprecision profile.** The imprecision profiles of the two assay methods are shown in Fig. 3. The lowest leptin concentrations that were measured with an imprecision <15% were ~0.78 and ~50 pg/assay for the ELISA and the RIA, respectively. For measurement of leptin concentrations in plasma and CSF, the lower limits of quantification in the ELISA were ~0.023 μg/L and ~0.016 μg/L, respectively, which were 20–30 times lower than the detection limit of the RIA (0.5 μg/L).

**Plasma interference.** To assess plasma interference, we prepared leptin-free plasma by charcoal treatment (13) and measured it using the RIA kit and the ELISA (Table 3). In each charcoal-treated sample, a slight signal (0.7–1.3 μg/L) was observed by the RIA kit, whereas no signal was observed by the ELISA. We also measured a plasma sample from C57BL/6J ob/ob mice, which lack a functional leptin gene. Even in this sample, a signal (1.0 μg/L) was observed by the RIA kit, whereas none was detected by the ELISA. These results showed that there is plasma interference in the RIA kit, which may lead to incorrect values in the low concentration range.

![Fig. 2. Comparison of the plasma leptin concentrations determined by our ELISA and the commercially available RIA kit.](image)

![Table 3. Determination of charcoal-treated plasma by two methods.](image)
**CONCENTRATIONS OF LEPTIN IN HUMAN PLASMA AND CSF**

**Plasma leptin concentrations in healthy adults.** Human leptin concentrations in plasma samples obtained from healthy men (n = 16) and women (n = 15) were determined by the ELISA. Regression analysis of leptin concentrations in relation to BMI separated by gender yielded high correlations (r = 0.90 for men, r = 0.79 for women). The rate of increase in plasma leptin concentrations in relation to BMI was greater in women than in men (1.52 μg/L vs 1.03 μg/L per unit of BMI, respectively) as reported previously by the RIA method (20).

**Plasma leptin concentrations in patients with AN.** Patients with AN, compared with the controls, had lower concentrations of plasma leptin concentrations measured both by the ELISA (0.343 ± 0.266 μg/L vs 8.48 ± 5.84 μg/L; P <0.001) and the RIA (1.54 ± 0.61 μg/L vs 6.91 ± 3.67 μg/L; P <0.001; Table 4). However, in patients with AN, plasma leptin concentrations measured by the RIA were fourfold higher than those measured by the ELISA. Moreover, the results from the ELISA showed a significant correlation with the BMI, whereas those from the RIA did not (Fig. 4).

**CSF leptin concentrations.** Concentrations of leptin in CSF (n = 8) were also measured by the two assay methods and are summarized in Table 5. All of the CSF samples could be measured by our ELISA (mean ± SD, 158 ± 67 ng/L), whereas none of them could be measured by the RIA because of their very low concentrations. Gel filtration analysis showed that most of the CSF leptin exists as 16-kDa protein (data not shown).

**Discussion**

We prepared monoclonal antibodies and developed a new sandwich ELISA to solve the problems related to RIA for leptin. There was a good correlation (r = 0.99) between plasma leptin concentrations measured by the ELISA and the commercially available RIA kit over the entire concentration range (Fig. 2). However, in the low concentration range (<3 μg/L), the RIA overestimated the concentration, and the two assay methods did not show any good correlation (r = 0.75). This difference was attributable to the insufficient limit of detection and plasma interference in the low range. For measurement of plasma leptin concentrations, our ELISA has a limit of detection ~20-fold lower than the RIA used (Fig. 3). In measurement of charcoal-treated plasma and plasma from C57BL/6J ob/ob mice, false-positive leptin values (~1 μg/L) were detected.

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**Table 4. Plasma leptin concentrations in healthy women and women with AN.**

<table>
<thead>
<tr>
<th></th>
<th>Women with AN* (n = 16)</th>
<th>Healthy women* (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>24 ± 8 (14–47)</td>
<td>28 ± 3 (26–35)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>13.3 ± 1.56 (11.5–16.0)</td>
<td>20.5 ± 3.1 (18.0–27.4)</td>
</tr>
<tr>
<td>Plasma leptin, μg/Lc</td>
<td>1.54 ± 0.61 (0.74–3.03)</td>
<td>6.91 ± 3.67 (1.81–14.0)</td>
</tr>
<tr>
<td>Plasma leptin, μg/Ld</td>
<td>0.343 ± 0.266 (0.024–0.749)</td>
<td>8.48 ± 5.84 (0.946–21.6)</td>
</tr>
</tbody>
</table>

* Results are expressed as the mean ± SD (range).

* P <0.001 for comparison with healthy women by the Student t-test.

* Determined by commercial RIA.

* Determined by present ELISA.

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**Table 5. Determination of CSF leptin by the two methods.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>RIA</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF 5</td>
<td>&lt;500</td>
<td>148</td>
</tr>
<tr>
<td>CSF 6</td>
<td>&lt;500</td>
<td>218</td>
</tr>
<tr>
<td>CSF 7</td>
<td>&lt;500</td>
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<tr>
<td>CSF 8</td>
<td>&lt;500</td>
<td>273</td>
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<tr>
<td>CSF 9</td>
<td>&lt;500</td>
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<tr>
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<td>&lt;500</td>
<td>119</td>
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<tr>
<td>CSF 12</td>
<td>&lt;500</td>
<td>119</td>
</tr>
</tbody>
</table>
by the RIA kit, whereas our ELISA did not detect any immunoreactivity (Table 3). In addition, Ma et al. (20) reported that no specimen in the study had a leptin concentration <1 μg/L by the RIA kit. These results imply that this RIA-specific false-positive values are caused by an unknown substance in plasma samples.

AN is characterized by the patient being underweight and suffering from amenorrhea and the specific psychopathological features of intense fear of becoming fat and gaining weight (26). AN patients have been reported to show a marked reduction in plasma leptin (27–29). In this study, we found that the ELISA revealed a more striking reduction in plasma leptin concentrations in AN patients than that observed by the RIA (Table 4). Furthermore, the ELISA demonstrated that leptin concentrations showed a significant correlation with the BMI even in a group of AN patients, whereas those measured by the RIA did not, as previously reported by Mantzoros et al. (29). These results were probably attributable to the overestimated values in the RIA. These findings suggest that the ELISA method would be more reliable when plasma leptin concentrations of AN patients are measured.

Measurement of leptin concentrations in the CSF may provide an important research probe for investigation of disorders of body weight regulation, because leptin acts directly on the central nervous system (6, 10). Previous reports have demonstrated a positive correlation between CSF and plasma leptin concentrations in individuals ranging from health-related weight to obese (30, 31). In overweight individuals, there was evidence for a decreased ratio of CSF to plasma leptin, suggesting that saturation of the leptin transporter at increased plasma leptin concentrations could contribute to leptin resistance in human obesity. However, the RIA kit does not permit direct measurement of CSF leptin. It requires concentration of the CSF samples and a longer incubation time (29). On the other hand, the present ELISA makes it possible to directly measure leptin concentrations in CSF (Table 5). Schwartz et al. (31) measured CSF leptin concentrations by a sandwich ELISA, in which affinity-purified polyclonal antibodies were used for both capture and signal. Their ELISA also had a lower detection limit (20 ng/L); however, it could not be easily adapted to routine determination of human leptin because the amounts of affinity-purified polyclonal antibodies were limited.

Recently, ELISA methods using monoclonal antibodies have also become commercially available (e.g., the Quantikine human leptin immunoassay, R&D Systems). However, for measurement of leptin concentrations in plasma, samples should be diluted at least 20-fold by the commercial ELISA kit to avoid plasma interference. As a result, the lower limit of quantification of the ELISA kit is 10-fold higher than that of our ELISA (data not shown).

Thus, our new ELISA is precise, sensitive, and simple enough to elucidate the physiological roles and clinical importance of leptin.

References


