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Markers of hepatic regeneration associated with surgical attenuation of congenital portosystemic shunts in dogs

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ABSTRACT

Dogs with congenital portosystemic shunts (CPSS) have liver hypoplasia and hepatic insufficiency. Surgical CPSS attenuation results in liver growth associated with clinical improvement. The mechanism of this hepatic response is unknown, although liver regeneration is suspected. This study investigated whether markers of liver regeneration were associated with CPSS attenuation. Dogs treated with CPSS attenuation were prospectively recruited. Residual liver tissue was collected for gene expression analysis (seven genes) from 24 CPSS dogs that tolerated complete attenuation, 25 dogs that tolerated partial attenuation and seven control dogs. Relative gene expression was measured using quantitative polymerase chain reaction (qPCR). Blood samples were collected before, 24 h and 48 h post-surgery from 36 CPSS dogs and from 10 control dogs. Serum hepatocyte growth factor (HGF) concentration was measured using a canine specific enzyme-linked immunosorbent assay (ELISA). HGF mRNA expression was significantly decreased in CPSS compared with control dogs ($P = 0.046$). There were significant increases in HGF ($P = 0.050$) and methionine adenosyltransferase 2 A (MAT2A; $P = 0.002$) mRNA expression following partial CPSS attenuation. Dogs with complete attenuation had significantly greater MAT2A ($P = 0.024$) mRNA expression compared with dogs with partial attenuation. Serum HGF concentration significantly increased 24 h following CPSS attenuation ($P < 0.001$). Hepatic mRNA expression of two markers of hepatocyte proliferation (HGF and MAT2A) was associated with the response to surgery in dogs with CPSS, and serum HGF significantly increased following surgery, suggesting hepatocyte proliferation. These findings support the concept that hepatic regeneration is important in the hepatic response to CPSS surgery.

Introduction

Dogs with congenital portosystemic shunts (CPSS) have liver hypoplasia associated with hepatic insufficiency. Successful CPSS attenuation results in resolution of clinical signs and improvement in hepatic function as assessed with dynamic bile acids or ammonia tolerance testing (Hunt and Hughes, 1999; Hunt et al., 2004). In the short term following CPSS attenuation, liver volume as measured by computed tomography (CT) or magnetic resonance imaging (MRI) increases (Stieger et al., 2007; Kummeling et al., 2010). These findings suggest that this rapid return of the liver to a normal size is achieved by hepatic regeneration, although evidence for this is circumstantial.

Liver regeneration is complex and involves a large number of factors, although hepatocyte growth factor (HGF) plays a key role. In experimental studies, HGF expression in liver and its serum concentration increase following partial hepatectomy (PH) in association with liver regeneration (Lindroos et al., 1991; Zarnegar et al., 1991). Serum HGF also increases following PH in humans, and it is suggested that this is associated with regeneration (Efimova et al., 2005).

No published studies have specifically investigated the mechanisms governing the hepatic response to CPSS attenuation in dogs. One study demonstrated that the main components of the HGF signalling pathway were reduced but intact in dogs with CPSS (Spee et al., 2005). If it can be demonstrated that liver regeneration occurs following CPSS attenuation, this could have important

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Table 1
Table showing details of reference gene and gene of interest primer pairs for quantitative polymerase chain reaction (qPCR).

Gene	Primer sequences	PCR amplicon length (bp)	GenBank accession number	Primer sequence reference
HMBS	Forward: TCACCATCGGAGCCATCT Reverse: GTTCCCACCACGCTCTTCT	112	XM546491	Peters et al., 2007
RPL13A	Forward: GCCGGAAGGTTGTAGTCGT Reverse: GGAGGAAGGCCAGGTAATTC	87	AJ388525	Peters et al., 2007
RPL32	Forward: TGGTTACAGGAGCAACAAGAAA Reverse: GCACATCAGCAGCACTTCA	100	XM_848016	Peters et al., 2007
RPS18	Forward: TGCTCATGTGGTATTGAGGAA Reverse: TCTTATACTGGCGTGGATTCTG	116	XM_532106	Peters et al., 2007
HGF	Forward: AAAGGAGATGAGAAACGCAAACAG Reverse: GGCCTAGCAAGCTTCAGTAATACC	92	NM_001002964	Kummeling et al.2012
HGFac	Forward: ACACAGACGTTTGGCATCGAGAAGTAT Reverse: AAACTGGAGCGGATGGCACAG	128	AY458142	Kummeling et al., 2012
cMET	Forward: TGTGCTGTGAAATCCCTGAATA/GAAATC Reverse: CCAAGAGTGAGAGTACGTTTGGATGAC	112	NM_001002963	Kummeling et al., 2012
MAT2A	Forward: TGCTTTTGGCGGGGAGGAG Reverse: TTTAAAAGCTGCCATCTGAGGTGA	121	XM_532980	Kummeling et al., 2012
TGFα	Forward: CCGCCTTGGTGGTGGTCTCC Reverse: AGGGCGCTGGGCTTCTCGT	136	AY458143	Spee et al., 2005
TGFβ	Forward: CAAGGATCTGGGCTGGAAGTGGA Reverse: CCAGGACCTTGCTGTACTGCGTGT	113	L34956	Spee et al., 2005
TGFβR2	Forward: GACCTGCTGCCTGTGTGACTTTG Reverse: GGACTTCGGGAGCCATGTATCTTG	116	XM_534237	Kummeling et al., 2012

implications for the development of therapy for this condition in dogs as well as in other species.

This study investigated whether markers of liver regeneration were associated with CPSS attenuation. The first aim was to measure the mRNA expression of genes associated with hepatic regeneration in liver biopsies from dogs with CPSS before and after partial attenuation. The second aim was to measure the serum concentration of HGF in dogs with CPSS before and after attenuation. The hypotheses tested were that the degree of hepatic development and the hepatic response to surgery would be significantly associated with gene expression and serum HGF concentration.

Materials and methods

Clinical management

Dogs with CPSS were prospectively recruited between August 2007 and October 2011. Our institution's Ethics Committee granted approval for the study and owners gave full informed consent. Dogs were treated surgically via suture attenuation of their CPSS as previously described (Lee et al., 2006). Dogs that could not tolerate complete attenuation due to intraoperative portal hypertension had partial attenuation. Dogs treated with partial attenuation had repeat surgery approximately 3 months post-operatively.

Healthy experimental Beagle dogs, which had been humanely euthanased for reasons unrelated to hepatic disease, were used as controls for the qPCR gene expression and serum HGF measurement. Dogs undergoing exploratory laparotomy for reasons unrelated to CPSS were included as controls for serum HGF measurement.

Gene expression

A liver biopsy was taken from CPSS dogs at each surgery for routine diagnostic purposes and a portion was placed in RNAlater (Sigma-Aldrich) and stored according to the manufacturer's instructions. Liver tissue was taken from Beagle control dogs immediately following euthanasia and stored similarly.

RNA was extracted from approximately 20–30 mg of each hepatic sample using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). The tissue was homogenised in 500 μL Lysis Solution using a Mixer Mill MM 300 (Retsch). An in-solution DNase digestion was performed using the Ambion TURBO DNA-free Kit (Life Technologies) to remove any contaminating DNA.

RNA quality and quantity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). The median RIN was 8.3 (range, 7.1–9.2). No samples had genomic DNA contamination. Two separate cDNA were synthesised from each RNA sample using a mixture of random hexamer and oligo $(dT)_{15}$ primers (Promega) and IMProm-II reverse transcriptase enzyme (Promega). Where possible, the amount of RNA template for cDNA synthesis was standardised at 1 μg. The cDNA was diluted to a final volume of 100 μL with nuclease free water and stored at –20 °C prior to further use.

Quantitative polymerase chain reaction (qPCR) was used to measure the relative hepatic expression of seven genes related to hepatic regeneration, including *HGF*, *HGF activator* (*HGFac*), *HGF receptor* (*cMET*), *methionine adenosyltransferase 2 A* (*MAT2A*), *transforming growth factor alpha* (*TGFα*), *TGF beta 1* (*TGFβ1*) and *TGFβ receptor 2* (*TGFβR2*). Previously published canine gene specific primers for the genes of interest (Spee et al., 2005; Kummeling et al., 2012) and four liver specific reference genes (Peters et al., 2007) *hydroxymethyl-bilane synthase* (*HMBS*), *ribosomal protein L13a* (*RPL13A*), *ribosomal protein L32* (*RPL32*) and *ribosomal protein S18* (*RPS18*) were used (Table 1).

For quantification each liver sample had two cDNA samples analysed in duplicate. Reactions were carried out in 25 μL using a Bio-Rad CFX96 Real-Time PCR Detection System thermocycler (Bio-Rad Laboratories). Each reaction consisted of 1 μL cDNA as the template with Immobuffer (1× concentration), Hi-Spec Additive (1× concentration), dNTP (final concentration 1 mM), magnesium chloride (final concentration 2.5 mM for genes of interest, 4.5 mM for reference genes), 1 unit Immolase DNA polymerase (all Bioline) and EvaGreen dye (Biotium) (0.06× diluted 1:4 with nuclease-free water). Samples were incubated at 95 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 10 s. An appropriate primer-dimer melting temperature for 1 s was programmed before fluorescence readings were taken at the end of each cycle. A melting curve analysis from 65 °C to 95 °C with a plate read every 0.5 °C was performed at the end of 40 cycles. Bio-Rad CFX Manager Software (Bio-Rad Laboratories) was used for the initial qPCR analysis.

Analysis of raw data was performed using GenEx professional version 4.4.2 software (Multid Analyses). Relative gene expression was quantified as previously described (Vandesompele et al., 2002). Quantification cycle (Cq) values were corrected using the calculated efficiencies for each primer set. Normalisation of each sample Cq for the genes of interest was performed relative to the geometric normalisation of the four reference genes. The relative expression of the mRNA of each genes of interest in each cDNA sample was then calculated using the normalised Cq of each sample relative to the average Cq of all of the samples.

Serum HGF concentration

Blood samples were taken from CPSS dogs and exploratory laparotomy controls preoperatively for diagnostic purposes and after surgery for post-operative monitoring and, where available, residual blood was collected for the study. Residual blood samples were also taken immediately before euthanasia in Beagle control dogs. The serum was separated and stored at –80 °C. A Canine ELISA Kit (Biorbyt) was used to measure the serum concentration of HGF. Samples were analysed in duplicate using an ELx808 absorbance microplate reader (BioTek Instruments). Sample concentration was calculated from the standard curve using Gen5 V1.07.5 software (BioTek Instruments).

Statistical analysis

Analysis was performed using PASW Statistics 18.0.0 statistical software package (Education SPSS). Continuous data were visually assessed for normality. Median and range were reported for skewed data, which was compared with the Mann–Whitney U test. Repeated measures were compared with the Friedman's