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SPONTANEOUSLY ARISING DISEASE

Pathological Features and Pathogenesis of the Endomyocardial Form of Restrictive Cardiomyopathy in Cats

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Summary

This study reports pathological and molecular features in 41 cases of feline restrictive cardiomyopathy (RCM). Grossly, there were patchy or diffuse areas of endocardial thickening affecting the left ventricle. The more common patchy endocardial lesions occurred as large trabecular or irregular broad bands of fibrous tissue bridging the left ventricular free wall and ventricular septum. Microscopically, regardless of the gross pattern, the thickened endocardium contained various numbers of stellate, spindle-shaped or elongated mesenchymal cells surrounded by fibrous connective tissue. Immunohistochemical findings were indicative of smooth muscle differentiation in mesenchymal cells. These cells proliferated vigorously and produced alcian blue-positive ground substance and collagen fibres; it was considered that the mesenchymal cells contributed to the formation of the endocardial lesions. In addition, multiple left ventricular 'false tendons' were invariably included within the trabecular or broad fibrous bands, providing a framework for formation of those bands. Evidence of endocarditis or endomyocarditis was lacking in all 41 cases, and no viral genomes were detected in any of the DNA or RNA samples obtained from 14 of the hearts. These observations suggest that any relationship between feline RCM and a virus-induced inflammatory response seems unlikely.

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Introduction

Restrictive cardiomyopathy (RCM) in man is characterized by restrictive filling and reduced diastolic volume of either or both ventricles, with normal or near-normal systolic function and wall thickness (Richardson *et al.*, 1996). A variety of specific pathological processes can cause RCM, including endomyocardial scarring, myocardial fibrosis or infiltrative disorders (Gallo and d'Amati, 2001). These conditions are divided into two distinct groups in man: those with predominant endocardial involvement (endomyocardial form) and others with predominant myocardial involvement (myocardial form) (Gallo and d'Amati, 2001). This classification of RCM also appears to be applicable to cats (Ferasin *et al.*, 2003; Fox, 2004) and the endomyocardial type, also known as endomyocardial fibrosis, is the most prevalent form of feline RCM (Bond and Fox, 1984; Kimura *et al.*, 2016).

A prominent pathological feature of the endomyocardial form of feline RCM is severe and extensive

fibrous thickening of the endocardium and chamber deformity (Fox, 2004). This process primarily affects the left ventricle (LV) or, rarely, both ventricles. There are two basic morphological patterns of endocardial fibrosis, although a degree of overlap in features may occur (Fox, 2004). In the first pattern, the LV shows diffuse, marked fibrosis, which appears as an opaque, white–grey, firm covering involving substantial portions of the left ventricular cavity. In the second pattern, large trabecular or irregular broad bands are observed bridging the left ventricular free wall and ventricular septum (VS) and often causing fixed stenosis or a fibrotic tube in the mid to apical left ventricular chamber. This is the most common form reported in cats with endomyocardial RCM (Fox, 2004).

The aetiology of endomyocardial RCM in cats is unknown, but is thought to be multifactorial, as there is a wide spectrum of clinical manifestations and pathological phenotypes (Fox, 2004). Possible hypotheses include viral or immune-mediated endomyocarditis followed by reparative fibrosis, or a consequence of the end stage of myocardial failure and infarction from hypertrophic cardiomyopathy (Fox, 1999, 2004).

The present report provides a fuller description of the range of pathological features in the endomyocardial form of RCM and presents a hypothesis to explain the pathogenesis of this disease in the cat.

Materials and Methods

Animals

As described in our previous report (Kimura *et al.*, 2016), the materials for the present study were obtained from consecutive feline necropsy examinations conducted at the Laboratory of Veterinary Clinical Oncology, Tokyo University of Agriculture and Technology, Tokyo, Japan, during the period 2005–2014. A total of 327 necropsy examinations were performed on cats with heart disease, and 41 cats were diagnosed as having the endomyocardial form of RCM. In these cases, the diagnosis was based on characteristic gross features of marked fibrosis focally or diffusely involving the endocardium of the LV. The hallmark gross findings included an enlarged heart with marked left atrial and often right atrial dilation and hypertrophy; severe and extensive endocardial fibrosis, frequently bridging the left ventricular free wall and VS; left ventricular hypertrophy commonly associated with wall and chamber deformity; and mural thrombi in the left atrium (LA) or LV (Fox, 1999, 2004).

Pathological Studies

A complete necropsy examination was performed on all 41 cats within 12 h of death. The hearts were examined and weighed, and the heart weight to body weight (HW/BW) was calculated (i.e. HW in g/BW in kg). The hearts were then placed in 10% neutral buffered formalin for a minimum of 5 days. After fixation, the ventricles were sliced transversely into serial sections approximately 5 mm thick, from the mitral valve to the cardiac apex. Each transverse ventricular slice was embedded in paraffin wax, sectioned (5 μm) and stained with haematoxylin and eosin (HE). Selected sections were also stained with Masson's trichrome for collagen fibres, elastic van Gieson for elastic fibres and alcian blue (pH 2.5) and toluidine blue for acidic glycosaminoglycans. The microscopical examination focused on any morphological changes in the endocardium of the LV.

Immunohistochemistry (IHC) was performed for identification of the endothelial marker CD34 (mouse monoclonal anti-human CD34 class II, Clone QBEnd-10; 1 in 100 dilution; Dako, Glostrup, Denmark), vimentin (mouse monoclonal antibody against vimentin, clone Vim 3B4; 1 in 100 dilution; Dako), α-smooth muscle actin (α-SMA; mouse monoclonal antibody against human smooth muscle actin, clone 1A4; 1 in 100 dilution; Dako), caldesmon (mouse monoclonal antibody against human caldesmon, clone h-CD; 1 in 100 dilution; Dako), platelet-derived growth factor (PDGF; rabbit polyclonal antibody against PDGF BB; 1 in 50 dilution; Abcam, Cambridge, UK) and transforming growth factor-β (TGF-β; rabbit polyclonal antibody against TGF-β; 1 in 100 dilution; Abcam) in the endocardium of the LV. The avidin–biotin–peroxidase method (Vectastain, Vector Laboratories, Burlingame, California, USA) was employed in order to identify the reaction product, with haematoxylin as a counterstain.

Molecular Biological Studies

Real-time polymerase chain reaction (PCR) was used to determine whether viral nucleic acids were detectable in cardiac tissues including the endocardium, myocardium and epicardium. DNA and RNA samples were extracted from wax-embedded tissue blocks of 14 cat hearts with endomyocardial RCM collected between 2012 and 2014 using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Life Technologies, Carlsbad, California, USA). Screening for a total of six viruses, including feline herpesvirus (FHV), feline panleukopenia virus (FPLV), feline calicivirus (FCV), feline coronavirus (FCoV), feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV), was carried out according to their

Table 1
Summary of primer–probe sets used for real-time polymerase chain reaction

Target name	Target gene name	Probe sequence (5′ FAM-3′ TAMURA)		Primer sequences (5′–3′)	GenBank accession number	Reference
FHV	ORF of gB protein	TATATG TGTCC ACCAC CTTCA GGATCT ACTGT CGA	F: R:	AGAGGCT AACGGA CCATCGA GCCCGT GGTGGC TCTAAAC	S66371	Vögtlin *et al.*, 2002
FPLV	VP	CGAGT GATGA ATTTGCT ACAGG	F: R:	TGGAAC TAGTGGC ACACCAA AAATGG TGGTAAG CCCAATG	HQ184203	André *et al.*, 2013
FCV	5′UTR	ACTCA CAGTG TCCGAA AGGAC TTTGTGC	F: R:	ATGTCT CAAACT CTGAGCT TCGT CGCCTC CGTGCAA GTGTTA	AY560115	–
FCoV	7b protein	CAGCG CGATG ACCAG TAATTCC TCGGA	F: R:	TGAGTT ATAAGGCA ACCCGATGT GTGCTT ACCATTC TGTACAA GAGTA	JN634064	–
FIV	gag protein	TATGCC TGTGGA GGGCC TTCCT	F: R:	AGAAC CTGGTG ATATACC AGAGAC TTGGGT CAAGTG CTACA TATTG	M25381	Dieter *et al.*, 1999
FeLV	U3 of LTR	CCAGC AGTCT CCAGG CTCCCCA	F: R:	AACAGC AGAAGTT TCAAGGCC TTATA GCAGAAA GCGCGCG	AB847301	Ravi *et al.*, 2005
ACTB	Chromosome E3, β-actin	TCGCTG TCCACCT TCCAGCA GATGT	F: R:	AGCGCA AGTACTC CGTGTG CGGACT CGTCGTA CTCCTGCTT	XM006941899	–

F, forward primer; R, reverse primer; FHV, feline herpesvirus; FPLV, feline panleukopenia virus; FCV, feline calicivirus; FCoV, feline coronavirus; FIV, feline immunodeficiency virus; FeLV, feline leukaemia virus; ACTB, feline β-actin.

prevalence, and the feline β-actin gene (ACTB) was selected as a positive control. Primer pairs and TaqMan probes labelled with the reporter dye FAM at the 5′ end and fluorescent dye TAMRA at the 3′ end for FHV, FPLV, FIV and FeLV were designed on the basis of the published sequences (Dieter *et al.*, 1999; Vögtlin *et al.*, 2002; Ravi *et al.*, 2005; André *et al.*, 2013). New primer–probe sets for FCV, FCoV and ACTB were designed to amplify the conserved region of each genome using PrimeQuest™ software (Integrated DNA Technologies, Coralville, Iowa, USA). All primer

sequences, target gene names and Genbank accession numbers are listed in Table 1. Real-time PCR reactions were performed using a LightCycler® Nano (Roche Diagnostics, Mannheim, Germany) and a one-step PrimeScript™ RT-PCR kit (TAKARA Biotech, Shiga, Japan). Nuclease-free water was used for negative controls in each run. The thermal cycling conditions were the same as those described previously (Tsuchiaka *et al.*, 2016).

Results

Necropsy Examination

Macroscopically, although some overlap was evident between them, two types of fibrotic lesion were observed in the endocardium of the LV. Most commonly, patchy endocardial lesions occurred as single or multiple large trabecular bands or irregular broad bands of fibrous tissue bridging the left ventricular lumen from the free wall to the septum ('patchy' pattern; $n = 34$) (Fig. 1A). The fibrous bands were usually located between the anterior and/or posterior papillary muscles (PMs) and the VS, frequently causing intraventricular partial obstruction and/or chamber deformity at different levels. Less commonly, the left ventricular endocardium showed diffuse and marked fibrosis, which appeared as a white—grey, smooth, firm and uniform covering over the inflow and outflow tracts, PMs, mitral valve apparatus and, occasionally, the aortic valves and

trunk ('diffuse' pattern; $n = 7$) (Fig. 1B). In this type, extensive fibrosis caused a moderate to marked reduction of left ventricular chamber size. The mitral valve apparatus and PMs were distorted and fused to surrounding structures.

Heart weights in the cats with endomyocardial RCM (HW/BW 6.0 ± 1.1 g/kg [mean $\pm$ SD]) were greater than those in cats with normal hearts (4.0 ± 0.3 g/kg; Liu and Tilley, 1980). The LA and its auricular appendage were markedly enlarged, often with apparent wall thickening. Mural thrombi occurred in the LA, left auricle or LV and were found in 29.3% of the affected cats. In addition, distal aortic thromboembolism was observed in 43.9% of cases.

Histopathology

Histologically, the endocardium of the LV was thickened by proliferation of hyaline and/or fibrous tissue in all 41 cases, regardless of the macroscopic pattern of endocardial thickening. The endocardial lesions were divided into three main histological types, although some cases with intermediate features were observed.

In three of the 41 cases, the thickened endocardium was covered by intact endothelial cells and contained a large number of stellate, spindle-shaped or elongated mesenchymal cells surrounded by various amounts of alcian blue-positive ground substance with delicate collagen fibrils (type 1) (Fig. 2A). These mesenchymal cells had large oval nuclei, prominent
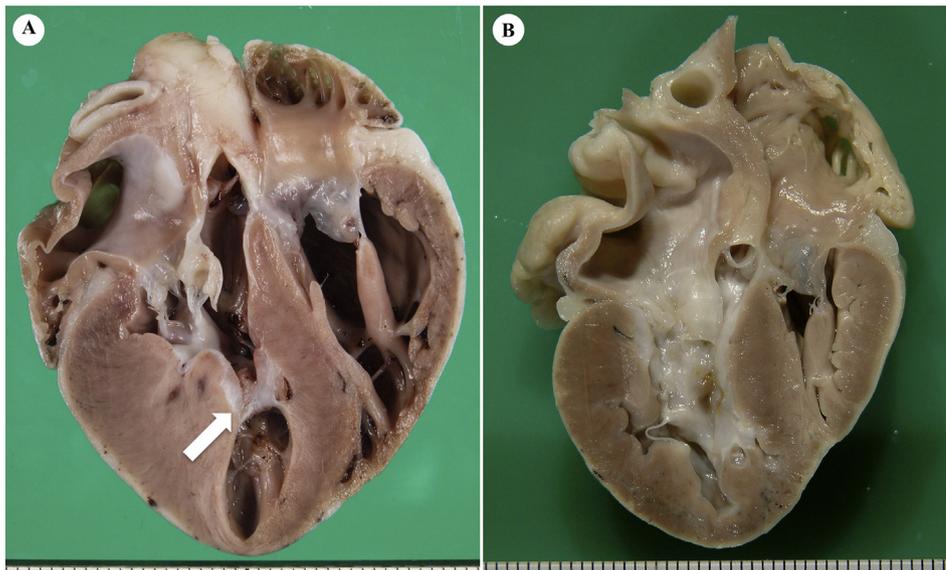
Fig. 1. (A) Longitudinal section of the heart of a 5-year-old domestic short-haired cat, showing a patchy pattern of endocardial thickening in the left ventricle (LV). Irregular broad bands of fibrous tissue bridge the anterior and posterior papillary muscles (PMs) and the ventricular septum (arrow). (B) Longitudinal section of the heart of a 6-year-old domestic short-haired cat, showing a diffuse pattern of endocardial thickening in the LV. White—grey, smooth, firm and uniform fibrous tissue covers the inflow and outflow tracts, PMs and mitral valve apparatus. Scale, 1 mm.

nucleoli, faintly eosinophilic cytoplasm and poorly defined cell margins, and were randomly arranged.

In the second type (type 2), which accounted for 19 of the 41 cases, the endocardial thickening revealed a two-layer structure (i.e. superficial and deep layers; Fig. 2B). These two layers were sometimes ill-defined, and there was some variation, even within the same chamber. The superficial layer consisted of polymorphic mesenchymal cells and a large amount of alcian blue-positive ground substance intermixed with delicate collagen fibrils. Some cells in this area were round to polygonal, had a metachromatic capsule when stained with toluidine blue, and sometimes were located within lacunae, showing chondrogenic differentiation. The deep layer was composed of densely arranged collagen fibres with frequently elongated mesenchymal cells. These cells had fusiform nuclei arranged in a uniform wavy pattern.

The third type (type 3) accounted for the other 19 cases. This type of lesion consisted of thick collagen fibres with sparse elastic fibres, occasionally containing foci of chondroid metaplasia (Fig. 2C). The mesenchymal cells interspersed among bundles of mature collagen fibres were oriented predominantly with their long axis parallel to the endocardial surface. These cells were similar in appearance to those seen in the deep layer of type 2 lesions, but were slightly less numerous.

Cats with type 1 lesions were young, with a mean age at death of 1.8 years (range = 0.3−3.0 years; SD = 1.4), while cats with type 2 and 3 lesions were usually older, with mean ages at death of 5.5 years (range = 2−11 years; SD = 2.6) and 10.2 years (range = 3−19 years; SD = 5.2), respectively. There were significant differences in the age at death among the three types (one-way analysis of variance; $P < 0.001$).

In the endocardial lesions showing a diffuse pattern, the line between the thickened endocardium and the underlying myocardium was usually well defined. There were no inflammatory cells in the endocardium or the myocardium, and there was no evidence of myocardial degeneration, necrosis or fibrosis (Fig. 2). In some cases, however, fibrous tissue of the endocardium extended for various distances into the myocardium, accompanying mild to moderate atrophy of myocardial fibres.
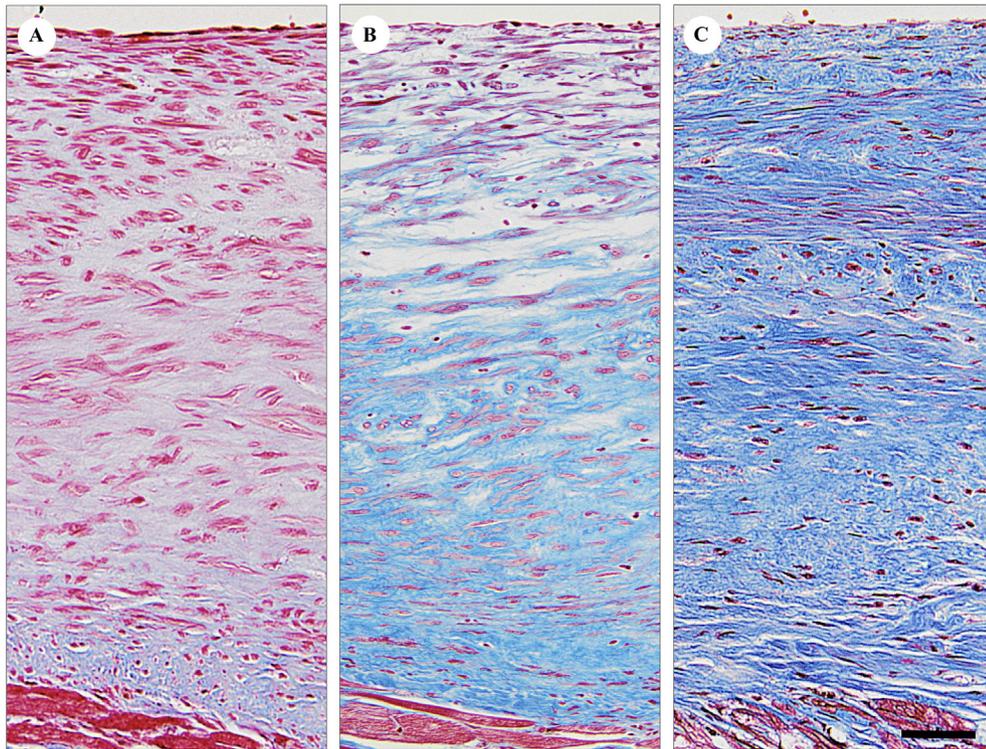
Fig. 2. Histological sections of the endocardium from 3-, 5- and 12-year-old domestic short-haired cats (A, B and C, respectively), showing a diffuse pattern of thickening. The endocardial lesions are divided into three histological types. (A) Type 1 lesions contain a large number of polymorphic mesenchymal cells surrounded by abundant amorphous substances with delicate collagen fibrils. (B) In type 2 lesions, the superficial layer of the thickened endocardium is qualitatively similar to the type 1 lesion, while the deep layer is composed of densely arranged collagen fibres with elongated mesenchymal cells. (C) Type 3 lesions consist of thick collagen bundles interspersed with elongated mesenchymal cells. Masson's trichrome. Bar, 100 μm.

In the endocardial lesions showing a patchy pattern, trabecular or broad bands of fibrous tissue always included multiple left ventricular false tendons (LVFTs), which were composed of central Purkinje fibres and surrounding collagen fibres. In lesions showing this pattern, mesenchymal cells derived from pre-existing LVFTs had proliferated vigorously, showing a characteristic concentric circular pattern centering on the LVFTs. These cells produced a large amount of alcian blue-positive ground substance and delicate collagen fibrils (type 1), resulting in fusion of the markedly thickened LVFTs (Fig. 3A). The cellular fibrous tissue then matured into densely arranged collagenous connective tissue (type 2) (Fig. 3B), and eventually comprised a large mass of fibrous scar tissue (type 3) (Fig. 3C). Also in this lesion pattern, no distinct inflammatory changes were evident.

Immunohistochemistry

In all 41 cases, positive labelling for CD34 was limited to the surface cells of the thickened endocardium and the capillary-like channel-lining cells. The stellate, spindle-shaped or elongated mesenchymal cells embedded within the fibrous connective tissue were diffusely positive for vimentin, as well as α-smooth muscle actin and caldesmon, although a small proportion of the cells were not labelled for either of the latter markers (Fig. 4A, B). PDGF and TGF-β were present frequently on the cell surface and in the sur-rounding fibrous connective tissue, especially in type 1 and 2 lesions (Figs. 4C, D).

Real-time Polymerase Chain Reaction

None of the viral genomes tested were detected in any of the DNA or RNA samples obtained from 14 cat hearts with this abnormality, while the *ACTB* genome was detected in all samples.

Discussion

The endomyocardial form of RCM in cats was first described in 1970 by Liu, who reported a retrospective series of 68 cases of focal endocardial and myocardial fibrosis in the LV as 'acquired cardiac lesions leading to congestive heart failure'. Thereafter, the clinical and morphological features of the abnormality were assessed systematically and reported as a distinct disease entity in 1980 by Liu and Tilley. In 2004, Fox reported the first comprehensive description of the disease. Since then, no additional pathological studies of the aetiology of feline endomyocardial RCM have been reported.

In the present study, the endocardial lesions were classified into three types according to their histological composition and structure. Type 1 lesions were found only in young cats aged between 4 months and 3 years, while type 2 and 3 lesions were observed predominantly in middle-aged and older cats, respectively. In addition, the number of cat hearts with type 1 lesions was much lower than the number with type 2
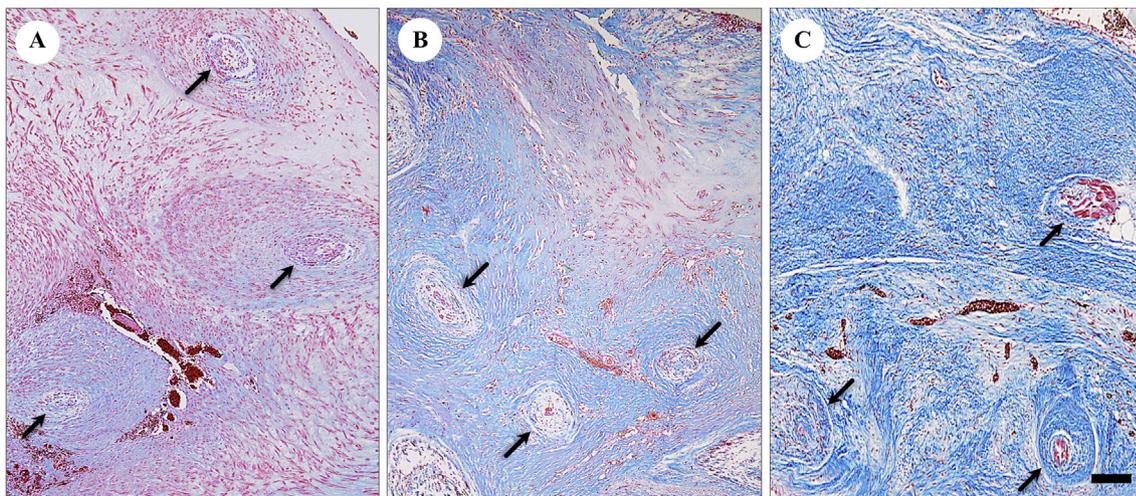
Fig. 3. Histological sections of the endocardium from 3-, 6- and 9-year-old domestic short-haired cats (A, B and C, respectively), showing a patchy pattern of thickening. The fundamental structural features are similar to those of lesions showing the diffuse pattern. Multiple left ventricular false tendons (LVFTs) are invariably embedded within trabecular or broad bands of fibrous tissue (arrows). (A) In type 1 lesions, mesenchymal cells proliferate around the LVFTs and produce abundant amorphous substances and delicate collagen fibrils. As a result, markedly thickened LVFTs are fused together. (B) In type 2 and 3 lesions, the cellular fibrous tissue matures into densely arranged collagenous connective tissue, and (C) eventually comprises a large mass of fibrous scar tissue. Masson's trichrome. Bar, 100 μm.

and 3 lesions. Considering the degree of fibrous tissue maturation in each lesion type, it is very likely that the type 1 lesion is an early form of this abnormality, which usually progresses to the more mature type 2 and 3 forms with age. The gradations of the endocardial lesions, the presence of intermediate cases, and the age relationship appear to indicate that the endomyocardial form of feline RCM develops after birth and progresses gradually with age. The fact that no reports appear to have documented the disease in newborn kittens lends further support to this idea.

In the immunohistochemical study, the mesenchymal cells embedded within fibrous connective tissue showed a positive reaction with antibodies against α-smooth muscle actin and caldesmon, indicating a tendency for smooth muscle differentiation; however, undifferentiated cells best classified as primitive mesenchymal cells were also present. In this connection, it is of interest that the subendocardial layer originally contains smooth muscle cells and undifferentiated mesenchymal cells, especially in the left ventricular septal region and the LA (John *et al.*, 2001). Furthermore, the presence of PDGF and TGF-β was confirmed immunohistochemically. It is well known that these growth factors regulate the proliferation of smooth muscle cells and their synthesis of extracellular matrix, leading to the development of arteriosclerotic intimal lesions (Gotlieb and Silver, 2001). Therefore, it is considered that the proliferation of pre-existing mesenchymal

cells, which are capable of undergoing smooth muscle differentiation, cause or contribute to the formation of endocardial lesions in the endomyocardial form of feline RCM.

Macroscopically, patchy endocardial thickening was the most common pattern of endomyocardial RCM in cats, as reported previously (Fox, 2004). The patchy lesions appeared as single or multiple large trabecular bands or irregular broad bands of fibrous tissue bridging the left ventricular lumen, usually from the anterior and/or posterior PMs to the VS. Histologically, the fibrous bands invariably included multiple LVFTs around which mesenchymal cells proliferated vigorously and produced alcian blue-positive ground substance and delicate collagen fibrils. These observations suggest that LVFTs provide a framework for the formation of fibrous bands, and it is noteworthy that a pathological condition associated with abnormal diffuse networks of LVFTs (referred to as 'excessive moderator bands') has been reported in 21 cats with heart failure (Liu *et al.*, 1982). The increased numbers of LVFTs most commonly connected the posterior PM or the anterior PM to the VS, showing arrangement patterns similar to those of the trabecular or broad bands observed in the present study. Considering that multiple LVFTs were embedded within the fibrous bands, it is possible that excessive LVFTs are closely related to the occurrence of this type of RCM. Ferasin (2009) believes that 'excessive moderator bands' or 'moderator
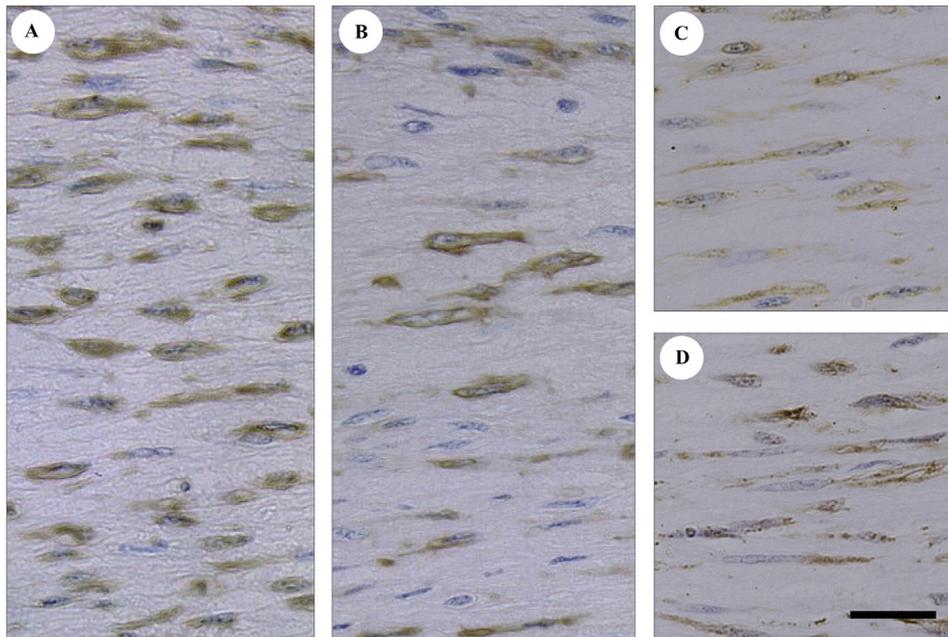
Fig. 4. Immunolabelling demonstrates (A) α-smooth muscle actin and (B) caldesmon in the cytoplasm of stellate, spindle-shaped or elongated mesenchymal cells within the thickened endocardium. (C) Platelet-derived growth factor and (D) transforming growth factor-β are present on the cell surface and in the surrounding fibrous connective tissue. IHC. Bar, 20 μm.

band cardiomyopathy' are simply lesions associated with endomyocardial RCM.

The aetiology of the endomyocardial form of feline RCM remains unresolved; however, it has been suggested that endomyocardial damage followed by reparative fibrosis might be associated with viral or immune-mediated endomyocarditis (Fox, 2004). Endomyocarditis of undetermined origin has been described as an important disease of cats since the early 1970s (Liu, 1970; Stalis *et al.*, 1995). In addition, parvoviral genomic material has been isolated from a small number of feline hearts with cardiomyopathy (including RCM) and myocarditis, although cause and effect has not been established (Meurs *et al.*, 2000). In the present study, histological evidence of endocarditis or endomyocarditis was lacking in all 41 cases. Furthermore, our molecular biological observations using real-time PCR detected no viral genomes in any of the DNA or RNA samples obtained from 14 cat hearts with this abnormality. These findings suggest that any relationship between a virus-induced inflammatory response and the occurrence of feline RCM is unlikely, but do not necessarily rule out this possibility because the present molecular biological study was limited by its small sample size, the localization of viral genomes within the heart and technical factors associated with real-time PCR. On the other hand, it remains to be seen whether an immune-mediated pathway contributes to the pathogenesis of endocardial or endomyocardial inflammation, and further studies will be required in order to address this issue.

The present observations confirm and extend the findings of previous studies and lead to the following conclusions. Firstly, patchy and diffuse patterns of endocardial thickening are evident macroscopically in the LV, the former being much more common. Secondly, from a histological viewpoint, stellate, spindle-shaped or elongated mesenchymal cells proliferate vigorously in the endocardium, producing alcian blue-positive ground substance and collagen fibres; these cells are thought to contribute to endocardial lesion formation. Thirdly, from an immunohisto-chemical viewpoint, the mesenchymal cells embedded within fibrous connective tissue show a tendency for smooth muscle differentiation. Fourthly, in lesions with a patchy pattern, multiple LVFTs are invariably included within large trabecular or irregular broad bands of fibrous tissue, providing a framework for the formation of the fibrous bands. Finally, the lack of any histological evidence of endocarditis or endomyocarditis and the absence of candidate viral genomes in cardiac tissue suggest that the occurrence of feline RCM is not significantly related to a virus-induced inflammatory response.

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