Feline Leptin: Immunogenic and Biological Activities of the Recombinant Protein, and Its Measurement by ELISA

Haruki SHIBATA¹⁾, Noriyasu SASAKI²⁾, Tsutomu HONJOH¹⁾, Iwao OHISHI²⁾, Mitsuyoshi TAKIGUCHI³⁾, Katsumi ISHIOKA⁴⁾, Mohamed AHMED⁴⁾, Mohamed SOLIMAN⁴⁾, Kazuhiro KIMURA⁴⁾ and Masayuki SAITO^{4)*}

¹⁾Morinaga Institute of Biological Science, Tsurumi, Yokohama 230–8504, ²⁾Department of Biochemistry, Nippon Veterinary and Animal Science University, Tokyo 180–0023 and ³⁾Departments of Veterinary Clinical Sciences and ⁴⁾Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

(Received 28 April 2003/Accepted 22 July 2003)

ABSTRACT. Leptin is a protein synthesized and secreted primarily by adipose tissue. The blood leptin concentration is known to reflect body fat content in rodents, humans and dogs, and thereby is useful for quantitative assessment of obesity. In the present study, we produced recombinant feline leptin in *Escherichia coli* transfected with feline leptin cDNA we cloned previously. The recombinant feline leptin with a molecular weight of 16 kDa induced phosphorylation of the signal transducers and activators of transcription 3 (STAT3) protein in the cells expressing rat leptin receptor. The anti-feline leptin antibody raised in rabbits reacted well to feline and human leptin and less to rodents' leptin in Western blot analysis. Sandwich enzyme-linked immunosorbent assay (ELISA) was developed, using rabbit anti-feline leptin antibody and recombinant feline leptin as a standard. In this ELISA system, cross-reactivity to human, rat and mouse leptin was 30.7%, 69.5% and 66.6%, respectively. The plasma leptin levels of 24 healthy cats were in a range from 0.3 to 29.7 ng/ml with the mean $\pm$ SEM of 4.5 ± 1.3 ng/ml, being positively proportional to body fat content. These results indicate that our ELISA system may be useful for assessment of obesity in cats.

KEY WORDS: ELISA, feline, leptin.

J. Vet. Med. Sci. 65(11): 1207–1211, 2003

Obesity is the most common nutritional disorder in small animal medicine, and enhances risks of diseases such as diabetes mellitus and hypertension, and decreases resistance to infection [13, 18, 19]. In cats, health risks of diabetes mellitus, lameness presumably related to osteoarthritis and soft-tissue injuries, and skin problems unrelated to allergies are known to associate with development of obesity [21].

Cats that are relative inactive, confined indoor and more than 3 years are often overweight or obese [22]. In addition, it is well known that gonadectomy may predispose to obesity by reducing energy expenditure and increased food intake [7, 17, 22]. Diet for weight reduction and/or restriction of food are central to successful treatment of overweight and prevention of obesity [9]. However, care must be taken because severe caloric restriction may cause hepatic lipidosis with blood biochemical abnormalities [4, 9].

To control diet of obese cats properly, it is necessary to develop the simple and reliable methods for accurate estimation of adiposity. The body condition score using a diagram of silhouettes is one of the diagnostic scales of obesity in cats [21], but this is a somewhat subjective estimate. Alternatively, dual energy X-ray absorptiometry and deuterium oxide dilution method are useful for quantitative estimation of body fat mass in cats [1–3, 15], but this is not easy for routine use.

Leptin, the product of the *ob* gene, is a protein synthesized and secreted primarily by adipose tissue [8, 27]. As in

human, rodents and dogs [5, 11, 16], the blood leptin concentration in cats determined by commercially available radioimmuno assay (RIA) kit with anti-human leptin antibody and human leptin as a standard was shown to positively correlate with body fat content and to be higher in obesity [1, 2]. These results suggest that blood leptin can be a quantitative marker of adiposity and obesity in cats, but validation of the feline leptin determination in blood is uncertain. Recently, we cloned feline leptin cDNA [20]. In the present study, we produced recombinant feline leptin in *Escherichia coli* (*E. coli*), examined its immunogenic specificity and biological activity, and established a sandwich enzyme-linked immunosorbent assay (ELISA) for feline leptin. Using this feline-specific ELISA method, we assayed plasma leptin concentrations of 24 privately owned healthy cats with a wide range of body fat contents and compared them with the values obtained by the conventional RIA kit.

MATERIALS AND METHODS

Production and purification of recombinant feline leptin
Feline leptin cDNA (GeneBank accession number: AB041360) was cloned as previously described [20]. Feline recombinant leptin was produced in *E. coli* (BL21(DE3)pLysS, Novagen, Madison, WI, U.S.A.) transfected with pET14-b plasmid (Novagen) containing a mature form of leptin sequence. In brief, the cells at an optimal concentration were treated with 0.4 mM isopropyl β -D-1-thiogalactopyranoside for 3 hr at 37°C. Inclusion body from the cells was then isolated using BugBuster reagent

*CORRESPONDENCE TO: SAITO, M., Laboratory of Biochemistry, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan.

(Novagen) according to the manufacture's recommended protocols, and incubated with buffer A [50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 10 mM DTT] containing 8 M urea for 1 hr at room temperature. After centrifugation (15,000 g for 15 min), the supernatant was dialyzed against buffer A containing 6 M urea, buffer A containing lower urea concentrations, and finally buffer A alone. The dialysate was applied onto the HiTrap Q HP column (5 ml, Amersham Bioscience Co., Piscataway, NJ, U.S.A.) equilibrated with buffer A, and the unbound passing through fractions containing recombinant leptin were collected and dialyzed against phosphate-buffered saline (PBS).

Cell responses to recombinant feline leptin: A clone of Chinese hamster ovary (CHO) cell stably expressing a predominant isoform of leptin receptor (OBRb) was obtained as described [12]. In brief, cDNA of rat OBRb was inserted into the pCAGGS vector using appropriate linkers. The expression plasmid with the pSV2neo plasmid was transfected into CHO cells, and cell clones were selected in the presence of G418 sulfate (Gibco-BRL, Gaithersburg, MD, U.S.A.).

The CHO cells were cultured in Ham's F-12 nutrient mixture (Gibco-BRL) supplemented with 10% fetal bovine serum in an atmosphere of 5% CO₂ in humidified air at 37°C. The subconfluent cells were cultured in the absence of fetal bovine serum for 24 hr, and then stimulated by 80 nM rat or feline leptin for 10 min. Phosphorylation of the signal transducers and activators of transcription 3 (STAT3) was measured by Western blot analysis as follows. After washing with an ice-cold buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM Na pyrophosphate, 2 mM NaVO₃, protease mixture (Complete™, Boehringer Mannheim, GmbH, Germany)], the cells were homogenized with the same buffer containing 1% Nonidet P-40, and centrifuged at 15,000 × g for 20 min. The resulting supernatant was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA, U.S.A.). After blocking by a buffer containing 0.1% Tween 20 and 5% skim milk, the membrane was incubated overnight in the blocking buffer containing either antibody against STAT3 or phospho-Tyr705-specific STAT3 (New England Biolabs, Beverly, MA, U.S.A.), washed and incubated for 1 hr in the blocking buffer containing horseradish peroxidase-linked goat anti-rabbit immunoglobulin (Zymed Laboratories, Inc. South San Francisco, CA). The bound antibody was visualized using an enhanced chemiluminescence system (Amersham).

Immunoreactivity of feline leptin: Rabbits were immunized with recombinant feline leptin (500 µg in Freund's complete adjuvant) and re-challenged two weeks later (300 µg in Freund's incomplete adjuvant). After confirming the presence of anti-feline leptin antibody in blood, serum was fractionated by 30% ammonium sulfate precipitation and the precipitate was dialyzed against PBS. The fraction was further purified by passing through DE-52 cellulose (Whatman International Ltd., Maidstone, England) and applied

onto feline leptin-conjugated HiTrap column (Amersham). Specific antibody to feline leptin was eluted with 0.1 M glycine/HCl (pH 2.5), and immediately neutralized with 1 M Tris/HCl (pH 8.6) and finally dialyzed to PBS. The immunoreactivity of recombinant feline leptin was examined by Western blot analyses, using antibodies against mouse leptin (OB1, Chemicon International Inc., Temecula, CA, U.S.A.), human leptin (lot 908-381, Morinaga Institute of Biological Science) and feline leptin. Recombinant human and mouse leptins were obtained from PeproTechEC Ltd. (London, England), and rat leptin was from R&D Systems (Minneapolis, MN, U.S.A.).

Sandwich ELISA for feline leptin: Each well of a 96-well microplate (Nunc, Tokyo) was coated with 100 µl of purified anti-feline leptin antibody (1 µg/ml) by incubating overnight at 4°C. After washing wells with PBS containing 0.05% Tween 20 (PBST), 300 µl of 1% bovine serum albumin in PBST was added to each well to block non-specific binding sites. After 2 hr incubation at room temperature, wells were washed three times with PBST, added with 5 µl of feline sera or 0.2–12.8 ng/ml recombinant feline leptin in a total volume of 100 µl, and further incubated overnight at 4°C. Then wells were washed three times with PBST, incubated with 100 µl of horseradish peroxidase-conjugated anti-feline leptin antibody (1 µg/ml) for 1 hr at room temperature, washed again, and finally incubated with a substrate solution (100 µl, DAKO TMB One-Step Substrate system, Dako Corporation, Carpinteria, CA, U.S.A.) for 30 min. H₂SO₄ (1 N, 100 µl) was added to stop color development and the absorbance at 450 nm and 630 nm as a reference were measured.

Blood sampling and leptin assays: Blood samples were obtained from 24 privately owned healthy cats: 10 males, age range 7 month to 10 years, body weight range 1.9 to 6.6 kg (average 4.3 ± 1.5 kg); 14 females, age range 10 month to 15 years, body weight range 2.2 to 6.8 kg (average 3.8 ± 1.2 kg) at the time of visit to Hiraoka Animal Hospital (Sapporo, Japan). The plasma were then isolated and stored at -80°C until use. Body fat content (%) of individual cats was roughly estimated by calculating feline body mass index (BMI) [10].

Plasma leptin concentrations were measured by the sandwich ELISA method as described above, and also by using multi-species leptin RIA kit with anti-human leptin antibody and human leptin as a standard (LINCO Research Inc., St. Charles, MO, U.S.A.).

RESULTS

Mature form of recombinant feline leptin was produced in *E. coli*, recovered from inclusion body with urea, and further purified by passing through HiTrap QHP column. The purified feline leptin migrated to the position of around 16 kDa in SDS-PAGE, having a similar molecular weight with other species leptin (Fig. 1, top panel). The feline leptin was detected not only by anti-feline leptin antibody, but also by anti-human and anti-mouse leptin antibodies (Fig. 1, second

Fig. 1. SDS-PAGE and Western blot analyses of recombinant feline leptin. Recombinant feline leptin (F, 0.5 μ g) was subjected to SDS-polyacrylamide gel (10%) electrophoresis, and detected by Coomassie brilliant blue R250 staining (top panel), anti-feline (second panel), anti-human (third panel) and anti-mouse (bottom panel) leptin antibodies. Recombinant human (H), rat (R) and mouse (M) leptins (0.5 μ g) were used for comparison.

to bottom panels). When the CHO cells expressing a long form of rat leptin receptor (OBRb) were treated with the feline leptin (80 nM), phosphorylation of STAT3 protein was induced without noticeable changes in the total amount of STAT3, similarly to those treated with rat leptin (Fig. 2). These results collectively suggest that the recombinant feline leptin obtained in this study is a biologically active form of protein.

We next developed a sandwich ELISA to measure plasma leptin levels of the cat. As shown in Fig. 3A, the developed ELISA enables to measure feline leptin ranging between 0.2 ng/ml and 12.8 ng/ml with a linear regression line. When plasma from 24 clinically healthy cats were measured, leptin concentrations were distributed in a range from 0.3 to 29.7 ng/ml with an average concentration of 4.5 ± 1.3 ng/ml, being positively correlated with respective body fat contents (Fig. 3B). Intra- and inter-assay variations and recovery of 12 samples were $3.9 \pm 1.4\%$, $3.3 \pm 0.9\%$, and $85.2 \pm 1.8\%$, respectively. Cross reactivity to human, rat and mouse lep-

tin in this ELISA system were 30.7%, 69.5% and 66.6%, respectively (Fig. 3C), although the anti-feline leptin antibody reacted well to feline and human leptin and less to rodents' leptin in Western blot analyses (Fig. 1).

Plasma leptin levels were also determined by using commercially available multi-species leptin RIA kit with anti-human leptin antibody and human leptin as a standard, which was used in other studies for determining feline leptin [1, 2]. The plasma leptin concentrations obtained by the RIA kit were highly correlated ($r=0.942$) with those obtained in the present ELISA system (Fig.3D). Cross reactivity to feline leptin in the RIA system was almost 100% if the concentration was less than 20 ng/ml of the recombinant protein (data not shown).

DISCUSSION

We previously cloned feline leptin cDNA, which contained a 501-bp open reading frame encoding a protein of 21 amino acids of N-terminal signal peptide and a mature peptide of 146 amino acids [20]. In good agreement with this predicted structure, recombinant feline leptin had a molecular mass of 16 kDa (Fig.1). The deduced amino acid sequence of feline mature leptin was 86.3%, 82.2%, and 81.5% identical to human, rat, and mouse leptin, respectively [20]. In the present study, we found the cross-reactivity of anti-feline leptin antibody to the four species' leptin, suggesting that the recombinant feline leptin shares its antigenicity with human and rodents' leptin. The differences in the reactivity between Western blot analysis and ELISA may be attributed to difference of leptin structure in the two conditions, denatured and native forms.

Leptin consists of four anti-parallel α -helixes arranged in a left-handed twisted bundle, and its ternary structure resembles other long-chain helical cytokines such as leukemia inhibitory factor and ciliary neurotrophic factor despite the absence of sequence similarity to leptin [26]. The predominant and largest isoform of leptin receptor (OBRb) has a 302-amino acid cytoplasmic domain which contains the binding sites for Janus kinase and STAT proteins [14, 23]. The binding of leptin to OBRb is known to induce phospho-

Fig. 2. Effect of recombinant feline leptin on STAT3 phosphorylation in the cells expressing leptin receptor. CHO cells expressing leptin receptor were stimulated with either feline or rat leptin (80 nM) in duplicate for 10 min, and the cell lysates were analyzed for phosphorylated and total STAT3 proteins.

Fig. 3. Determination of feline leptin by a sandwich ELISA. A: Recombinant feline leptin (0.2–12.8 ng/ml) was measured by the sandwich ELISA using purified anti-feline leptin antibody. B: Leptin concentrations in plasma of 24 healthy cats were determined by the ELISA using recombinant feline leptin as a standard, and plotted against estimated body fat content. C: Recombinant human, rat and mouse were measured by the ELISA using purified anti-feline leptin antibody. D: Plasma leptin concentrations were also measured by a commercially available RIA kit using anti-human leptin antibody and human leptin as a standard, and plotted against the values obtained by the ELISA.

rylation of STAT3, an isoform of the STAT family [6, 24]. In confirmation of these previous reports for other species' leptin, the recombinant feline leptin induced phosphorylation of STAT3 in the cells expressing rat OBRb. Thus it is likely that the feline leptin may form the ternary structure like other species' leptin and conserve critical amino acids for receptor binding such as Arg¹²⁸ [25].

There have been reports on the plasma leptin concentration in cats [1, 2]. In these previous studies, however, it was assayed using a multi-species RIA kit with human leptin as a standard. In the present study, we established a sandwich ELISA with anti-feline leptin antibody and recombinant feline leptin as a standard. Our ELISA enabled to measure feline leptin ranging between 0.2 ng/ml and 12.8 ng/ml. The average plasma leptin concentration in cats was 4.5 ± 1.3 ng/ml, comparable with those in previous reports (6.4 ± 2.2 ng/ml (n=26, range: 0.92–11.9 ng/ml) [1] and 2.9 ± 0.2 ng/ml (n=19, range: 1.6–4.9 ng/ml) [2]). In addition, the plasma leptin concentrations in cats correlated closely with body fat content estimated by BMI, being consistent with previous reports [1, 2]. Positive correlation between plasma leptin

concentration and body fat content has also been confirmed in rodents, humans and dogs [5, 11, 16].

In summary, we have succeeded to produce a biologically active recombinant feline leptin protein in *E. coli*, and established a sandwich ELISA for feline leptin, which would be useful for the measurement of blood leptin in the cat without using radioisotope and possibly for assessment of body fat content.

ACKNOWLEDGMENTS. We thank Drs. Yuriko Terada and Michiko Sekiguchi, Hiraoka Animal Hospital for their help to obtain blood samples. This work was supported by grants from the Ministry of Education, Science and Culture of Japan, the Ministry of Agriculture, Forestry and Fisheries of Japan, and PROBRAIN from the Bio-oriented Technology Research Advancement Institution, Japan.

REFERENCES

1. Appleton, D. J., Rand, J. S. and Sunvold, G. D. 2000. Plasma leptin concentrations in cats: reference range, effect of weight

- gain and relationship with adiposity as measured by dual energy X-ray absorptiometry. *J. Feline Med. Surg.* **2**: 191–199.
2. Backus, R.C., Havel, P.J., Gingerich, R.L. and Rogers, Q.R. 2000. Relationship between serum leptin immunoreactivity and body fat mass as estimated by use of a novel gas-phase Fourier transform infrared spectroscopy deuterium dilution method in cats. *Am. J. Vet. Res.* **61**: 796–801.
 3. Burkholder, W.J. and Thatcher, C.D. 1998. Validation of predictive equations for use of deuterium oxide dilution to determine body composition of dogs. *Am. J. Vet. Res.* **59**: 927–937.
 4. Butterwick, R. F. and Hawthorne, A. J. 1998. Advances in dietary management of obesity in dogs and cats. *J. Nutr.* **128** (Suppl): 2771S–2775S.
 5. Considine, R. V., Sinha, M. K., Heiman, M. L., Kriauciunas, A., Stephens, T. W., Nyce, M. R., Ohannesian, J. P., Marco, C. C., McKee, L. J., Bauer, T. L. and Caro, J. F. 1996. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New Engl. J. Med.* **334**: 292–295.
 6. Darnell, J. E. 1996. Reflection on STAT3, STAT5, and STAT6 as fat STATs. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 6221–6224.
 7. Fettman, M. J., Stanton, C. A., Banks, L. L., Hamar, D. W., Johnson, D. E., Hegstad, R. L. and Johnston, S. 1997. Effects of neutering on body weight, metabolic rate and glucose tolerance of domestic cats. *Res. Vet. Sci.* **62**: 131–136.
 8. Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Lallone, R. L., Burley, S. K. and Friedman, J. M. 1995. Weight-reducing effects of the plasma protein encoded by the *obese* gene. *Science* **269**: 543–546.
 9. Hand, M.S., Armstrong, P.J. and Allen, T.A. 1989. Obesity: occurrence, treatment, and prevention. *Vet. Clin. North America - Small Anim. Pract.* **19**: 447–474.
 10. Hawthorne, A.J. and Butterwick, R.F. 2001. Feline body mass index. *Waltham Focus* **10**: 32–33.
 11. Ishioka, K., Soliman, M.M., Sagawa, M., Nakadomo, F., Shibata, H., Honjoh, T., Hashimoto, A., Kimura, K. and Saito, M. 2002. Experimental and clinical studies on plasma leptin in obese dogs. *J. Vet. Med. Sci.* **64**: 349–353.
 12. Iwase, M., Kimura, K., Sasaki, N., Komagome, R., Ishioka, K., Morimatsu, M., Murakami, T. and Saito, M. 2000. Canine leptin: molecular cloning, expression and functional properties of recombinant protein. *Res. Vet. Sci.* **68**: 109–114.
 13. Krook, L., Larsson, S. and Rooney, J.R. 1960. The interrelationship of diabetes mellitus, obesity, and pyometra in the dog. *Am. J. Vet. Res.* **21**: 120–124.
 14. Lee, G. H., Proenca, R., Montez, J. M., Carroll, K. M., Darvishzadeh, J. G., Lee, J. I. and Friedman, J. M. 1996. Abnormal splicing of the leptin receptor in diabetic mice. *Nature (Lond.)* **379**: 632–635.
 15. Lukaski, H.C. and Johnson, P.E. 1985. A simple, inexpensive method of determining total body water using a tracer dose of D₂O and infrared absorption of biological fluids. *Am. J. Clin. Nutr.* **41**: 363–370.
 16. Maffei, M., Halaas, J., Ravussin, E., Pratley, R. E., Lee, G. H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., Kern, P. A. and Friedman, J. M. 1995. Leptin levels in human and rodent - measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. *Nat. Med.* **1**: 1155–1161.
 17. Martin, L., Siliart, B., Dumon, H., Backus, R., Biourge, V. and Nguyen, P. 2001. Leptin, body fat content and energy expenditure in intact and gonadectomized adult cats: a preliminary study. *J. Anim. Physiol. Anim. Nutr.* **85**: 195–199.
 18. Newberne, P.M. 1966. Overnutrition and resistance of dogs to distemper virus. *Fed. Proc.* **25**: 1701–1710.
 19. Rocchini, A. P., Moorehead, C. P., DeRemer, S. and Bondie, D. 1989. Pathogenesis of weight-related changes in blood pressure in dogs. *Hypertension* **13**: 922–928.
 20. Sasaki, N., Shibata, H., Honjoh, T., Kimura, K., Saito, M. and Ohishi, I. 2001. cDNA cloning of feline leptin and its mRNA expression in adipose tissue. *J. Vet. Med. Sci.* **63**: 1115–1120.
 21. Scarlett, J.M. and Donoghue, S. 1998. Associations between body condition and disease in cats. *J. Am. Vet. Med. Assoc.* **212**: 1725–1731.
 22. Sloth, C. 1992. Practical management of obesity in dogs and cats. *J. Small Anim. Pract.* **33**: 178–182.
 23. Tartaglia, L. A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T. and Deeds, J. 1995. Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83**: 1263–1271.
 24. Vaisse, C., Halaas, J.L., Horvath, C.M., Darnell, J. E., Jr., Stoffel, M. and Friedman, J. M. 1996. Leptin activation of STAT3 in the hypothalamus of wildtype and *ob/ob* mice but not *db/db* mice. *Nat. Genet.* **14**: 95–97.
 25. Verploegen, S. A., Plaetinck, G., Devos, R., Van der Heyden, J. and Guisez, Y. 1997. A human leptin mutant induces weight gain in normal mice. *FEBS Lett.* **405**: 237–240.
 26. Zhang, F., Basinski, M. B., Beals, J. M., Briggs, S. L., Churgay, L. M., Clawson, D. K., DiMarchi, R. D., Furman, T. C., Hale, J. E., Hsiung, H. M., Schoner, B. E., Smith, D. P., Zhang, X. Y., Wery, J. P. and Schevitz, R. W. 1997. Crystal structure of the obese protein leptin-E100. *Nature (Lond.)* **387**: 206–209.
 27. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. M. 1994. Positional cloning of the mouse *obese* gene and its human homologue. *Nature (Lond.)* **372**: 425–432.