Ultrastructural morphologic evaluation of the phenotype of valvular interstitial cells in dogs with myxomatous degeneration of the mitral valve

Alexander Black, MMedSc; Anne T. French, MVB; Joanna Dukes-McEwan, BVMS, MVM, PhD; Brendan M. Corcoran, MVB, PhD

Objective—To evaluate morphologic changes in valvular interstitial cells of dogs and to find evidence for disease-associated phenotypic changes in these cells.

Animals—5 clinically normal dogs and 5 dogs with severe mitral valve endocardiosis.

Procedure—Mitral valve leaflets were evaluated by use of transmission electron microscopy. Differences in cell type and cell location were identified.

Results—A change in cell type toward a myofibroblast or smooth muscle cell phenotype was detected, with the smooth muscle cell type being most common. These cells had long amorphous cytoplasmic extensions, fibrillar cytoplasm, incomplete basal lamina, few mitochondria, and eccentrically placed nuclei but lacked smooth endoplasmic reticulum or Golgi complexes. Remaining valvular interstitial cells had heterochromatic nuclei and produced only minimal quantities of collagen. Compared with normal valves, myxomatous valves had many interstitial-like cells located adjacent to the endothelium. Deeper within the abnormal valves, cells with a heterogenous phenotype formed groupings that appeared to be anchored to adjacent collagen.

Conclusions and Clinical Relevance—Myxomatous degeneration of the mitral valve in dogs is associated with phenotypic alteration, changing from an interstitial to a mixed myofibroblast or smooth muscle cell phenotype. A closer association between interstitial cells and the endothelium is evident in diseased valves. In response to the disease process, valvular interstitial cells of dogs appear to change toward a smooth muscle phenotype, possibly in an attempt to maintain valve tone and mechanical function. (Am J Vet Res 2005;66:1408–1414)

Myxomatous disease of the mitral valve (commonly known as mitral valve endocardiosis) is the single most commonly acquired cardiac disease of dogs. The disease involves progressive myxomatous degeneration of the mitral valve and chordae tendineae and has close pathologic similarities to myxomatous degeneration of the mitral valve in humans.

Myxomatous degeneration results in disturbance of collagen deposition and organization and increased amounts of acid mucopolysaccharides. The gross and histopathologic appearance of affected valve leaflets in dogs has been reported. Similarities between forms of this disease in humans and dogs may involve similar pathologic processes, suggesting that they may be the same disease entity. However, the cause of myxomatous mitral valve disease in dogs and humans is unknown. Evidence emerged from a study in humans that documents an increase in numbers of valvular interstitial cells and also phenotypic alteration to a more active and secretory myofibroblast type of cell.

Valvular interstitial cells are the major cell type of the valve and are enmeshed in the valve matrix. Valvular interstitial cells can have fibroblast-like and smooth muscle cell characteristics and are often described as myofibroblasts because of this mixed cell phenotype. They produce the extracellular matrix of the valve (collagen, elastin, and proteoglycans), and it can be presumed they have a role to play in the development of the disease in dogs and humans. Whether there is a similar phenotypic alteration of cells in dogs with myxomatous disease of the mitral valve is not known. Therefore, the objective of the study reported here was to use transmission electron microscopy (TEM) to identify phenotypic changes in valvular interstitial cells of affected dogs.

Material and Methods

Animals—Mitral valve tissues were collected from 5 clinically normal client-owned dogs and 5 client-owned dogs affected by mitral valve endocardiosis. For all dogs, mitral valve tissues were collected during postmortem examination after dogs were euthanatized; all owners provided informed consent for use of the tissues.

The 5 clinically normal dogs were young adult mixed-breed dogs (3 males and 2 females; estimated age of 1 to 2 years) that were euthanatized for reasons other than cardiac disease (unaffected group). These dogs did not have evidence of a murmur during auscultation and had no evidence of endocardiosis on gross inspection of the valve leaflets. Young
Dogs were used for this group because we believed it unlikely that normal tissues could be obtained from a population of older dogs.

Dogs with clinical evidence of advanced mitral valve endocardiosis (affected group; n = 5) were euthanatized because of intractable congestive heart failure. Affected dogs were between 9 and 15 years of age and included 3 male Cavalier King Charles Spaniels, 1 female Cocker Spaniel, and 1 male mixed-breed dog. Affected dogs had a left apical sys-

Figure 1—Transmission electron micrograph of a section of the mitral valve from a clinically normal dog. The valvular interstitial cell has a typically large central nucleus with a high degree of euchromatin and a prominent nucleolus (N). There are distinct lamellae of rough endoplasmic reticulum and scattered mitochondria (arrows). Notice the dense ordered collagen surrounding the cell. Uranyl acetate and lead citrate; bar = 10 µm.

Figure 2—Transmission electron micrograph of a section of the mitral valve from a clinically normal dog revealing a valvular interstitial cell (IC) in close proximity to a second IC. The top right corner reveals a nucleus with a thin rim of heterochromatin and scattered perinuclear mitochondria in close association with free ribosomes. The bottom left corner presents an area with distinct rough endoplasmic reticulum, lamellae, and cytoplasmic filaments. Elastic extracellular matrix (E) is evident as amorphous foci. Uranyl acetate and lead citrate; bar = 0.5 µm.

Figure 3—Transmission electron micrographs of a section of an abnormal mitral valve obtained from a dog with mitral valve endocardiosis. A—Overview of an area of a diseased valve revealing 2 cell profiles (arrows). These cells are atypical of ICs seen in unaffected valves. The cells appear multinucleated and are embedded in a sparse extracellular matrix. Lack of collagen is particularly noticeable. B—Higher magnification of banded collagen in the valve reveals some disintegration of typical collagen morphologic characteristics. Notice also the fine fibrillar nature of extracellular matrix elements in the area adjacent to the banded collagen. Uranyl acetate and lead citrate; bars = 0.5 µm.
tolic heart murmur and clinical and radiographic signs of congestive heart failure; they were in the advanced stages of the disease. Mitral valve endocardiosis was confirmed by echocardiography performed before the dogs were euthanized as well as by gross inspection of the valve leaflets during postmortem examination. All affected dogs had severe mitral valve endocardiosis (type III or IV).

TEM—Excised mitral valve tissues were immediately immersed in a fixative solution of 4% glutaraldehyde in 0.1M sodium cacodylate (pH, 7.4). Mitral valves were then dissected and removed by use of a standard dissecting microscope; removed valves were rinsed for 30 minutes in 0.1M sodium cacodylate buffer. For TEM, samples from the valve leaflets were then fixed in 1% osmium tetroxide solution, dehydrated through a series of graded ethanol solutions, and embedded in resin.

Evidence of myxomatous degeneration was initially determined on the basis of a gross pathologic description reported elsewhere. Semithin (thickness of 1 µm) sections were obtained and stained with toluidine blue; these sections were then viewed under light microscopy to confirm abnormalities. On the basis of results for this preliminary examination, areas were selected for additional evaluation. Ultrathin (thickness of 60 nm) sections with a gold inference color were cut from these areas by use of an ultramicrotome, mounted on carbon-slot grids (1 X 2 mm), and stained with uranyl acetate (45 minutes at 40°C) followed by lead citrate (6 minutes at 20°C). Stained ultrathin sections were then viewed in a TEM at an accelerating voltage of 75 kV.

Internal ultrastructure of the valve leaflets was examined, and representative photomicrographs were obtained. All sections were reviewed concurrently by 2 of the authors (AB and BMC). For each dog, 3 sections obtained from various sites were assessed and described. These composite descriptions were used to compare the types of changes found in abnormal valves with results for valves from clinically normal dogs.

In affected dogs, samples for TEM were selected from the parts of the leaflet (cranial [septal] or caudal [mural]) that visibly were most abnormal, rather than from specific predetermined parts of the valve. In clinically normal dogs, samples were obtained from the free edge of the caudal valve leaflet. Additional samples were also processed for light microscopy and stained with H&E. Myxomatous changes were confirmed by use of descriptions reported elsewhere.

Results

Histologic examination—For affected and unaffected dogs, examination of H&E-stained sections revealed typical histologic features. For valves from affected dogs, there appeared to be an increase in cell numbers in the myxomatous areas of the valve leaflet, compared with results for valves from unaffected dogs. In all cases, abnormal areas had typical destruction and disorganization of the fibrosa and expansion of the spongiosa.

TEM of unaffected valves—Mitral valves from unaffected dogs had a typical appearance on TEM

Figure 4—Transmission electron micrographs of a section of an abnormal mitral valve from a dog. A—An elongated cell with many cytoplasmic extensions possesses a highly folded nucleus with a high euchromatin-to-heterochromatin ratio, which indicates cellular activity. B—Higher magnification of the cytoplasm (inset of panel A) reveals that it is packed with fibrils. Many coated vesicles are also evident in the perinuclear area. This cell has a mostly mixed fibroblast-smooth muscle cell phenotype (myofibroblastic phenotype). Uranyl acetate and lead citrate; bar = 3 µm for panel A and 0.5 µm for panel B.

Figure 5—Transmission electron micrograph of a section of an abnormal mitral valve from a dog revealing an endothelial cell (EC) and an IC in close proximity. The nucleus of the EC has a distinct rim of heterochromatin with many nuclear pores. Mitochondria are scattered in the left perinuclear region, and the cytoplasm has a clearly developed cytoskeleton. Notice that the EC forms a distinct junction with an adjacent cell (arrowhead). The IC has a prominent oval nucleus with a high heterochromatin-to-euchromatin ratio, which is indicative of relative inactivity. The IC has few organelles, although there are abundant ribosomes on the far right side of the cell. The membrane on the far left has some vesicular transport, which is indicative of a low amount of secretion of extracellular matrix. Uranyl acetate and lead citrate.
These valves consisted mainly of chains of banded collagen. These bundles of collagen were dense and compact in the fibrosa but more loosely arranged and sparser in the spongiosa. Valvular interstitial cells were embedded in the matrix they produced. Cells had varying amounts of activity, with some cells having obvious swollen rough endoplasmic reticulum and numerous vesicles. The cells appeared spherical in shape or elongated and had thin processes that extended from the cytoplasm to adjacent cells; these processes formed junctions with the adjacent cells (Figure 2). Cells typically had centrally located nuclei, which were highly euchromatic, and a prominent nucleolus. The nuclei were crenated with visible pores on the nuclear envelope. Mitochondria, rough endoplasmic reticulum, smooth endoplasmic reticulum, free ribosomes, and Golgi complexes were randomly scattered throughout the cytoplasm or sometimes located in 1 part of the cell. Secretory vesicles were readily visible in the cytoplasm; some fused with the cell wall to form coves from which collagen precursors were secreted. Organized bands of collagen were visible adjacent to these sites. However, other cells, although morphologically similar, had evidence of quiescence typified by heterochromatic nuclei. Many of these less active cells were deeply embedded in dense, tightly packed collagen bundles. A few cells that still maintained a fibroblast phenotype were identified in close proximity to the endothelium, and some cells appeared to form close contacts with the endothelial cells. However, neither simple nor complex junctions between the 2 cell types could be identified.

**TEM of affected valves**—Valves of affected dogs had a characteristic appearance on TEM (Figure 3). The valves lacked organized structure with degeneration of collagen bundles and an excessive abundance of ground substance. Fragments of interstitial cells and remnants of cytoplasmic processes could be seen within this disorganized matrix. Compared with results for the unaffected valves, collagen bundles appeared...
swollen, separated from each other, and with varying degrees of disintegration. The overall arrangement of collagen bundles was erratic and the collagen sparse. Collagen fiber precursors in the cell surface coves, when identified, also lacked organization and formed thick fibers of unequal size, shape, and width. The fibers could be seen exiting the coves in haphazard directions, which contrasted with the results for the unaffected cells. However, large amounts of elastin could also be seen at some sites within the extensive mucoid areas.

The cellular composition of the abnormal valves differed from that of the unaffected valves. Subjectively, it appeared there was an increase in cell numbers (also evident on H&E-stained sections), but this was not quantified. It also appeared that there was an increase in the number of cells located in the vicinity of the endothelium. Changes in cellular ultrastructural features were indicative of altered phenotype.

Many of the cells had structural characteristics typical of a smooth muscle phenotype (Figure 4). The cells had long amorphous cytoplasmic extensions into the valve matrix. Many of these extensions were orientated parallel to the collagen bundles, and some cells appeared to have produced small quantities of poorly formed collagen. In the body of these cells, there were varying numbers of spherical, oval, and elongated mitochondria in the cytoplasm, but few other organelles were visible. The nuclei were eccentrically located and had numerous coated vesicles. Typically, rough endoplasmic reticulum fragments were located only in the vicinity of the perinuclear area. There was little evidence of smooth endoplasmic reticulum or Golgi complexes, suggesting a reduced amount of secretory activity, compared with that for cells in the unaffected valves. The cytoplasm of these cells was highly filamentous, and the cytoskeleton appeared less organized than in the unaffected valves; a distinct incomplete basal lamina also was evident. For some of the cells, nuclei were typically electron-dense, more irregularly shaped, and had a higher concentration of heterochromatin than the cells in unaffected valves.

The exact phenotype of these cells was difficult to assess, but they appeared to have characteristics of both fibroblast and smooth muscle cells. Some of the cells appeared inactive, whereas others had morphologic evidence of secretory activity.

Many more cells were found in close proximity to the endothelium in the myxomatous valves, compared with results for the unaffected valves (Figure 5). The cells were orientated in a semicontinuous layer of several cells in some places, whereas the cells were in close apposition to the endothelial cells with local adhesions in other places. Some of these cells appeared quite active and retained a fibroblastic phenotype, whereas others were relatively inactive but appeared to have continued to produce small quantities of extracellular matrix.

Finally, a consistent finding in the abnormal valves was groups of cells (typically 3 cells in a row) in close contact with each other that had varying phenotypes (Figure 6). These cells were located deep within the valve, located away from the endothelial surface, and often abutted bundles of collagen. The cells were in differing states of activity. There was evidence that the adjacent collagen bundles were produced by the cells, but at other sites, this was not as apparent and the cells appeared to be attached (anchored) to the collagen bundles. Such arrangements were not seen during examination of any of the unaffected valves.

**Discussion**

In the study reported here, we identified alterations of the valvular interstitial cells from a predominantly fibroblast phenotype to that of more mixed myofibroblast and smooth muscle cell phenotypes. Furthermore, analysis of our results documented that there is profound alteration in morphologic characteristics and activity of valvular interstitial cells, and we believe these changes in overall valve cell phenotype and function will have important bearing on the maintenance of normal valve structure and therefore normal mechanical function. Valvular interstitial cells are responsible for the production of the valve matrix (collagen, elastin, and acid-mucopolysaccharides), and it is the ability of valvular interstitial cells to continually regulate valve remodeling that probably determines valve durability in the face of loading forces. There is also evidence that valvular interstitial cells may be important in maintaining the structure and deformability of valve leaflets through a combination of passive structural support and inherent contractility.

Variations in valvular interstitial cell phenotype have been recognized in experimental cell culture systems and in humans with naturally developing mitral valve disease. Two basic phenotypes are identified (ie, fibroblast or smooth muscle), but a combined myofibroblast phenotype can also exist. The myofibroblast phenotype may be an intermediary stage between the other 2 phenotypes. In normal human mitral valves, the fibroblast phenotype (vimentin-positive) is the predominant cell type, but in diseased valves, the mixed myofibroblast cell type (vimentin- or α-smooth muscle actin-positive or vimentin-desmin-positive) also appears. Of major interest is the fact that these activated myofibroblasts express catabolic enzymes such as collagenases, and it is has been suggested that this enzymatic activity is important in the collagen destruction evident with this disease. In experimental cell culture systems, similar cell phenotypic variation is recognized, but the effect of disease on such variation has not been reported.

In the study reported here, we identified similar disease-associated changes in the interstitial cell phenotype in the mitral valve of dogs. We were able to identify an increase in cell numbers and an alteration from a fibroblastic-like phenotype to more of a smooth muscle cell phenotype. We also recognized intermediate phenotypic stages and identified quiescent cells that had morphologic characteristics of interstitial cells in both affected and unaffected valves. Of particular interest was the identification of an apparent migration of interstitial cells toward the endothelial surface in both affected and unaffected valves and the development of a close association between groups of cells that appeared to be anchored to bundles of collagen deeper within the abnormal valves.
Proliferation and migration of valvular interstitial cells toward the endothelium have been reported for experimental models of mitral valve injury and are dependent on fibroblast growth factor-2. Additionally, endothelial damage as a feature of myxomatous disease of mitral valves has been identified in humans and dogs with the disease. Although the exact reasons for such damage are not known, involvement of repeated trauma along the line of valve closure must be considered because this is the site that is typically affected by the disease. It would be interesting to speculate that endothelial damage stimulates proliferation and migration of interstitial cells, but whether it has an effect on cell phenotype and the dissolution and destruction of valve matrix is not known.

The apparent reduction in fibroblast phenotype and replacement with a smooth muscle or myofibroblast phenotype was a consistent finding in the study reported here. Cells with mixed phenotypes contained abundant stress filaments. Although they may also produce elements of the extracellular matrix, this did not appear to be their primary function. The predominance of cells with a mixed smooth muscle-myofibroblast phenotype may be an adaptation to the loss of extracellular matrix in that these cells were able to maintain some degree of valve tone and thus help maintain valve competence. In 1 study, investigators used immunophenotyping and mainly found myofibroblasts in affected human valves, with little evidence for the appearance of smooth muscle cells. The results of that study differ from our findings in dogs. However, although we used ultrastructural morphologic criteria to identify cells, we cannot state with certainty that the cells we identified were a pure smooth muscle phenotype. Furthermore, irrespective of the phenotyping technique used, there are particular problems with valvular cells because of their obvious phenotypic heterogeneity.

Another consideration is that any difference in cell type between humans and dogs may represent differences in disease severity. Valve samples are more likely to be obtained in dogs with advanced disease, whereas material is often collected from humans earlier during the disease process at the time of valve replacement. All the affected dogs in the study reported here had advanced disease. Therefore, it is possible that a more smooth muscle phenotype may be identified in humans with advanced disease and, similarly, a myofibroblast phenotype may be identified in dogs during earlier stages of the disease.

An interesting observation in the study reported here was the identification of groups of cells in abnormal valves with a predominantly smooth muscle appearance that were in close association with each other and that appeared to be attached to bundles of collagen. Because we could not identify similar changes in the unaffected valves, we would speculate that the development of such cell groupings may be an additional attempt to provide contractile mechanisms that will help sustain valve tone and support valve mechanics.

This was a limited descriptive study; thus, we did not seek to ascertain regional differences in cell numbers or cell phenotype. This issue will be addressed in ongoing studies. Instead, we targeted the most visible area of diseased valves in the affected dogs, but it would be of interest to ascertain whether there are subtle differences among areas and zones of diseased leaflets, compared with results for unaffected valves, as well as differences between the 2 leaflets in the same affected dog.

Irrespective of alterations in cell phenotype that have been identified in mitral valves of dogs and humans, the underlying reason for collagen destruction and accumulation of proteoglycans is unknown. Increased expression of collagenases by myofibroblasts could contribute to loss of extracellular matrix. What can be stated is that there is a change in the valve cell phenotype consequent to the disease in both species, but it does not identify the underlying cause. Because endothelial damage has also been documented in humans and dogs with the disease, it would be reasonable to suspect that endothelial dysfunction may be implicated.

Limitations of the study reported here were that it was observational in nature and used relatively small numbers of samples, which did not permit quantitative analysis. To further elucidate the mechanisms for cell phenotypic alteration in dogs with myxomatous degeneration of the mitral valve, studies are required to phenotype cells by other means (eg, immunophenotyping) and investigate the temporal and regional changes in phenotypic expression.

Interstitial cells in the mitral valves of dogs with myxomatous degeneration alter from a fibroblast phenotype toward a smooth muscle phenotype. In addition, there is evidence of increased cell localization close to the endothelium and that other cells attach to each other and adjacent collagen-forming smooth muscle contractile units. However, the overall cellular phenotypic characteristics of abnormal valves can be best described as mixed with variations in cell phenotype and cell activity.

References


