cDNA Cloning of Feline Leptin and Its mRNA Expression in Adipose Tissue

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ABSTRACT. Leptin, the product of the obese (ob) gene, is an adipocyte-derived hormone involved in regulating food intake and energy expenditure in humans and rodents. To determine the primary structure of feline leptin, we cloned the feline leptin cDNA using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of complementary DNA (cDNA) ends (RACE) methods. The full-length feline leptin cDNA was 2935 bp with a 501 bp open reading frame encoding the precursor peptide of 167 amino acids including 21 residues of signal peptide. The sequence of a 146-amino acid mature leptin was 81.5–91.8% homologous to those of other species. RT-PCR analysis revealed that the leptin mRNA was expressed in adipose tissues and not detected in liver, heart, kidney, lung, pancreas, brain and skeletal muscle. These data show that feline leptin is highly homologous to leptins of other species, and expressed in adipose tissues in cats.

KEY WORDS: adipose tissue, cDNA cloning, feline, leptin.

Leptin, the product of the obese (ob) gene, is a 16 kDa polypeptide synthesized and secreted mainly by adipocytes. The ob gene was originally identified by positional cloning to seek a mutation that caused extreme obesity in the ob/ob mouse [27]. It is well known that leptin is a hormonal signal of adipocyte mass to regulate appetite and energy expenditure [6, 11]. In human and rodents, the circulating levels of leptin are positively correlated with body fat content, implying that blood leptin is a quantitative marker of adiposity [4, 5, 10, 16–18, 21]. In addition, exogenous administration of recombinant leptin reduces food intake and adiposity in rodents [3, 9, 22, 24, 26]. Thus, leptin would be one of the promising molecules not only for diagnosis but also for treatment of obesity in most mammals including companion animals.

There have been several reports on complementary DNA (cDNA) cloning of leptin in various species [8, 13, 15, 19, 23], and we also reported cDNA cloning of canine leptin and the activity of its recombinant protein [14]. In this study, we cloned feline leptin cDNA using reverse transcription-polymerase chain reaction (RT-PCR) and examined tissue distribution of its mRNA by RT-PCR analysis.

MATERIALS AND METHODS

Tissue collection and RNA extraction: Two adult male cats were euthanized by intravenous administration of sodium pentobarbital. Within 30 min after euthanasia, various tissues including adipose tissue were removed and frozen immediately in liquid nitrogen. Total RNA was isolated by using a phenol-guanidine-isothiocyanate reagent (TRIzol, Gibco BRL, Rockville, MD, U.S.A.). The yield of RNA was assessed by measuring absorbance at 260 nm and 280 nm.

Cloning of feline leptin cDNA: Full-length feline leptin cDNA was cloned by the RT-PCR and rapid amplification of cDNA ends (RACE) methods [7] as follows. The mRNA was purified with oligo(dT)-latex beads (Oligotex-dT30 <Super>, Roche Diagnostics, Tokyo) from total RNA extracted from omental adipose tissue, then 1 µg of mRNA was reverse transcribed with oligo(dT)18-adaptor and avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences Inc., St. Petersburg, FL, U.S.A.). For preliminary RT-PCR, four primers (COB1, COB2, COB3, COB4; Table 1) were designed based on the sequence of murine (GeneBank accession number; U18812) and canine (DDBJ accession number; AB020986) leptin cDNA. PCR amplification was performed for 35 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 1 min in a solution (50 µM each primer, and 1.25 units LA Taq DNA polymerase (TAKARA)) containing 1 x PCR buffer, 0.2 µM deoxynucleotide, 2.5 mM magnesium chloride, 0.2 µM each primer, and 1.25 units LA Taq DNA polymerase (TAKARA Shuzo CO., Shiga). The PCR product of the expected size was subcloned into pT7-Blue T vector (Novagen, Madison, WI, U.S.A.). The ligated plasmid was transformed into NovaBlue E. coli strain (Novagen), and then isolated.

5' - and 3'-RACE: RACE amplification using modified adaptor-primers was performed to obtain the both ends of leptin cDNA as shown in Fig. 1 (FL3R and FL5R). To generate 3'-partial cDNA end, 1 µg of mRNA was reverse-transcribed using an oligo(dT)18-adaptor primer. First amplification was performed using FOB1 primer and A1 adaptor primer (Table 1). The PCR conditions were: 94°C for 30 sec, 57°C for 30 sec and 72°C for 3 min (35 cycles). Nested PCR was then carried out using FOB2 primer and A2 primer under the conditions of 94°C for 30 sec, 57°C for 30 sec and 72°C for 3 min (20 cycles). The amplified PCR product of the expected size was subcloned into pT7-Blue T vector.
The ligated plasmids were transformed into E. coli strain, and then isolated. For preliminary sequencing of the FL3R fragment, the inserted plasmid was digested with BamHI, and the digested fragments were self-ligated or ligated to another vector. After partial sequencing of these fragments, feline leptin cDNA-specific primers were designed to determine the entire sequence of FL3R fragment (Table 1).

To generate 5'-partial cDNA end, 1 μg of mRNA was reverse transcribed using COB4 primer and AMV reverse transcriptase. Then, a poly (A) tail was added to this synthesized cDNA using terminal deoxynucleotidyltransferase (TOYOBO CO., Osaka) and 2'-deoxyadenosine 5'-triphosphate. PCR amplification was performed with an oligo(dT)18-adaptor primer, A2 adaptor primer and COB2 primer. The PCR conditions were: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min (35 cycles). The obtained fragments were subcloned and sequenced.

**Table 1. Oligonucleotide sequences used as primers for RT-PCR and RACE method**

| Clone name | Primer name | Primer type | Sequences(5’–3’)
|------------|-------------|-------------|----------------
| FL1        | COB1        | Forward     | TTCCGTGGAGCTGTGAGGA
|            | COB2        | Reverse     | GCCACACCTGTCGAGTGA
| FL5R, FL3R | Oligo(dT) 18-adaptor | Oligo (dT) | ACGGGCCCTCGAAGATTCT(18)
| A1         | Adaptor     | BamHI       | CCGGATCCCTCGAGCTGACGA
| A2         | Adaptor     | BamHI       | CCCTCGAGCTGACGA
| COB3       | Forward     | BamHI       | CCGGATCCCTCGAGCTGACGA
| COB4       | Reverse     | BamHI       | CCGGATCCCTCGAGCTGACGA
| FOB1       | Forward     | BamHI       | GCCTCTAGAAACGTTGCTC
| FOB2       | Forward     | BamHI       | CCGGATCCCTGGAGAGACCTCGGGGA
| FOB3       | Forward     | BamHI       | CATGACGCTACAGATCCAC
| FOB6       | Reverse     | BamHI       | CCTCTAGGTGGAAAGGCG
| FOB7       | Forward     | BamHI       | AGCAATGCTGAGTGC
| FOB10      | Reverse     | BamHI       | TCGTCTGCTTTGCTG
| FOB11      | Forward     | BamHI       | GAGACACCAGGGTGAAG

*Additional restriction enzyme sites (BamHI) are underlined.

**Fig. 1.** Schematic representation of the structure and sequencing strategy for feline leptin cDNA. The open reading frame is depicted by closed boxes. PCR products (solid bar) and sequencing length and directions (arrow) are also indicated.
were sequenced and consensus sequence was adopted. At least three independent PCR fragments (5'-TAATACGACTCACTATAGGG-3') were used for sequencing. RT-PCR analysis of feline leptin mRNA expression: Total RNA (10 µg) was treated with 10 units deoxyribonuclease I (DNase I) (TAKARA Shuzo Co.) to eliminate any contaminating genomic DNA. One microgram of RNA was transcribed using an oligo(dT)18-adaptor, and resulting cDNA was amplified using COB1 and COB2 primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was also evaluated using the primers for human GAPDH (CLONTECH Laboratories Inc., Palo Alto, CA, U.S.A.) as an internal standard. PCR was performed for 35 cycles at 94°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

RESULTS

cDNA cloning and sequence analysis of feline leptin: In order to clone the full length feline leptin cDNA, a cDNA fragment corresponding to a main part of the coding region was firstly amplified from omental adipose tissue mRNA. RT-PCR was performed using primers designed from the sequence of murine and canine leptin cDNA. After confirming the nucleotide sequence of this fragment, feline leptin-specific primers for 3’- and 5’-RACE were designed (Table 1). Next, 3’-RACE amplification was performed between FOB1 primer and A1 primer, and then between FOB2 primer and A2 primer. The PCR product of about 2.4 kbp (FL3R fragment) was digested with BamHI and sequenced. To confirm the complete sequence of the FL3R fragment, it was divided into five parts, amplified with newly designed primers (FOB3, FOB6, FOB7, FOB10, FOB11) and sequenced from both directions (Fig. 1).

Figure 2 shows the complete nucleotide sequence of the cloned feline leptin cDNA and deduced amino acid sequence. The cDNA sequence was submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB041360. The cDNA consisted of a 5’-untranslated region (30 bp), a single open reading frame (501 bp) and the following 3’-untranslated region (2401 bp). A polyadenylation signal (AATAAA) was present at nucleotides 2914–2919. The coding region of feline leptin cDNA was 82.7–93.5% identical to those of other species so far reported (Table 2). The feline leptin cDNA encoded a predicted 167 amino acid polypeptide with a putative signal peptide of 21 amino acids (Fig. 2). The amino acid sequence of feline mature leptin was 81.5–91.8% identical to those of other species (Fig. 3, Table 2). Two cysteine (Cys) residues that are thought to form an intramolecular disulfide bond were conserved in the same positions (Cys117 and Cys167) as in the other species’ sequence (Fig. 3).

Another cDNA clone lacking the codon of CAG at nucleotide 162–164, was also obtained. This cDNA coded a leptin lacking glutamine at position 49 (Gln 49).

Tissue distribution of feline leptin mRNA: To examine the distribution of leptin in feline tissues, feline leptin mRNA was detected by RT-PCR analysis of RNA extracted from various tissues of adult male cats. RNA samples were treated with DNase I to eliminate contaminating genomic DNA (Fig. 4). The expected amplified product (467 bp) was found in omental adipose tissue, but not in heart, lung, liver, kidney, skeletal muscle, pancreas and brain. GAPDH mRNA, used as positive control for RT-PCR, was detected in all tissues.

DISCUSSION

The cDNA cloned from feline white adipose tissue 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. Since the deduced amino acid sequence was highly homologous to leptin of other species so far reported, this was concluded to be feline leptin. Two cysteine residues (Cys 117 and Cys 167) forming an intramolecular disulfide bond were also conserved in feline leptin at the same position of other species, although the C-terminal disulfide bond is not essential for the biological activities of leptin [12].

A cDNA lacking the sequence of CAG encoding Gln 49 was also obtained. The same missing sequence at the identical position was also reported in murine and canine leptins [14, 19, 27]. Since the CAG codon included a possible AG splice-acceptor sequence, there might have been slippage at this site. Although Gln 49 is located in a highly conserved region of the leptin molecule, it has been suggested not to be essential for biological activities of leptin [14].

RT-PCR analysis revealed that leptin mRNA was expressed in adipose tissue of cats, but not in other tissues so far examined. These results are consistent with those reported in other species. Recently, it was reported that leptin mRNA was detected in gastric epithelium [2], placental trophoblast [20], and skeletal muscle [25] in rodents. However no amplified product was detected in cat skeletal muscle by RT-PCR analysis. Although we did not examine the stomach and the placenta tissues in this study, it is likely that adipose tissue is the major site of leptin production in feline, as in other species.

Leptin is now a target molecule for the study and treatment of human obesity since leptin is well known for its role in regulating appetite and whole body energy balance. Many suppliers have been providing leptin determination systems for many species including human, rat, mouse and primates. Most these assay systems for human, for rat, for mouse leptin recognize only human, rat and mouse, respectively. For example, a commercially available radioimmunoassay (RIA) kit for rat leptin shows 100% crossreactivity in rat, but less than 0.2% in human (manufacturer’s instruction). In addition, we confirmed that anti-canine leptin antibody showed differences in immunoreactivity among other species as compared to the canine leptin [14]. Recently, Backus et al. [1] reported serum leptin-like immunoreactiv-
ity in cats was poorly measurable with a RIA based on antisera against mouse, rat, human, and primate leptins, suggesting the low immunoreactivity of these antisera to feline leptin. These facts indicate that species-specific leptin and/or anti-leptin antibody is required for better understanding the physiological and pathological functions of leptin in detail. In this context, in preliminary experiments, we have tried to express the recombinant feline leptin in bacterial cells. The resulting recombinant protein was revealed in the size of approximately 16 kDa, but unfortunately almost all recombinant protein was present in inclusion body. Further studies are underway to obtain the soluble and active feline recombinant leptin, and to prepare for an antibody raised against it.

Fig. 2. Nucleotide and deduced amino acid sequences of feline leptin. Nucleotides are numbered from 5'-cDNA end and amino acid residues from the initiation codon (ATG). The CAG codon of Gln 49 is indicated by hatched box. Polyadenylation signal is underlined. The italic amino acid residues indicate the signal peptide.
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REFERENCES


12. Imagawa, K., Numata, Y., Katsuura, G., Sakaguchi, I., Morita, K. 1998. Feline mature leptin cDNA cloning and deduced amino acid sequence identities with those of other species. Table 2. Nucleotide and deduced amino acid sequence identities of feline mature leptin with those of other species

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Fig. 3. Comparison of amino acid sequence of feline leptin with those of leptins of other species. Dots indicate identical amino acid to feline leptin. Signal peptide of 21 amino acids is boxed and hatched. Two cystein residues at 117 and 167 are indicated by asterisks.


