Cloning of cDNA Encoding Canine Endothelin Receptors and Their Expressions in Normal Tissues

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ABSTRACT. The receptors for endothelin (ET) family, ET_A and ET_B, were molecularly cloned and the expression of ET_A and ET_B as well as preproendothelin-1 (PPET-1, precursor of ET-1) was examined in normal canine tissues by RT-PCR. The entire open reading frames of the canine ET_A and ET_B were shown to encode 427 and 442 amino acid residues, respectively, showing from 87.4 to 97.3% sequence similarity to human, mouse, and rat counterparts. ET_A and ET_B mRNAs were ubiquitously expressed in a variety of canine tissues in this study and PPET-1 mRNA was detected in the tissues except for heart and liver. It was speculated that ET could play an important role in physiological events in most of the organs.

KEY WORDS: canine, endothelin-1, endothelin receptor.

NOTE

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Endothelins (ETs) are peptide hormones composed of 21 amino acids, which are known to be synthesized by various kinds of cells [17]. There are three members of the family (ET-1, ET-2, and ET-3) derived from distinct chromosomal location [22, 25], among which ET-1 is well known as a potent vasoconstrictor and produced by endothelial cells [26]. In the synthesis of ET-1, preproendothelin-1 (PPET-1) is first translated from the ET-1 mRNA, and then cleaved to generate a propeptide, big endothelin-1 [25]. The big endothelin-1 is further processed by the endothelin-converting enzymes, finally forming the mature form of ET-1 [25]. ET-1 binds to the heptaherical G protein-coupled-receptors, endothelin receptor A (ET_A) and endothelin receptor B (ET_B), to show their physiological functions in an autocrine or paracrine manner [2, 17, 20]. Besides the roles as vasoconstriction, ET-1 relates to various functions such as cell proliferation [21] and chemotaxis [24]. Additionally, plasma level of ET-1 was shown to increase in human patients with chronic heart failure [12] and various solid tumors [6]. The concentration of ET-1 was also found to increase in the bronchoalveolar lavage fluid from human patients with asthma [11]. Moreover, a number of antagonists for ET receptors have been applied for the treatment of congestive heart failure and prostate cancer in human [13, 16]. Thus, regulation of ET-1 and its receptors can be considered as a new therapeutic approach for specific diseases in dogs.

Dogs have been used to evaluate the efficacy of ET receptor antagonists for the control of heart failure, because an experimental heart failure model in the dog showed increased ET-1 concentration in the plasma [3, 4]. However, in dog, only a partial coding sequences, corresponding to ET receptors, have been available and there is little information about physiological roles of ET-1 in dogs. Therefore, we sequenced full-length cDNA of ET receptors and investigated expression of PPET-1, ET_A, and ET_B mRNAs in normal tissues as a first step to elucidate the functions of ET system in dogs.

Under the permission of Animal Use Committee at the University of Tokyo, two healthy dogs (one male and one female) were euthanized with intravenous injection of thiopental at the dosage beyond 100 mg/kg in order to obtain tissue samples of various organs for the molecular cloning and the analysis of mRNA expressions. Tissue samples were immediately frozen in liquid nitrogen and kept at −80 °C until use. Total RNAs of these tissue samples were isolated by BioRobot EZ1 System (Qiagen, Santa Clarita, CA, U.S.A.). Additionally, RNA was isolated from blood sample with QIAamp RNA Blood Mini Kit (Qiagen, Santa Clarita, CA, U.S.A.). DNase treatment was carried out for elimination of contaminated genome DNA in these systems. Single-strand cDNAs were synthesized from the total RNAs by GeneAmp RNA PCR Core Kit (Applied Biosystems, Branchburg, NJ, U.S.A.). To obtain the unknown sequences of canine ET_A and ET_B cDNAs, rapid amplification of the 5'- and 3'- cDNA ends (RACE) method was employed with a Gene Racer Kit (Invitrogen, Carlsbad, CA, U.S.A.). For the 5'- and 3'- RACE, the primers and nested primers were designed based on reported sequences of canine ET_A [9], human ET_A (GenBank accession number: BC022511), and canine ET_B (GenBank accession number: AF034530).
Using these primers, 5'- and 3'- regions of canine ET_A and ET_B cDNAs from the sample of thymus were amplified with a TAKARA Ex Taq (Takara, Shiga, Japan) according to the manufacturer's instruction. The PCR was carried out as follows: 2 min at 94°C; 35 cycles of 35 sec at 94°C, 1 min at 60°C, and 2 min at 72°C, followed by 2 min at 72°C. The PCR generated a single DNA fragment and the product was cloned into a plasmid using a pGEM-T Easy Vector system (Promega, Madison, WI, U.S.A.), followed by transformation of *Escherichia coli* competent cells (Invitrogen). The PCR products cloned into plasmids were sequenced by the dye-termination method (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). At last, the primers for the full length cDNA of canine ET_A and ET_B were designed to confirm consistency for the resultant sequences.

The entire open reading frames for canine ET_A and ET_B were 1281 and 1329 bp long and encoded 427 and 442 amino acid residues, respectively (Fig. 1). The deduced amino acid sequence of canine ET_A had 94.1, 92.0, and 91.6% similarity with that of human (GenBank accession number: AAQ87880), mouse (GenBank accession number: XP_134499), and rat (GenBank accession number: AAA41114) counterpart, respectively, while that of canine ET_B showed 97.3, 88.3, and 87.4% similarity with that of human (GenBank accession number: AAP32295), mouse (GenBank accession number: NP_031930), and rat (GenBank accession number: NP_031930), and rat (GenBank accession number: NP_031930).
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Bank accession number: NP_059029) counterpart, respectively. Based on the deduced amino acid sequences, it was suggested that ET_A and ET_B peptides should consist of seven hydrophobic segments with an extracellular N-terminus and a cytoplasmic C-terminus in a manner similar to other G protein-coupled receptors as shown in the counterparts of other species including human [2, 20] and rat [1, 19].

The mRNA expressions for canine PPET-1, ET_A, and ET_B were examined by RT-PCR as described above in blood and various normal tissues including skin, thymus, lung, heart, liver, pancreas, spleen, adrenal gland, kidney, testis, uterus, bladder, stomach, duodenum, colon, and mesenteric lymph node. A part of these cDNAs was amplified with TAKARA Ex Taq. The sequences of the primers for the RT-PCR was based on the sequences previously identified (Table 1). The PCR products were electrophoresed through a 2% (w/v) agarose gel, and stained with ethidium bromide for visualization. In the results, a distinct band for a part of cDNA of canine PPET-1 was detected in the samples of skin, thymus, lung, pancreas, spleen, adrenal gland, kidney, testis, uterus, bladder, stomach, duodenum,

Fig. 1. Comparison of the deduced amino acid sequence of canine ET receptor cDNA with those of human, mouse, and rat homologues. Asterisks indicate identities of amino acids with the sequences of canine ET_A and ET_B. Numerals at the right ends of each line show the total numbers of amino acid residues. a) ET_A; b) ET_B.
colon, and mesenteric lymph node, whereas it was not detected in the samples of heart and liver. Distinct bands of ETA and ETB were identified in all the tissues examined in this study (Fig. 2). In the blood sample, mRNA expressions for these genes were not detected (Fig. 2), indicating that the bands detected on canine tissue samples were not influenced by blood cells possibly contained in those tissue samples. Since, in all of the samples, GAPDH mRNA was not amplified without reverse transcriptase, a possible genomic contamination in the samples was excluded. The mRNA expression patterns of PPET-1, ETA, and ETB in the dogs were very similar to those in the other mammalian species such as human [14, 17] and rat [5, 17]. Interestingly, the expressions of PPET-1, ETA, and ETB mRNAs were found in lymph node in this study, although the role of ET-1 in lymph node is still unknown. Since it has been reported that dendritic cells (DCs) express ET-1, ETA, and ETB according to their maturation and subsequently produce IL-12 in human [7], it can be speculated that an interaction between ET-1 and its receptors plays a role in regulating the immune system to direct Th1-skewed immune responses. From these results, it was speculated that ET-1 and its receptors might have important roles in physiological events in various tissues in dog as shown in other mammalian species.

As reported in the rat and human [8, 10], PPET-1 mRNA expression was not detected in the normal heart and liver tissues in the dogs. Since the expression of ET-1 was reported to be markedly increased in the heart of patients with heart failure [23] and the liver of those with cirrhosis [15], suggesting that high expression of ET-1 in these tissues might indicate some pathological processes of these diseases. Indeed, it was found that up-regulated ET system contributed to initiation of ventricular dysfunction in an in vivo experiment using rats [18] and aberrant activation of hepatic stellate cells in patients with liver cirrhosis [15]. Therefore, it would be possible that ET system could be associated with the development of heart and liver diseases in dogs.

In conclusion, in the present study, full length canine ETA and ETB cDNAs were sequenced and PPET-1, ETA, and ETB mRNAs were found to be ubiquitously expressed in the normal canine tissues. These results provide a fundamental knowledge for the further investigation of the physiological and pathological roles of ET in dog.

REFERENCES