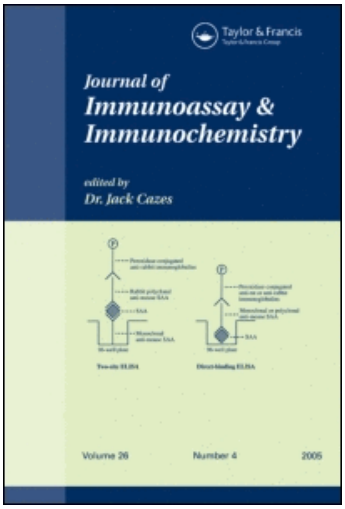

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ABSTRACT

The highly effective antibody has been obtained by immunizing rabbits with recombinant leptin many times. The leptin is iodinated with the chloramine-T method and purified with a Sephadex-G25 chromatography column. The reaction between antigen and antibody is carried out by a one-step balance method and cultured at 4°C for 24 h; the binding and free antigen was then separated by PR reagent. The determining range of this method is about 0.5–24 ng/mL; limited detection level is 0.45 ng/mL, relative standard deviation in a group, and among groups, are less than 5.4% and 8%, respectively. The level of blood leptin in 277 samples of

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normal persons, in 112 samples of overweight persons (weight/hieght $m^2 \geq 25$) and 224 samples of hyperlipidemic patients have been measured by this method. It is demonstrated that the level of blood leptin in males is much lower than that of the females, and becomes elevated with increased age. Serum leptin level in overweight persons and hyperlipidemic patients is also much higher than that of normal groups ($P < 0.01$). Serum leptin of 21 workers in our lab at 8:00AM and 4:00PM has been tested. It was found that there are no differences between the two time points. The same results are obtained within age groups. Leptin levels of pregnant women's serum is higher than those of the control group ($P < 0.001$). Leptin in newborn's serum is significantly lower than those of mothers ($P < 0.01$). There is no obvious correlation between leptin level of mother and newborns by correlation analysis ($r = 0.19$, $P > 0.05$). The body weight and body weight index of pregnant women are well correlated with their serum leptin levels ($r = 0.33$ and 0.35, $P < 0.05$). The body weight and body weight index of newborns are well correlated with their serum leptin levels ($r = 0.54$ and 0.49, $P < 0.001$). The serum leptin level of pregnant women is not correlated with newborn's body weight ($r = 0.10$). These results have shown that the proposed method is stable, simple, and specific, being sensitive enough to determine leptin levels in human serum or plasma.

INTRODUCTION

Leptin is a type of protein activating factor which is extensively presented in adipose tissue, and expressed by an obesity gene under the feedback regulation of the endocrine nervous system and energy metabolism.[1] It has been demonstrated that leptin is maintained at a definite level and participates in the regulation of ingestion, energy metabolism, and equilibrium of body weight.[2,3]

Several investigations indicate that leptin has a multiple effect like cytokines, involved in diabetes of the insulin confused type,[4] hypertension resulting from nerve-endocrine factors, and thyroid function confused difference endocrine disease.[5,6] Leptin also has been shown to play an important role in energy metabolism chaos resulting from trauma infection.[7–9] This paper describes a radioimmunoassay method which is simple, specific, and highly sensitive for leptin in human serum.

EXPERIMENTAL

Materials

Three large eared rabbits (weight, ~1.2 kg) were supplied by the experimental animal center of our hospital. Human recombinant leptin is from Peprotech, Inc., England. Freund's complete and incomplete adjuvants are from Life Technologies, Inc., New York, USA. Cytokines of IL-1, IL-6, IL-8, and EPO (erythropoietin) granulocyte-macrophage colony stimulating factor (GM-CSF) are from Sigma, St. Louis, MO, USA. ^{125}NaI is from the Atomic Energy Academy of China. Other reagents were purchased locally and are of analytically pure grade.

Preparation of Antiserum

One hundred and twenty micrograms of purified antigen of leptin were intimately mixed with 3 mL of Frenud's complete adjuvant for immunization of three New Zealand rabbits by intradermal injection at multiple points of their backs. Enhanced immunization was carried out the same way by using 70 μg Leptin to mix with 3 mL of incomplete adjuvant once every 4 weeks. After seven days since the fourth enhanced immunization, the serum from ear edge vein was tested for the antibody titer. After obtaining a high titer antiserum from the rabbits, blood was collected from the carotid artery and the separated serum was preserved at −80°C while waiting for analysis.[10]

Iodination of Antigen

Three micrograms leptin is dissolved in 30 μL of 0.2 mol/L, pH 7.5, phosphate buffer; sodium iodide (^{125}I), 0.5 mCi (3.7×10^7 Bq) was added, following 10 μg chloramines T in 10 μL to start the reaction for 30 s. The iodination reaction is terminated by adding 20 μg sodium metabisulfite in 20 μL solution. The reaction solution is added to the top of a Sephadex G-25 gel column that has been equilibrated with the same buffer solution and washed with 0.1 mol/L pH 7.4 phosphate buffer, then the solution is collected at the rate of one tube per minute. After reacting with anti-serum, those iodinated leptins with high specific binding rate and low fault binding rate are taken as successful iodinated leptin and stored at −20°C mixed with 3% BSA (v : v = 1 : 1).[10]

Radioimmunoassay

Hundred microlitre standard Leptin (0.5, 1.5, 3.6, 12, 24 ng/mL) and samples, are mixed with 100 μL rabbit anti-serum (1 : 10 000) as well as 100 μL ^{125}I-Leptin (about 2×10^4 cpm), respectively, and allowed to react at 4°C for 24 h. After adding 500 μL PR reagent (combined reagent of polyethylene glycol and anti-serum of donkey anti-rabbit IgG) at room temperature, lasting for 15 min, all tubes are centrifuged for 15 min at 3500 rpm, then the supernatants are discarded and the radioactivity of precipitate in the tubes is measured. All the data are treated by radioimmunoassay software and the binding rate of each point is calculated; sample concentrations are obtained by a standard curve.[10]

Optimizing Analysis Conditions

The incubation times at 3–6 h at 37°C and 16–24 h at 4°C are studied for optimized reaction conditions. Three different concentrations of iodinated marker were compared to optimize binding rate, and no specific binding, for the best standard curve shape.

Measurement of Leptin in Serum and Blood Plasma

Leptin level in the serum of 277 healthy persons, 112 obese persons (weight Kg/height $m^2 \geq 25$), 224 samples of high cholesterol and triglyceride persons were measured and leptin in blood plasma of 20 researchers in our laboratory, at different times, also were measured. Fifty cases of pregnant women without disease of gestation, and their newborns, ($n = 50$) were selected as samples for leptin studies; 29 cases of healthy, non-pregnant women were taken as controls. The persons in the research group averaged 28 years old, with body weight 54.9 ± 7.3 kg and body weight index average 21.2 ± 2.5 kg/m^2 before pregnancy. The persons in the control group were 27 years old with body weight 52.7 ± 6.8 kg and body weight index 20.1 ± 2.1 kg/m^2. Body weight and body weight index showed no differences in the above mentioned two groups of women. Four millilitre blood of research group patients were taken from a vein at 37–38 weeks of pregnancy, and 4 mL blood of the newborn hilum vein were taken at the time of childbirth. Serum was isolated by centrifugation and kept at −20°C for leptin tests.

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RESULTS

Standard and Antibody Dilution

The best curve shape has been achieved at 4°C for 24 h incubation through the testing of 3–6 h at 37°C and 16–24 h at 4°C. Adding 20 000 cpm of iodinated marker can yield a suitable binding rate (45%) and no-specific binding (3.2%). Final antibody dilution is 1 : 30 000 and a good binding curve has been obtained in the standard field of 0.5–24 ng/mL, as Fig. 1 shows.

Sensitivity and Precision

Calculated as $B_0 \pm 2S$, the minimum measurable value of leptin is 0.45 ng/mL. In addition, three human serum samples have been tested at the same time for 5 times, respectively; one sample has been tested 5 times, and it has been demonstrated that variance in the group is less than 6.4%, and variance among groups is less than 9.6%.

Specificity and Validity

There are no cross reactions among anti-serum of leptin with IL-1, IL-2, IL-3, IL-6, IL-8, TNF, and GM-CSF in the 25–200 ng/mL range.

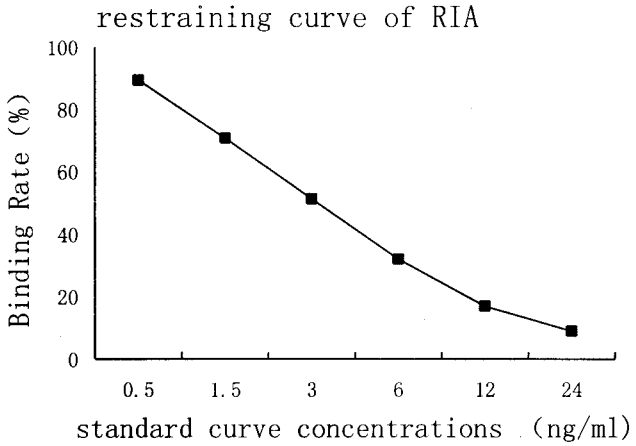

Figure 1. Standard curve.

The recovery rates range from 86.3 to 100% when adding 0.5, 2.5, and 10 ng/mL of leptin standards to 1 mL normal serum. There has been good linearity between the tested values and dilution times when one high concentration of leptin serum is diluted from 1/2 to 1/32 times.

Serum Leptin of Healthy Humans with Different Ages

From 227 samples of healthy human serum, it has been observed that leptin is increased significantly following age increase and, furthermore, leptin in female groups is obviously greater than that in male groups. In females, serum leptin levels in overweight (weight kg/height $m^2 \geq 25$) and high blood-lipid levels (cholesterol and triglycerides) persons is obviously greater than that of healthy controls in different age groups. In males, serum leptin level is significantly elevated following age increase and is obviously higher in overweight and high blood-lipid persons than those of healthy controls. But, there are no significant differences from serum leptin among simple overweight and high blood-lipids in male and female at different age groups.

These results have strongly demonstrated that serum leptin level is higher in female than in male, and is greater in aged than in middle and youth groups, and is higher in fat and high blood-lipids persons than in healthy persons.

Blood Plasma Leptin of Healthy Humans at Different Times

Leptin levels in 20 samples of healthy persons with EDTA and aprotinin anti-clotting is 4.91 ± 2.14 ng/mL in the morning (8:00 AM) and is 4.64 ± 2.05 ng/mL in the afternoon without significant differences. There are no differences of leptin levels between serum and blood plasma. These results indicate that leptin level is almost the same and no differences between morning and afternoon are seen.

Changes of Leptin and Cholesterol Level in Patients After Taking AOB132

It was discovered that serum leptin and cholesterol levels were significantly reduced a half year after taking AOB132 (a kind of blood-lipids adjustor).

Leptin Level of Pregnant Women and Newborns' Serum

Leptin level of pregnant serum is 13.62 ± 3.68 ng/mL, and is 6.60 ± 3.04 in the control group ($P < 0.001$). Leptin in newborns' serum was 8.05 ± 4.61, which is significantly less than that of mothers ($P < 0.01$). There is no obvious correlation between leptin level of mother and newborns by correlation analysis ($r = 0.19$, $P > 0.05$). The body weight (72.3 ± 8.5 kg) and body weight index (27.43 ± 2.66 kg/m^2) of pregnant women are well correlated with their serum leptin levels ($r = 0.33$ and 0.35, $P < 0.05$). The body weight (3.38 ± 4.14 kg) and body weight index (13.32 ± 1.07 kg/m^2) of newborns are well correlated with their hilum vein leptin level ($r = 0.54$ and 0.49, $P < 0.001$). The pregnant women serum leptin level is not correlated with newborns body weight ($r = 0.10$, $P > 0.05$). The control group women leptin level is well correlated with their body weight and body weight index ($r = 0.72$ and 0.78, $P < 0.001$).

DISCUSSION

Zhang has successfully cloned the obese gene from mouse, and its coding protein is called leptin; there are 84% amino acid sequences in common between human and mouse.[1] Leptin is a type of protein which consists of 167 amino acids, existing extensively in fat tissue of humans and other mammals. It mainly works as a kind of signal peptide, influencing receptors of leptin in hypothalamus tissues through blood feedback.[2] It has been proven that leptin regulates the ingestion/energy metabolism and consumes fat tissues and influences body weight.[5] Studies have already identified that animals significantly reduce food intake and weight after repeated injections of leptin via abdominal cavity; this may cause an increase of neuropeptide Y (NPY) in brain tissues.[4]

Additionally, leptin has been demonstrated to activate Jak signal way by its receptor binding and altering many endocrine cell functions through stimulating STATS activation. It is demonstrated that leptin receptors belong to the IL-6/gp 130 receptor family. Furthermore, leptin has been described to produce more by LPS stimulation, playing an important role in regulating cell differentiations, macrophage functions, and participates in inflammation response as well as internal environment maintenance.[8,9]

More studies have been focused on the mechanism of leptin activity in clinical disease processes, especially as related to the research of high blood lipids, diabetes, hypertension, abnormal thyroid function, and arteriosclerosis.[5–7] Studies in this field have progressed very rapidly, but the results are not consistent at all. Very little is known about its mechanism

Table 1. The Level of Human Serum Leptin (ng/mL)

	Normal		Fat		High Blood Lipoids	
Age	Male	Female	Male	Female	Male	Female
15–30	2.7 ± 0.8 $N = 21$	5.3 ± 1.3 $N = 32$	5.4 ± 1.2 $N = 7$			
31–50	3.6 ± 1.3# $N = 84$	6.2 ± 2.0# $N = 80$	4.8 ± 1.6* $N = 55$	8.1 ± 2.9* $N = 22$	4.6 ± 1.7* $N = 120$	9.3 ± 4.3* $N = 18$
51–70	3.5 ± 1.2# $N = 39$	7.5 ± 2.3# $N = 21$	5.4 ± 1.8* $N = 25$	9.6 ± 3.7 $N = 10$	4.9 ± 1.6* $N = 67$	10 ± 4.7* $N = 19$

Compared in age group of normal human $\#P < 0.05$, compared in same age group $*P < 0.01$.

Table 2. The Changes of Serum Leptin and Cholesterol After Taking ABO132 One Half Years Later

Group	Before ABO132	After ABO132	*T* Value	*P* Value
Cholesterol	6.8 ± 0.9 mmol/L	5.7 ± 0.9 mmol/L	7.52	$P < 0.001$
Leptin	8.9 ± 7.0 ng/mL	7.6 ± 4.8 ng/mL	2.429	$P < 0.05$

because leptin is present at a very low level in serum and ordinary immunoassay methods are not sensitive enough to satisfy the needs of clinical research. Leptin also cannot be tested by a bioactivity method as can other kinds of cytokines. Since leptin is a type of conservative protein, only having minimal antigenicity; the expected high specific and affinity leptin antibody have not been obtained yet and only one method of rat leptin radioimmunoassay has been reported.[4]

Through repeated studies, we have used low dosages of leptin (40 μg) for rabbits immunizations and obtained a high titer antiserum of leptin, which has been proven without cross reactions with other cytokines having the same molecular weight as leptin. By optimizing the method, the best experimental results have been obtained at 4°C for 24 h incubation using a one-step equilibrium method. The sensitivity of leptin testing in human serum by our method is higher than that in rats serum leptin reported in another paper. Iodinated leptin can been kept at room temperature for two months after lyophilization. Lyophilized standards and antibody can provide a good curve shape for one-half year at room temperature, and for two years at −20°C.

Leptin has been well studied to show correlation with energy metabolism and fat accumulation, and it is also very interesting because of its influence on development and growth of embryos. Our results have shown that pregnant women's serum leptin is significantly higher than that of healthy women, and well correlated with body weight. This result indicates the leptin level is related to the fat accumulation during the late period of pregnancy. Placenta-secreted leptin is also one of reasons causing the leptin increase in pregnancy.[11]

Our results also have shown that the correlation of leptin in pregnant women's serum and their body weight are lower than those of healthy, nonpregnant women. This may be caused by the embryo, thru retention of body fluids, which is also one reason for body increase in pregnancy and a decrease in the proportion of fat tissue in the body weight. The serum leptin level of newborns has been provn to have good positive correlation with body weight, which is consistent with another report.[12] However, there is no correlation in a mother's serum leptin with the newborn's serum leptin and body weight, which is also consistent with another report.[13] Thus, late period pregnant women's serum leptin cannot serve as an index of newborn body weight, but the hilum vein blood may serve as an index of newborn body weight.

By our initial clinical applications, it has been discovered that leptin levels are significantly different among males and females, as well as in the aged groups. The leptin level in obese persons and those with high blood lipid levels are obviously higher than that of normal persons in corresponding age groups. It is more persuasive that cholesterol and leptin are significantly reduced after using blood lipid adjustors in patient serum. The above results have shown that the described method may be used in humans for leptin measurement in blood plasma or serum; it offers a reliable technique for clinical and research studies in this field.

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