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SPONTANEOUSLY ARISING DISEASE

Expression of the Thrombopoietin Gene in Tissues from Healthy Dogs

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Summary

Thrombopoietin (THPO) is the major cytokine that regulates megakaryopoiesis and platelet production. Several human and murine studies have demonstrated that THPO is primarily synthesized in the liver, but the kidney, spleen and bone marrow are also sites of expression. The aim of this study was to determine THPO mRNA levels in a range of canine tissues by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Samples of bone marrow ($n = 5$), liver ($n = 10$), lung ($n = 10$), renal cortex ($n = 10$), renal medulla ($n = 5$) and spleen ($n = 10$) were obtained from 10 healthy, hound-cross dogs aged 6–8 months. The highest THPO mRNA levels were found in the liver, followed by the bone marrow, spleen, lung and kidney. There was a 13-fold difference in expression between liver and kidney. The bone marrow showed high levels of THPO mRNA in the absence of disease. The liver and bone marrow are likely to be the major sites of THPO production in the dog.

Keywords: dog; thrombopoietin; tissue mRNA; RT-qPCR

Introduction

Thrombopoietin (THPO) is the major cytokine responsible for regulating megakaryocyte production via signalling through its receptor, Mpl (Kaushansky, 1995). Moreover, THPO stimulates megakaryocytes to increase in size and ploidy, and triggers the formation of proplatelet processes that then fragment into single platelets (Kaushansky, 2005). In addition to its importance in the maintenance of circulating levels of platelets, THPO also regulates haemopoietic stem cell (HSC) expansion and maintenance of adult quiescent HSCs (Qian *et al*., 2007).

The liver is the primary site for megakaryopoiesis during human fetal development, although the bone marrow becomes increasingly important in the postnatal period and ultimately becomes the major site for megakaryopoiesis in adults. However, the liver remains the major source of THPO production throughout life (Sungaran *et al*., 1997). THPO gene expression also occurs in several human, mouse and rat tissues in addition to the liver, such as the kidney, bone marrow, lung, spleen and central nervous system (McCarty *et al*., 1995; Sungaran *et al*., 1997; Qian *et al*., 1998; Dame *et al*., 2003; Kazama *et al*., 2011). Although the THPO gene is expressed in a wide range of organs and tissues, specific cell types within these tissues have been shown to be responsible for the production of THPO mRNA. In the liver, the production takes place primarily in hepatocytes of the periportal regions, and in the kidney production occurs in both proximal and distal renal tubular cells. Stromal cells are the major source of THPO mRNA in the bone marrow (Sungaran *et al*., 1997).

The importance of the liver in the production of THPO mRNA was evidenced by the significant (>50%) decrease in platelet count when the normal livers of wild-type mice were replaced by livers from THPO-knockout mice (Qian *et al*., 1998). Additionally, it has been suggested that the liver is responsible for 95% of THPO mRNA production in human fetuses (Wolber *et al*., 1999). The impact of THPO production by other organs and tissues on

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megakaryopoiesis is currently unknown (Deutsch and Tomer, 2006).

Recombinant THPO or THPO peptide mimetics have been used to treat thrombocytopenic disorders and have resulted in increased platelet counts in dogs (Peng *et al*., 1996; Case *et al*., 2000) and man (Vadhan-Raj *et al*., 1997; Desjardins *et al*., 2007).

To our knowledge, there is no information concerning THPO gene expression levels in canine tissues. A clearer understanding of the primary production sites and the regulatory mechanisms involved in THPO expression in the dog is the first step towards the development of therapies with THPO mimetics for thrombocytopenic conditions in this species and towards developing potential comparative models. Therefore, the aim of the present study was to quantify THPO mRNA in the tissues of healthy dogs.

Materials and Methods

Tissue Samples

Tissue specimens were obtained from 10 healthy research dogs following a terminal procedure. The health status of the dogs was determined by checking the vaccination history of each animal and undertaking a full haematological examination and heartworm test. The dogs were all hound crosses, aged 6–8 months. Samples of bone marrow ($n = 5$), liver ($n = 10$), lung ($n = 10$), renal cortex ($n = 10$), renal medulla ($n = 10$) and spleen ($n = 10$) were stored at −80°C immediately after collection.

RNA Extraction and Quality Assessment

Tissue samples were weighed, sectioned into 100 mg slices and dispersed in 1 ml TRIzol®-Reagent (Life Technologies, Grand Island, New York, USA) using Tissuemizer® High Torque (Tekmer Co., Cincinnati, Ohio, USA). After a second phase separation with chloroform, total RNA was isolated according to the manufacturer's protocol (Life Technologies) and further purified by on-column cleanup (Qiamp RNA Blood Mini Kit, Qiagen, Valencia, California, USA). The RNA concentration was measured spectrophotometrically using NanoDrop® ND-100 (Thermo Scientific, Wilmington, Delaware, USA) and RNA quality was assessed by determining the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230}.

Oligonucleotides and Hydrolysis Probes

Primer Express software version 3.0 (Applied Biosystems, Foster City, California, USA) was used for designing three oligonucleotides for each target gene: forward, reverse and an internal oligonucleotide as a hydrolysis probe (Table 1). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and THPO oligonucleotides were costume synthesized and purchased from Life Technologies and Eurofins MWG Operon (Hunstville, Alabama, USA), respectively.

Polymerase Chain Reaction

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed using GoTaq® Probe 1-Step RT-qPCR System (Promega, Madison, Wisconsin, USA) to a final volume of 20 µl containing 0.4 µl of GoScript™ RT Mix, 10 µl of Go Taq® Probe Master Mix, 0.9 µM final concentration of forward primer, 0.25 µM of probe, 30 nM of carboxy-X-rhodamine (CXR) reference dye and 200 ng of RNA per reaction. Reverse transcription for 30 min at 50°C was followed by a denaturation step at 95°C for 2 min and 40 cycles of 95°C for 15 sec and 60°C for 60 sec. All reactions were run in triplicate and included a no-template control (NTC). The amplification efficiencies of THPO (102.5%) and GAPDH (95.2%) assays were determined in triplicate using serial fivefold and 10-fold sample dilutions, respectively. For relative quantification the comparative Cq method (Livak and Schmittgen, 2001) was employed.

Statistical Analysis

Data are presented as mean ± standard deviation (SD) and were analyzed by analysis of variance

Table 1
Description of primers and probes

Name of primer or probe	*Sequence (5′–3′)*	*Amplicon length (base pairs)*	*GenBank accession number*
TPO forward	TGTGACCCCCGTCTCCTAAAT	68	TPO XM_003640131
TPO reverse	CACTGGCTCAATCTGCTGTGA		
TPO probe	6-FAM-AAATGCTTCGTGACTCC-MGBNFQ		
GAPDH forward	TGTCCCCACCCCCAATG	69	GAPDH NM_001003141
GAPDH reverse	TCGTCATATTTGGCAGCTTTCTC		
GAPDH probe	6-FAM-CAGTTGTGGATCTGACCTGCCGCC-BHQ1		

FAM, 6-carboxyfluorescein; MDBNFQ, minor groove-binding non-fluorescent quencher; BHQ1, black hole quencher-1.