

CULTURE AND PHENOTYPE OF CANINE VALVULAR INTERSTITIAL CELLS

by

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B.S., Kansas State University, 1997
D.V.M., Kansas State University, 1999

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Clinical Sciences
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2007

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Abstract

Degenerative valve disease is the most common cardiac affliction facing our canine population. To date, canine research has focused on characterizing the disease itself and the histopathological features. Because of the ability to routinely repair or replace diseased valves in human medicine, research focus in humans has been on perfecting these techniques rather than elucidating etiology. The recent interest in valvular interstitial cells has been primarily due to their capacity to degrade collagen with the knowledge that disorganized collagen is a hallmark characteristic of degenerative valve disease. In this project, an easily reproducible cell culture protocol for canine valvular interstitial cells was developed. These cells were phenotyped by utilization of RT-PCR and immunocytochemistry. The use of these cells in a research project looking at response to endothelin exposure with and without protection of vitamin E is demonstrated as an example of the unlimited possibilities for these cells to elucidate not only the etiology of the disease process but also the response to therapy.

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Acknowledgments

I would like to acknowledge my committee for all of their hard work and support. Specifically, I would like to acknowledge Tom Schermerhorn for endless hours of advice, counseling, and pep talks throughout the project as well as his expertise in the area of bench top techniques and his technician Vicky Donley who truly made every question seem like a good one. I would like to acknowledge Chris Ross for the use of his lab space and his talented technician, Ling Zheng, as well as his valuable insight. Last but clearly not least, I would like to acknowledge my major professor, Barret Bulmer, who provided me the opportunity in the form of a residency to pursue my dreams clinically and encouraged me to pursue a clinically oriented basic science project. Without his encouragement, I would not have tackled this project. Funding for this project was provided by the Waltham Foundation, the Kansas State University Clinical Resident Research Grant, and the ACVIM Cardiology Resident Research Grant.

Dedication

I would like to dedicate this thesis to my family including my parents who always believed in me and often more than I believed in myself. My sister, Bridget, who was so enthusiastic about learning what I was doing, it made even the boring details seem cool. Thank you all for being a constant source of love and support in my life. I also would like to dedicate this thesis to my husband, Adam, who has accepted my dreams as his own. Thank you for chasing my dreams with me from one coast to the other. Thanks also for being a fountain of energy and encouragement during this endeavor. Here's to a new chapter!

CHAPTER 1 - Literature Review

Introduction – Background and Importance

Chronic degenerative valve disease (CDVD) remains the most common acquired cardiac disease faced in the canine domestic population. In a 1965 study of almost 5,000 dogs it was found that 545 had reliable evidence of cardiovascular disease and 72% of those patients had clinical or port-mortem evidence of chronic valvular disease (391/545).¹ Similarly, Pensinger's study during that same year revealed 73.2% of dogs presented to a cardiology service with evidence of cardiovascular disease had chronic mitral valve disease.² It is estimated that three-fourths of all canine patients that present in heart failure do so as a sequelae of CDVD and the prevalence of the disease increases almost linearly with age.^{1,3} Mitral murmurs were audible in one study in 20-25% of examined dogs in the 9- to 12- year age group and approximately one-third of dogs over the age of 13 years. Males were somewhat overrepresented.¹

Approximately 60% of cases have only mitral involvement with the mitral and tricuspid valves afflicted concurrently in about 30% of cases. Sole tricuspid valve involvement is much less frequent (7.5%) while the aortic (2.1%) and pulmonary (0.4%) valves are rarely affected.⁴ This disease and similar disease processes have been labeled with many names describing function, clinical signs and pathologic appearance in human and veterinary literature. It is likely that floppy mitral valve,⁵ redundant cusp syndrome,⁶ billowing sail deformity,^{7,8} and ballooning mitral cusp⁹ are synonyms used in literature for the disease currently known as mitral valve prolapse¹⁰ in both the veterinary and human literature. Evidence of this disease historically is substantiated by a specimen mounted in the Pathological Museum of St. George's Hospital in 1936 of this same pathology with the name 'parachute deformity of the mitral valve'. This name created confusion with the congenital anomaly of the same name where the chordae insert into one papillary muscle and has fallen out of favor.¹¹ When describing the gross examination of the post mortem floppy mitral valve, Davies et al. defined the lesion as 'significant elongation of the chordae and expansion of the cusp area to allow prolapse into the atria on applying a pressure load in the ventricle to close the valve'.¹¹ Mitral

valve prolapse has been described in some canine populations as a precursor to degenerative valve disease.¹²

Other terms used to describe this disease process include myxomatous degeneration^{5, 13} and mucinous degeneration¹⁴ which clearly emphasize the pathology of the degenerative valve. Endocardiosis is used frequently in the veterinary literature despite being somewhat of a misnomer. Although similar diseases occur in other species including humans, the incidence of severe valvular distortions and atrioventricular insufficiency is much greater in dogs with advancing age than any other species.³

Gross Pathology

Pomerance and Whitney described a classification system for canine CDVD in 1970 that divides the pathology into four groups.¹⁵

-Type 1: The presence of small nodules on the valve leaflet edges in the area of contact with other leaflets as well as diffuse opacity in the proximal portion of the valve.

-Type 2: Larger nodules evident in the area of contact that tend to coalesce with their neighbors with potential areas of diffuse opacity.

-Type 3: Large nodules to coalescing irregular, plaque-like deformities that extend to involve the proximal portions of the chordae tendineae.

-Type 4: There is gross distortion and ballooning of the valve cusp with chordae tendineae that are thickened proximally.

The authors report that these lesions are seen as separate conditions in man but not the apparent continuum in the dog. Type 2 lesions are consistent with 'senile' sclerosis reported in man. Mucoïd degeneration of the fibrosa which gives rise to type 3 and type 4 canine lesions is also common in man in the form of mitral valve prolapse but is rarely of clinical consequence. The area of the mitral valve cusp is increased both longitudinally and transversely which allows for the upward doming of the valve towards the left atrium.¹¹ The pathology is nearly identical to man where the normally dense collagenous fibrosa is replaced by loose metachromatically-staining mucoïd material.¹⁵

Other authors have described the gross appearance of the mitral valve apparatus as being thickened, distorted and often nodular with retracted leaflets that can curl under at the free margin and compromise the chordae tendineae.^{3, 4, 15}

The alterations in the geometry of the mitral valve apparatus result in loss of normal apposition of the valve leaflets. This results in the valve being “insufficient” and causes regurgitation of blood from the left ventricle into the low pressure left atrium during systole. Therefore, the left atrium is often dilated in cases of significant mitral regurgitation. Because this high velocity regurgitant flow can be directed towards the endocardial surface of the left atrial wall, jet lesions can be visualized on the left atrial wall.² Occasionally, endomyocardial tears can occur.

Histology

Normally, the leaflet is composed of endothelial cells that line both the atrial and ventricular surfaces of the leaflet. Elastic fibers are found in the atrialis layer which provide the valve its extensibility.¹⁶ A few bundles of smooth muscle cells are present below the level of the atrial endocardium. The spongiosa layer is a loosely arranged layer of collagen and proteoglycans located on the atrial side of the leaflet. The fibrosa layer is a dense layer of collagen that is located on the ventricular side of the leaflet and is continuous with the central portion of the chordae tendineae as well as the valve annulus.^{11, 17} Valvular interstitial cells are fibroblast-like cells located in both the spongiosa and fibrosa. In dogs, the vasculature of the mitral valve is thought to be contained to the basilar 1/3 of the leaflet.^{18, 19}

The earliest changes in diseased valves appear to be an accumulation of water and mucopolysaccharides in the ground substance. This results in thickening of the valve giving it a swollen, translucent appearance. Eventually, there is an increase in collagen and elastic fibers which are often fragmented.⁴ The collagen is abnormal in the fibrosa layer with large areas showing apparent loss of fibrous tissue and collagen bundles that are fragmented, coiled, and disrupted. Destruction is concentrated around sites of chordal insertion but the changes may extend into the body of the cusp. Areas of destruction of collagen usually show easily demonstrable pools of mucopolysaccharide within which residual strands of collagen lie.¹¹

Olsen et al. were the first to recognize the thickening of the spongiosa layer in human floppy mitral valve disease. They first measured the thickness of the spongiosa layer in mitral valves from normal hearts and realized a variable non-age related

thickening of the spongiosa not occupying more than 60% of the total thickness of the valve. In 43/50 floppy mitral valve patients this spongiosa thickness involved 62-94% of the thickness of the valve. They also suggested that humans are born with either a thin or thick spongiosa and patients with a prominent spongiosa have an inherently weaker valve and are likely to develop this syndrome if other factors are present. Once doming has taken place, secondary thickening of the valve leaflet occurs with extension of the myxomatous tissue into the fibrosa. Functional or anatomical abnormality of any component contributing to mitral valve function is an important contributory factor in the development of this syndrome.¹⁶ The replacement of dense collagen by loose myxomatoid tissue allows stretching of the cusp under normal hemodynamic pressure (for which the terms floppy valve, ballooning, parachute, or billowing sail clearly describe) and the cusps prolapse into the atrium in systole producing mitral incompetence.¹⁵ The myxomatous valve's expanded spongiosa is composed of loose amorphous extracellular matrix staining strongly for proteoglycans with diminished staining for collagen and elastic fibers. Diseased valves are significantly thicker than normal valves. Inflammatory cells are negligible in diseased valves.¹⁷ Myofibroblast-like cells accumulate in myxomatous leaflets but not in normal leaflets. This has been shown by the population of cells altering their expression from just vimentin (V Phenotype) in normal cells to cells that stained with vimentin as well as α -actin or desmin (VA, VD, VAD). The cell staining for desmin and α -actin are considered activated interstitial cells.²⁰

In addition to the mitral valve pathology, histologic changes have been found in the coronary arteries of patients suffering from myxomatous degeneration. The intramural coronary arteries can display intimal thickening that greatly reduces the lumen. Areas of focal myocyte necrosis are present near severely arteriosclerotic coronary arteries. Early authors considered this triad of findings: valve fibrosis, intimal thickening of the coronary arteries, and myocyte necrosis, characteristic of CDVD.^{1, 3, 4} More recently, authors have speculated that the coexistence of the coronary artery changes and CDVD should be expected due to the high prevalence of both conditions in older dogs.²¹

Progression of Disease

Not only is the degeneration of the valve a progressive process but “mitral regurgitation begets further mitral regurgitation”. This is because the two principle determinants of the volume of mitral regurgitation are the regurgitant orifice area and the pressure difference between the left ventricle and the left atrium. The left ventricle is initially able to compensate for short term mitral regurgitation by increasing preload via the renin-angiotensin-aldosterone system and capitalizing on the Frank-Starling mechanism. Also the increase in heart rate due to sympathetic stimulation contributes to maintaining cardiac output. The heart’s response to long standing volume overload is eccentric hypertrophy, which increases the diameter of not only the left ventricle, but also the annular portion of the mitral valve. This increases the size of the regurgitant orifice area and, in combination with malalignment of the papillary muscles, further volume overloads the heart.^{19, 22} Patients experience an average asymptomatic period of 3 years before decompensation develops.²³ During the subclinical period, medical intervention has not proven to slow the progression of disease.

Manifestations of Canine Valvular Disease

With progression of disease many patients are no longer able to compensate for the volume overload of the left heart. As patients decompensate, most commonly they present in left heart failure with coughing as their most common clinical sign. This occurs due to elevations in left atrial pressure which is dependent on the volume of the regurgitant flow, the output of the right heart, the diastolic pressure of the left ventricle, and the left atrial compliance.¹⁹ Left atrial pressures can increase acutely with rupture of a chordae tendineae increasing the regurgitant orifice area without time for the left atrium to dilate. Left atrial enlargement can predispose to atrial fibrillation which causes loss of atrial contribution to left ventricular filling. This loss of “atrial kick” can cause a patient to decompensate rapidly. Occasionally syncopal episodes can occur. Less commonly these patients present in right sided heart failure late in the course of the disease due to long standing elevation in pulmonary venous pressures causing reactive pulmonary hypertension. Right heart failure symptoms can occur acutely in the situation of a left atrial tear leading to hemopericardium and tamponade or even less commonly due to an

interatrial septal tear causing a left to right shunting atrial septal defect. Regardless of the particular manifestation of an individual patient, once they have developed decompensated degenerative valve disease they are subject to lifelong medication and a relatively high likelihood of dying from this disease.

Human Therapeutic Considerations

In human cardiology, mitral regurgitation is considered a surgical disease.²⁴ Surgical repair or replacement of the mitral valve is considered when there is “functional disability despite optimal medical management and/or for patients with only mild symptoms but with progressively deteriorating left ventricular function as documented by noninvasive studies.”²² More recent studies have supported early surgical intervention and suggest surgery be performed at the earliest onset of symptoms, in the face of any left ventricular dysfunction, and in the presence of atrial fibrillation.²⁴ The trend to earlier surgical intervention is due to improved surgical success rates and the increasing prevalence of mitral valve repair. The luxury of having a surgical option in these patients, as well as the lower incidence of clinically significant mitral valve prolapse in humans, has limited research interest in delineating its etiology and finding methods of early medical intervention.

Canine Therapeutic Considerations

Currently, therapy for dogs with CDVD involves managing congestive heart failure. This is primarily due to the lack of readily available experienced surgical centers as well as the cost associated with valve replacement or repair surgeries. The baseline therapy includes a diuretic, most commonly furosemide, and an angiotensin converting enzyme inhibitor, most commonly enalapril. Additional medications are frequently used for refractory cases including spironolactone, digoxin, amlodipine, hydrochlorothiazide, and pimobendan. This is drastically different than management in humans where valve repair or replacement is the pillar of therapy. In the two studies that have evaluated mitral valve repair and replacement in canine patients, the long term results were disappointing.^{25, 26} Of 8 patients in the mitral valve replacement study, one died during surgery, one died during an attempted electrical cardioversion, and the remaining 6 died

of mitral valve prosthesis thrombosis within approximately 1 year of the procedure.²⁷ While this complication rate may be improved upon with experience and more aggressive anticoagulation, ideally disease prevention or slowing the progression of the disease would be a preferable target in our veterinary patients. A procedure to perform external annuloplasty of the mitral valve without cardiac bypass was developed by Buchanan and Sammarco.²⁸ The intraoperative mortality rate was high (40%) for the first 15 dogs who underwent the procedure. Three dogs were considered “long term survivors” with survival ranging from 6-26 months. At this time, elucidating the etiology of the disease via basic science techniques may provide new approaches to medical management of these patients with an emphasis on prevention or slowing progression as opposed to the current state of managing symptoms of decompensated disease.

Historical Speculation on Etiology

Detweiler et al. suggested the possibility that CDVD in dogs had a generalized connective tissue component, due to the importance of connective tissue elements in valvular fibrosis, intramural coronary arteriosclerosis and myocardial necrosis and fibrosis which were all associated as a triad of post-mortem findings in degenerative valve disease dogs.^{1, 3, 4} They also suggested that the onset of valvular fibrosis preceded the intramural coronary arteriosclerosis and focal myocardial necrosis and fibrosis.³

Pomerance performed a study exploring the pathogenesis of mitral valve prolapse in humans by evaluating the valves of all necropsied hearts in human patients over ten years of age. In this study, the author found that all adult mitral valves had some degree of nodular thickening and proposed that the plaques were ‘a response to repeated trauma, analogous to cutaneous callous formation’.²⁹ The author found no correlation between history of systemic hypertension and degree of mitral thickening. The only correlation considered to be significant was the presence of pulmonary disease correlating with lesions on the mitral and tricuspid valve suggesting a potential role for hypoxia.²⁹ Others have suggested hypertension, stress, prior valve infection, or endocrinopathies as potential causes. No direct evidence has supported any of these causes.¹⁹

Because dogs often have degenerative valve disease as an isolated condition, a generalized connective tissue disorder seems unlikely. The overrepresentation of certain

breeds such as the Cavalier King Charles Spaniel suggests a genetic role. In dachshunds and Cavalier King Charles Spaniels a polygenic mode of inheritance has been suggested.^{12, 30}

Recent Insight into Etiology

A few recent studies have focused on histopathologic evaluation as a method to elucidate etiology. Examples of this include Olsen et al. who showed an increased NADPH-d activity in areas of the mitral valve with myxomatous degeneration which indicates an increase in NOS expression in such areas. These authors suggest that nitric oxide plays a role in the pathogenesis of canine chronic degenerative valve disease by serving as a signal to the subendothelium and regulating extracellular matrix.³¹ Another study described the appearance of mitral valve endocardiosis using electron microscopy. Affected dogs showed marked thickening and swelling of the free edge of the valve and marked damage to the endothelial surface was evident. This damage exposed the underlying collagen, elastin and VIC remnants.³² Transmission electron microscopy was used by Black et al. to show that the VIC phenotype is dramatically different in dogs with degenerative valve disease when compared to unaffected dogs. In the normal leaflets the VICs were characterized as a fibroblast phenotype whereas in the diseased leaflets the cells were a mixed myofibroblast or a smooth muscle cell phenotype.³³ This study is particularly interesting with regard to this thesis as it reveals that there are *in vivo* differences in VICs with CDVD. While these studies are useful to study the result of the disease process and to identify important players for further studies, it becomes difficult to ascertain cause versus effect in such a setting. The study by Black et al. provides support for the importance of studying canine VICs as a means to elucidate the etiology and pathophysiology of CDVD.

As will be discussed later, recent theories suggest that CDVD results from an imbalance between collagen production and collagen degradation in the mitral valve apparatus. Because the valvular interstitial cells are responsible for maintaining the extracellular matrix in the valve, recent attention has been given to this cell type.

Valvular Interstitial Cells (VICs)

Valvular interstitial cells are the most prevalent cell type in cardiac valves and substantial research interest has focused on their participation in response to injury.³⁴ The VICs are derived from the endocardial cells embryologically and have undergone a transformation from epithelial to mesenchymal cells. Some of the cells have migrated from extra-cardiac sites.³⁵ Interstitial cells are responsible for synthesizing the extracellular matrix (collagen, elastin, and proteoglycans) and mediating its ongoing repair and remodeling which is the basis of the valve's durability.¹⁷ The VICs are known to be phenotypically heterogeneous based on cell morphology, immunohistochemical characteristics, age and location within the valve.³⁴ One cell morphology is small islands of cuboidal cells while the other is spindle shaped elongated cells.³⁵ The cuboidal shaped cells are a matrix regulation subtype that have prominent synthetic and secretory granules and are associated with expression of prolyl-4-hydroxylase, which is an enzyme necessary for the stabilization of the collagen triple helix. The spindle shaped cells are a myofibroblast phenotype which have prominent stress fibers as well as smooth muscle α -actin expression and are considered to be the contractile, or activated, phenotype.³⁵ Mulholland and Gotlieb reviewed the cell biology of VICs and recognized the heterogeneity but it was only with later studies that these different cell types were related to alterations in function (normal versus activated interstitial cells).^{17, 36} Activated myofibroblasts are found in fetal human and sheep valves as well as in valves with evidence of myxomatous degeneration, suggesting that during times of disease and repair these cells are transformed to their more fetal-like state.³⁷

In vitro studies have proven VICs contract suggesting that they provide more support for the valve than merely passive structural support.³⁶ These interstitial cells are embedded in a three-dimensional matrix and they are responsible for secreting the matrix as well as interacting with that matrix.³⁴ VICs are capable of synthesizing collagen, elastin, proteoglycans, fibronectin, growth factors, cytokines, chemokines as well as matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs).³⁵

MMPs are members of the large extracellular matrix protease family which are divided into four major classes: the serine proteases, the cystyl proteases, aspartyl proteases, and the matrix metalloproteinases.³⁸ The activity of MMPs is tightly regulated

on three levels including transcription, activation of latent zymogens, and inhibition of their proteolytic activity. Disruption in any of these control steps can lead to excess activity by these enzymes. MMP-2 and MMP-9 degrade type IV collagen which is present in the basement membrane and disruption of this membrane may initiate the inflammatory response.³⁹ In a study on chondrocytes from articular cartilage from rabbits, nitric oxide mediated upregulation of MMP-2 and -9 resulted in release of basic fibroblast growth factor from the extracellular matrix which operates in an autocrine fashion.⁴⁰ This response to injury supports exploring the role of MMPs in the pathogenesis of chronic degenerative valve disease.

Human valves have collagen types I, II, and IV, and express MMPs -1, -2, and -3.³⁴ In a study of normal valves, MMP-1 was expressed in all valves while MMP-2 expression was low in the aortic and pulmonary valves but absent in the atrioventricular valves. MMP-3 was not present in any normal valves.³⁸ In that same study TIMP-1 and TIMP-2 were found in all four normal valve leaflets but TIMP-3 was only expressed in the tricuspid valve. They also found that MMP and TIMP expression was most concentrated at the commissure region of the valve leaflet.³⁸ Interestingly, in some diseased states, including nonrheumatic stenosis, the aortic valve is known to increase expression of these MMPs and begin novel expression of MMP-9.³⁹ In a study performed by Rabkin et al., VICs from myxomatous valves expressed MMP -1, -2, -9, and -13 with the collagenases (MMP-1 and MMP-13) especially elevated. The VICs had increased collagenolytic enzymes but were still capable of synthesizing collagen suggesting that the collagen abnormalities were a result of excessive collagen degradation rather than from decreased collagen synthesis.¹⁷ These findings support the idea of altered MMP expression or altered balance between MMPs and TIMPs as having a role in degenerative valve disease.

The increased incidence of CDVD on the left side of the heart (mitral and aortic valves) compared to the incidence on the right side of the heart (tricuspid and pulmonary valves) has remained an intriguing mystery. A recent study in sheep heart valves showed significantly greater cell stiffness, cytoskeletal protein content and collagen production in aortic and mitral VICs compared to VICs from the tricuspid and pulmonary valves.⁴¹

Many studies have been performed on both cultured VICs and valve organ culture in various species. An elegant study by Lester et al. used a bovine mitral valve organ culture technique to demonstrate that interstitial cells were capable of migrating and proliferating at the damaged portion of the lateral edge of the valve surface in six days.¹⁸ As of yet, a culture technique has not been described for canine valves. The studies that have been performed in other species have provided great insight into our understanding of the valve's response to injury and applying this knowledge to the species with the highest prevalence of the disease process will only serve to advance this understanding.

Endothelin

Of the potential vasoactive factors that may play a role in initiating or perpetuating degenerative valve disease, endothelin lends itself as a prime suspect. First, it is known that endothelin is released from the endothelial tissue in response to shear stress and it has long been suspected that shear stress at the level of the valve may play a role in valvular degeneration. In a study by Mow and Pedersen⁴², using autoradiographic techniques it was shown that endothelin receptor density is increased in myxomatous canine mitral valve leaflets. The increased receptor density also correlated with the areas of the diseased valves that appeared grossly abnormal and was confined to the distal 1/3 of the valve leaflets.⁴² In human heart valves endothelin 1 can cause an increase in intracellular levels of calcium suggesting VICs can change calcium levels and activate cell signaling pathways.⁴³ Because of the ability of endothelin to exert a localized effect, evaluating circulating endothelin levels may not truly reflect its activity at the level of the valve. Determining the effect endothelin exposure has on valvular interstitial cells, and more specifically alterations in the balance between MMPs/TIMPs, may reveal great insight into its potential role in this disease process.

CHAPTER 2 - Thesis Objectives

The objectives of this thesis are:

1. To develop methods for isolation and culture of canine valvular interstitial cells.
2. To characterize the cellular and molecular phenotype of valvular interstitial cells isolated from canine heart valves.
3. To characterize the effects of endothelin and alpha tocopherol on MMP 9 and 13 exposure in VICs.

CHAPTER 3 - Methods and Materials

Valve Tissue Source

Canine valve tissue was obtained from animals either at the time of necropsy or euthanasia. Canine heart valves were from animals submitted to the Kansas State University Veterinary Medical Teaching Hospital (KSU-VMTH) for necropsy examination. Immediately after harvesting, the tissues were processed for cell culture.

Cell Culture Method

Modified from the methods of Katwa et al.⁴⁴ and Lester et al.⁴⁵, the distal one-third of the mitral and aortic valve leaflets were harvested from canine patients undergoing gross necropsy within 36 hours of death or euthanasia. The samples were transported in cold phosphate buffered saline. The endothelial surface of the valve was abraded with a scalpel blade and the tissue was divided into approximately 1 mm square sections. Each tissue sample was placed into an individual well in a 6-well culture plate coated with type I collagen to promote adherence to the plate. The sample was covered with one drop of DMEM with 20% fetal bovine serum (FBS) and 1% antibiotic/antifungal. After 12 hours, 1 ml of media was added to each well and the media was subsequently changed every 3-4 days. The original tissue sample was removed once a colony of explanted cells developed. When the cells were 75-100% confluent they were passaged using standard trypsinization techniques. This technique involved removing the media from each well, replacing it with 1 ml of 0.25% trypsin for 5-8 minutes (until cells began to release from the plate), and then adding 1 ml of media to the plate pipetting up and down to release the remaining cells. Once cells were released from the plate, the suspended cells were placed in a 15 ml conical tube and centrifuged for 5 minutes at 1050 RPM with a slow acceleration and deceleration. The supernatant was discarded and the pellet of cells was resuspended in culture media. The cells were split 1:4 and re-plated into either new 6-well plates or T-75 flasks, depending on the intent of use, and maintained in the DMEM with 20% fetal bovine serum (FBS) and 1%

antibiotic/antifungal added. Cells from each passage were placed in freeze media (the above growth media with 7.5% DMSO added) and frozen in liquid nitrogen for further experiments.

Collagen Coating

Collagen coating of the 6 well cell culture plate with type I collagen was achieved by using a filter set to purify a solution of 16 ml collagen (Bovine Collagen, Type I, BD Biosciences, Bedford, MA) with 2 ml of 10X PBS and 0.2 ml 5M NaOH that was titrated to a pH of 7 with HCl. The filtered collagen solution was used to thinly coat the wells of the plate and then the plates were incubated for 1 hour at 37°C with 95% O₂ and 5% CO₂. The coated plates were then placed in the cell culture hood overnight to dry. The plates were sealed and stored at 4°C until use. Prior to use the collagen coating was rinsed well with sterile water to remove salt and acid.

Phenotypic Characterization

RT-PCR protocol – Primers for vimentin were developed based on a known rat vimentin sequence (GenBank Accession number X62952) using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Canine specific primers were not used as the canine genome had not yet been released when these studies were performed.

Vimentin Primers:

Sense: 5' – AGA TCG ATG TGG ACT TTT CC – 3'

Antisense: 5' – TCC GGT ATT CGT TTG ACT CC – 3'

Expected product: 198 bp

Primers for PECAM (platelet endothelial cell adhesion molecule) were based on human RNA from a paper by Sauer et al.⁴⁶

PECAM primers:

Sense: 5' – CAT TTT GCA TTT CTC TCC ACC – 3'

Antisense: 5' – GCA GGG CAG GTT CAT AAA TAA G – 3'

Expected product: 218 bp

GAPDH Primers (positive control):

Sense: 5' – ATC TTC CAG GAG CGA GAT – 3'

Antisense: 5' – TGG TCA TGA GTC CTT CCA CGA TA – 3'

Expected product: 300bp

Total RNA was prepared from cells using Tri Reagent RNA Isolation Reagent (Sigma-Aldrich). 0.3 ml of Tri Reagent was added for each well of the 6 well plate and allowed to incubate for 5 minutes. 0.2 ml chloroform was added and agitated for 15 seconds and then allowed to incubate at room temperature for 10 minutes. The sample was then centrifuged at 12,000 g for 15 minutes at 4 C. The supernatant was removed and placed into a new tube. 0.5 ml isopropanol was added and allowed to incubate for 10 minutes. The sample was centrifuged at 12,000 g for 10 minutes at 4 C. An RNA pellet was visible at this time and the supernatant was discarded. The pellet was rinsed with 70% ethyl alcohol and 60 µl of dH₂O was added and the sample stored at 4° C. The dog kidney total RNA was isolated with the same method to serve as PECAM positive control.

cDNA synthesis and amplification of vimentin and PECAM by RT-PCR was carried out using the TGradient Thermocycler (Biometra, Goettingen, Germany) by using a mixture (total reaction volume was 25 µl) that included: Access Quick Mastermix (AccessQuick™ RT-PCR System, Promega, Madison, WI) (12.5 µL), 1 µl of each specific oligonucleotide primer, total RNA (3 µl), AMV Reverse Transcriptase (0.5 µl), and filtered dH₂O (q.s. to 25 µl). The mixture was incubated for 45 min at 48 C and then at 95 C for 1 min and 15 sec. For amplification the mixture was cycled 30 times through the following three conditions: 95 C for 45 sec., 55 C for 45 sec., and 72 C for 1 min. and 15 sec. The reaction was terminated by heating the reaction to 72 C for 5 min. and then the reaction was held at 4 C. Products from the PCR reaction were resolved by electrophoresis at 75 V for 30 to 45 minutes on a 1.5 % agarose gel and detected with ethidium bromide staining and UV light. Products were isolated from the agarose gel (DNA Gel Extraction Kit, Millipore Corporation, Bedford, MA) and the identity of the

PCR products was confirmed by sequence analysis performed using an automated DNA analyzer (ABI 3700 and ABI 3730 analyzers, KSU Dept Plant Pathology).

Immunocytochemistry protocols – Cells were applied to microscope slide via cytopsin for both protocols. The cytopsin preparation consisted of collecting a T-75 flask of VICs for cytopsin analysis. Aliquots of VIC suspensions (200ul) were placed into individual wells of a Shandon Cytospin® cytocentrifuge. The samples were spun at 700 rpm for 3 min onto a glass slide. Slides were air-dried for at least 4 hrs prior to fixation and staining for vimentin and factor VIII.

Vimentin protocol: Primary antibody was applied [Vimentin prediluted by manufacturer (DAKO)] by hand and incubated for 32 minutes. The slide was rinsed and one drop of biotinylated IgG was applied and incubated for 8 minutes. The slide was rinsed again and one drop of avidin-alkaline phosphatase was applied and incubated for 12 minutes. After rinsing one drop of Enhancer was applied and incubated for 4 minutes followed by one drop of Fast Red A and one drop of Naphthol and incubated for 8 minutes. One drop of Fast Red B was then applied and incubated for 8 minutes and then the slide was rinsed again. One drop of Hematoxylin counterstain was added and incubated for 2 minutes followed by one drop of Bluing Reagent as a post counterstain and incubated for 2 minutes. The slide was then rinsed by 20 dips in water with 3 drops of Dawn dishwashing liquid and then 20 dips in tap water. The slide was dehydrated by 3 changes in 100% EtOH and then 2 changes in Xylene and then a cover slip was placed.

Factor VIII protocol: Primary antibody was applied [Factor VIII diluted 1:400 (DAKO)] by hand and incubated for 32 minutes. After rinsing, one drop of avidin-HRPO was added to the slide and incubated for 8 minutes. The slide was rinsed again and then one drop of DAB and one drop of DAB H₂O₂ was applied and incubated for 8 minutes. After rinsing again, one drop of copper was added and incubated for 4 minutes. Following another rinse, the slide was incubated with one drop of Hematoxylin as a counterstain for 2 minutes. The slide was rinsed again prior to incubating for 2 minutes with one drop of Bluing Reagent as a post counterstain. The final rinse and the dehydration steps were performed as for the vimentin slides.

Western Blot Protocol

Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Briefly, lysates were loaded onto SDS-acrylamide 4-12% gels. The 4% stacking/12% resolving gel was made with the following protocol:

4% Stacking Gel:

6.2 ml ddH₂O
1.3 ml 30% Acrylamide/BIS
2.5 ml 0.5 M Tris-HCl, pH 6.8
0.1 ml 10% SDS
50µl Ammonium Persulfate (APS)
10µl TEMED

12% Resolving Gel:

3.4 ml ddH₂O
4.0 ml 30% Acrylamide/BIS
2.5 ml 1.5 M Tris- HCl, pH 8.8
0.1 ml 10% SDS
50µl Ammonium Persulfate (APS)
5µl TEMED

For multiscreen western blots, a gel was made that had a single large well for lysates and a smaller well for the marker. The protein sample was prepared by combining 250 µg of protein with 16.7 µL Laemmli sample buffer and 5% β mercaptoethanol and boiling for 5 minutes. The gel was loaded with 20 µl marker added to the small well. Electrophoresis was performed at 160 volts for 1 hour at constant voltage and then transferred to a PVDF membrane at 80 mA overnight at a constant mA. The membrane was blocked with Tris Buffered Saline (TBS) buffer + 0.01% Tween-20 (TBST) with 5% evaporated milk. The membrane was incubated for two hours at room temperature with one of four primary antibodies: rabbit polyclonal against the hinge region of MMP1 (AB806, Chemicon® International), mouse monoclonal against MMP2 (RDI-MMP2abm-5D, RDI, Concord, MA), rabbit polyclonal antibody against the C-terminal domain of MMP9 (AB804, Chemicon® International), and polyclonal antibody against MMP13 (AB8114, Chemicon® International) at dilutions of either 1:500 or 1:1000. Next, the membrane was washed again with TBST followed by incubation with

HRP-conjugated anti-rabbit IgG or anti-mouse IgG (secondary antibody) for 2 hours. Protein detection was accomplished using a chemiluminescence detection system (SuperSignal West Pico Substrate, Pierce, Rockford, IL) and recorded on photographic film.

For routine Western blots, 20 µg of protein (sample protein concentration determined by BCA assay) was added to each well with Laemmli sample buffer and 5% β mercaptoethanol added at 33% of the volume of the sample. All SDS-Page was performed using a 4% stacking/12% resolving gel made for each blot as described above. Resolved proteins were transferred to a PVDF membrane for routine immunoblot analysis. After the transfer step, the membrane was washed three times with TBST and blocked for at least one hour in TBST + 5% evaporated milk. After blocking, the membrane was washed three times with TBST and incubated for 2 hours at room temperature with the primary antibodies (1:500) against MMP9 and MMP13 as previously described. Anti-actin antibody was used in two of the experiments as the internal control at a concentration of 1:1000. Next the membrane was washed again with TBST followed by incubation with HRP-conjugated anti-rabbit IgG for 2 hours at room temperature. Protein detection was performed using a chemiluminescence detection system (SuperSignal West Pico Substrate, Pierce, Rockford, IL) and recorded on photographic film.

Endothelin Exposure and Vitamin E

To examine the effects of vitamin E on MMP protein expression in the presence of endothelin exposure, VICs were exposed to either endothelin 1 (ET1) alone or ET1 and Vitamin E for 24, 48 and 72 hours. Vitamin E media was prepared by first dissolving 31.25 mg of alpha tocopherol (T3001, Sigma-Aldrich) into 32.5 µl of 100% ethanol and then sonicating this solution at high speed into 25 ml of FBS. This solution was used as the 20% FBS in the antioxidant media. The “control” media consisted of the normal VIC media with the addition of 37.5 µl 100% ethanol. For experiments, the media was removed from 9 T-75 flasks of confluent cells. Six of the flasks had 10 ml of the antioxidant media added and 3 of the flasks had 10 ml of control media added. Endothelin 1 (E-7764, Endothelin 1-Human, Porcine, Sigma-Aldrich) was diluted in 1%

acetic acid to make a 1mM stock solution. To the ET1 alone flasks and the ET1 in combination with vitamin E flasks, 50 µl of the 1 mM ET1 solution was added. To the control flasks 50 µl of 1% acetic acid was added. At 24, 48, and 72 hours, one flask from each group was collected for protein analysis. Samples were fractionated into nuclear and cytoplasmic lysates. Nuclear fraction was obtained using a kit (Nuclear Extraction Kit, Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the cytoplasmic fraction was collected by washing the cells with cold phosphate buffered saline and a phosphatase inhibitor (PBS/Phosphatase I). The cells were removed from the flask with a cell scraper and suspended in cold PBS/Phosphatase I. The suspension was centrifuged at 500 RMP at 4 C for 5 min. The supernatant was discarded and the cells resuspended in hypotonic buffer and incubated on ice for 15 min. A detergent was added and the sample vortexed for 10 sec. before centrifuging for 30 sec. at 14,000g at 4 C. The supernatant consisting of the cytoplasmic fraction was collected and used for the western blot studies.

Image Analysis

Digital images were analyzed using ImageJ image analysis software (National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>). Actin, MMP9 and MMP13 areas were determined by manually outlining regions of interest using drawing tools included in the software. Grey level units were obtained for each band and the MMP9 and MMP13 blots were normalized to the intensity of the actin bands.

CHAPTER 4 - Results

Cell Culture Results

In an experiment to determine the most appropriate substrate for cell culture, 9 sets of aortic and mitral valve samples were plated on plastic (non-coated) wells and 8 sets of valves were plated on collagen-coated plastic wells. (Figure 4.1) The results showed that the success of adherence to the non-coated plate and eventual migration of valvular interstitial cells from the tissue was much lower than the success rate with plates coated with collagen. Of 9 sets of aortic and mitral valves that were plated without collagen coating, only 1 had growth and only the aortic VICs were cultured. Interestingly, the canine mitral VICs were not successfully cultured on the plastic plates. Of the 8 canine valve sets plated on collagen coated plates, 6 of these 8 samples successfully cultured aortic and mitral VICs. The two sets of valves that were unsuccessful adhered to the plate, but both samples were contaminated within 2-7 days.

When comparing media containing 10% FBS with media containing 20% FBS, the samples cultured with 10% FBS had sluggish migration of the cells with the samples frequently becoming contaminated due to the length of time that the initial tissue sample remained in the well before a population of VICs was established on the plate. Increasing the FBS to 20% allowed the tissue to be removed within 2-3 days which minimized the time for contamination.

It was found that leaving the single drop of media on the tissue for 12 hours was very important because adding media at 6 hours and 8 hours often led to dislodgement of the sample. This timeline was somewhat less important with the collagen coating as the adherence of the tissue to the plate was greatly aided by the presence of the collagen. Different concentrations of DMSO were used in the freeze media (5%, 7.5%, and 10%) with no apparent effect on the viability of the cells.

Canine valvular interstitial cells from the mitral and aortic valves were repeatedly cultured and passaged. Cells were cultured that had been stored in liquid nitrogen with most cells surviving the stored time period. It took nearly a week in the culture

environment after being removed from liquid nitrogen for the cells to regain their initial appearance in culture and begin to undergo cell division. It was evident that when the cells were not confluent they appeared multipolar and well spread out on the culture surface (Figure 4-1). Once confluency was achieved they became more cobblestone in appearance and developed the characteristic whorling pattern characteristic of fibroblast-like cells (Figure 4-2). These were cells cultured directly from animal tissue; therefore contamination was a considerable concern. The utilization of the antibiotic/antimycotic solution in the cell culture media prevented contamination unless aseptic technique was broken.

RT-PCR Results

The VIC retains significant plasticity and is able to be phenotypically described as a fibroblast, myofibroblast, or smooth muscle like cell. This variability lends itself to unique challenges when attempting to confirm the phenotype of the primary cells isolated from canine valves. Because all of the possible phenotypes express vimentin but not necessarily smooth muscle actin or desmin, vimentin was selected as the positive control for both RT-PCR and immunocytochemistry. PECAM is not expressed by VICs and is found only in endothelial cells that line the valve tissue. PECAM was used as the negative control for the VIC phenotype. Because the kidney is a highly vascular structure and the endothelium of the vessels in the kidney should express PECAM, this was used as the positive control. GAPDH was run as an internal positive control. The presence of vimentin in these cells was confirmed by PCR (Figure 4-3). A 131 bp partial sequence of vimentin was obtained from total RNA isolated from cultured cells. Vimentin partial sequence: 5' - gctgcctgc gtgatgtacg ccagcaatat gaaagcgtgg ctgccaagaa ccttcaggag gccgaggaat ggtacaagtc caagtttgcc gacctctctg aggctgctaa ccggaacaat gatgcctctgc g - 3'. This partial sequence was 100% homologous with a predicted canine vimentin sequence (GenBank accession number XM535175) as well as a partial primary sequence of canine vimentin (GenBank accession number DR103772). The primary sequence obtained has been submitted to GenBank under the accession number EF432321.

Although the PECAM product in the dog kidney sample appeared to be at the correct size based on the marker, the product was not able to be effectively sequenced.

There was not a band at the appropriate molecular weight in the VIC samples suggesting that the VICs are negative for PECAM which would be expected for VICs. (Figure 4.3)

Immunocytochemistry Results

Immunocytochemistry revealed that all VICs stained positively for vimentin (Figure 4-4) and none of the cells stained positively for factor VIII (Figure 4-5). Staining observed on slides stained for factor VIII was scant and non-specific, similar to that observed in control slides. These results confirm that the cells cultured from canine valves contain the intermediate filament, vimentin as expected for VICs and lack factor VIII, indicating that they are not of endothelial origin.

Western Blot Results

Four samples were performed for each antibody with the concentrations of primary antibody either 1:500 or 1:1000 and the concentration of the secondary antibody of either 1:3000 or 1:6000 to determine the optimal protocol (Figure 4.7). MMP1 immunoblotting at all concentrations was negative. MMP1 has been shown to be present in valvular interstitial cells in other studies. This result was likely the result of outdated antibodies. MMP2 immunoblotting resulted in nonspecific uptake and was likely the result of either too high a concentration of polyclonal antibody or insufficient washing. MMP 9 and MMP 13 had bands at the appropriate molecular weights and revealed that the optimal antibody concentrations for these two antibodies were 1:500 for the primary antibody and 1:3000 for the secondary antibody. These two antibody concentrations were utilized for the additional western blots.

Western blots against MMP9 and MMP 13 normalized to actin were performed on samples that had been exposed to endothelin for 48 and 72 hours as well as samples at those same time points that had been exposed to endothelin and Vitamin E. When normalized to actin, cytoplasmic MMP9 and MMP 13 levels were lower at each time point when concurrently exposed to Vitamin E (Figure 4.8 and 4.9).

CHAPTER 5 - General Discussion

In this study, a successful cell culture protocol was developed for canine valvular interstitial cells. This provides a simplified system to study these cells directly and may help elucidate their role in this disease process. The information achieved from VIC studies can later be applied back to the valve and even the canine patient as a whole.

The reproducibility of the protocol increased dramatically throughout the study. This is likely due to the modifications that were made. Although fibroblast-like cells are thought to be strongly adherent to cell culture plates, it was found that pretreating the culture surface with collagen was very important in promoting this adherence. After the VICs migrated from the tissue and began to replicate, the plate coating was unnecessary. This observation suggests that the collagen coating is actually more important to keep the valve tissue in place for the cells to migrate out and not necessarily a requirement for the VICs themselves. It was also interesting that the cells replicated more quickly with a higher serum concentration (20% FBS). This suggests that in the cell culture environment, these cells have a relatively high nutrient need at least for the constituents of fetal bovine serum.

As previously mentioned, the molecular markers of these cells are variable depending on their conditions. This presented a unique challenge for establishing the phenotype of these cells. Vimentin was chosen as the positive marker as it is present in all variations of the cell. In both the RT-PCR studies as well as the immunocytochemical studies, markers were chosen that should only be expressed on the endothelial cells within the valve. In both situations, the cells were negative for endothelial markers. Although a significant portion of the project revolved around phenotyping these cells prior to subjecting them to experiments, it is highly unlikely that anything but valvular interstitial cells were cultured. One reason for this is that very few cells in the valve would have the potential to migrate from the valve tissue (primarily VICs and endothelial cells). Because the growth environment would be unlikely to support endothelial cell growth, this population should only be VICs. The cellular characteristics in culture

appeared and behaved the way that one would expect VICs to in the cell culture environment. In the preliminary studies performed, these cells responded the way that would be expected from VICs. For all of these reasons in addition to the phenotyping that was performed, the cells cultured via this technique can be confidently considered VICs.

The lower levels of MMP 9 and MMP 13 found in these preliminary studies suggest that vitamin E may have a protective effect on the VICs when they are concurrently exposed to endothelin. If increased collagen turnover due to an imbalance between MMPs and TIMPs is central to the etiology of CDVD then this information may provide insight into a potential therapeutic role for vitamin E. Improvements could have been made to this study. Adding a control population not exposed to either endothelin or vitamin E would have clarified whether endothelin does indeed increase MMP levels. Also, exploring the levels of MMP -1, and -2 as well as TIMP-1 and -2 would have provided further insight into this balance. Clearly more investigation needs to be performed in this area but these early results are intriguing.

A limitation of this cell culture technique is that it may select for VICs that are more motile and is therefore not an adequate representation of the cells in the valve tissue. But because previous studies have shown the plasticity of these cells, it seems likely that given the correct environment all VICs have the potential to become motile. The ease of reproducing this technique allows for consistent populations of VICs from all mitral and aortic valve tissue cultured as long as strict aseptic technique is performed. The benefit of the reproducibility of the technique far outweighs any potential selection for more motile cells.

Any research projects involving cell culture techniques are subject to oversimplifying the system. While this would be a concern of employing this model to study CDVD, the value of being able to study multiple conditions inexpensively and without the ethical concerns of using live patients makes this model an attractive research option.

A limitation of the PCR protocol is that the PECAM was unable to be appropriately sequenced. While this is a significant limitation, this was the primary stimulus for the immunocytochemistry to be performed. The presence of vimentin in all

of the stained cells and the absence of factor VIII suggests that these cells are fibroblast-like with the absence of endothelial cells.

Although the potential applications of this model are somewhat limitless, there are a few projects that are logical next steps. It would be prudent to characterize these cells further. This could include evaluating markers like vimentin, desmin, and smooth muscle actin over passages to determine when a stable phenotype exists. It would be useful to explore the presence of other receptors, like angiotensin II, endothelin receptor subtypes, and serotonin, on these cells in an effort to elucidate major players in CDVD. Also, evaluating MMP/TIMP levels while concurrently evaluating collagenase activity may help clarify the role of this balance in CDVD.

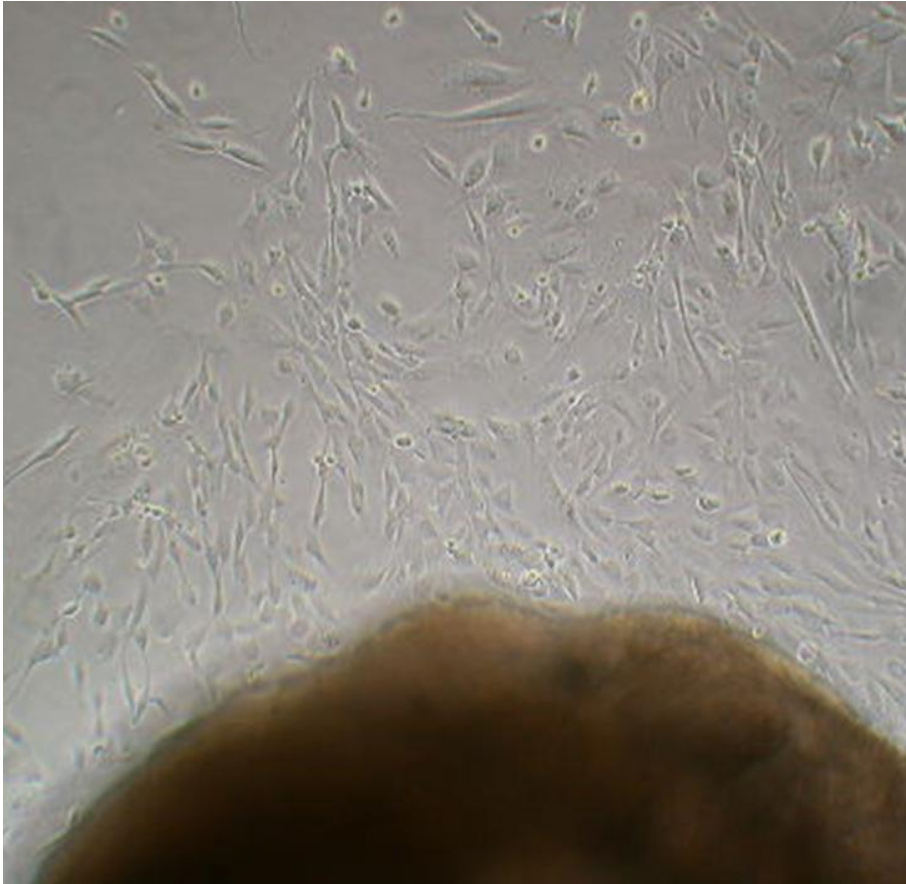
In summary, this project established a reliable cell culture protocol for canine VICs. This is the first documentation of a canine VIC culture protocol in the literature. Decreased cytoplasmic levels of MMP9 and MMP13 were demonstrated when cells exposed to endothelin were pretreated with vitamin E. These preliminary studies suggest further exploration into the role of endothelin and the potential benefit of vitamin E is warranted. Using this cell culture method has the potential to elucidate the role of VICs in CDVD and potentially aid in determining the etiology of this disease process.

Figures and Tables

Figure 4.1 Chart of successful culture with and without collagen coating

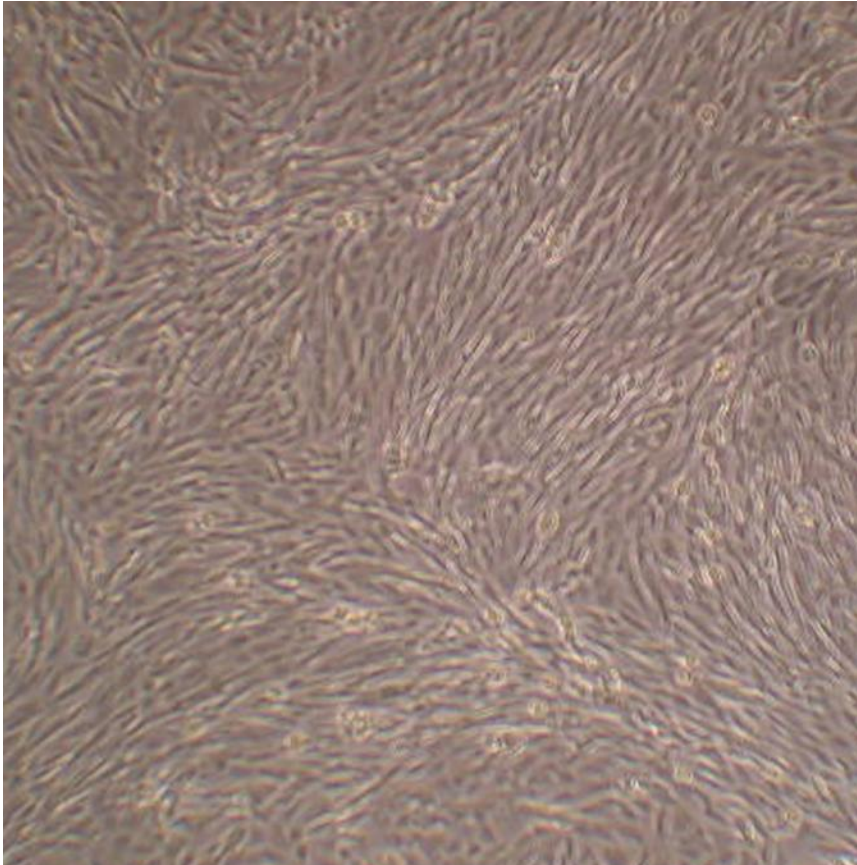
	Without Collagen Coating	With Collagen Coating
Failed Cultures	8	2 Became contaminated within 2 days
Successful Cultures	1 Aortic valve cells only	6

Figure 4.2 Valvular interstitial cells 4 days in culture



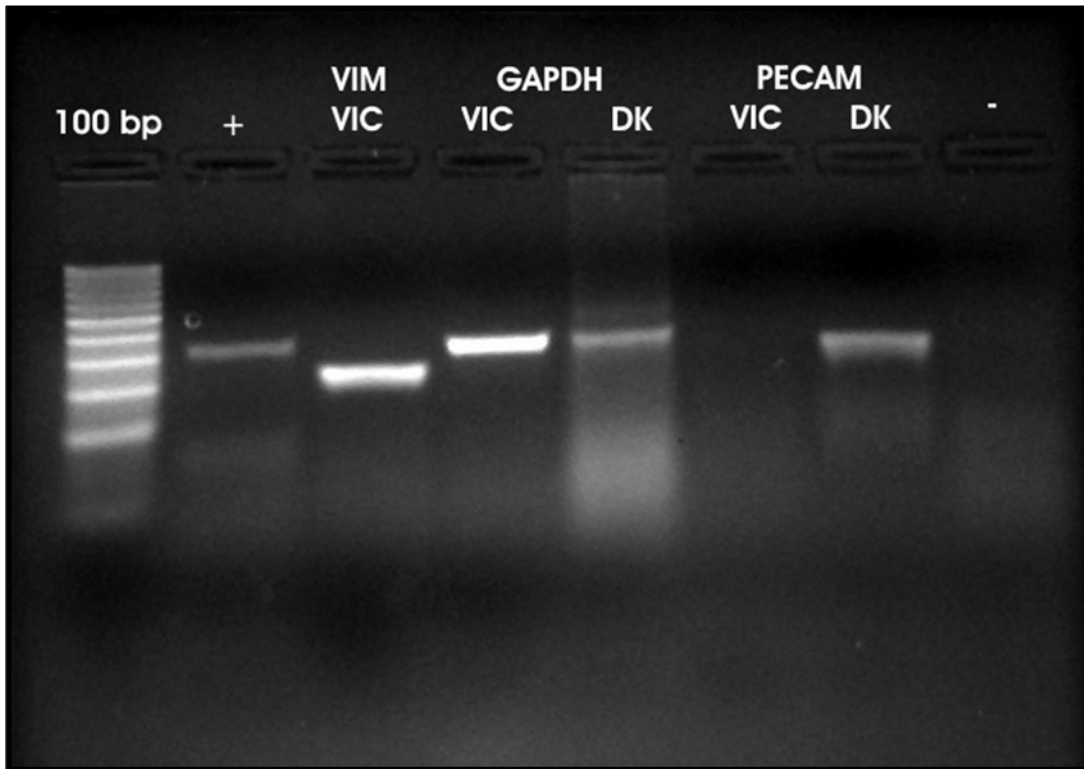
Photograph taken 4 days after tissue placed in 6-well culture plate showing fibroblast-like cells migrating from the valve tissue in culture. At this non-confluent state the cells are multipolar and spread out on the plate.

Figure 4.3 Confluent Cell Culture



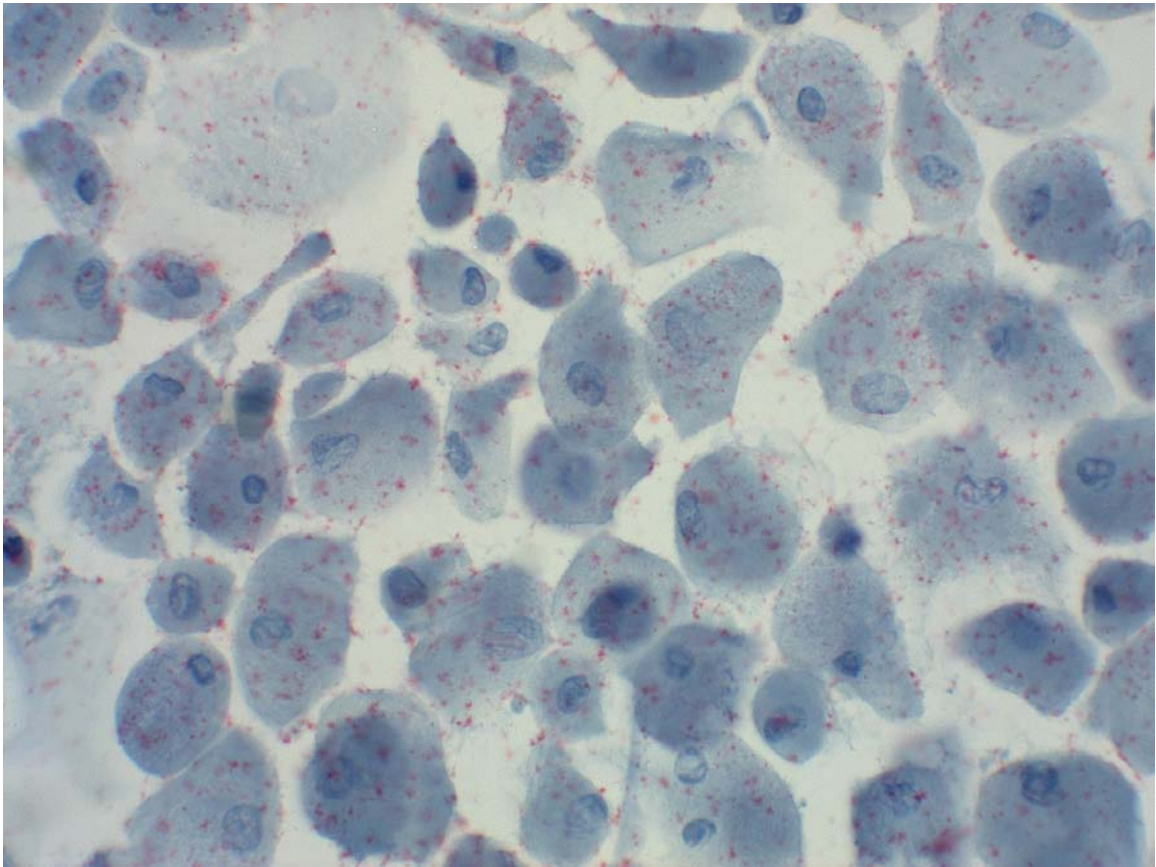
Photograph showing confluent cell population with characteristic parallel arrays and whorls that are characteristic of fibroblast-like cells.

Figure 4.4 RT-PCR gel



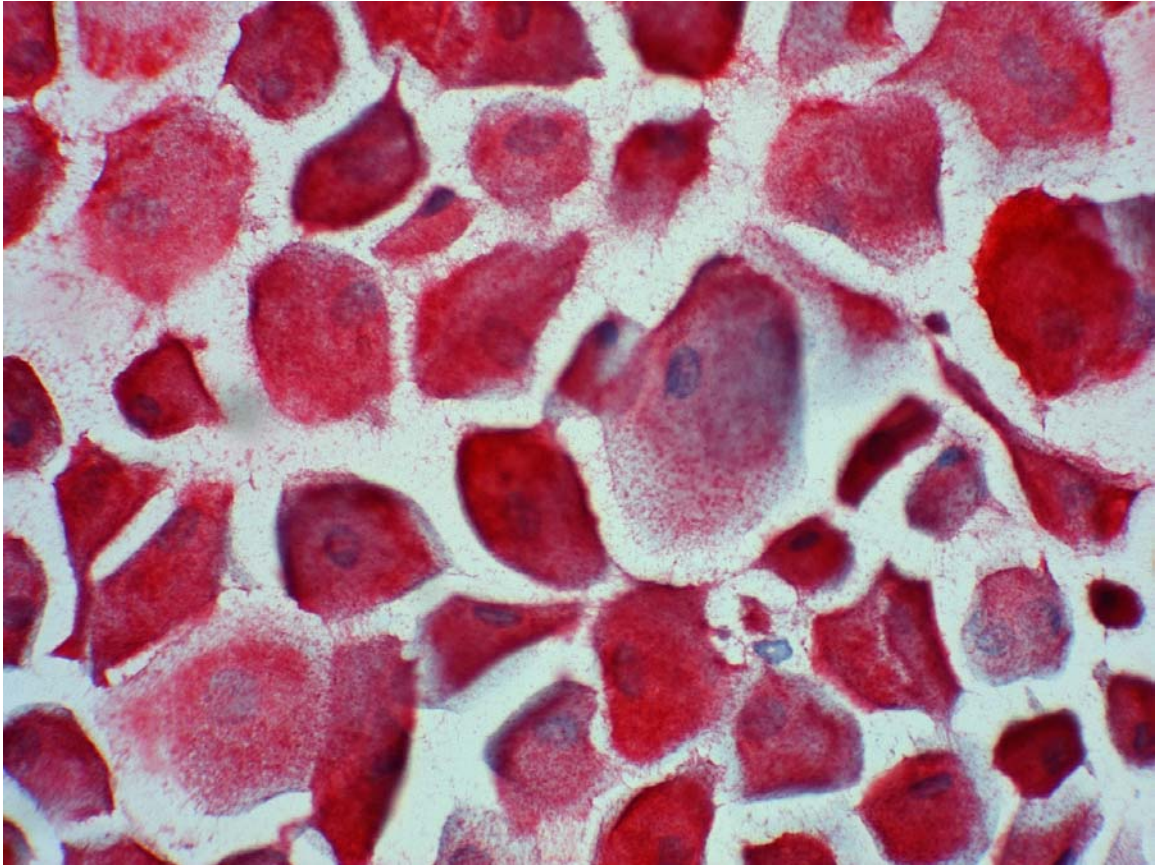
One step RT-PCR performed using total RNA from cultured cells (VIC) and from total RNA harvested from homogenized canine kidney (DK). From left to right: Lane 1 is positive control RNA supplied by Promega with primers to amplify product of 320 bp. Lane 2 is vimentin (VIM) using primers to amplify 198 bp product. Lanes 3 and 4 show GAPDH utilized as internal control with primers meant to amplify 300 bp present in both cultured cells (VIC) and kidney tissue (DK). In lanes 5 and 6 PECAM is included to rule out the presence of endothelial cells. PECAM is expressed in canine kidney (DK) but not in VICs. Negative control is vimentin primers with proposed VICs without transcriptase.

Figure 4.5 VICs stained with Factor VIII (50X)



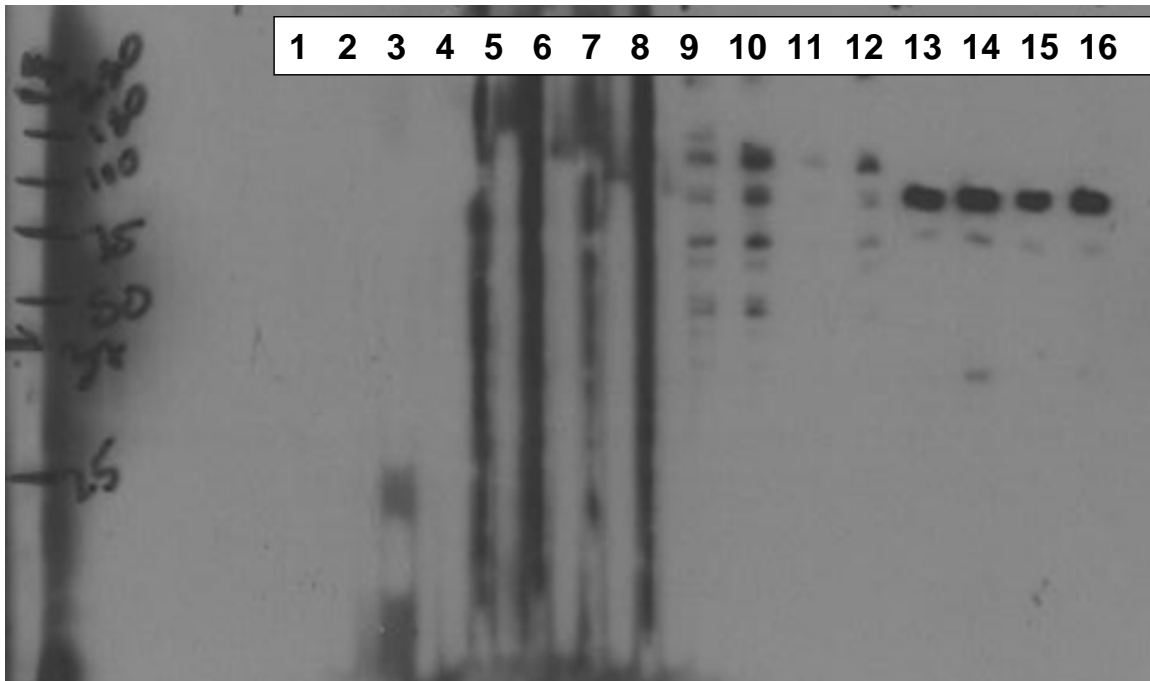
Immunocytochemistry performed with primary antibody against factor VIII. Cells are negative for factor VIII and nonspecific stain uptake is also present on negative control.

Figure 4.6 VICs stained with Vimentin (50X)



Immunocytochemistry performed with primary antibody against vimentin. All cells stained heavily for this intermediate filament.

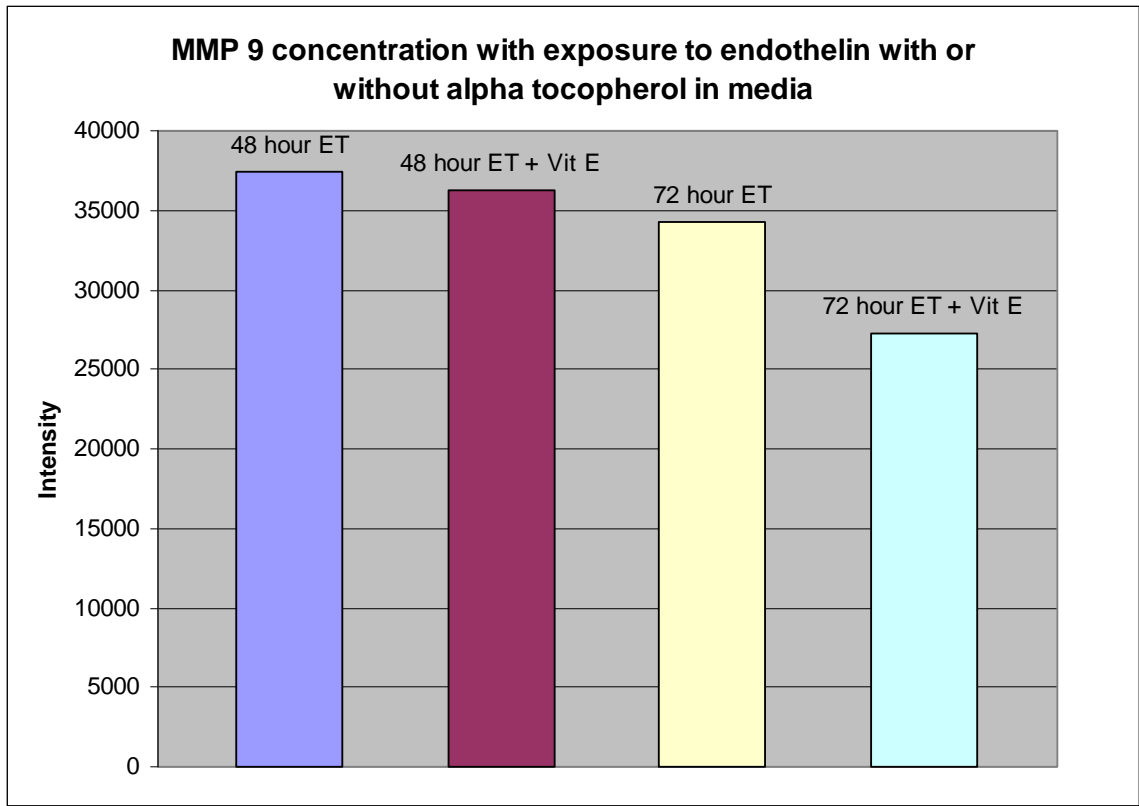
Figure 4.7 Multiscreen Western



Western blot performed to optimize antibody conditions. MMP9 and MMP13 antibodies were effective and optimal concentrations were 1:500 for the primary and 1:3000 for the secondary.

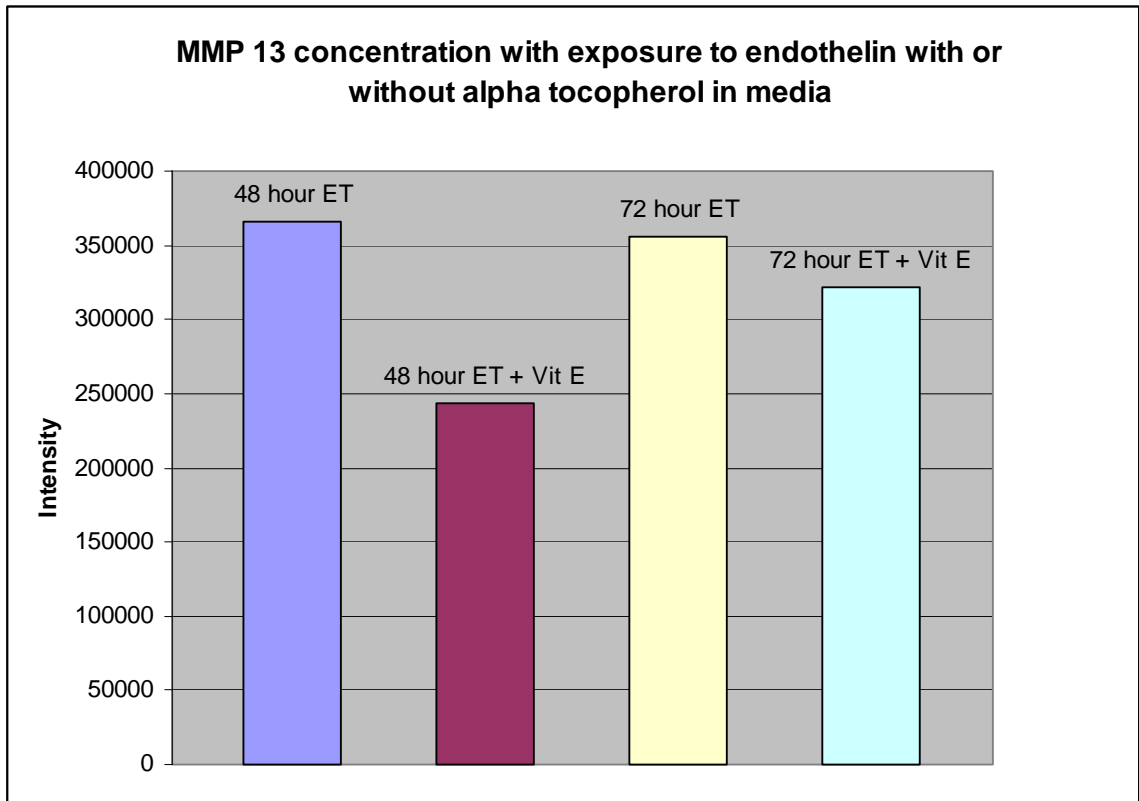
	<u>Primary Conc.</u>	<u>Secondary Conc.</u>
1) MMP1	1:1000	1:3000
2) MMP1	1:500	1:3000
3) MMP1	1:1000	1:6000
4) MMP1	1:500	1:6000
5) MMP2	1:1000	1:3000
6) MMP2	1:500	1:3000
7) MMP2	1:1000	1:6000
8) MMP2	1:500	1:6000
9) MMP9	1:1000	1:3000
10) MMP9	1:500	1:3000
11) MMP9	1:1000	1:6000
12) MMP9	1:500	1:6000
13) MMP13	1:1000	1:3000
14) MMP13	1:500	1:3000
15) MMP13	1:1000	1:6000
16) MMP13	1:500	1:6000

Figure 4.8 MMP9 concentration normalized to actin



Intensity of immunoblot (from Image J) normalized to actin for MMP9 cytoplasmic concentration. Cells were exposed to 10^{-6} M endothelin 1 for 48 and 72 hours with and without antioxidant media (200 μ g/ml vitamin E added to cell culture media). At both time points, the intensity (and thus MMP9 concentration) is lower in the cells in the antioxidant media.

Figure 4.9 MMP13 concentration normalized to actin



Intensity of immunoblot (from Image J) normalized to actin for MMP13 cytoplasmic concentration. Cells were exposed to 10^{-6} M endothelin 1 for 48 and 72 hours with and without antioxidant media (200 μ g/ml vitamin E added to cell culture media). At both time points, the intensity (and thus MMP13 concentration) is lower in the cells in the antioxidant media.

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