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Cytokine expression in peripheral blood mononuclear cells of dogs with mitral valve disease

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ABSTRACT

Inflammation plays an important role in the pathogenesis of congestive heart failure (CHF). In humans with CHF, increased production and high plasma concentrations of tumour necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1, IL-8 and transforming growth factor-β (TGF-β) have been associated with disease progression and a negative prognosis. The aim of this study was to investigate whether differences in cytokine blood mRNA expression exist between clinically healthy dogs and dogs with myxomatous mitral valve disease (MMVD); to determine if the expression was related to the severity of MMVD, and to detect any correlations with echocardiographic parameters of cardiac remodelling. Twenty-three dogs with MMVD of varying severity and six clinically healthy dogs were included in the study. Whole blood samples were obtained for measurement of mRNA expression of IL-1α, IL-1β, IL-6, IL-8, TGF-β1, TNF-α by reverse transcriptase-PCR (RT-PCR).

There were statistically significant differences between clinically healthy dogs and dogs with MMVD for IL-8 and TGF-β1 gene expression. IL-8 expression increased with increasing MMVD severity and TGF-β1 expression was higher in asymptomatic dogs with echocardiographic signs of cardiac remodelling (American College Veterinary Internal Medicine class B2) than in all other groups. These results could suggest the involvement of these cytokines at different stages of the disease.

Introduction

Since the first report in 1990 (Levine et al., 1990), numerous studies have shown that inflammation is involved in the pathogenesis of heart failure in human patients. The production of inflammatory mediators, such as cytokines, in association with the activation of various neurohormonal systems, such as the sympathetic nervous system and the renin-angiotensin-aldosterone system, is known to contribute to progression of chronic heart failure in people (Packer, 1992; Chatterjee, 2005).

Cytokines are a group of low-molecular weight proteins responsible for autocrine and paracrine signaling influencing the function of neighbouring cells and involved in endocrine signaling throughout the body. Cytokines are produced by cells of the immune system and by all cell types in the myocardium, including the cardiac myocytes (Parissis et al., 2002; Bozkurt et al., 2010).

In human medicine, increased expression of cytokines has been demonstrated in patients with congestive heart failure (CHF) regardless of aetiology (Testa et al., 1996; Aukrust et al., 1999; Hartupee and Mann, 2013) and has been correlated to increased mortality and disease progression (Anker and Von Haehling, 2004; Demyanets et al., 2011).

Over the past few years, there has been increasing interest in the role of pro-inflammatory cytokines in heart failure in veterinary patients. Several studies have focused on myxomatous mitral valve disease (MMVD) (Aupperle et al., 2008; Disartian and Orton, 2009; Obayashi et al., 2011; Zois et al., 2012), as this is the most common cause of CHF in small breed dogs (Pedersen and Häggström, 2000). MMVD is characterised by non-inflammatory, non-infectious valvular degeneration that leads to mitral regurgitation (Pedersen and Häggström, 2000; Aupperle and Disartian, 2012). Although the disease appears to be non-inflammatory, upregulation of numerous cytokines has been demonstrated in the mitral valve and myocardium of dogs with MMVD (Oyama and Chittur, 2006; Paslawska et al., 2006; Kiczak et al., 2008). Later studies have measured peripheral cytokine expression and circulating concentrations in dogs with heart disease. Increased blood mRNA expression of interleukin (IL)-1β and IL-2 was detected in dogs with CHF while other cytokines showed lower (tumour necrosis factor [TNF]-α, transforming growth factor [TGF] – β3) or not significantly different (TGF-β1, TGF-β2, IL-8, IL-4, IL-10) expression in dogs with CHF compared to control dogs (Fonfara et al., 2012). Peripheral concentration of several cytokines (IL-6, TNF-α, IL-10) were non-quantifiable (Mavropoulou et al., 2010; Zois et al., 2012) in dogs with different

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MMVD stages, while others (IL-2, IL-7 and IL-8) decreased with disease severity (Zois et al., 2012). These results differ from those reported in human medicine, where increased cytokine levels (particularly IL-6 and TNF-α) have been consistently reported in patients with heart failure and in patients with chronic mitral regurgitation (Oral et al., 2003; Oikonomou et al., 2011).

TGF-β is a cytokine that has gained attention and was implicated in canine MMVD (Aupperle et al., 2008; Obayashi et al., 2011). It belongs to the family of growth factors and three distinct isoforms (TGF-β1, TGF-β2 and TGF-β3) have been identified in mammals (Akhust et al., 1990). TGF-β has an important role in the regulation of cell growth, differentiation and repair in several tissues (Lim and Zhu, 2006) and there is evidence that it may contribute to valve degeneration (Aupperle et al., 2008; Obayashi et al., 2011). Elevated levels of TGF-β have been demonstrated in valvular heart diseases in humans (Waltenberger et al., 1993; Kim et al., 2008) and also in canine MMVD (Aupperle et al., 2008; Obayashi et al., 2011).

The aim of this study was: (1) to determine levels of cytokine expression in peripheral blood mononuclear cells (PBMC) of dogs with MMVD at different stages and in healthy dogs; and (2) to investigate potential relationships between cytokine expression and echocardiographic indices of MMVD severity, left ventricular (LV) function and remodelling. For this purpose, IL-1, IL-6, IL-8, TGF-β1 and TNF-α PBMC expression was measured by RT-PCR.

Materials and methods

Recruitment and examination of dogs

Cases were recruited prospectively from client-owned dogs presenting for cardiovascular examination to the cardiology service of the Veterinary Teaching Hospital of the University of Parma from January 2009 to July 2010. Informed consent was obtained from all owners and the study protocol was approved by the University of Parma Institutional Animal Care Committee (Protocol number 22/13, 4 February 2013).

Only dogs 5 years or older and with a bodyweight (BW) between 5 and 20 kg were enrolled. Dogs with echocardiographic evidence of MMVD (mitral valve thickening and/or valve prolapse resulting in mitral regurgitation detected with colour Doppler) were included in the MMVD group. The control group was composed of dogs that were presented to the Veterinary Teaching Hospital of the University of Parma for non-therapeutic ovariohysterectomy/orchiectomy or for preventative cardiac screening. These dogs were enrolled on the basis of a history of good health and no abnormal findings on physical examination, electrocardiography, thoracic radiography, echocardiography, haematology and biochemistry.

Dogs with evidence of cardiac disease (acquired or congenital) other than MMVD, systemic arterial hypertension (systolic blood pressure >160 mmHg), clinical signs and/or haematological/biochemical abnormalities compatible with organ dysfunction, inflammatory, infectious or neoplastic disease and dogs receiving any medication at presentation were excluded from the study.

For all dogs signalment was recorded and clinical history was taken. A complete physical examination, thoracic radiography (right lateral and dorsoventral views), echocardiography, systolic blood pressure measurement (Doppler method), routine haematology and biochemistry were performed. Dogs with MMVD were divided into groups (Stage B1, B2, C), according to the classification system used by the American College of Veterinary Internal Medicine (ACVIM), Specialty of Cardiology (Atkins et al., 2009). Echocardiography was performed without sedation, with dogs positioned in lateral recumbency, using a Megas CPV (Esaote Biomedica) equipped with electronic, phased array transducers of variable frequency (from 2.5 to 7.5 MHz). Standard transthoracic right and left parasternal and subcostal views were obtained for echocardiographic evaluation and measurements (Thomas et al., 1993). Mitral valve morphology was examined, lesions identified and an estimation of regurgitation was performed using the right parasternal long axis and left parasternal apical 2D views. The left atrium/aortic root ratio (LA/Ao) was obtained from the right parasternal short axis 2D view as previously described (Boon, 2011). The left ventricular internal dimension in diastole (LVIDd) and in systole (LVIDs) were measured from the M-mode echocardiogram, which was obtained from the right parasternal short axis 2D view. The transmitral diastolic flow and mitral regurgitation Doppler recordings were obtained from the left apical 4-chamber view. For the classification of dogs according to the stage of heart failure and for statistical analysis, the following echocardiographic measurements relating to left ventricular function, cardiac remodelling and mitral valve disease severity were recorded: LA/Ao ratio, transmitral inflow E wave velocity to A wave velocity ratio (E/A ratio) and E wave velocity, the LVIDd (Thomas et al., 1993) and LVIDd normalised (nLVIDd) according to allometric scaling ($VDd/BW^{0.294}$), the LVIDs and the LVIDs normalised (nLVIDs) according to the allometric scaling ($VDs/BW^{0.315}$; Cornell et al., 2004). Hearts with LA/Ao ratio > 1.6 and nLVIDd ≥ 1.73 were considered remodelled (Hansson et al., 2002; Borgarelli et al., 2012). Evidence of distended pulmonary veins and pulmonary infiltrate compatible with cardiogenic oedema on thoracic radiography were considered signs of left sided congestive heart failure.

Blood sampling and processing

The expression levels of mRNA for relevant cytokines (IL-1α, IL-1β, IL-6, IL-8, TGF-β1 and TNF-α) were determined in canine PBMC. Blood samples (1.5 mL) were obtained via jugular venepuncture and collected in lithium heparin tubes. Immediately after blood collection, PBMC were isolated by density gradient using Histopaque-1077 (Sigma-Aldrich) according to manufacturer's instructions. After isolation, PBMC samples were washed twice in phosphate buffered saline (PBS), resuspended in RPMI-1640 complete medium supplemented with 40% heat-inactivated (56 °C for 30 min) foetal calf serum (FCS) and 10% dymethylsolfoxide (DMSO) and immediately frozen at –80 °C using a Mr Frosty (Sigma) device gradient and stored in liquid nitrogen the following day. Further processing was performed within 2 weeks of collection. All PBMC samples were thawed and cell viability was evaluated by trypan blue dye exclusion with a result >96%. At that time, 4×10^6 cells were processed for total cellular RNA extraction using TRI-reagent (Ambion-Life Technologies) according to the manufacturers' instructions. Purity and concentration were assessed by UV-spectrophotometry at 260/280 and 260 nm respectively (GeneQuant Pro, Amersham Pharmacia Biotech-GE Healthcare Life Sciences). RNA integrity and quality were assessed using an Agilent Bioanalyzer 2100 and RNA 6000 Labchip kit (Agilent Technologies). RNA samples were stored at –80 °C until reverse-transcription (RT) phase commenced.

All RNA samples were DNAse-treated (Sigma) prior to cDNA synthesis. Total RNA (1 μg/20 μL) was reverse-transcripted using a high-capacity cDNA RT kit (Applied Biosystems). RT was performed using a PTC-100 Peltier thermal cycler (MJ Research) StepOne according to the manufacturer's instructions under the following thermal conditions: 5 min at 25 °C, 30 min at 42 °C followed by 5 min at 85 °C. All cDNA samples were stored at –20 °C until PCR processing.

The cDNA obtained from each sample was used as a template for PCR performed using a PTC-100 Peltier thermal cycler (MJ Research) and amplified in duplicate. For PCR amplification, 2 μL of cDNA were used in the reaction buffer containing $MgCl_2$ (2 mM), 1 μL dNTPs (0.2 mM), 0.5 μL DreamTaq Green DNA polymerase (0.05 U/μL; Fermentas Life Science), 5 μL of both forward and reverse primers (2.5 μM; MWG-Biotech) with a final volume of 50 μL. The primers were designed based on published gene sequences (Rottmann et al., 1996; Hegemann et al., 2003) and purchased from Eurofins MWG Operon. Details of each primer set for detection of cytokine gene expression are reported in Table 1. The reaction was run for 3 min at 94 °C followed by 32 cycles (when the reaction was in the middle of the linear range, before reaching the amplification plateau) and a final elongation step at 72 °C for 10 min. For TNF-α, IL-1α, IL1β and IL-8, each cycle consisted of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. For TGF-β1 each cycle consisted of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min and extension at 72 °C for 1 min. For IL-6 and GAPDH each cycle consisted of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min 30 s and extension at 72 °C for 2 min.

PCR products were separated by electrophoresis on 2% agarose gel in Sybr Safe (Invitrogen), and visualised under UV light. The average intensity of each band was determined by densitometric analysis with Scion Image (Scion Capture Driver 1.2 for Image-Pro Plus, Scion) in a grey-scale mode. The density of selected band was calculated after background subtraction and values were presented as the ratio of band intensities of each cytokine RT-PCR product over those of the corresponding housekeeping gene GAPDH RT-PCR product. The cytokine/GAPDH ratio was expressed in relative arbitrary units (RAU).

Statistical analysis

Descriptive statistics were performed and median and interquartile ranges were calculated for echocardiographic parameters and cytokine expression in the different

Table 1
Primer sequences and final product size of IL-1α, IL-1β, IL-6, IL-8, TGF-β, TNF-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hegemann et al., 2003).

Gene	Sequences	Amplicon
IL-1α	S: 5′-TTGGAAGACCTGAAGAACTGTTAC-3′ A: 5′-GTTTTTGAGATTCTTAGAGTCAC -3′	545 bp
IL1β	S: 5′-CACAGTTCTCTGGTAGATGAGG -3′ A: 5′-TGGCTTATGTCCTGTAACTTGC -3′	262 bp
IL-6	S: 5′-CTATGAACTCCCTCTCCACAA -3′ A: 5′-TGCCCAGTGGACAGGTTTCT -3′	711 bp
IL-8	S: 5′-AGGGATCCTGTGTAAACATGACTTCC -3′ A: 5′-GGAATTCACGGATCTTGTTTCTC -3′	330 bp
TGF-β	A: 5′-TTCCTGCTCCTCATGGCCAC -3′ A: 5′-GCAGGAGCGCACGATCATGT -3′	393 bp
TNF-α	S: 5′-CTCTTCTGCCTGCTGCAC -3′ A: 5′-GCCCTTGAAGAGGACCTG -3′	288 bp
GAPDH	S: 5′-CCTTCATTGACCTCAACTACAT -3′ A: 5′-CCAAAGTTGTCATGGATGACC-3′	400 bp