The Veterinary Journal 197 (2013) 388–394

Contents lists available at SciVerse ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Morphological changes to endothelial and interstitial cells and to the extra-cellular matrix in canine myxomatous mitral valve disease (endocardiosis)

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ARTICLE INFO

Article history:
Accepted 23 January 2013

Keywords:
Dog
Myxomatous mitral valve disease
Electron microscopy
Endocardiosis

ABSTRACT

Morphological and functional changes in endothelial and interstitial cells are considered central to myxomatous degeneration of the canine mitral valve (endocardiosis). The aim of this study was to describe and quantify changes in valve endothelial cells (VECs), interstitial cells (VICs) and the extra-cellular matrix (ECM) of the sub-endothelial zone of diseased valves using a combination of transmission electron microscopy, stereology and computer-aided image analysis.

Marked degradation of the endothelium was evident in diseased valves, which coincided with significant degradation of the local ECM ($P < 0.001$). There were decreases and increases in the numbers of VECs and VICs, respectively, in diseased valves, with particular accumulation of VICs subjacent to the valve surface ($P < 0.01$). Overall, VICs were more pleomorphic than VECs in both normal and diseased valves, but for VECs, the degree of pleomorphism was significantly different in diseased valves ($P < 0.0001$). The findings of the study confirm that canine myxomatous mitral valve disease is associated with marked endothelial damage, with attendant proliferation of subjacent activated myofibroblasts. The fact that similar endothelial changes are present in normal valves suggests these processes not only contribute to valve pathology, but may also represent life-long valve remodelling.

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Introduction

Myxomatous mitral valve degeneration (MMVD) or endocardiosis, is the most common cardiac disease in the dog, is particularly prevalent in certain breeds, and is closely associated with advancing age (Buchanan, 1977; Beardow and Buchanan, 1993; Han et al., 2008). As the disease progresses, there is destruction and derangement of the valve stroma with loss of collagen bundle organisation and accumulation of glycosaminoglycan in the leaflets, predominantly towards the valve edge (Pomerance and Whitney, 1970; Buchanan, 1977; Kogure, 1980; Black et al., 2005; Han et al., 2009; Culshaw et al., 2010).

The cellular changes in canine MMVD have been partially characterised and predominantly involve myofibroblast activation (Black et al., 2005; Han et al., 2008, 2009; Disatian et al., 2008, 2010). The end result is a valve stroma with excess proteoglycans, collagen loss, disorganisation of a proportion of the remaining collagen, and a reduction in mature collagen cross-linking (Hadian et al., 2007, 2010; Han et al., 2009). These changes also include a relative loss of valvular interstitial cells (VICs) from an overtly myxoid stroma and in proliferation of activated myofibroblasts subjacent to the endothelial surface (Black et al., 2005; Disatian et al., 2008; Han et al., 2008, 2009).

Whether or not this is a manifestation of cell migration towards the endothelium or local sub-endothelial cell proliferation is not known. However, there are also clear changes to the valve endothelium of affected dogs which involve both structural and functional alteration of the endothelial cells, including apoptosis, cell detachment and loss, along with increased nitric oxide synthesis and endothelin receptor expression (Mow and Pedersen, 1999; Olsen et al., 2003; Black et al., 2005; Corcoran et al., 2004).

The structural changes affecting the endothelium and subjacent stroma have, to date, only been partially described (Mow and Pedersen, 1999; Olsen et al., 2003; Corcoran et al., 2004; Black et al., 2005). The aim of this study was to address this information deficit by describing and quantifying changes to valvular endothelial cells (VECs), VICs and the subjacent extra-cellular matrix (ECM) of canine valves affected by MMVD using a combination of transmission electron microscopy, stereology and computer-aided image analysis.

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1090-0233/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tvjl.2013.01.027

Materials and methods

Tissue selection

Mitral valves from three normal dogs (a 2-year old male Labrador retriever, a 3-year old male mixed breed dog, and a 6-year old male Staffordshire bull terrier), and from three animals with severe MMVD (an 8-year old female German shepherd, a 13-year old male Cavalier King Charles spaniel, and a 16-year old male Jack Russell Terrier) were examined (Whitney, 1974). Only the Cavalier King Charles spaniel had been in heart failure and been treated with a combination of furosemide, (Hoechst), pimobendan (Boehringer Ingelheim), and enalapril (Merial). The dogs were euthanased using IV pentobarbitone and the valves collected and fixed within 10 min. These tissues were collected with full owner consent and the project had the approval of the Veterinary Ethical Research Committee of the Royal (Dick) School of Veterinary Studies, The University of Edinburgh.

Transmission electron microscopy

Samples from the mid-point of the anterior leaflet of the mitral valve were fixed at 4 °C for 18 h in 2.5% glutaraldehyde (Agar Scientific) in 0.1 M sodium cacodylate buffer (Agar Scientific), rinsed three times in 0.1 M sodium cacodylate buffer (30 min for each rinse), and then rinsed twice in distilled water (2 min for each rinse). Specimens were post-fixed in 1% osmium tetraoxide (Electron Microscopy Science) for 1.5 h, rinsed three times in distilled water (2 min for each rinse), dehydrated in ascending concentrations of ethanol (Fisher Scientific) (50% for 15 min, 70% for 15 min, 90% for 15 min, and 100% for 30 min), and were then rinsed twice (30 min each time) in acetone (Fisher Scientific).

The specimens were then incubated at room temperature in a Spurr resin (Agar Scientific) and acetone mixture (1:2) for 1 h, followed by Spurr resin and acetone (1:1) for a further 1 h, and were then 'cured' in a silicone mould with Spurr resin at 70 °C overnight. The embedded specimen blocks were trimmed and sections cut on a Leica Ultracut UCT microtome (Reichert), initially at 1 μm semi-thin, and stained with Toluidine Blue.

The semi-thin sections were viewed under a Leitz Laborlux light microscope (Leica) and areas of interest were selected for ultra-thin sectioning. The ultra-thin sections of 60 nm, with gold inference colour, were collected on Formvar-coated single-hole copper grids (Agar Scientific) and stained with 2% uranyl-acetate (Merck) for 45 min followed by lead citrate (Electron Microscopy Science) for 6 min. The ultra-thin sections were then examined in a Philips CM12 transmission electron microscope (Philips) at 75 kV beam acceleration voltage, photographed (Kodak Electron Microscope Film 4489, Eastman Kodak) and qualitatively described.

Quantitative stereology

From each of the six blocks, three ultra-thin sections were cut at assigned points along the length of the anterior leaflet: the first close to the edge (tip of leaflet) and the next 50 μm and 100 μm from the first section site in the direction of the annulus. Ten images were randomly taken from each of the 18 sections at points along the length of the section following the line of direction of the endothelial edge (a total of 180 images at 3000× magnification).

Electron micrographs were scanned into digital format. A 1 mm scaled grid was used to count the grid intersections (nodes) overlying the VECs and VICs. This process was repeated for the collagenous and non-collagenous ECM. For the total grid score the number of nodal points counted equated to the volume of the structure being examined. In the case of cells this was an equivalence measurement of cell numbers not an actual cell count and for non-cellular structures was equivalent to total content of that structure.

From the 180 transmission electron micrographs, a total of 176 VECs and VICs were identified. The outline of each cell was traced and a circularity score was calculated using ImageJ[1] based on the formula 4π (area/perimeter^2), where a perfect circle has a value of '1' and any value deviating towards zero has an elongated shape. Circularity was then used as an index of cell pleomorphism.

Statistical analysis

Data were expressed as means ± standard error of the means. Inferential statistical analysis involved one-way analysis of variance ANOVA testing with $P < 0.05$ taken as statistically significant. Means comparisons between groups were subjected to a pair-wise Tukey–Kramer test.

Results

Qualitative assessment of valves

Normal valves

The endothelium formed a continuous, flat and smooth single-cell layer with cells in close apposition to each other linked by desmosomal-type junctions. The endothelium overlaid a distinct and intact basement membrane marking the border with the stromal ECM (Fig. 1A). A prominent nucleus occupied the majority of the cytosolic space and often appeared flattened. Dense heterochromatin was spread evenly throughout the lighter granulated euchromatin in the nucleus, and was not concentrated at the nuclear periphery. Occasionally, vesicles near the cell surface exhibited endocytosis and/or exocytotic activity.

Apoptotic cells with achromatic swollen nuclei that contained flocculated material were occasionally seen and were partially covered by normal appearing VECs. These cells had degraded cytoplasm, large vacuoles, many small vesicles and loss of granular, electron-dense cytoplasmic contents and damaged cell membranes with leakage of contents into the extracellular space (Fig. 1B). Immediately subjacent to the basement membrane were isolated collagen fibres and occasional VICs, some in close proximity to the endothelium and others in direct contact with the VECs through extended cytoplasmic processes (Fig. 1C). Slightly deeper into the valve stroma, organised collagen bundles with a typical banding pattern, running in different directions and some extending towards the surface endothelium and in close association with the VECs, were observed. In some damaged areas there was: disruption of the basement membrane, endothelial detachment and surface denuding; the presence of degenerating cells with swollen nuclei and lack of tight junctions (desmosomal attachments only); and an amorphous subendothelial ECM with leakage though the damaged basement membrane (Fig. 1D).

Diseased valves

There was extensive denuding of the endothelium on affected valves with loss of surface continuity and affected cells had prominent 'doming' towards their luminal surface, which was roughened with increased numbers of micro-appendages (Fig. 2A). Doming was very pronounced in some cases (Fig. 2B), while other cells were completely detached from the basement membrane (Fig. 2C). Affected cells also had a clearer distinction between their nuclear euchromatin and heterochromatin compared to normal cells.

Changes to the basement membrane were varied. In areas where endothelial cells were still present the basement membrane exhibited small gaps exposing the underlying ECM. Similar to damaged areas on normal valves, the basement membrane was thickened and in some areas split into two layers (Fig. 2D). There were areas where the basement membrane appeared intact despite loss of the overlying VECs. In other, more severely affected, areas there was total loss of the basement membrane, exposing the underlying ECM which had an amorphous or translucent degraded appearance. Interstitial cells were clearly seen in close proximity to the damaged endothelium, with many more cells present compared to normal. Some VICs were in direct contact with VECs while others appeared lodged between endothelial cells (Fig. 3A). There was disarray of collagen bundles, which were sparsely arranged within the abundant ground substance. The typical 'banding pattern' appearance of densely packed collagen was no longer visible, and there was splintering of some bundles, with many fibrils angled rather than straight and with increased inter-fibrillar space (Fig. 3B).

There was marked pleomorphism of the VICs which were often located 'side-by-side'. Many cells were elongated in shape, contained numerous vacuoles and secretory vesicles, and were actively producing matrix. Their nuclei had central light granulated euchromatin surrounded by dark heterochromatin, typical of metabolically active cells. Binucleated cells were commonly seen and were actively producing ECM (Fig. 3C). Small numbers of round cells with pro-collagen containing vacuoles, a more granular cyto-

[1] See: http://rsb.info.nih.gov/ij/.

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