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Myocardial collagen deposition and inflammatory cell infiltration in cats with pre-clinical hypertrophic cardiomyopathy

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ABSTRACT

The histological features of feline hypertrophic cardiomyopathy (HCM) have been well documented, but there are no reports describing the histological features in mild pre-clinical disease, since cats are rarely screened for the disease in the early stages before clinical signs are apparent. Histological changes at the early stage of the disease in pre-clinical cats could contribute to an improved understanding of disease aetiology or progression. The aim of this study was to evaluate the histological features of HCM in the left ventricular (LV) myocardium of cats diagnosed with pre-clinical HCM. Clinically healthy cats with normal ($n = 11$) and pre-clinical HCM ($n = 6$) were identified on the basis of echocardiography; LV free wall dimensions (LVFWd) and/or interventricular septal wall (IVSd) dimensions during diastole of 6–7 mm were defined as HCM, while equivalent dimensions <5.5 mm were defined as normal. LV myocardial sections were assessed and collagen content and inflammatory cell infiltrates were quantified objectively. Multifocal areas of inflammatory cell infiltration, predominantly lymphocytes, were observed frequently in the left myocardium of cats with pre-clinical HCM. Tissue from cats with pre-clinical HCM also had a higher number of neutrophils and a greater collagen content than the myocardium of normal cats. The myocardium variably demonstrated other features characteristic of HCM, including arteriolar mural hypertrophy and interstitial fibrosis and, to a lesser extent, myocardial fibre disarray and cardiomyocyte hypertrophy. These results suggest that an inflammatory process could contribute to increased collagen content and the myocardial fibrosis known to be associated with HCM.

Introduction

Hypertrophic cardiomyopathy (HCM) is a primary myocardial disease characterised by concentric hypertrophy of the left ventricle (LV). In human beings and cats, HCM is caused by mutations in genes that encode for the myofilament sarcomeric proteins, Z-disc proteins, calcium-handling proteins and other proteins related to the sarcomere (Ferrantini et al., 2009; Lehrer and Geeves, 2014). To date, 20 genes with over 400 missense mutations have been identified in human beings; there is strong evidence for pathogenicity for some of these mutations but less evidence for others (Ferrantini et al., 2009; Tian et al., 2013; Marsiglia and Pereira, 2014).

In cats, two single nucleotide substitutions in the myosin-binding protein C gene have been identified, but the broad genetic spectrum in human beings suggests that many sarcomeric genes could also be implicated in cats (Wess et al., 2010). In both species, there is marked phenotypic heterogeneity and LV hypertrophy can be global or regional. Papillary muscle hypertrophy, systolic anterior motion of the mitral valve and/ or left atrial dilatation have also been identified (Liu et al., 1981; Kittleson et al., 1999; Fox, 2003). In cats, the functional implications of this pathology include diastolic dysfunction that can result in congestive heart failure, systemic thromboembolism and fatal arrhythmias (Fox et al., 1995).

In human beings, the mechanism by which the genetic mutation in the sarcomere translates to the phenotype remains poorly understood. Direct investigation of the effect of the HCM sarcomeric mutation is difficult because human tissue is limited to autopsy samples of patients with terminal disease, small biopsy samples, or myectomised tissue from patients with LV outflow tract obstruction. For the latter, tissue samples are modified by secondary changes associated with altered haemodynamic and mechanical stress independent of the disease-causing mutation. While transgenic mouse models afford more readily accessible tissue, the HCM phenotype in this species develops without LV outflow tract obstruction and microvascular pathology (Maass and Leinwand, 2000; Shephard and Semsarian, 2009). Studies of the pathogenesis of feline HCM from sarcomeric mutation to phenotypic expression are lacking and histological description is limited to post-mortem investigations of cats with severe and spontaneously terminal disease (Liu et al., 1981, 1993; Fox et al., 1995; Kittleson et al., 1999).

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As in human beings, a post-mortem diagnosis of feline HCM is based on the identification of a hypertrophied, non-dilated LV and an increase in absolute and relative heart weight (Liu et al., 1993). Histological changes of LV myocardial tissue stained with haematoxylin and eosin (H and E) include myocardial fibre disarray, intramural coronary arteriosclerosis and myocardial fibrosis (Liu et al., 1981, 1993; Kittleson et al., 1999; Fox, 2003). Cardiomyocytes have been described as hypertrophied, with large, rectangular, hyperchromic nuclei (Fox, 2003), but similar changes have not been identified in cats with HCM (Kershaw et al., 2012).

The aim of the present study was to report the LV histological changes in cats with pre-clinical HCM compared with normal cats. Additional staining techniques were utilised to quantitatively assess the LV myocardium of cats with pre-clinical HCM for collagen content and infiltration by inflammatory cells.

Materials and methods

Animals

Unowned cats scheduled for euthanasia were obtained from a local animal shelter; their use was approved by the University of Queensland Animal Ethics Committee (approval number SVS/040/09). Cats that appeared overtly healthy and were considered to be normal on clinical examination ($n = 28$) were sedated SC with 0.1 mg/kg acepromazine (ACP 2, Delvet) and 0.1 mg/kg hydromorphone (Dilaudid, Mundipharma) for echocardiography. Cats without cardiac disease and those with pre-clinical HCM were then recruited for further study.

Normal cats

Cats were identified as normal if the following criteria were met: (1) physical examination was unremarkable, they were well hydrated and had a body condition score of 3–5/5 (Laflamme, 1997); (2) thoracic auscultation identified a regular heart rhythm and no heart murmur; (3) six-lead electrocardiogram identified normal sinus rhythm or sinus tachycardia with a mean electrical axis between –10° and +140° (Harvey et al., 2005); and (4) echocardiography identified LV wall symmetry from the right parasternal short-axis view by continuous base-to-apical sweep with LV free wall (LVFWd) and interventricular septal wall (IVSd) dimensions during diastole of <5.5 mm (Fox et al., 1995), left atrial (LA) to aortic (Ao) root ratio (LA:Ao) <1.37 (Abbott and MacLean, 2006), subjectively normal right heart with no more than trivial insufficiencies of the pulmonic and tricuspid valves, no insufficiency of the aortic and mitral valves, ventricular outflow velocities determined by pulsed-wave Doppler echocardiography of <1.5 m/s and pulsed-wave tissue Doppler velocity of the lateral mitral valve annulus determined from the left apical four-chamber view of >5.8 cm/s (Koffas et al., 2006).

Cats with pre-clinical hypertrophic cardiomyopathy

Cats were identified as having pre-clinical HCM if the following criteria were met: (1) physical examination was unremarkable and body condition score was 3-5/5 (Laflamme, 1997); (2) thoracic auscultation was unremarkable or identified a systolic heart murmur of grade IV/VI or less; (3) six-lead electrocardiogram identified normal sinus rhythm or sinus tachycardia irrespective of mean electrical axis (Harvey et al., 2005) and; (4) echocardiography identified LVFWd and/ or IVSd dimensions during diastole of 6–7 mm (MacDonald et al., 2006), LA:Ao ratio >1.37 (Abbott and MacLean, 2006), subjectively normal right heart with no more than trivial insufficiencies of the pulmonic and tricuspid valves, no insufficiency of the aortic valve and right ventricular outflow velocity determined by pulsed-wave Doppler <2.4 m/s (Rishniw and Thomas, 2002).

Echocardiography

Echocardiographic (Phillips iE33, Phillips Healthcare) examination was performed with the cat lightly restrained in lateral recumbency on a purpose-built table, which allowed placement of the transducer (12 MHz) on the dependent side of the thorax. Electrodes attached to the skin overlying the stifles and right elbow allowed the simultaneous recording of a lead II electrocardiogram (ECG) that was displayed on the ultrasound monitor. All examinations were performed by the same experienced echocardiographer (FEC).

Dimensional measurements of the LV were made from a right parasternal short axis view (Thomas et al., 1993) at the level of the papillary muscles from two-dimensional short-axis images using the leading-edge method (Sahn et al., 1978) and included IVSd and LVFWd and the internal diameter of the LV in diastole and systole (LVIDd and LVIDs, respectively). Calipers were positioned at the onset of the QRS complex on the simultaneously recorded ECG for determination of diastolic measurements. Systolic measurements were made from the frame with smallest chamber dimension immediately preceding ventricular expansion.

Using a modification of a previously described technique (Rishniw and Erb, 2000), LA and aortic root (Ao) dimensions were determined from a right parasternal two-dimensional short-axis view at the heart base by directing the calipers in a line along the commissure between the non-coronary and the left coronary aortic valve cusps through the Ao and LA. All LA and Ao measurements were determined immediately preceding atrial systole at the onset of the P wave on the ECG (Abbott and MacLean, 2006).

Echocardiographic examination also included colour-flow Doppler assessment of all valves and pulsed-wave Doppler assessment of both outflow tracts to identify any significant valvular insufficiencies or outflow obstruction suggestive of non-HCM cardiac disease and to quantify peak LV outflow tract velocity when systolic anterior motion of the mitral valve was present with HCM.

Transmitral flow was recorded from the left apical four-chamber view with the 2 mm pulsed-wave sample volume placed between the tips of the open mitral leaflets. Peak early (peak E) and late (peak A) diastolic flow wave velocities were measured. When rapid heart rates produced E and A wave summation, peak velocity of the summed waveform (summed EA) was recorded. Pulsed-wave tissue Doppler imaging of the LV myocardium was used to determine early (Peak E′) and late (Peak A′) diastolic velocity of the lateral mitral annulus from the left parasternal four-chamber view. When rapid heart rates produced E′ and A′ wave summation, peak velocity of the summed waveform (summed E′A′) was recorded. For tissue Doppler imaging, the gate of the 12 MHz transducer was placed perpendicular to myocardial movement, the Nyquist limit was set at 10–15 cm/s, sweep speed was 160 cm/s and the filter was set at 50 MHz (Koffas et al., 2006). All measures were made from four to five consecutive cardiac cycles and averaged.

Selection and preparation of tissue sections

Hearts were collected, sectioned and prepared according to a standardised protocol. Cats were humanely euthanased by sodium pentobarbitone (30 mg/kg IV; Lethabarb Euthanasia Injection, Virbac) and heparin was administered (320 IU/kg IV) to prevent thrombosis. Right thoracotomy was immediately performed and the heart was excised from the mediastinum, and peripheral fat and loose connective tissue were removed. The wet heart weight was recorded, then the LV was excised, weighed and fixed in 10% neutral buffered formalin.

Full thickness tissue sections of the LV were taken perpendicular to the long axis of the LV from: (1) the IVS at the point of maximal thickness, and (2) the posterior LVFW about one-half the distance between the mitral valve annulus and the LV apex. These sections were dehydrated in graded ethanol series, embedded in paraffin wax and processed for histopathology. Sections with a thickness of 10 μm for picrosirius red staining, and 6 μm for H and E and Leder (chloroacetate esterase) staining, were mounted onto slides.

Routine histopathological examination

LV tissues stained with H and E from normal cats ($n = 11$) and cats with pre-clinical ($n = 6$) HCM were evaluated histologically by a single blinded veterinary pathologist (HO). Subjective description of tissue sections included: (1) semi-quantification of myocardial fibre disarray visually estimated as absent (occupying <5% or 5–15% of the tissue section); (2) presence or absence of myocyte hypertrophy (assessed subjectively as number fold increase in fibre diameter compared with normal healthy tissues), fragmentation and vacuolation; (3) presence or absence, type and degree of infiltrative leucocytes; (4) subjective description of arteriosclerosis with mural thickening of the small arterioles and the presence or absence of arteriole thrombi; and (5) presence or absence of myocardial fibrosis.

Quantification of collagen by picrosirius red stain staining

Dehydrated tissue sections (10 μm thickness) from normal cats and cats with pre-clinical HCM were rehydrated by immersion in lithium carbonate (saturated in distilled water) for 3 min. Slides were rinsed in a water bath (5 min) and washed in distilled water (30 s) before being immersed in phosphomolybdic acid (0.2% in distilled water, 4 min) to reduce non-specific binding of the stain to the section. After rinsing in distilled water, slides were transferred into the picrosirius red stain (0.1% sirius red F3BA in saturated picric acid) and incubated for 45 min. Slides were then placed in 0.01 M hydrochloric acid for 2 min, removed and mounted on Depex with a cover slip and allowed to dry overnight (Allan et al., 2005). Analysis of stained sections was performed using a laser-scanning confocal microscope (Model LSM 510 Meta, Carl Zeiss) with a HeNel laser. Slides were exposed to a red filter (excitation wavelength of 543 nm, emission wavelength of 560–615 nm).

Since any fibrosis in the normal and pre-clinical cases appeared to be relatively uniform in distribution on the basis of examination of H and E sections, five randomly selected regions of each tissue section were chosen for evaluation. Images were acquired at 40× magnification and analysed for pixel intensity to ascertain the extent of collagen deposition. The data were compiled using Image J software (National Institute of Health). Collagen deposition, given as a percentage of tissue evaluated, was averaged from five images (Fenning et al., 2005).

Identification and quantification of neutrophils

Tissue sections (6 μm thickness) from normal cats and cats with pre-clinical HCM were stained by the Leder method using the 91C-1KT – Naphthol AS-D Chloroacetate