รายงานวิจัยฉบับสมบูรณ์

โครงการ

การตายของเซลล์และการปรากฏของโปรตีนในตระกูลบีซีแอลสอง และทรานสฟอร์มมิ่ง โกรว์ท แฟคเตอร์ เบต้า 1 ในโรคลิ้นหัวใจไมทรัลเสื่อมในสุนัข

Apoptosis and the expression of Bcl-2 family and transforming growth factor β1 (TGF β1) signaling proteins in canine myxomatous mitral valve disease

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วันที่ 14 กันยายน 2554

สัญญาเลขที่ MRG5380171

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Apoptosis and the expression of Bcl-2 family and transforming growth factor β1 (TGF β1) signaling proteins in canine myxomatous mitral valve disease

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

เงินทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่ ตามโครงการความร่วมมือระหว่างสำนักงานคณะกรรมการการอุดมศึกษากับสำนักงานกองทุนสนับสนุนการวิจัย ที่สนับสนุนเงินทุนสำหรับดำเนินงานวิจัยชิ้นนี้

หน่วยพยาธิวิทยา ภาคพยาธิวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ที่เอื้อเฟื้อสถานที่และอำนวยความสะดวกในการเก็บตัวอย่างและทำการทดลอง

รศ.ดร. บรรจบ ศรีภา และหน่วยปฏิบัติการวิจัยพยาธิชีววิทยา ภาคพยาธิวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ที่เอื้อเฟื้อสถานที่อุปกรณ์และวัสดุในการทำ Tissue microarray

Abstract

Project Code: MRG5380171

Project Title: Apoptosis and the expression of Bcl-2 family and transforming growth factor β1 (TGFβ1) signaling proteins in canine myxomatous mitral valve disease

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Project Period: 1 year

Myxomatous mitral valve disease (MMVD) is the most common acquired heart disease in dogs. An increased cell density has been reported in myxomatous valves. Cell proliferation is unlikely a major mechanism of increased cellularity. Decreasing in cell death or anti-apoptosis may play roles implicating persistence of cells in diseased valves. To determine apoptosis, the expression of cleaved caspase–3, DNA fragmentation (TUNEL marker) and apoptotic bodies was evaluated in normal (n=15), early stage (n=20) and late stage (n=20) MMVD valves. Cells in normal and both stages of MMVD expressed the TUNEL marker and cleaved caspase-3, but not apoptotic bodies. The percentage of TUNEL marker and cleaved caspase-3 positive nuclei was non-significantly different in three groups of dogs ($p<0.05$). To determine the relationship between TGFβ1 signaling and apoptosis, the expression of activated TGFβ1 signaling protein, phosphorylated Smad2/3 (p-Smad2/3) and Bcl-2 family proteins (*bax* and *bcl–2*) was reviewed. P-Smad2/3 and pro-apoptotic protein, *bax* were up-regulated in myxomatous valve; whereas, anti-apoptotic protein, *bcl-2* was decreased. P-Smad2/3 expression increased only in late stage MMVD. These data suggest that cells in myxomatous valves undergo pro-apoptotic condition; however, these cells do not undergo execute apoptosis. The apoptosis remains unchanged during disease progression. The role of TGFβ1-Smad signaling pathways in inducing pro-apoptotic state is unclear.

Keywords: apoptosis, *bax/bcl-2*, dogs, myxomatous valves, TGFβ

บทคัดย่อ

รหัสโครงการ: MRG5380171

ชื่อโครงการวิจัย: การตายของเซลล์และการปรากฏของโปรตีนในตระกูลบีซีแอลสอง และทรานสฟอร์มมิ่งโกรว์ท แฟคเตอร์ เบต้า 1 ในโรคลิ้นหัวใจไมทรัลเสื่อมในสุนัข

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ระยะเวลาโครงการ: 1 ปี

โรคลิ้นหัวใจไมทรัลเสื่อมเป็นโรคหัวใจที่เป็นภายหลังกำเนิดที่พบได้บ่อยในสุนัข มีรายงานการเพิ่มขึ้นของจำนวนเซลล์ในลิ้นหัวใจที่เสื่อม โดยที่การเพิ่มการแบ่งตัวของเซลล์ (cell proliferation) อาจไม่ใช่กลไกสำคัญในการเพิ่มจำนวนเซลล์ การตายที่ลดลงหรือการยับยั้งกระบวนการตายของเซลล์ (apoptosis) อาจเป็นกลไกที่ทำให้เซลล์ในลิ้นหัวใจที่เป็นโรคยังคงอยู่ การศึกษานี้ทำการประเมินการตายของเซลล์ โดยดูจากการปรากฏของ cleave caspase-3 การแตกของสายดีเอ็นเอ (TUNEL marker) และ apoptotic bodies ในลิ้นหัวใจของสุนัขปกติ (n=15) สุนัขที่มีปัญหาลิ้นหัวใจเสื่อมช่วงแรก (n=20) และช่วงท้าย (n=20) ผลการศึกษาพบการปรากฏของ TUNEL marker และ cleaved caspase-3 ในลิ้นหัวใจปกติและลิ้นหัวใจที่เป็นโรค แต่ไม่พบการปรากฏของ apoptotic bodies ร้อยละการปรากฏของ TUNEL marker และ cleave caspase-3 ไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติในสุนัขทั้งสามกลุ่ม ($p<0.05$) ทำการศึกษาความสัมพันธ์ระหว่าง TGFβ1 signaling และการตายของเซลล์ โดยดูการปรากฏของ phosphorylated Smad2/3 (p-Smad2/3) และโปรตีนในตระกูล Bcl-2 (*bax* และ *bcl-2*) พบว่า p-Smad2/3 และ *bax* ซึ่งเป็นโปรตีนเหนี่ยวนำการตาย มีการปรากฏเพิ่มขึ้นในลิ้นหัวใจที่เกิดการเสื่อม ในขณะที่ *bcl-2* โปรตีนที่ทำหน้าที่ในการยับยั้งการตายของเซลล์มีการปรากฏลดลง พบว่าการปรากฏของ p-smad2/3 จะมีการเพิ่มขึ้นเฉพาะในลิ้นหัวใจที่เกิดการเสื่อมช่วงท้าย โดยสรุปจากข้อมูลที่ได้ เซลล์ในลิ้นหัวใจเสื่อมน่าจะมีการเปลี่ยนแปลงเข้าสู่ภาวะเหนี่ยวนำให้เกิดการตาย (pro-apoptotic) อย่างไรก็ตามเซลล์เหล่านี้ไม่ได้เข้าสู่กระบวนการตายโดยสมบูรณ์ การตายของเซลล์ไม่มีการเปลี่ยนแปลงในระหว่างการเกิดโรค ส่วนบทบาทของ TGFβ1-Smad signaling ในการเหนี่ยวนำการตายของเซลล์ยังไม่เป็นที่ทราบแน่ชัด

คำหลัก: การตายของเซลล์ *bax/bcl-2* สุนัข ลิ้นหัวใจเสื่อม TGFβ1

Introduction

Myxomatous mitral valve disease (MMVD) is the most common acquired heart disease in dogs (Whitney, 1979). MMVD accounts for approximately 75% of dogs affected with cardiovascular diseases (Kvart and Haggstrom, 2000). The prevalence of MMVD is reported approximately 20 to 25 % in dogs 9 years or older (Delweiler and Patterson, 1965). The pathology of degenerative valves mainly involves thickening of mitral valve leaflets with elongated and occasionally ruptured of associated chordae tendineae. Myxomatous degeneration is a common cause of mitral valve regurgitation and left sided congestive heart failure (Braunwald, 1997). The etiology of MMVD is unclear. Several factors such as mechanical stress, heredity, vasoactive substances, extracellular matrix abnormalities and protein signaling have been proposed to be involved (Sweson et al, 1996; Olsen et al, 1999; Mow and Pederson, 1999; Pederson and Häggström, 2000; Rabkin et al, 2001; Disatian and Orton, 2009). The definitive treatment for MMVD in human is surgical repair or replacement of damaged valves (Braunwald, 1997). However, secondary to the expense of cardiac bypass surgery, mitral valve surgery has been performed in just a few hospital case dogs (Boggs et al, 1996; Griffiths et al, 2004; Orton et al, 2005). No drugs or treatments can slow the progression of myxomatous degeneration. Thus, the current treatment goal of dogs affected by MMVD is to reduce the effects of circulatory disturbance rather than to treat the actual lesions. The better understanding in pathogenesis of this disease may help in developing treatment protocols to prevent or slow disease progression. The histopathologic changes of degenerative valves include increased cellularity, cell phenotypic transformation, increased enzymatic activity and valve structure destruction secondary to glycosaminoglycan accumulation as well as elastic and collagen fiber fragmentation. The cell density has been found increasingly in the area underneath the valve surface on the atrial side of the leaflet and gradually increases with disease progression (Disatian et al, 2008). The mechanisms by which degeneration induced the increased cellularity remain unclear. A previous study by our group suggested that an enhancement of cell proliferation is unlikely a major aspect of increased cell density in canine degenerative valves since the proliferative marker, Ki-67 was hardly seen in affected valves (Disatian et al, 2008). It is reasonable to speculate that other mechanisms such as decreased cell death due to anti apoptosis may play a role implicating persistence of cells in diseased valves.

Apoptosis or program cell death is a process of cell death morphologically characterized by cell volume reduction, chromatin condensation, DNA and nuclear fragmentation, and apoptotic body formation (Lossi and Merighi, 2003). Apoptosis is mediated by two distinct pathways: the extrinsic or caspase pathways and intrinsic or Bcl-2 family pathways. The extrinsic pathway is activated when death ligands, such as Fas ligand or TNF-α bind to their cognate receptors at the plasma membrane causing recruitment of specific adaptor proteins and leading to activation of initiator caspases such as caspases-8,9 and 10 which cleave and activate effector caspases-3,6 and 7. Once the process progresses to the cleavage of effector caspases, it results in irreversible cell death (Fischer et al, 2005). The intrinsic pathway of apoptosis is regulated by Bcl-2 gene family. There are 2 types of proteins in Bcl-2 family members which has either pro-apoptotic (e.g. *bax*, *bad*) or anti-apoptotic (e.g. *bcl-2*,*bcl-XL*) effect. During the process of apoptosis, pro-apoptotic proteins translocate into the mitrochondria and mediate molecular pathways leading to cell death. On the other hand, anti-apoptotic proteins inhibit cell death by antagonizing effect of pro-apoptotic proteins. Thus, an imbalance between *bcl-2* and *bax* can cause either apoptotic or anti apoptotic effect on cells. The Bcl-2 family members have been shown to regulate cell death in cardiovascular system (Gustafsson and Gotlieb, 2007). The expression of Bcl-2 proteins has been reported in adult human heart (Gustaffson and Gotlieb, 2007) and vasculature (Fox and Patel, 1998). Bcl-2 proteins have been implicated in various human cardiac diseases including myocardial infarction (Boldi et al, 2002), dilated cardiomyopathy (Di Napoli et al, 2003) and ischemic heart diseases (Latif et al, 2000).

The evidence of transforming growth factor beta 1 (TGFβ1) signaling mediating valve degeneration has been proposed (Disatian and Orton, 2009). TGFβ1 is a growth factor belonging to TGFβ superfamily signaling through cell surface serine-threonine kinase receptors including TGFβ1 receptors I and II (TGFβ1 RI and RII). TGFβ1 is secreted from cells in a latent form comprised of latency associated peptide and mature TGFβ1. TGFβ1 signaling requires active TGFβ1, a cleaved form of latent TGFβ1. The complex of active TGFβ1 and TGFβ1 RII phosphorylate the TGFβ1 RI which in turn phosphorylate and activate Smad 2 and 3. Activated Smad 2/3 bind to Smad 4, translocate into nucleus and activate transcription of specific genes implicating extracellular matrix production, cell proliferation, and cell apoptosis (Massague, 1998). Overexpression of TGFβ1 has been demonstrated in several human heart valve diseases including carcinoid heart disease (Jian et al, 2002), calcific aortic stenosis (Jian et al, 2003), rheumatic heart disease (Kim et al, 2008) and MMVD (Park et al, 2009). An up-regulation of TGFβ1 signaling proteins and mRNA has been reported in naturally occurring canine MMVD (Oyama and Chittur, 2006; Disatian and Orton, 2009). In cultured valve interstitial cells, TGFβ1 can increase α-smooth muscle actin (α-sma) expression (a putative maker of phenotype transformation) (Walker et al, 2004; Cushing et al, 2005; Park et al, 2009), as well as increased collagen and GAG synthesis (Jian et al, 2002). TGFβ1 has also been found to regulate cultured valve interstitial cell proliferation and apoptosis (Liu and Gotlieb, 2008). Roles of TGFβ1 signaling in regulating cell density in spontaneous MMVD have been unexplored. We hypothesize that TGFβ1 signaling may implicate anti-apoptosis on valve interstitial cells contributing to increased cell density in canine spontaneous MMVD.

The objective of this study is to determine whether TGFβ1 signaling implicate anti-apoptosis in canine myxomatous mitral valves. The following specific aims of this study are addressed.

1. To determine cell apoptosis in spontaneous canine myxomatous mitral valves.
2. To determine the relationship between temporal and spatial expression of pro-apoptotic protein (*bax*), anti-apoptotic protein (*bcl-2*) and activated TGFβ1 signaling proteins, phosphorylated Smad2/3 (p-Smad2/3).

Materials and methods

Tissue collection and preparation: Septal (anterior) mitral valve leaflets were obtained post-mortem from normal dogs (n=15) and dogs affected by early stage (n=20) and late stage (n=20) MMVD at the Department of Pathology, Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, Thailand. Normal valves were collected from dogs died for reason other than cardiac disease and did not have gross lesions of myxomatous degeneration. All normal valves had thickness less than 1 mm. Myxomatous valve tissues were obtained from dogs with gross evidence of MMVD at necropsy including enlarged thickened leaflets with rolling up edges (Kvart and Haggstrom, 2000). Early stage and late stage disease valves were 1-2 mm and >2 mm, respectively. After dissection, tissues were fixed in 10% formalin for 24 hours and embedded in paraffin.

Construction of mitral tissue microarray (modified from Rosen et al, 2004): Mitral valve tissue microarrays were constructed with 55 samples of mitral valve tissues by using tissue microarray facility at Khonkan University, Khonkan, Thailand. Paraffin embedded tissue blocks from 55 consecutive cases of early stage and late stage myxomatous valves as well as normal valves were cut at 4 μm. Serial sections were stained with Hematoxylin and Eosin and reviewed by 3 pathologists to determine general morphology, confirm evidence of MMVD and identify representative areas for constructing tissue microarray. Cylindrical 1.5 mm in diameter and 4 mm in depth core was used to cut 9 representative areas from each valve. Each core sample was assembled into a recipient paraffin block. One recipient block consisted of 123 tissue spots with a 1 mm sample space.

Histology: 4 μm serial sections of microarray tissues were stained with Hematoxylin and Eosin for determining general morphology and apoptotic cells. Apoptotic cells were characterized by cell volume reduction, chromatin condensation, nuclear fragmentation, and apoptotic body formation (Lossi and Merighi, 2003). The amount of apoptotic cells subjectively evaluated from 6 random areas at 400x magnification (High Power Field; HPF) in each leaflet was recorded. Sections were then stained with Periodic Acid Schiff (PAS) and Alcian blue for extracellular matrix morphology. Myxomatous valve sections were evaluated for morphologic changes including mucopolysaccharide accumulation, collagen bundle disruption, elastic fiber fragmentation, and loss of normal valve architecture.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining: To determine the expression of DNA fragmentation, TUNEL reaction was performed with TdT-FragEL^{TM} DNA fragmentation Detection kits (S1700, chemicon, CA, USA) according to manufacturer's instructions. In brief, 4 μm tissue sections were deparaffinized in xylene, rehydrated in graded alcohol solutions and antigen unmasked with proteinase K 10 mM Tris pH 8.0 at room temperature for 20 minutes. Sections were incubated sequentially with 3% hydrogen peroxide/methanol for 5 minutes and TdT equilibration buffer for 10 minutes at room temperature. Slides were rinsed with phosphate buffered saline after each incubation step. Sections were incubated with TdT labeling reaction mixture at 37 ºC for 90 minutes. The labeling reaction was terminated with stop buffer solution. Labeled DNA was detected with peroxidase conjugated streptovidin solution for 30 minutes. Peroxidase activity was visualized with 3,3' diaminobenzidine (DAB). Finally, slides were counter stained with Hematoxyllin for 3 minutes and mounted with toluene solution. Canine lymphoma was used as a positive control. 6 areas randomly selected from each leaflet were examined at 400x magnification (High Power Field; HPF) under light microscope. A number of TUNEL- positive nuclei were counted. As TUNEL sometimes labeled nonspecific DNA degradation in necrotic cells and transient DNA strand breaks in mitotic cells (Grasl-Kraup et al, 1995), as well as DNA fragmentation in apoptotic cells, care was taken to exclude nuclei. The difference in average positive nuclei among disease stages was determined by One-way ANOVA. Pair comparisons were evaluated with Fisher least significant difference test. The Ryan-Joiner test was used to test the normality of data. $p < 0.05$ was considered significant.

Immunohistochemistry for cleaved caspase-3 staining: After deparaffinization and rehydration steps, tissue sections were treated with 1M citrate buffer pH 6 at 95 ºC for 20 minutes to unmask antigen epitopes. Sections were pre-incubated with 0.3% hydrogen peroxide in methanol for 5 minutes to block endogenous peroxidase activity. The monoclonal antibody anti cleaved caspase-3 (Cell signaling, Danvers, MA) in a dilution of 1:800 was incubated for 90 minutes. Sections were applied with peroxidase conjugated goat anti-mouse (Envision+ Dako) for 45 minutes. Slides were rinsed with phosphate buffered saline between each incubation step. Peroxidase activity was visualized with 3,3' diaminobenzidine solution freshly prepared in 0.03% hydrogen peroxide in 0.1M Tris HCl buffer. Sections were counterstained with Hematoxylin for 2 minutes and mounted with permount solution. Negative controls were created by substituting universal negative control for N-series mouse antibodies (Dako, Carpenteria, CA, USA). Positive control was cleaved caspase-3 against canine lymphoma. The percentage of positive cells was counted and compared between each disease stage as previously described for TUNEL staining.

Immunohistochemistry for phosphorylated Smad2/3, *bcl-2* and *bax*: The immunohistochemical staining was followed the same protocol as described for cleaved caspase-3 staining. Mouse monoclonal antibodies against 1:1500 p-Smad2/3 (Santa cruz biotechnology, Santa cruz, CA), 1:100 *bcl-2* (Sigma-aldrich, St.Louis, MO, USA), or 1:100 *bax* (Sigma-aldrich, St.Louis, MO, USA) were used to review the expression of these proteins. Slides applied with universal negative control for N-series mouse antibodies were served as negative controls. Positive control included p-Smad2/3 against canine kidney tissues, *bcl-2* and *bax* against canine lymphoma. The spatial and temporal distributions of positive cells were examined. The percentage of cells expressed p-Smad2/3, *bcl-2* and *bax* was counted on random 6 high power fields in each valve leaflet. Average percentage of positive cells was calculated and compared between disease stages. One way-ANOVA following by Fisher least significant test was used to determine differences in the percentage of positive cells between disease stages. The normality was tested by the Ryan-Joiner test. $p < 0.05$ was considered significant. The correlation between the expression of p-Smad2/3, *bcl-2* and *bax* was evaluated.

Results

Animals: Mitral valves were collected from 15 normal dogs and from 20 dogs each with early and late stage MMVD. Therefore, a total 55 dogs (23 females, 32 males) was studied. Breeds and gender of dogs included in this study are summarized in table 1. The average age of normal dogs and dogs with early stage and late stage MMVD were 3.75±2.55, 8.6±2.82 and 10.33±1.61 years, respectively (Table 2). The average age of normal dogs was significantly difference from that of MMVD dogs ($p<0.0001$). The mean thickness of normal valves, early stage and late stage MMVD were 0.81±0.13, 1.61±0.23 and 2.64±0.54 mm, respectively. The cause of death was related to MMVD in 4 dogs. In all other dogs, the cause of death was unrelated to cardiovascular disease.

Table 1 Breed and number of samples in this study

Breed	Samples(n)						
	Normal		Early staged MMVD		Late staged MMVD		Total
	Male	Female	Male	Female	Male	Female	
Mixed	3	3	4		2	2	14
Dushchund		1					1
Miniature Pincher	1	1					2
Shi-Tzu		1	1	1			3
Bulldog	1		1				2
Thai				1			1
Rottweiler			1				1
Poodle			1		2		3
Siberian Husky			2				2
Pomeranian			1				1
Labrador retriever	1	1		2	1		5
Sharpie		1			1		2
Cocker spaniel						3	3
Dalmatian						1	1
Bull terrier			1		1		2
Pekingese					1		1
Boxer					1		1
Golden retriever					2	1	3
Aus heeler	1						1
Bangkaew			1				1
Pug				2			2
Chihuahua				1			1
German Shepherd					1	1	2
Total	7	8	13	7	12	8	55

Table 2 Stages of disease, ages of dogs and thickness of valves included in this study

Stage / Parameter	Normal valve (n=15)	Early stage MMVD (n=20)	Late stage MMVD (n=20)
Average thickness (mm)	0.81 ± 0.13^{a}	1.61 ± 0.23^{b}	2.64 ± 0.57^{c}
Average age (yrs)	3.56 ± 2.69^{a}	8.71±3.15	10.34±1.87

Value are mean±SD

^{a,b,c}Indicate significant difference; $p<0.0001$

Due to the high prevalence of this disease in small breed dogs, the study of cell apoptosis and protein expression were performed in valves from small to middle size dogs. 5 dogs were selected from each group. Breeds of dogs included Shi-Tzu (3), Dushchund (2), Miniature pincher (2), Bulldog (2), Poodle (2), Pomeranian (1), English cocker spaniel (1), Pekingese (1) and mixed breed (1). The average age of dog and valve thickness from each group were concluded in table 3.

Table 3 Stages of disease, ages of dogs and thickness of valves included in the study of cell apoptosis and protein expression

Stage / Parameter	Normal valve (n=5)	Early stage MMVD (n=5)	Late stage MMVD (n=5)
Average thickness (mm)	0.95 ± 0.08^{a}	1.60 ± 0.34^{b}	2.89 ± 0.67^{c}
Average age (yrs)	2.60 ± 1.67^{a}	8.00 ± 4.64^{b}	10.4 ± 1.82^{b}

Value are mean±SD

^{a,b,c}Indicate significant difference; $p<0.005$

Valve morphology and histology: The gross morphology of mitral valves were compatible to normal and myxomatous valves with early and late stage disease. Macroscopically, the normal leaflets were thin, translucent and soft. The early stage myxomatous mitral valves became thickened with rolling up free edge. In late- stage MMVD, the entire cusp of leaflets was marked thickening (up to 2 mm) and deformed. Hematoxyllin and Eosin staining as well as special staining including Periodic Acid- Schiff and Alcian blue staining revealed 3 well-defined tissue layers, each containing valvular interstitial cells (VIC) and extracellular matrix (ECM): (1) the atrialis layer, which was composed of elastic fibers. (2) the centrally located spongiosa layer, which consisted of loosely arranged mucopolysaccharides and (3) the fibrosa layer, which was comprised predominantly of collagen fibers arranged parallel to the free edge of the leaflet. The elongation of chordae tendineae was generally seen in both stages of MMVD (Figure 1). Histopathologically, early stage myxomatous mitral valves were characterized by mild fragmentation of elastic fibers in the atrialis layer and collagen bundles in the fibrosa layer, mild increase in VIC and accumulation of mucopolysaccharide within the spongiosa layer. The histopathologic changes occurred mainly in the distal half of the valves. Late stage MMVD was characterized by a marked increase of mucopolysaccharide accumulation in the spongiosa layer and increased in number of VIC in the atrialis layer. The complete disruption and fragmentation of collagen bundles and fibroelastin were seen in the atrialis and fibrosa layer. The macrophages were sparsely observed in both normal and myxomatous mitral valves. In addition, there was focally calcification in the fibrosa layer in 2 myxomatous mitral valves.

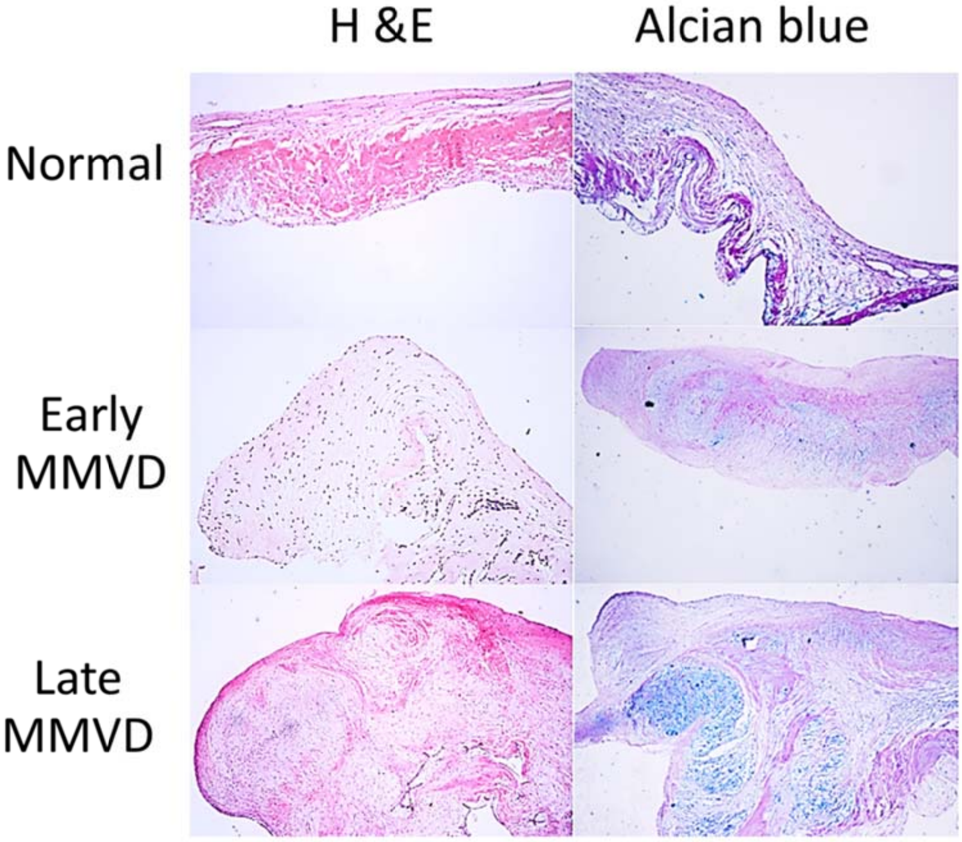


Figure 1 The histopathology of normal, early and late stage MMVD valves (100x magnification)

Apoptosis: Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed to detect DNA damage or DNA fragmentation in VIC. The association of TUNEL marker with apoptotic bodies was also reviewed. The result showed TUNEL staining in normal and myxomatous valves. The positive intranuclear staining was mainly seen in the spindle-shape VIC. These cells had no apoptotic bodies within the nuclei (Figure 2). The average percentage of positive nuclei was 27.72±8.60%, 37.37±12.94% and 34.68±12.78% in normal, early stage and late stage myxomatous valves, respectively (Table 4). The number of positive nuclei was not significantly different between normal and each stage of MMVD. The cellular morphology of some VIC in myxomatous valves was rounding up. Some of these cells had dense and small nuclei. The halo space was also seen in cytoplasm of some of these cells (Figure 3). The apoptotic bodies were hardly seen in both stages of MMVD. To detect the irreversible and terminal stages of apoptosis the immunohistochemistry of cleaved caspase -3 was performed. The result showed expression of intranuclear and intracytoplasmic staining against cleaved caspase-3 (Figure 4). The number of positive cells was not significantly different between each group of valves (*p*<0.05). The percentage of positive cells was 77.34±8.86%, 66.22±11.59% and 73.04±5.04% in normal, early stage and late stage MMVD, respectively.

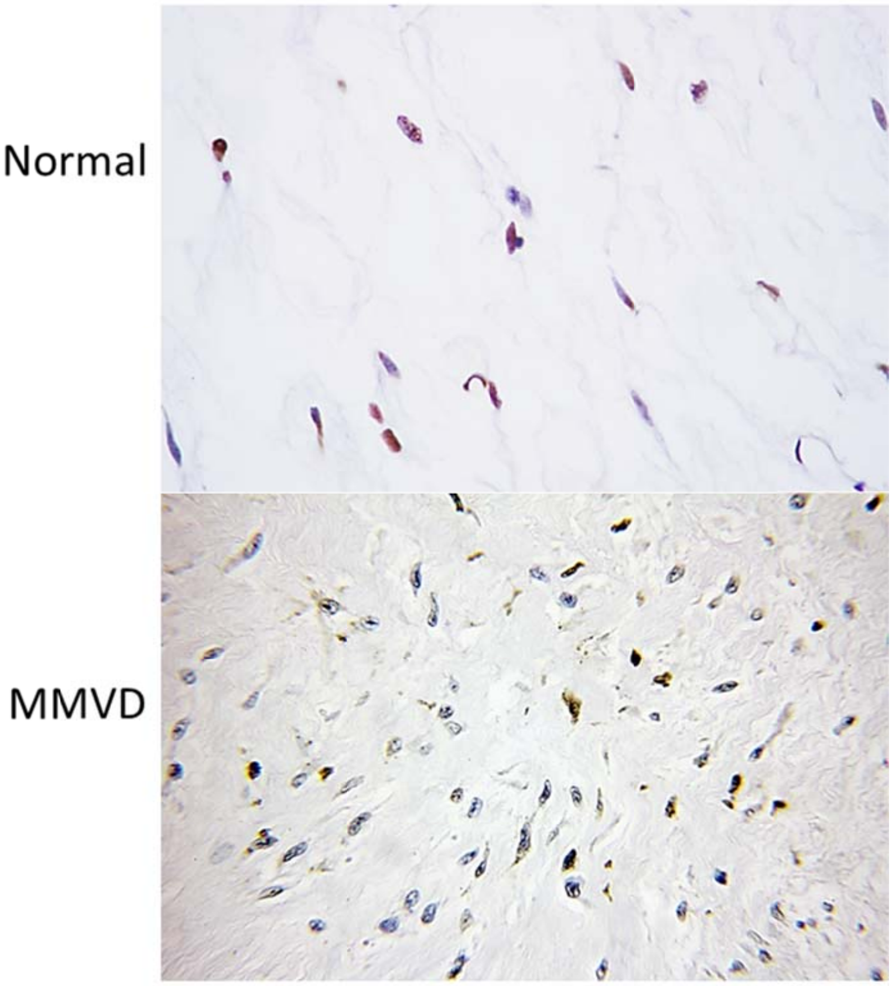


Figure 2 TUNEL positive nuclei in normal (above) and MMVD (below) valves (400x magnification).

<u>Table 4</u> Percentage of positive cells to TUNEL, cleaved caspase 3, p-Smad 2/3, *bax* and *bcl-2* staining

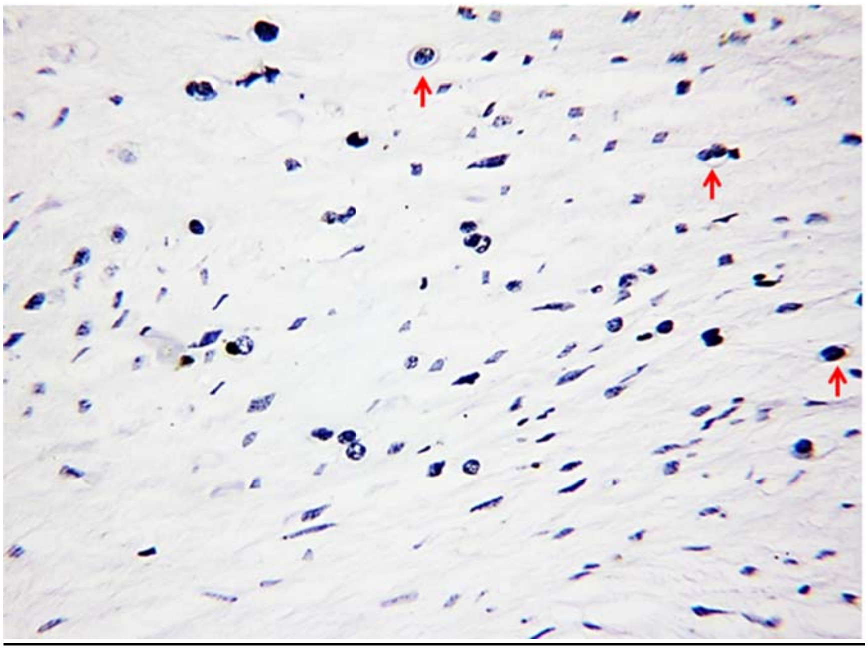
	Percentage of Positive Cells of Immunohistochemistry Staining					
	TUNEL	Cleaved caspase-3	p-Smad2/3	*bax*	*bcl-2*	*bax/bcl-2* *ratio*
Normal	27.72±8.60	77.34±8.86	28.59±9.32	55.06±5.59$^{\#}$	84.38±3.45^{+}	0.65±0.07$^{\#}$
Early	37.37±12.94	66.22±11.59	23.73±5.71	74.92±7.85	51.04±7.38	1.49±0.24
Late	34.68±12.78	73.04±5.04	38.10±8.25*	75.00±8.82	52.34±8.27	1.45±0.23

Value are mean%±SD

*Indicate significant difference; $p<0.05$

$^{\#}$ Indicate significant difference; $p<0.005$

$^{+}$ Indicate significant difference; $p<0.001$



<u>Figure 3</u> Valve interstitial cells in late stage myxomatous valves. Cells with round appearance, dense nuclei and halo space in cytoplasm are shown (red arrows) (400x magnification).

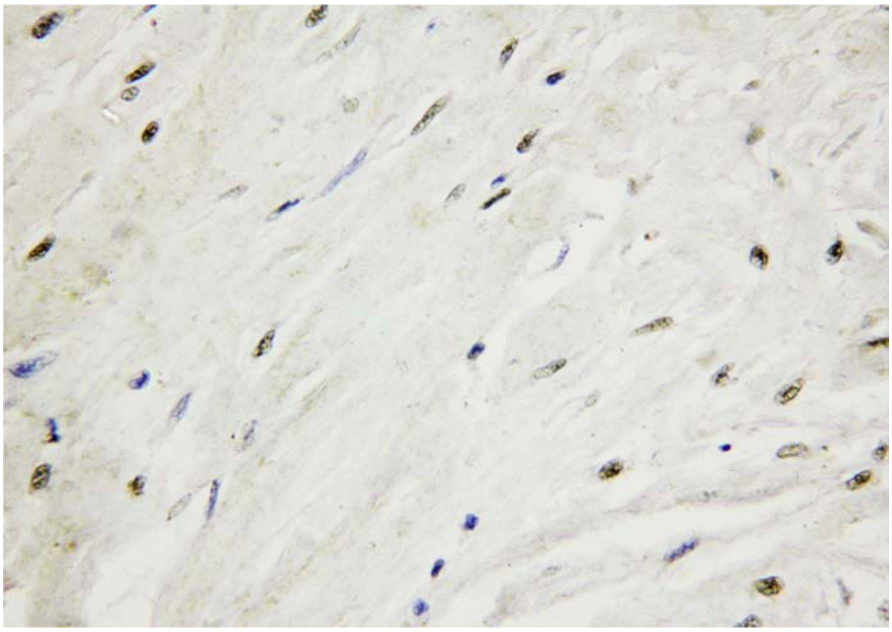


Figure 4 Cleaved caspase -3 positive nuclei in late stage myxomatous valves (400x magnification)

The relationship between expression of pro-apoptotic protein (*bax*), anti apoptotic protein (*bcl-2*) and activated TGF-β1 signaling protein, phosphorylated Smad2/3 (p-Smad2/3): The expression of p-Smad2/3, key transcription factors forming in the TGF β1 signaling pathway, anti-apoptotic protein, *bcl*-2 and pro-apoptotic protein, *bax* was determined by immunohistochemistry technique. The result showed the expression of intranuclear and intracytoplasmic staining against p-Smad2/3 in VIC (Figure 5). The positive staining was also seen in ECM of normal and myxomatous valves. The percentage of positive cells was significantly increased in late stage MMVD (38.10±8.25%) compared to early stage MMVD (23.73±5.71%) and normal dogs (28.59±9.32%) (p<0.05). The expression of *bax* in early and late stage myxomatous mitral valves was significantly higher than normal mitral valves as shown by the average percentage of positive cells 74.92±7.85%, 75.00±8.82% and 55.06±5.59%, respectively (p<0.005). On the other hand, the expression of *bcl*-2 significantly decreased in myxomatous valves compared to normal valves (*p*<0.001). The VIC in normal valves expressed *bcl-2* 84.38±3.45%; however, the average percentage of positive cells in early and late stage myxomatous valves was 51.04 ±7.38% and 52.34±8.27%, respectively. The ratio of *bax/bcl-2* increased significantly between normal (0.65±0.07) and myxomatous valves. There was no difference between early (1.49±0.24) and late stage MMVD (1.45±0.23) (*p*<0.005).

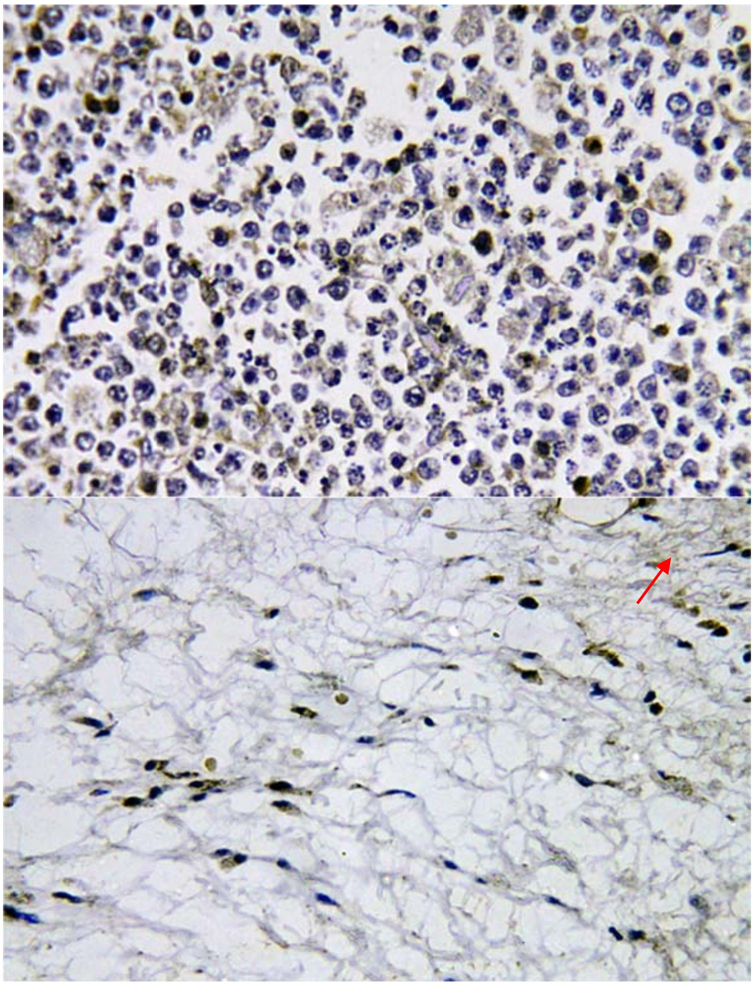


Figure 5 Positive nuclei to p-Smad2/3 in canine kidney tissue (above) and intranuclei and intracytoplasmic positive staining to p-Smad2/3 in late stage myxomatous valves (below) are demonstrated. The ECM positive staining is also seen (red arrow).

Discussion

The cell morphologic study in the present study revealed that some VIC transformed their appearance from spindle like in normal valves into more round or cuboidal like cells in myxomatous valves. The spindle VIC in normal and myxomatous valves had large oval nuclei; on the other hand, small dense nuclei with halo space between nuclear membrane and cell membrane were seen in round VIC from myxomatous valves. However, most of cells in both normal and myxomatous valves had a spindle like appearance. This result matches to the previous finding by Han et al (2010) which occasionally found the round VIC throughout the myxomatous valve leaflets. The number of spindle VIC increased in myxomatous valves and accumulated mostly in the atriallis layer (Disatian et al, 2008; Han et al, 2010). Most of these cells were α-smooth muscle actin positive cells (Disatian et al, 2008). The mechanisms initiate and promote increased cellularity within this region of myxomatous mitral valves remain unknown. Previous study demonstrated that the expression of Ki-67, a cell proliferative marker was nearly undetectable in myxomatous valves (<0.05%) (Disatian et al, 2008) suggesting that VIC persist in myxomatous valves are not in the proliferative state. Previous *in vitro* study demonstrated that a spindle like cell was the motile type of cells (Liu and Gotlieb et al, 2007). Thus, an increased cell number within the atrialis layer may occur due to the migration of cells from the inner to accumulate in the marginal area of the valves. However, several studies showed that the number of cells within the central part of the myoxomatous valves or the spongisa layer was unchanged during disease progression suggesting that there is no cell recruitment in or migration out of this area (Disatian et al, 2008; Han et al, 2010).Taken together, the cell proliferation seem not to be a major aspect of increased cellularity in the disease valves. The evidence of the cell migration in naturally occurring MMVD is uncertain and still needs further elucidation. We therefore hypothesized that the other mechanisms such as anti-apoptosis may implicate an increase in cell density.

In this study, the expression of *bax* increased; whereas, *bcl-2* decreased in myxomatous valves compared to normal valves. The *bax* protein is an apoptosis promoting factor (Oltvai et al, 1993); however, the *bcl-2* protein acts as an apoptosis suppressing factor (Hockenberry et al, 1990). The result of present study suggests that VIC in myxomatous valves may be more sensitive to apoptotic stimuli and may have ability to respond to an apoptotic signal (Yang and Korsmeyer, 1996). However, the study of the cell morphology as well as the expression of TUNEL marker and cleaved caspase 3 suggests that VIC in myxomatous valves do not undergo execute apoptosis. The caspase-3 is known to act downstream of *bax/bcl-2* control and have an important role in the completion of apoptosis (Rudel, 1999). An increase *bax/bcl-2* ratio suggests a high activation and a low inhibition of apoptotic process. However, the activation of caspase-3 was failed to demonstrate in this study since the expression of cleaved caspase-3 was not significantly different between normal and MMVD dogs. The lack of caspase-3 activation resulting to apoptosis via chromosome condensation, cell membrane blebbing and DNA fragmentation (Krajewska et al, 1997) was also demonstrated in the study of cell morphology and the expression of TUNEL marker. A non-significant difference in the expression of TUNEL marker between normal and myxomatous valves confirms that the DNA damage or DNA fragmentation within VIC did not increase during degenerative process. Taken together, these results suggest that the VIC in affected valves may undergo pro-apoptotic condition and change their cellular structure as well as phenotype. However, these cells may not die because they do not pass the final stages of apoptosis.

An increased expression of phosphorylated Smad2/3 (p-Smad2/3), the downstream activation factor forming in the TGFβ1 signaling pathway confirms the previous of TGF β1 signaling (Disatian and Orton, 2009) in MMVD and suggests the activation of this signaling through the Smad pathway in diseased valves. However, the Smad-dependent TGFβ1 signaling may be not a key pathway to regulate the *bax* and *bcl-2* protein expression in canine MMVD since the expression of p-Smad 2/3, was increased only in the late stage MMVD; whereas, *bax* and *bcl-2* expression changed primarily in the early stage MMVD. TGF β1 signaling has been found to have several roles in MMVD including cell phenotypic transformation (Walker et al, 2004; Cushing et al, 2005; Park et al, 2009), extracellular matrix synthesis (Jian et al, 2002), as well as cell proliferation and apoptosis (Liu and Gotlieb, 2008). The TGFβ actually activates Smad and several Smad-independent pathways including the mitogen-activated protein kinase, ρ-like Rho –GTPase, and PI3K-AKT pathway (Zhang, 2009). One of these TGFβ downstream pathways may have roles in Bcl-2 family protein regulation. The previous study of VIC culture found that TGFβ1 decreased VIC proliferation but had no effect on apoptosis (Li and Gotlieb, 2011). This result is similar to the finding in this study which the activation of TGFβ1 increased the pro-apoptotic condition, but the apoptosis remained unchanged in myxomatous valves. Roles of TGFβ1 in regulating apoptosis in MMVD are unclear. Further studies are needed to complete the understanding in relationship between TGFβ1 signaling and pathogenesis of MMVD in dogs.

In conclusion, our major findings are that the apoptosis does not change in normal and MMVD dogs; therefore, the anti-apoptosis may not explain the increase in cell density seen in myxomatous valves. VIC in myxomatous valves may be in pro-apoptotic state. An increased expression of p-Smad2/3 confirms the activation of TGFβ1 signaling in degenerative valves.

The role of TGF β1-Smad signaling in inducing a pro-apoptotic state is unclear.

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