



NOTE

Immunology

Gene and protein expression of a soluble form of CTLA-4 in a healthy dog

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ABSTRACT. Cytotoxic T lymphocyte associated gene-4 (CTLA-4) is a costimulatory molecule, expressed on the surface of activated T cells that negatively regulates T cell activation. In humans, alternative splicing of the *CTLA-4* gene generates two major isoforms of mRNA, and a soluble form of CTLA-4 (sCTLA-4) was detected in normal human serum. We describe alternatively spliced mRNA expressed in peripheral blood mononuclear cells obtained from a healthy dog lacking the transmembrane domain coded by exon 3 of the *CTLA-4* gene. Immunoprecipitation and western blotting of dog serum revealed a band of approximately 23-kDa, which is consistent with the predicted size, based on the amino acid sequence of the canine sCTLA-4 obtained in this study.

KEY WORDS: CD152, CTLA-4, dog, soluble form, splicing variant

T cells require two signals to become fully activated. The first signal occurs when an antigenic peptide is presented to the antigen-specific T cell receptor (TCR) by the major histocompatibility complex molecules, and the second, a costimulatory signal, is mediated by the interaction of CD28 with CD80 (B7-1) and/or CD86 (B7-2) expressed by antigen-presenting cells (APCs) [1]. Cytotoxic T lymphocyte associated gene-4 (CTLA-4, CD152), a costimulatory receptor expressed on the surface of activated T cells, is a negative regulator of T cell activation [22]. It is a member of the immunoglobulin superfamily and binds to the same B7 family members with much higher affinity [22]. In addition, it has been reported that CTLA-4 is similarly expressed on regulatory T cells, B cells, monocytes and dendritic cells, and that it acts to downregulate cell functions [20].

Alternative splicing of the *CTLA-4* gene generates two major isoforms of mRNA in humans: a full length (fCTLA-4) and a soluble form (sCTLA-4) that lacks exon 3, which encodes the transmembrane domain of the CTLA-4 molecule [8]. sCTLA-4 is a 23-kDa protein and contains a conserved motif (MYPPPY) located in the extracellular domain that is critical for binding to B7 molecules [8]. While low levels of sCTLA-4 have been detected in normal human serum [8], recent studies in humans demonstrated that high concentrations of sCTLA-4 were observed in the serum of patients with various autoimmune diseases, such as systemic lupus erythematosus [7], myasthenia gravis [21], autoimmune thyroid disease [11] and systemic sclerosis [15]. sCTLA-4 is capable of disrupting the B7/CTLA-4/CD28 signaling pathway and has *in vitro* immunomodulatory function [19]. The effect of sCTLA-4 might depend on the activation state of the T cells involved. It was suggested that sCTLA-4 could block B7-CD28 interactions on resting T cells, thereby interfering with T cell costimulation. Alternatively, inhibition of B7-CTLA-4 interactions on activated T cells (the condition under which the full length form of CTLA-4 is expressed) may prevent down-regulation of T cell responses [12, 19]. In patients with autoimmune diseases, it was suggested that sCTLA-4 blocks B7-CTLA-4 interactions, and thereby enhances T cell activation and autoreactivity [15].

Although previous studies revealed genome and amino acid sequences of CTLA-4 in the dog [5], alternatively spliced variants of CTLA-4 and protein expression of sCTLA-4 have not yet been reported in veterinary medicine. Thus, the aim of this study was to evaluate gene and protein expression of sCTLA-4 in the dog.

This study was approved by the Institutional Animal Care and Use Committees at the Obihiro University of Agriculture and Veterinary Medicine. Heparinized peripheral blood was obtained from a clinically normal beagle (female, 5 years of age) that was cared for at the Obihiro University of Agriculture and Veterinary Medicine and diluted with an equal volume of saline. Peripheral

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blood mononuclear cells (PBMCs) were separated using Ficoll-Paque (Lymphoprep™, Axis-Shield PoC AS, Oslo, Norway) density gradient centrifugation [6]. Following centrifugation at $800 \times g$ for 20 min at room temperature, PBMCs were collected and washed twice with saline.

Total RNA was extracted from PBMCs, using the TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. The extracted total RNA was treated with the TURBO DNA-free Kit (Ambion, Austin, TX, U.S.A.) to remove contaminating genomic DNA. Reverse transcription of 50 ng RNA was performed using the PrimeScript™ RT reagent Kit (Takara Bio Inc., Otsu, Japan) in a total volume of 50 μ l at 37°C for 15 min followed by denaturation at 85°C for 15 sec in a thermal cycler (Applied Biosystems, South San Francisco, CA, U.S.A.). Complementary DNA was stored at -30°C until use.

For reverse transcriptase (RT) PCR, oligonucleotide primers were designed based on canine *CTLA-4* gene sequences (NM001003106 and NC006619) obtained from the GenBank Database. The primer set was f100 (5'-TTCTCCAAAGGGATGCATGT-3'), r694 (5'-TCACATTCTGGCTCAGTTGG-3') and the expected amplicon was 536 bp in length. To prevent amplification of chromosomal DNA, the primers were designed to anneal at the junctions of two exons, respectively. The 20 μ l PCR reaction mixture contained 4.0 μ l of 5 \times buffer, 2.0 μ l of 2 mM dNTP, 0.5 U of Taq polymerase (Promega Corporation, Madison, WI, U.S.A.), 10 pmol of each primer, 10.9 μ l of distilled water and 1.0 μ l of cDNA template. Cycling conditions were as follows: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 90 sec; and a final extension at 72°C for 5 min and cooling to 4°C. All amplicons were electrophoresed on a 1.2% agarose gel in Tris/Borate/Ethylenediaminetetraacetic acid (TBE) buffer and visualized by the Midori Green DNA stain (NIPPON Genetics, Tokyo, Japan) under 470 nm green LED light, using the FAS-Digi system (NIPPON Genetics). The PCR amplicon was analyzed by plasmid sequencing, as described below.

To determine the entire coding sequence of *CTLA-4*, a PCR was performed using the following primer set: f11 (5'-GGTTTYGCTCTRCTTCCTGA-3') and r793 (5'-AAAGTTYAATTGCCTCAGCT-3') as previously described [10]. The product was cloned in the pGEM-T easy vector (Promega Corporation) and then subjected to Hokkaido System Science (Sapporo, Japan) for DNA sequencing. Nucleotide sequence results were confirmed using the BLAST search program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) for comparison with other known sequences and aligned using CLUSTAL W software (<http://www.genome.jp/tools/clustalw/>).

Serum sCTLA-4 protein was evaluated by immunoprecipitation and western blotting as follows: Dynabeads Protein G (Life Technologies, Carlsbad, CA, U.S.A.) were conjugated to 5 μ g anti-human CTLA-4 polyclonal antibody (H-126, Santa Cruz Biotechnology, Dallas, TX, U.S.A.) according to the manufacturer's protocol. Briefly, 800 μ l of healthy dog serum was incubated, with rotation for 10 min at room temperature, with anti-human CTLA-4 pAb-dynabeads. The beads were washed three times with phosphate-buffered saline (PBS), bead-bound immune complexes were resuspended in 20 μ l of elution buffer, and the beads were removed magnetically. The supernatant was combined with an equal volume of 2 \times sodium dodecyl sulphate (SDS) sample buffer (EzApply, Takara Bio Inc.) and boiled at 95°C for 5 min. Isotype-matched antibody (Santa Cruz Biotechnology) was conjugated in the same experiment as the control. The samples were subjected to western blotting. Recombinant human CTLA-4 protein encoding the extracellular domain of the CTLA-4 molecule (Sino Biological Inc., Beijing, China) was used as a positive control. In addition, antibody cross-reactivity was assessed using the recombinant canine CTLA-4 protein (Sino Biological Inc.).

Following immunoprecipitation, eluted samples were separated on 4–15% SDS-polyacrylamide gel electrophoresis (PAGE) gels using a Mini-PROTEAN Tetra system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The separated components were electroblotted onto polyvinylidene fluoride (PVDF) membranes, which were then blocked with EzBlock Chemi (ATTO Corporation, Tokyo, Japan) in PBS at room temperature for 60 min and incubated with primary antibody against human CTLA-4 (H-126) at a dilution of 1:200 in PBS at room temperature for 60 min. After washing, the membrane was incubated with horseradish peroxidase (HRP)-labeled mouse anti-rabbit IgG antibody (TrueBlot®, Rockland Immunochemicals, Limerick, PA, U.S.A.) at a dilution of 1:5,000 in PBS at room temperature for 60 min. After washing, the blots were developed using a commercially available chemiluminescence detection kit (EzWestBlue, ATTO Corporation) according to the manufacturer's instructions.

To characterize the alternate splice variant of canine *CTLA-4* mRNA, RT-PCR was performed using the f100/r694 primer set. Amplification by RT-PCR revealed two bands of approximately 450 and 550 bp (Fig. 1). The product obtained from another RT-PCR that was used to determine the entire coding sequence of canine *CTLA-4* was subcloned into the cloning vector and sequenced. As a result, two cDNA fragments were obtained. One fragment shared 100% identity with the canine *CTLA-4* mRNA (NM 001003106), and the other was generated by alternative splicing of the canine *CTLA-4* mRNA where exon 3 (that encodes the transmembrane region) is spliced out (Fig. 2). The sequence of the full length and alternatively spliced canine *CTLA-4* genes were submitted to the DNA data bank of Japan (DDBJ; <http://www.ddbj.nig.ac.jp>) under accession numbers LC191194 and LC191195, respectively. Two amplicons obtained by RT-PCR using the f100/r694 primer set were confirmed as identical to the full length and alternatively spliced canine *CTLA-4* by sequencing, and the splicing variant of canine *CTLA-4* has been observed in three additional healthy dogs. These sequences were identical to the alternatively spliced canine *CTLA-4* gene obtained in this study (data not shown). A comparison of the canine sCTLA-4 molecule with its full length form is presented in Fig. 3. Immunoprecipitation and western blotting using an anti-CTLA-4 antibody were performed to determine whether a sCTLA-4 was detectable in canine serum. A band of approximately 23-kDa was observed, which is consistent with the predicted size based on the amino acid sequence of canine sCTLA-4 obtained in this study (Fig. 4a). In addition, the antibodies assessed here demonstrated cross-reactivity with the recombinant canine CTLA-4 protein (Fig. 4b). The band of approximately 23-kDa has been observed in two additional healthy dogs (data not shown).

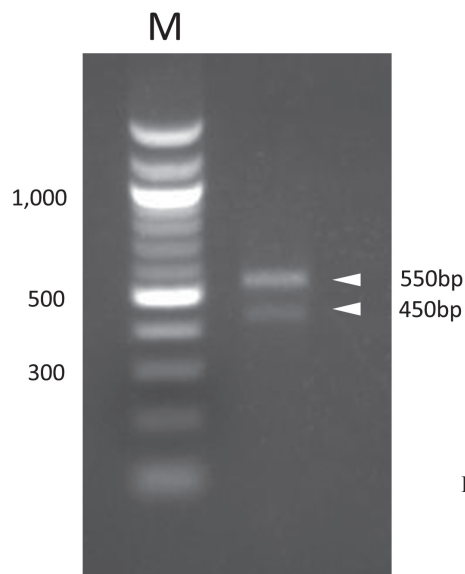


Fig. 1. RT-PCR amplification of canine *CTLA-4* mRNA. Bands corresponding to 450 and 550 bp were observed. M; 100 bp DNA maker.

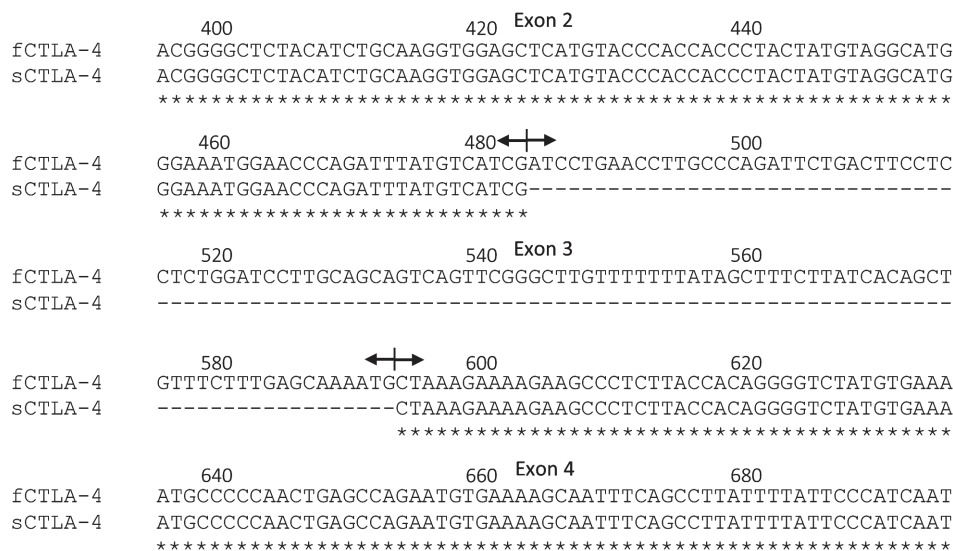


Fig. 2. Comparison of the partial nucleotide sequence of the full length (fCTLA-4) and soluble (sCTLA-4) forms of *CTLA-4* transcripts.

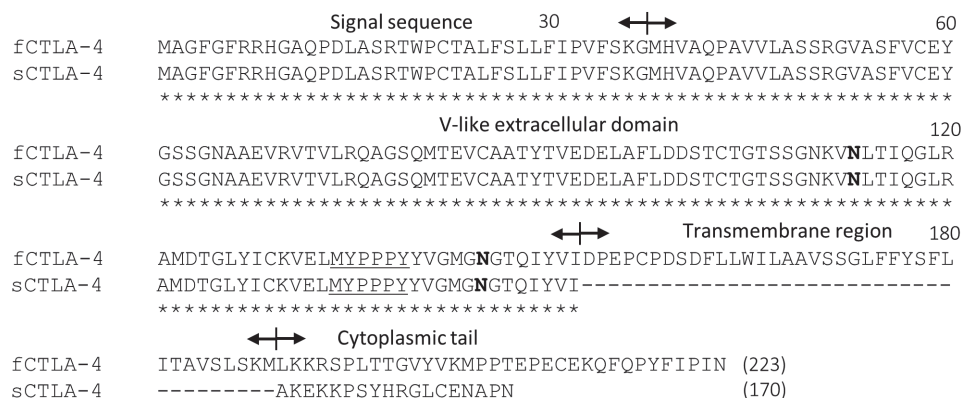


Fig. 3. Comparison of the predicted amino acid sequences of the full length (fCTLA-4) and soluble (sCTLA-4) forms of CTLA-4. Expected signal sequence, V-like extracellular domain, transmembrane region and cytoplasmic tail are shown. The MYPPPY hexapeptide motif is underlined. Bold amino acids represent predicted sites for N-linked glycosylation. The numbers at the end show the total number of amino acid residues.

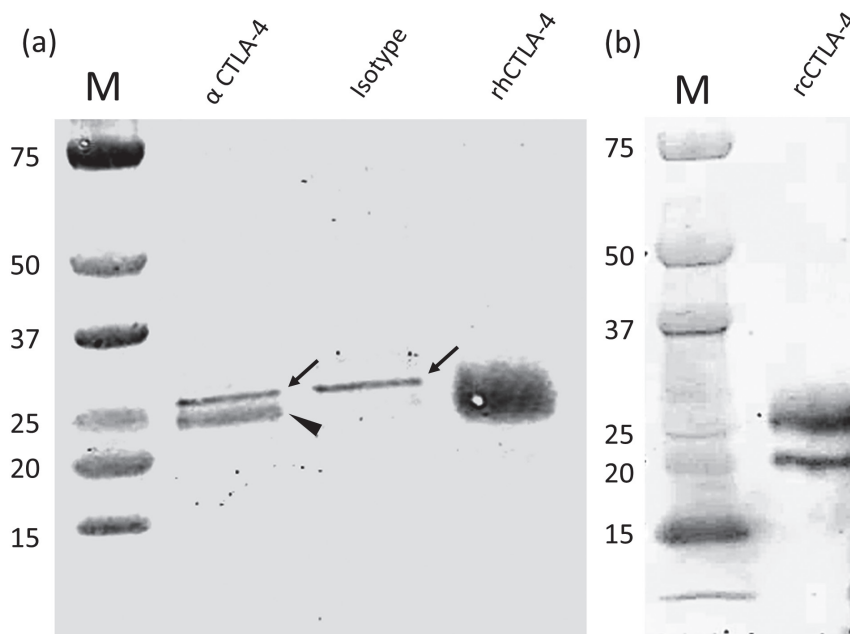


Fig. 4. a: Western blot of sCTLA-4 derived from healthy dog serum using the anti-human CTLA-4 antibody (α CTLA-4). Arrowhead; the 23-kDa soluble species. The band in the lane labeled Isotype indicates the negative control using an isotype-matched antibody. rhCTLA-4 is the recombinant human CTLA-4 protein. The 25-kDa band represents rabbit Ig carried over in the precipitation (arrow). b: Antibody cross-reactivity was assessed using recombinant canine CTLA-4 protein (rcCTLA-4). M; protein molecular marker (kDa).

CTLA-4 is an immunoregulatory receptor primarily expressed on the surface of activated T cells [1]. CTLA-4 transmits signals that inhibit T cell activation by binding to CD80 and CD86 [22]. The mRNA encoding CTLA-4 consists of four exons: exon 1 encodes a leader peptide, exon 2 the ligand-binding domain, exon 3 the transmembrane domain and exon 4 the cytoplasmic tail. Recently, a soluble form of CTLA-4 was described, which is generated by alternative splicing of exon 3 mRNA, and was found to be a functional molecule with the ability to bind to CD80 and CD86 [8, 19]. This splicing event introduces a 110-bp deletion corresponding to the entire transmembrane domain of the CTLA-4 molecule and a frameshift mutation within the cytoplasmic domain of sCTLA-4 [12]. The present study demonstrated that PCR amplification of the coding sequence of CTLA-4 in PBMCs obtained from a healthy dog revealed two transcripts, and sequencing demonstrated that the larger transcript is the full length of CTLA-4 and the shorter transcript is a spliced variant in which exon 3, that codes for the transmembrane region is deleted. In addition, it was known that the splicing event introduces a frameshift mutation into the cytoplasmic domain of human sCTLA-4, and the change in the reading frame produces an amino acid tail that is unique to the sCTLA-4 molecule [12]. In this study, the same frameshift mutation was observed in canine sCTLA-4, and the unique cytoplasmic tail produced by the spliced transcript was highly similar to that of human sCTLA-4. However, the tail of the canine sCTLA-4 was four amino acids shorter than that of the human sCTLA-4 owing to the introduction of a TGA termination codon at nucleotide position 511 of the canine gene. Although a previous study described the nucleic acid sequences of alternate transcripts of CTLA-4 in the human, mouse and rat [12], the shortening of the cytoplasmic tail in the dog transcript was not observed. The sCTLA-4 molecules contain a MYPPPY peptide motif located in the extracellular domains of the full length CTLA-4, and sCTLA-4 can bind to B7 costimulatory ligands on APCs [14]. The canine sCTLA-4 obtained in this study contains the same motif, and thus it was believed that the canine sCTLA-4 could also bind to B7 ligands. It is known that the intracellular portion of the full length CTLA-4 mediates signal transduction and intracellular trafficking [10], however, the role of the sCTLA-4 cytoplasmic tail has not been described, and it is unknown whether the absence of the four amino acids in the C-terminus of the canine sCTLA-4 protein affects the function of the canine sCTLA-4.

Western blotting of serum from a normal dog demonstrated an immunoreactive species of approximately 23-kDa when assessed using anti-human CTLA-4 antibodies. It was known that the spliced transcript of the human sCTLA-4 produces a 23-kDa product, and low levels of sCTLA-4 are detected in normal human serum [8]. On the other hand, increased levels of serum sCTLA-4 are observed in several human autoimmune diseases [14, 16], and in some, serum sCTLA-4 levels correlate with disease severity and concentration of serum autoantibodies [2, 15, 21]. Although the mechanism of increased serum concentrations of sCTLA-4 in patients with autoimmune diseases has not been fully elucidated, the presence of sCTLA-4 was suggested to be a relevant mechanism for the perpetuation of immunological injury and possibly related to disease outcome [17]. In addition, an individual's genetic background, including CTLA-4 polymorphism, might be associated with the expression of the sCTLA-4 transcript in some autoimmune diseases [16]. Various autoimmune diseases are also recognized in nonhuman species, specifically domestic animals [4], and a further study is necessary to evaluate the usefulness of the serum sCTLA-4 level as a biomarker for the management of autoimmune diseases in the dog.

The biological significance of increased serum sCTLA-4 levels is not yet fully understood. A recent study suggested that sCTLA-4 may have important immunoregulatory functions and its effect may be dependent upon the activation state of the cells involved. In short, on resting cells, sCTLA-4 could block CD80/CD86-CD28 interactions, which could interfere with T cell costimulation. Alternatively, inhibition of CD80/CD86-CTLA-4 interactions on activated T cells may prevent downregulation of T cell responses [19]. Abatacept, a fusion protein consisting of the extracellular domain of CTLA-4 and a modulator of T cell activation, is an approved therapeutic agent for treatment of human rheumatoid arthritis and several other autoimmune diseases [3, 9, 13]. In addition, previous studies documented in the veterinary literature indicated that recombinant canine CTLA4Ig protein might be effective in autoimmune disease therapy [18] and that sCTLA-4 could be a novel therapeutic agent for autoimmune diseases in the dog.

In conclusion, our data demonstrate mRNA and protein expression of a soluble CTLA-4 variant in a healthy dog. A further study is required to evaluate serum sCTLA-4 levels in various autoimmune diseases in dogs.

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