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Studies of cocktail therapy with multiple cytokines for neoplasia or infectious disease of the dog I. cDNA cloning of canine IL-3 and IL-6

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This paper describes the cloning and sequence analysis of the cDNAs encoding the canine homologues of interleukin-3 (IL-3) and interleukin-6 (IL-6). The coding sequences for canine IL-3 and IL-6 were obtained by using the reverse transcription polymerase chain reaction (RT-PCR) with RNA harvested from canine peripheral blood mononuclear cells (PBMCs). Canine IL-3 cDNA includes a single open reading frame of 432 nucleotides, which encodes a 143 amino acid polypeptide and has 44.7, 42.4, 37 and 23.7% homology with the cow, sheep, human and rat IL-3 sequences, respectively. Canine IL-6 cDNA (GenBank accession number; AF275796) encodes a putative 20-amino acid signal peptide followed by a 187-amino acid mature protein. The predicted amino acid sequence of canine IL-6 shares 60.4, 77.2, 71.0, 55.8 and 42.0% sequence identity with those of human, feline, porcine, sheep and rat IL-6, respectively.

Key words: Cytokine, cDNA, cloning, PCR, IL-3, IL-6, dog

Introduction

Interleukin-3 (IL-3) is a glycoprotein which has a broad spectrum colony stimulating effect. IL-3 acts on primitive pluripotent stem cells and progenitor cells of every lineage, except for those committed to the T-lymphoid and B-lymphoid lineages. IL-3 is also called multi-colony stimulating factor (CSF), mast-cell growth factor or stem cell activating factor and has many other names [15]. Thus, IL-3 can stimulate the generation and differentiation of macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytes and erythroid cells [24]. Furthermore, IL-3 synergizes with other cytokines to support the complete and amplified development of several hematopoietic lineages, including those of eosinophils and macrophages [16,20]. Activated T-cells are mainly responsible for secreted IL-3 [9,22,23] though small amount is produced by activated natural killer (NK) cells, mast cells and eosinophils. IL-3 is a important growth factor that ligand the immune system and homeostasis in non-immune tissues. Genes coding for mouse, rat, human, gibbon, rhesus monkey and sheep IL-3 have been cloned and the recombinant protein expressed [2,4,5,7,14,28]. IL-3 is a relatevely small protein, with a polypeptide chain ranging from 140 to 166 amino acids.

IL-6 also has been called by a variety of names, such as interfeon-β_2 (IFN-β_2), T-cell replacing factor (TRF)-like factor, B-cell differentiation factor (BCDF), BCDF2, 26-kDa protein, B-cell stimulatory factor-2 (BSF-2), hybridoma-plasmacytoma growth factor (HPGF or IL-HP1), hepatocyte stimulating factor (HSF) and monocyte-granulocyte inducer type 2 (MGI-2). However, molecular cloning of IFN-β_2, 26-kDa protein and BSF-2 revealed that all these molecules are identical. IL-6 is a multifunctional cytokine, which is produced by both lymphoid and non-lymphoid cells and regulates immune responses [26], acute-phase reactions [8,21] and haematopoiesis [10]. IL-6 also has roles as an autocrine growth stimulator in a number of tumors, most notably plasmacytomas and myelomas [17,25] and for some normal cell types. It also has a number of functions in the endocrine and nervous systems. Over-expression of IL-6 is known to be an important feature of the pathogenesis of a number of inflammatory diseases, such as rheumatoid arthritis, glomerular nephritis and psoriasis.

We cloned the full coding region of canine IL-3 and IL-6 from peripheral blood using PCR (polymerase chain reaction) assay and performed nucleotide sequence analysis to allow comparison to be made with other species.

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Materials and Methods

Part A. Canine IL-3 cDNA amplification

Preparation of blood cells

A normal healthy adult dog served as a blood donor. Peripheral blood mononuclear cells (PBMCs) were seperated from approximately 30 ml of venous blood supplemented with 3.5 ml citrate phosphate dextrose acid (CPDA) as an anti-coagulant. PBMCs were separated by Ficoll Paque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation at 1800 rpm, 20 min [3]. Cell viabiliy was determined by the trypan blue dye exclusion method. Cells were washed twice with phosphate-buffered saline (PBS) and plated to a concentration of 1×10^6 cells per ml in RPMI 1640 (GIBCO, Grand Island, USA) supplemented with 10% fetal calf serum (FCS) and 50 μg/ml of gentamicin. In order to stimulate the T-lymphocytes, each of the following reagents-1) 10 μg/ml concanavaline A (ConA), 2) 10 μg/ml lipopolysaccharide (LPS) or 3) 10 ß¹/ml ConA plus 10 ng/ml phorbol 12, 13-myristate acetate (PMA)-was added to the medium. These T-lymphocytes were then cultured for 2 h, 4 h and 7 h at 37°C in a humidified incubator with a 5% CO_2 atmosphere. After cultivation, the canine cells were collected by centrifugation and then quickly frozen in liquid nitrogen.

Isolation of RNA and the preparation of cDNA

Total RNA was isolated from lymphocytes stimulated with ConA, LPS or ConA plus PMA using TRIzol (Gibco, NY, USA). The RNA concentration was approximately 0.25 μg/μl and 2 μg of RNA was used in the synthesis of first strand cDNA using moloney murine leukemia virus reverse transcriptase (M-Mulv RT) and oligo$(dT)_{18}$ primer, according to the manufacturer's instructions.

Polymerase chain reaction

The following primer pairs were used for the PCR reaction. The foward BamHIcaIL3-F primer (5'-CCG GGA TCC AGC AGC TTC CCC ATC CTG CAC-3' nt; 52-71) and the reverse HindIIIcaIL3-R primer (5'-CCG AAG CTT AGG CCC CAT GAT GAG AAG GCC-3' nt; 505-525) were synthesized based on the canine IL-3 sequence (GeneBank accession number; AF250764) and BamHI and HindIII restriction sites were added to the 5'-ending region of these two primers for vector ligation. Using these primers, a 480 bp fragment, including the whole coding sequence of canine IL-3, was expected to be amplified. The PCR amplification was performed for 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min with an additional extension step at 72°C for 10 min.

Part B. Canine IL-6 cDNA amplification

Preparation of canine blood cell

A normal healthy adult dog served as a blood donor. PBMCs were separated from approximately 10 ml of venous blood supplemented with 1.14 ml of CPDA as anti-coagulant. Ficoll-Paque gradient centrifugation was then performed. PBMCs were washed twice with PBS and adjusted to 1×10^6 viable cells per ml in RPMI 1640 medium supplemented with 10% FCS and 50 μg/ml of gentamicin, and stimulated with 5 μg/ml of LPS at 37°C in a humidified incubator with 5% CO_2 atmosphere. After cultivating for 4 hours, the canine PBMCs were collected by centrifugation and then quickly frozen in liquid nitrogen. Total RNA extraction and cDNA preparation was performed as described above.

Polymerase chain reaction

Primer pairs were prepared based on the sequence of canine IL-6 mRNA (GenBank accession number; CFU12234). The primer sequences used for amplifying of canine IL-6 were 5'-ATG AAC TCC CTC TCC ACA AG-3' (il6S; nt. 58-77) and 5'-CTA CAT TAT CCG AAC AGC CC-3' (il6R; nt. 662-681). Using these primers, we expected about 620 bp fragments, including the whole coding sequence of canine IL-6, to be amplified. The cDNA was amplified by PCR in a final volume of 50 μl, using the primer pairs (1.0 uM each), *Taq* polymerase (1.5 units), and the reagents recommended by the manufacturer (Takara, Otsu, Japan). Samples were subjected to an initial denaturation at 94°C for 5 min followed by 30 cycles of amplification, each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with an additional final 10-min incubation at 72°C to complete all extensions.

Cloning and nucleotide sequence analysis

Eight microliter aliquots of PCR products were separated electrophoretically on a 1.5% agarose gel stained with ethidium bromide (EtBr) and visualized under UV light. The amplified DNA was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA), and the recombinant vector transformed into *Escherichia coli* TOP10 (Invitrogen). Transformed *E. coli* TOP10 cells were then plated onto Luria-Bertani (LB) agar plates containing ampicillin (50 μg/ml) and incubated overnight at 37°C. Several clones were sequenced using the M13 forward and the M13 reverse universal primers derived from the vector sequence.

Results

Using ConA-stimulated canine PBMC cDNA as a template, PCR amplification of cDNA was performed using the BamHIcaIL3-F and HindIIIcaIL3-R primer pair

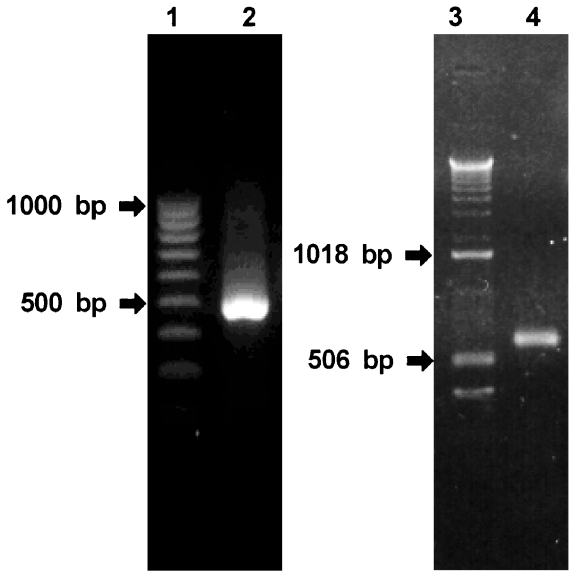

Fig. 1. Electrophorectic analysis of canine IL-3 and IL-6 PCR products with 1.5% agarose gel. 100 bp DNA ladder (lane 1), the amplified 480-bp PCR product using PBMC-cDNA stimulated ConA as a template with BamHIcaIL3-F and HindIIIcaIL3-R primers (lane 2), 1kb DNA ladder (lane 3) and the 620-bp PCR product using PBMC-cDNA stimulated LPS as a template with il6S and il6R primers (lane 4).

to determine the whole sequence of canine IL-3. ConA stimulation with an incubation time of 4h produced a more sensitive PCR reaction than obtained with LPS or ConA plus PMA stimulation with incubation times of 2h, 4h and 7h (data not shown). As expected from the design of the primers BamHIcaIL3-F and HindIIIcaIL3-R, a single band of approximately 480-bp was observed (Fig. 1, lane 2).

To determine the canine IL-6 protein coding sequence, single stranded cDNA was made from total RNA of LPS-stimulated PBMCs and amplified using the polymerase chain reaction with il-6S and il-6R primers derived from the sequences of canine IL-6 mRNA. Agarose gel electrophoresis of the IL-6 PCR reaction products also revealed a single band of about 620-bp, as expected (Fig. 1, lane 4).

Products obtained by PCR were purified and ligated directly into pCR2.1 vector (Invitrogen) and the nucleotides of each PCR product were sequenced in both directions to determine their identities using M13 forward and reverse universal primers derived from the vector sequence.

The canine IL-3 PCR product of 480 bp that encompassed the full coding region was also amplified. The coding region of the canine IL-3 gene includes a single open reading frame of 432 nucleotides, which encodes a 143 amino acid polypeptide. Comparing sequence homology at the nucleotide level with other species in the coding region we obtained 67.1, 63.2, 56.3

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001  ATG AGC AGC TTC CCC ATC CTG CAC CTT CTC TTG CTC CTG CTT GGA TGC CAA GTC CCA CAG
       M   S   S   F   P   I   L   H   L   L   L   L   L   G   C   Q   V   P   Q

061  GCA CAG GGG AGG CCT TTT TCG ACA GAC CTG CCT AAG CAA TAC TTC ACA ATG ATC AAT GAA
       A   Q   G   R   P   F   S   T   D   L   P   K   Q   Y   F   T   M   I   N   E

121  ATT ATG GAG ATG TTA AAC AAG TCA CCC TCG CCT TCA GAA GAA CCC TTG GAC TCA AAT GAG
       I   M   E   M   L   N   K   S   P   S   P   S   E   E   P   L   D   S   N   E

181  AAA GAG ACC TTG CTG GAA GAT ACC CTT CTG AGG CCA AAC CTG GAT GTA TTC TTG AAT GCT
       K   E   T   L   L   E   D   T   L   L   R   P   N   L   D   V   F   L   N   A

241  TCC AGC AAA TTC CAC AAA AAT GGA TTG CTA ATC TGG AAT AAT CTT AAG GAA TTC CTG CCA
       S   S   K   F   H   K   N   G   L   L   I   W   N   N   L   K   E   F   L   P

301  CTC CTG CCC ACT CCC ACA CCC AGG GGA GAA CCA ATC TCT ATC ATG GAG AAT AAT TGG GGT
       L   L   P   T   P   T   P   R   G   E   P   I   S   I   M   E   N   N   W   G

361  GAT TTC CAA AGG AAA TTG AAA AAA TAT CTG GAA GCC CTT GAT AAC TTT CTG AAT TTC AAG
       D   F   Q   R   K   L   K   K   Y   L   E   A   L   D   N   F   L   N   F   K

401  AAC AAA CCC TGA GTC CTA GAA TTC TCA GTC TGA GGC CTT CTC ATC ATG GGG CCT T
       N   K   P   *
```

Fig. 2. The nucleotide sequence and the predicted amino acid sequence of canine IL-3. The deduced amino acid sequence is shown by the single-letter amino acid code under the nucleotide sequence and the stop codon is indicated by an asterisk (*). The predicted amino acid terminus (Arg 24) of mature IL-3 is marked with a triangle (▶). Potential N-glycosylation sites are marked with stars (★).

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001  ATG AAC TCC CTC TCC ACA AGC GCC TTC TCC CTG GGG CTG CTC CTG GTG ATG GCT ACT GCT
       M   N   S   L   S   T   S   A   F   S   L   G   L   L   L   V   M   A   T   A

061  TTC CCT ACC CCG GGA CCC CTG GCA GGA GAT TCC AAG GAT GAT GCC ACT TCA AAT AGT CTA
       F   P   T   P   G   P   L   A   G   D   S   K   D   D   A   T   S   N   S   L

121  CCA CTC ACC TCT GCA AAC AAA GTG GAA GAA CTG ATT AAG TAC ATC CTC GGC AAA ATC TCT
       P   L   T   S   A   N   K   V   E   E   L   I   K   Y   I   L   G   K   I   S

181  GCA CTG AGA AAG GAG ATG TGT GAC AAG TTT AAC AAG TGT GAA GAC AGC AAA GAG GCA CTG
       A   L   R   K   E   M   C   D   K   F   N   K   C   E   D   S   K   E   A   L

241  GCA GAA AAT AAC CTA CAT CTT CCC AAA CTG GAG GGA AAA GAT GGA TGC TTC CAA TCT GGG
       A   E   N   N   L   H   L   P   K   L   E   G   K   D   G   C   F   Q   S   G

301  TTC AAT CAG GAG ACC TGC TTG ACA AGA ATC ACT ACC GGT CTT GTG GAG TTT CAG CTA CAC
       F   N   Q   E   T   C   L   T   R   I   T   T   G   L   V   E   F   Q   L   H

361  CTG AAT ATC CTC CAG AAC AAC TAT GAG GGT GAT AAG GAA AAT GTC AAG TCT GTG CAC ATG
       L   N   I   L   Q   N   N   Y   E   G   D   K   E   N   V   K   S   V   H   M

421  AGT ACC AAG ATC CTG GTC CAG ATG CTA AAG AGC AAG GTA AAG AAT CAG GAT GAA GTG ACC
       S   T   K   I   L   V   Q   M   L   K   S   K   V   K   N   Q   D   E   V   T

481  ACT CCT GAC CCA ACC ACA GAC GCC AGC CTG CAG GCT ATC TTG CAG TCG CAG GAT GAG TGG
       T   P   D   P   T   T   D   A   S   L   Q   A   I   L   Q   S   Q   D   E   W

541  CTG AAG CAC ACA ACA ATT CAC CTC ATC CTG CGG AGT CTG GAG GAT TTC CTG CAG TTC AGT
       L   K   H   T   T   I   H   L   I   L   R   S   L   E   D   F   L   Q   F   S

601  CTG AGG GCT GTT CGG ATA ATG TAG
       L   R   A   V   R   I   M   *
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Fig. 3. The nucleotide sequence and predicted amino acid sequence of canine IL-6. The deduced amino acid sequence is shown by the single-letter amino acid code under the nucleotide sequence and the stop codon is indicated by an asterisk (*). The positions at which the previously reported canine nucleic acid and amino acid sequences (GeneBank accession number; U12234) slightly differ from the sequences of PCR results are indicated above and below the PCR sequences. This canine IL-6 cDNA sequence was deposited in the GeneBank nucleotide database under accession number AF275796. The predicted amino acid terminus (Phe 21) of mature IL-6 is marked with a triangle (▶).

and 37.7% in cow, sheep, human and rat, respectively. The deduced amino acid sequence includes one possible N-glycosylation site (Fig. 2, marked with a star). Canine IL-3, like bovine and ovine IL-3, lacks the cystein residues found in human and rat IL-3 proteins.

The determined nucleotide sequence for canine IL-6, produced by PCR amplification, is shown in Fig. 3. It is almost identical with the previously reported canine IL-6

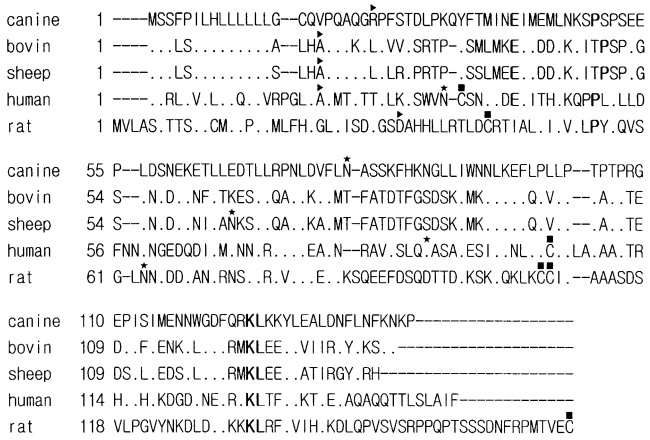

Fig. 4. The amino acid sequence of canine IL-3 gene was aligned with those of cow, sheep, human and rat. Amino terminus of the mature proteins (▶), potential N-glycosylation sites (★), and conserved cysteine residues (■) are indicated. Dots indicate identities with amino acids of the canine IL-3 sequence. Gaps were introduced in sequences to maximize alignment (–). Those amino acids reported to play a important role in regulating the activity of human protein, together with the overlapping bovine, ovine and canine residues are shown in bold.

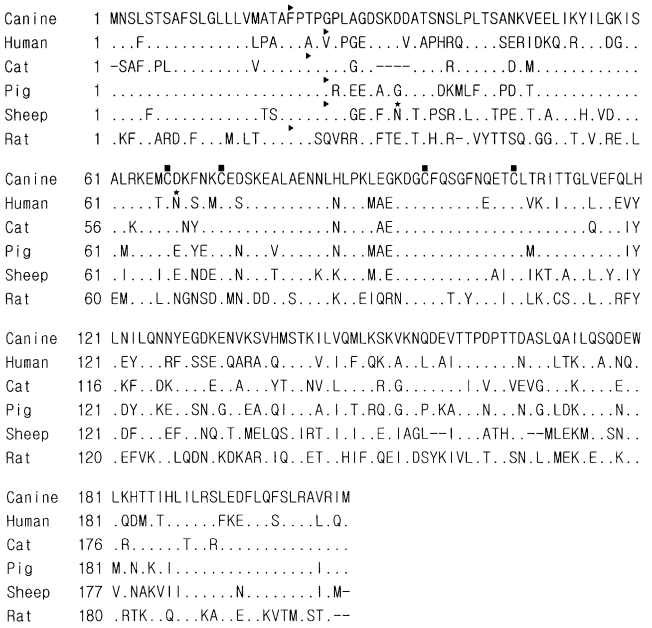

Fig. 5. The amino acid sequence of canine IL-6 gene was aligned with those of its human, cat, pig, sheep and rat counterparts. Amino terminus of the mature proteins (▶), potential N-glycosylation sites (★), and conserved cysteine residues (■) are indicated. Dots indicate identities with amino acids of the canine IL-6 sequence. Gaps were introduced in sequences to maximize alignment (–).

mRNA (Genebank accession number; U12234) except for two positions, a G at position 540 instead of a C residue and a C at position 541 instead of G residue. The resulting peptide has two substitutions of a Cys residue for Trp 180 and a Val residue for Leu 181, which suggests a higher degree of homology than observed in other species. The canine IL-6 cDNA sequence elucidated in this study was deposited in the GeneBank nucleotide database under accession number AF275796. An open reading frame begins with the start codon ATG and ends at the stop codon TAG, which is 621 bp long and has a 75, 84, 80, 74 and 58 % sequence homology with those of human [27], feline [19], porcine [13], sheep [1] and rat [18] IL-6, respectively. The deduced amino acid sequence does not include a possible N-glycosylation site. It encodes a 20-amino acid signal peptide followed by a 187-amino acid mature protein (Fig. 3, triangle).

Discussion

We amplified the full coding region of canine IL-3 and IL-6 from the blood cells of a dog by PCR. The canine IL-3 open reading frame begins with an ATG start codon and ends at a TGA stop codon. A computer-assisted alignment of the canine, bovine, ovine, human and rat IL-3 amino acid sequences (Fig. 4) revealed a low level of identity among these species. IL-3 codes for a polypeptide of 143 amino acids, which is 1, 3, 9 and 23 amino acids shorter, respectively, than the deduced bovine, ovine, human and rat IL-3 sequences. Moreover, the nucleotide sequence homology varied from 37% to 67% among these species while at the amino acid level canine IL-3 shows identities of 44.7%, 42.4%, 37% and 23.7%, with the cow, sheep, human and rat IL-3 sequences. This inter-species divergency made it difficult to amplify canine IL-3 cDNA with primer pairs based on the sequences of the other species.

The putative encoded protein consists of a leader peptide of 23 amino acids, which is probably cleaved between the glycine and arginine residues. Canine IL-3 signal peptide shares 66.7% and 70.8% homology with bovine and ovine IL-3, but less than 50% with human IL-3 and no significant homology with rat IL-3. The mature protein has one potential glycosylation site but previous studies have shown that glycosylation dose not influence the role of the mature protein [2]. The cysteine residues conserved in human and rat IL-3 are absent in canine, bovine and ovine protein but the predicted secondary structure of canine IL-3 protein is a four alpha-helix topology, as is human protein. Though inter-species comparison showed low identity some of the residues are reported to play a critical role in modulating the biological activity of the human protein, such as proline, lysine and leucine, and these are also conserved in the canine IL-3 protein [11,12].

A comparison of the amino acid sequences of IL-6 in other species is shown in Fig. 5. The canine IL-6 cDNA consists of an open reading frame of 207 amino acids, in

which a putative 20-amino acid signal sequence precedes Phe-21 at the amino terminus of the 187 amino acid mature protein. The predicted amino acid sequence of canine IL-6 shares 60.4, 77.2, 71.0, 55.8 and 42.0% sequence identity with those of human, feline, porcine, sheep and rat IL-6, respectively. IL-6 sequences are not extensively conserved between species, but four cystein residues forming disulfied bridges at positions 67-73 and 97-106 are well conserved.

Acknowledgments

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