NOTE *Internal Medicine*

Molecular Cloning of Canine Thrombomodulin cDNA and Expression in Normal Tissues

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ABSTRACT. Thrombomodulin (TM) is a glycoprotein localized mainly on endothelial cell surfaces, and is a major regulator of vascular thromboresistance. The entire open reading frame of canine TM cDNA comprises 1737 bp, encoding 578 amino acid residues. Comparison of the deduced amino acid sequence from canine TM with those of human, mouse, rat, rabbit and bovine (partial) TM sequen ces revealed 73.1%, 69.1%, 65.8%, 74.3% and 69.5% identity, respectively. Canine TM mRNA expression was confirmed by RT-PCR analysis in lung, liver, spleen, kidney, pancreas and lymph node, and was relatively low in heart, cerebrum, urinary bladder and ute rus. The present results provide valuable data for research into canine coagulation disorders.

KEY WORDS: canine, cDNA cloning, thrombomodulin.

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Thrombomodulin (TM), a glycoprotein localized mainly on endothelial cell surfaces, is a key regulator of thrombin activities and the protein C (PC) anticoagulant pathway. TM forms a complex with thrombin in a 1:1 ratio, preventing thrombin activities such as fibrin formation, platelet aggregation, coagulation factor activation and endothelial cell activation [7]. Moreover, thrombin combined with TM activates PC 1000- to 2000-fold more strongly than thrombin alone [5, 7]. TM thus converts thrombin from a procoagulant protease into an anticoagulant.

TM comprises five domains: a lectin-like domain, an epidermal growth factor (EGF)-like domain with six EGF-like structures, an *O*-glycosylation site-rich domain, a transmembrane domain, and a cytoplasmic domain [16]. The region including the fourth, fifth and sixth EGF-like structures of the EGF-like domain is the minimum necessary for anticoagulant and PC-activating cofactor activity [21].

In humans, down-regulation of TM reportedly is one cause of thrombosis and DIC [5]. Recombinant human soluble TM (rhs-TM) comprising the lectin-like domain, EGF-like domain and *O*-glycosylation site-rich domain, and without the transmembrane and cytoplasmic domains, has been produced. This rhs TM has prevented thrombosis and DIC in animal models [1, 9, 10, 13, 14]. However, rhs-TM has no ability to activate PC in dog plasma [13]. Species-specific recombinant TM is required to treat canine DIC in veterinary medicine.

The present study describes molecular cloning of the canine TM gene and expression in various canine tissues to provide information for synthesizing recombinant canine TM and facilitate prospective studies on canine coagulation disorders.

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Canine lung, liver, spleen, heart, kidney, pancreas, cerebrum, urinary bladder, uterus and lymph node tissues were obtained from a healthy male beagle dog. Tissue samples were immediately frozen in liquid nitrogen and preserved at –80°C until used.

Total RNA for cloning canine TM was extracted from normal canine lung using an RNeasy Mini Kit (Qiagen, CA, U.S.A.). Subsequently, total RNAs were treated to remove contaminating DNA with a DNA-free™ kit (Ambion, TX, U.S.A.). A cDNA sample was transcribed using an Omniscript™ Reverse Transcriptase kit (Qiagen) and oligo $(dT)_{16}$ primer.

To clone the canine partial TM gene, primer sequences for the canine TM gene were constructed based on conserved nucleotide sequences between human [20] and mouse [6] TM genes (Table 1). Using primer pairs (hTM 4S and hTM 4R) (Fig. 1), a partial sequence of canine TM cDNA was amplified from canine lung cDNA by polymerase chain reaction (PCR) using an Advantage™-GC 2 PCR kit (Clontech, CA, U.S.A.). PCR amplification was performed in accordance with the manufacturer's instructions. Conditions for PCR cycles were as follows: 1 cycle at 94°C for 3 min; 35 cycles at 94°C for 30 sec, 55°C at 30 sec and 72°C for 1 min; and 1 cycle of 72°C for 7 min. PCR product from the partial canine TM gene was cloned into the pCR2.1 vector using a TA Cloning kit (Invitrogen, CA, U.S.A.). Competent cells, INVαF' (Invitrogen), were transformed using ligation mixture. Plasmid DNAs were extracted from bacterial cultures grown in LB broth using a Quantum prep kit (Bio Rad, CA, U.S.A.). PCR products were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, U.S.A.) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Table 1. Primers used for cloning and cDNA amplification of canine TM and GAPDH

Primers	Primer sequences	Purpose	Primers position in canine TM cDNA
hTM1S[a)]	5'-CGTCGAGCACGACTGCTT-3'	cloning	135–152
cTM1R[b)]	5'-GCCGGTGACCCACTGGAA-3'	cloning	381–364
hTM2S[a)]	5'-TAATGACAGTGCGCTCCTC-3'	cloning	221–239
cTM2R[b)]	5'-GCCTGCAGGTAGGTGTCA-3'	cloning	872–855
hTM3S[a)]	5'-TGGGACTGCAGCGTGGAGAA-3'	cloning	775–794
cTM3R[b)]	5'-GGCACTCTCCGTTTTCGCA-3'	cloning	1399–1381
hTM4S[a)]	5'-TGTGAGTGCCCTGAAGGCT-3'	cloning	1321–1339
hTM4R[a)]	5'-CTGCAGCACTACCTCCTTGG -3'	cloning	1746–1727
cTM5S[b)]	5'-GTGCTCATTGGCATCTCCAT-3'	cloning	1603–1622
hTM5R[a)]	5'-TAATGCCAGCTAAGGTGC-3'	cloning	1962–1945[c)]
cTM6S[b)]	5'-AGCACGACTGCTTCCAGCTCTTCCGA-3'	cloning	140–165
cTM6R[b)]	5'-TCTTAGAGTTTCTGAGGCATCTGCTCAGT-3'	cloning	1784–1756
cTM RACE1[b)]	5'-TGCTGTAGCTGGTGCGGTT-3'	5'RACE method	403–385
cTM RACE2[b)]	5'-GCCGGTGACCCACTGGAA-3'	5'RACE method	381–364
cTM RACE3[b)]	5'-GCTCAGTAGCAGGGAAATGACA-3'	5'RACE method	273–252
RTcTMS[b)]	5'-GTGAGCCAGACCGACTATC-3'	RT-PCR	1189–1207
RTcTMR[b)]	5'-GGCACTCTCCGTTTTCGCA-3'	RT-PCR	1399–1381
cGAPDH S	5'-GGAGAAAGCTGCCAAATATG-3'	RT-PCR	–
cGAPDH R	5'-ACCAGGAAATGAGCTTGACA-3'	RT-PCR	–

a) The sequence of the primers were constructed based on human TM [20]. b) The sequence of the primers were constructed based on the PCR fragment of canine TM obtained in this study. c) Position in human TM [20].

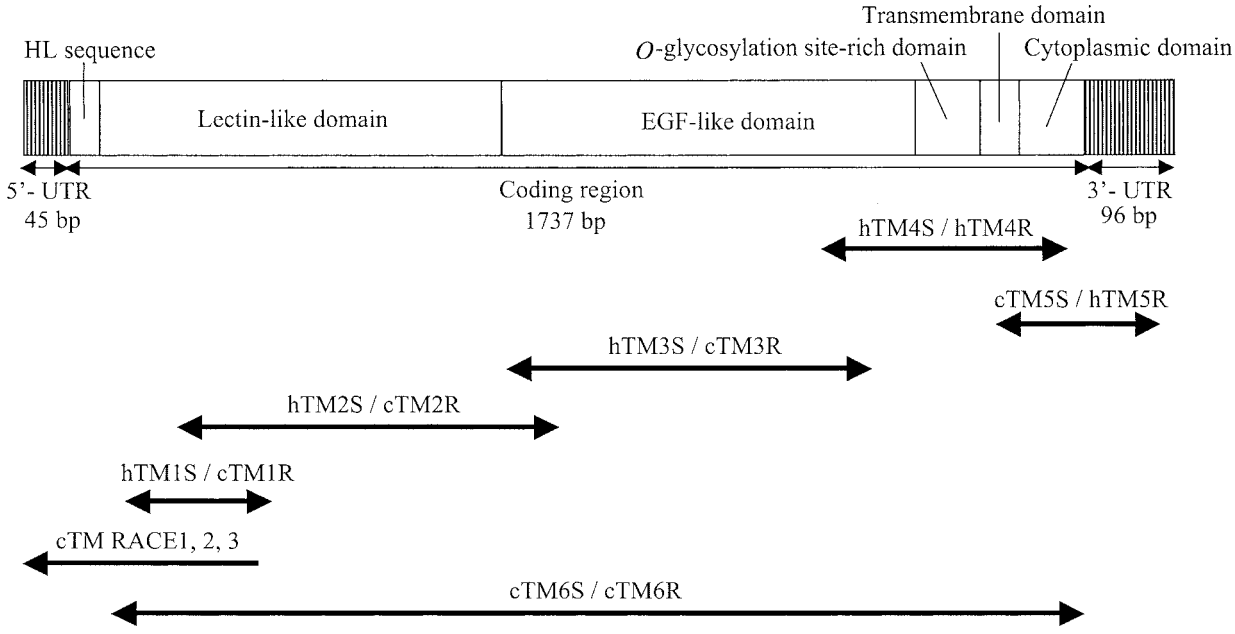

Fig. 1. Strategy for cloning of the coding region of canine TM gene. The fragments with dual arrows were obtained by PCR. The fragment identified by a single arrow was obtained by the 5'-RACE method. Primers are shown above the arrows and are listed in Table 1. Each domain in the coding region is represented by open frames while the 5'- and 3'-UTRs are represented by vertical shading. UTR: untranslated region; HL: hydrophobic leader; EGF: epidermal growth factor.

Furthermore, to amplify the remaining region of canine TM cDNA, primer sequences were designed from the sequences of progressively amplified products beginning with the sequences of canine TM gene fragments, or were constructed based on conserved nucleotide sequences between human [20] and mouse [6] TM genes (Table 1). Using primer pairs (hTM1S and cTM1R, hTM2S and cTM2R, hTM3S and cTM3R, cTM5S and hTM5R) (Fig. 1), PCR and sequencing methods were performed as described above.

In addition, a series using 5' rapid amplification of cDNA ends (RACE) was used to clone the 5' end of the gene.

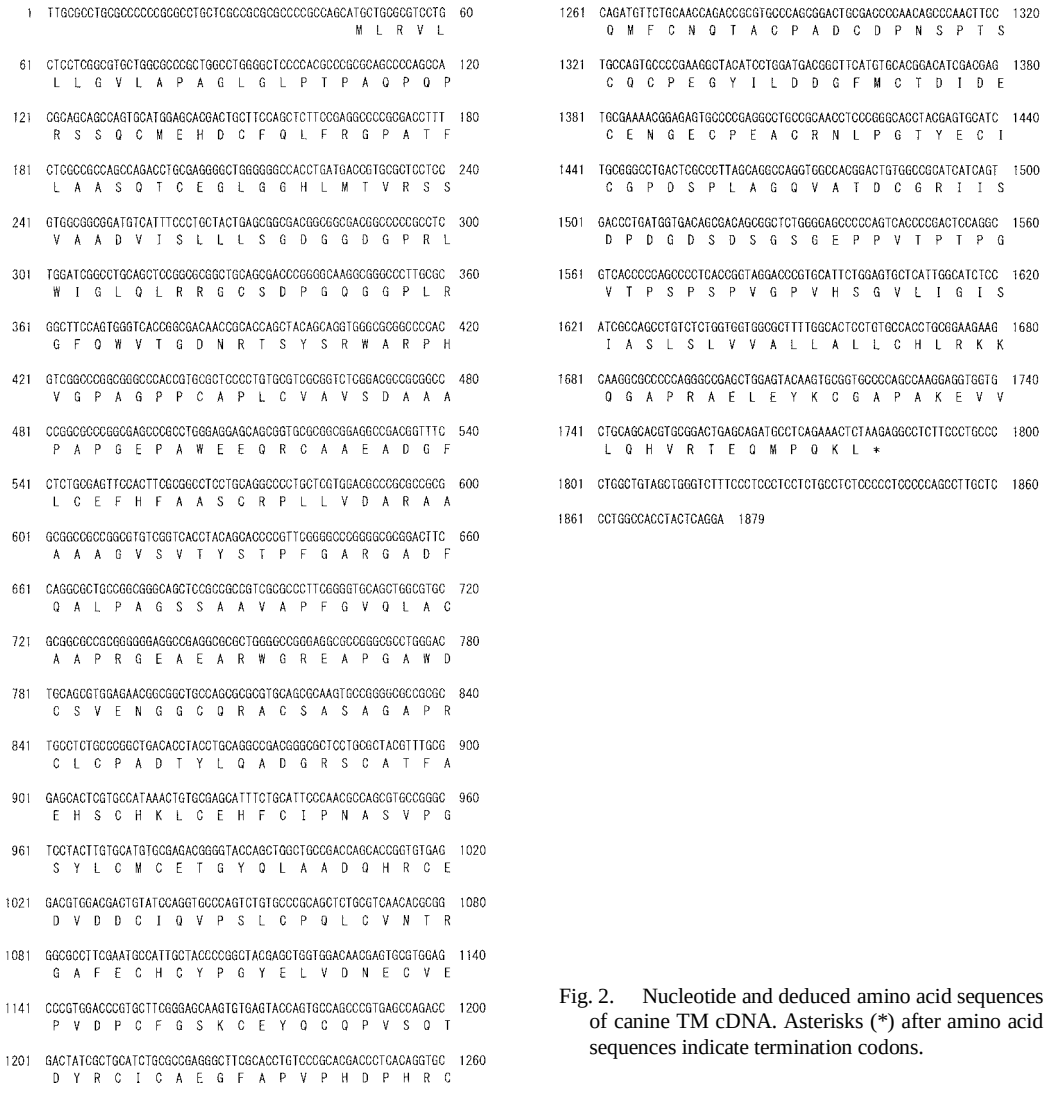

Fig. 2. Nucleotide and deduced amino acid sequences of canine TM cDNA. Asterisks (*) after amino acid sequences indicate termination codons.

Sequences of gene-specific primers for 5' RACE were designed from sequences of progressively amplified products beginning with the sequences of canine TM gene fragments (Table 1) (Fig. 1). PCR products of the 5' side of the canine TM gene were sequenced as described above.

Finally, to confirm the linear gene cloned in this study, we also amplified and cloned amino acids of the conserved region. Primer sequences (cTM6S and cTM6R) for the canine TM gene are shown in Table 1. These primers were expected to amplify a 1645 bp fragment consistent with the canine TM amino acid conserved gene (Fig. 1). Canine lung cDNA was amplified by PCR in a reaction mixture (25 μl) of an Advantage^{TM}-GC 2 PCR kit (Clontech) containing 0.4 μM of each primer. PCR amplification was performed as follows: 1 cycle at 96°C for 3 min; 35 cycles at 96°C for 30 sec and 68°C for 3 min; and 1 cycle at 68°C for 3 min. PCR products were sequenced as described above.

Expression of TM mRNA in normal canine tissues was examined by reverse transcription-PCR (RT-PCR). Total RNA samples were extracted from lung, liver, spleen, heart, kidney, pancreas, cerebrum, urinary bladder, uterus, and lymph node tissue. The cDNA samples were prepared as described above. As an internal control, canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GeneBank accession no. AB038240) mRNA was amplified in each sample. Sequences of primer pairs (RTcTMS and RTcTMR for canine TM, cGAPDH S and cGAPDH R for canine GAPDH) are shown in Table 1. PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 1 cycle of 95°C for 9 min; 35 (TM) or 30 (GAPDH) cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min; and 1 cycle of 72°C for 7 min. PCR products were electrophoresed through 3% agarose gel, and stained with ethidium bromide for visualization. Amplified DNA fragments in RT-PCR were sequenced to confirm fragments of canine TM gene.

Combining the sequences of partial overlapping cDNA fragments obtained in this study, a linear sequence of 1879

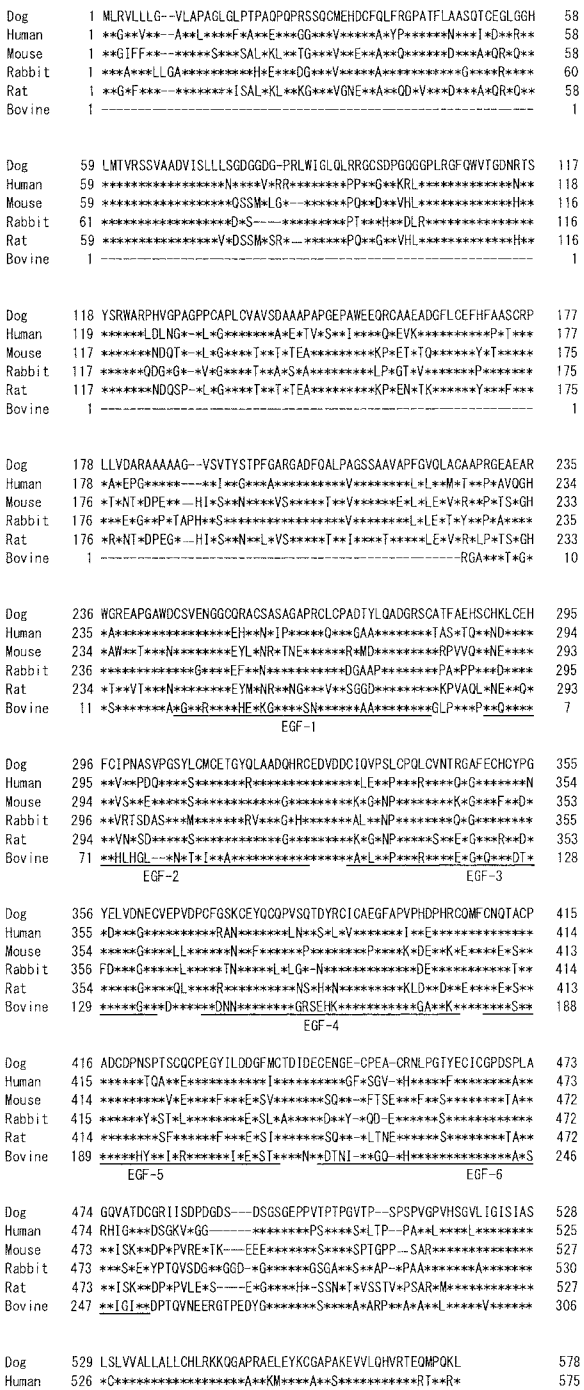

Fig. 3. Comparison of deduced amino acid sequences for canine TM cDNA with human, mouse, rat, rabbit and partial bovine homologues. Asterisks (*) indicate identity with canine TM sequence and dashes (–) indicate gaps. Underlined sequences correspond to EGF-like domains.

bp corresponding to canine TM cDNA was obtained. This sequence contained all 1737 bp of the entire open reading frame, encoding 578 amino acid residues (Fig. 2). Comparison of the nucleotide sequence in the open reading frame of canine TM gene with that of human, mouse, rat, rabbit and bovine (a partial of the open reading frame) TM genes revealed 79.7%, 72.6%, 71.6%, 79.2% and 77.4% identity, respectively. Comparison of the deduced amino acid sequence of canine TM with those of human, mouse, rat, rabbit and bovine (partial) TM revealed 73.1%, 69.1%, 65.8%, 74.3% and 69.5% identity, respectively (Fig. 3).

Primer pairs of cTM6S-cTM6R amplified the 1645-bp PCR product with a sequence corresponding to canine TM cDNA. The result suggested that a linear canine TM cDNA had been cloned and sequenced.

The deduced amino acid sequence of canine TM is similar to those of other species, containing a hydrophobic leader sequence (amino acid 1–18) that could represent a signal peptide [18], a lectin-like domain (amino acid 19–245), an EGF-like domain with six EGF-like structures (amino acid 246–480), an *O*-glycosylation site-rich domain (amino acid 481–517) that was the most heterogeneous region among the various species, a transmembrane domain (amino acid 518–540), and a cytoplasmic domain (amino acid 541–578) (Figs. 1, 3). Three-dimensional conformation of the EGF-like domain is stabilized by disulfide bonds [18]. All cysteine residues of the EGF-like domain in other species TM were conserved (Fig. 3), suggesting that the canine EGF-like domain might conformationally resemble those of other species TM. Since mRNA in canine TM was highly conserved and the protein structure was also similar to that of other species TMs, canine TM could possess anticoagulant functions similar to other species TMs. However, rhs-TM had no ability to activate PC in dog, rat, rabbit, mouse, hamster or guinea pig plasma [13]. Species-specific recombinant TM is thus required for treatment of canine DIC in veterinary medicine.

Canine TM mRNA expression was confirmed by RT-PCR analysis in lung, liver, spleen, kidney, pancreas and lymph node, and was relatively low in the heart, cerebrum, urinary bladder and uterus (Fig. 4).

Human TM mRNA is most expressed in the heart, followed by the pancreas, lung, skeletal muscle, kidney, liver, placenta and brain [2]. Furthermore, rat TM mRNA was shown to be most highly expressed in the lung, followed by the kidney, brain, intestine and liver [19]. The present study on canine TM mRNA expression also indicates that TM is commonly detected in organs that contain many capillaries, such as the lung, kidney and spleen. Proinflammatory cytokines such as TNF-α and IL-1β could activate endothelial cells and monocytes to increase expression of tissue factors, leading to the activation of coagulation. These cytokines further decrease endothelial expression of TM to about half normal levels by suppression of transcription and translation, thus reducing its endothelial anticoagulant potential [3, 8, 12, 15, 17].

The present results should provide valuable information

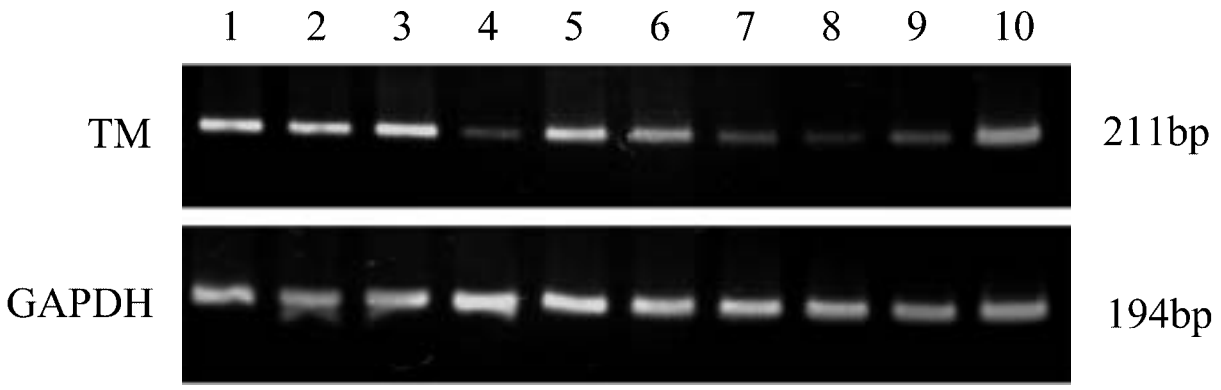

Fig. 4. Detection of canine TM mRNA in various normal dog tissues. Canine TM mRNA (upper lanes) and canine GAPDH mRNA (lower lanes) were detected using RT-PCR with primers specific to canine TM and GAPDH cDNAs, respectively. Lane 1, lung; lane 2, liver; lane 3, spleen; lane 4, heart; lane 5, kidney; lane 6, pancreas; lane 7, cerebrum; lane 8, urinary bladder; lane 9, uterus; lane 10, lymph node.

to facilitate synthesis of recombinant canine TM for treatment of canine DIC, and for examination of expression patterns in dogs with coagulation disorders.

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