Effects of Nitric Oxide on Aortic Valve Calcification *in vitro*

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ABSTRACT

Aims: Aortic stenosis (AS) is characterized by accelerated aortic valve (AV) calcification, but the pathogenesis of this process is poorly understood. The aim of this study was to evaluate the potential impact of NO supplementation on valve matrix calcification in a tissue culture model, and the interaction between superoxide anion release and the process.

Methods: Interstitial cells were isolated from porcine AV leaflets and grown to 95% confluence in 10% serum. Medium was changed to low serum (0.67%) ± transforming growth factor-beta1 (TGF-β1) (5ng/ml) ± study drugs, replenished every 48 h for 4-14 days. Both spontaneous nodule formation and that induced by TGF-β1 over 4-14 days. Experiments were conducted in triplicate in at least 4 cultures at cell passages 4. Nodules were counted by an observer blinded to treatment. In parallel experiment, dihydroethidium staining was utilized to measure superoxide formation in pig aortic valve fibroblasts just to parallel to nodule formation.

Results: Exogenous TGF-β1 elicited a marked increase in calcific nodule formation compared to paired controls. This was inhibited by co-incubation with the NO donor DETA-NONOate (1-100μM), 8-Br-cGMP (1mM) and by the superoxide scavenger, TEMPOL (100μM). In addition, L-NAME (100μM) had no effect on TGF-β1 induced nodule formation. TGF-β1 elicited a marked increase in intracellular superoxide formation compared to paired controls. DETA-NONOate (20μM) and TEMPOL (100μM) blocked intracellular superoxide formation in the presence of TGF-β1.

Conclusions: From these data, we have established that TGF-β1 both induced superoxide anion release and calcific nodule formation in this preparation, while NO donor DETA-NONOate reverses both these effects and cGMP analogue inhibits nodule formation. Furthermore, TEMPOL, a superoxide scavenger, also limits nodule

formation. Therefore, these data suggest that superoxide anion release, induced by TGF-β1 contributes to calcific nodule formation, and that NO can limit this process possibly via the production of cGMP. It remains to be determined whether these effects of NO are relevant to the impact of valvular endothelial dysfunction and/or to a potential therapeutic role for NO donors in prevention of AS.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1

INTRODUCTION
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1.1 Introduction

Although aortic stenosis (AS) had been thought of as a result of "wear and tear" with aging, a recent review by Rajamannan et al (2003) has pointed out that the possibility that it might be an active process was suggested as far back as 1854 As they state: "In 1854, William Stokes described in his textbook, *The disease of the heart and aorta,* a special description of calcific aortic valve stenosis, including: (1) permanent patency of the valve where the diameter may be increased or decreased; (2) an extreme sclerotic growth along the calce surrounding the left ventricle, where the valve is always destroyed; (3) an atheromatous deposit on the ventricular surface of the valve which is often seen in the context of fatty degeneration of the heart (Stokes, 1845)." Aortic valve stenosis involves progressive calcium deposition within the aortic valve, resulting in increased stiffness and narrowing of the valve. The earliest stages of this process are called aortic valve sclerosis, implying the absence of severe obstruction.

1.2 aortic valve stenosis as an emerging clinical epidemic

1.2.1 Pathogenesis of aortic valve stenosis

Aortic valve stenosis is a progressive disease. In mild to moderate aortic stenosis, patients are always asymptomatic and the condition is well tolerated for several years. When it becomes severe, which means that the valve orifice area is reduced by more than 75% of its normal area (3-4 cm^2), the transvalvular pressure between left ventricle and aorta may be more than 100mmHg. As a result, the left ventricle faces significantly increased afterload caused by severe obstruction to normal blood flow across the valve during systole, and the left ventricle becomes concentrically hypertrophied in response to increased afterload. Although left ventricular hypertrophy has a significant compensatory role in reducing left ventricular wall tension, it also reduces the compliance of the ventricle and makes it prone to ischemia and hypertrophied myocardium increases oxygen demand. As a result, many patients with severe aortic stenosis have angina, even though they have normal coronary

anatomy (Carabello and Crawford, 1997).

Symptoms of angina, syncope, and congestive heart failure appear relatively late in the natural history of aortic stenosis. Atrial fibrillation and pulmonary hypertension are rare in aortic stenosis. Early in aortic stenosis, the mean atrial pressure and pulmonary capillary wedge pressure remain normal. However, hypertrophy is eventually associated with impaired left ventricular relaxation. As a result, this leads to marked elevation in left atrial and pulmonary pressure, which produce the symptom of congestive heart failure in the late stages of the disease (Carabello and Crawford, 1997).

Recently, the early lesions of mild aortic stenosis have been well demonstrated. The main characteristics are subendothelial thickening with disruption of basement membrane, accumulation of intracellular and extracellular lipids, and cell infiltration of macrophages (Wareen and Young, 1997; Roberts, 1970; Campbell, 1968; Otto et al., 1994). The adjacent fibrosa becomes thickened and demonstrates accumulation of protein, lipid, and calcium. Both foam cells and non-foam cells such as macrophages and T cells are present. The abnormalities are concentrated on the aortic side of the non-coaptational portion of the aortic valve cusps (Anegelini et al., 1994; Pomerance, 1972; Wareen and Young, 1997; Roberts, 1970; Campbell, 1968; Otto et al., 1994). In the valve cusps of severe aortic stenosis, diffuse thick and degenerated structural components are present. Macroscopic calcific nodules are also present and located at the area of cusp flexion. These abnormalities lead to thicker and stiff cusps, resulting in the obstruction of left ventricular outflow (Anegelini et al., 1994; Pomerance, 1972; Wareen and Young, 1997; Roberts, 1970; Campbell, 1968).

In adult patients, the main feature of severe AS, including both bicuspid aortic (BAV) and tricuspid aortic valves (TAV), is the presence of extensive calcification (Davies et al., 1996; Subramanian et al., 1984; Peterson et al., 1985; Dare et al., 1993). In BAV, calcification is nodular and is located at the raphe and the base of valve pockets. However, in TAV, calcification is diffuse rather than nodular, and is localized in the valve pockets and the commissures (Lindroos et al., 1993; Nistal et al., 1994; Beppu et al., 1993).

1.2.2 Epidemiology of aortic valve stenosis in the elderly

Aortic valve stenosis (AS) is now the most common valvular disease in the western world (Davies et al., 1996; Subramanian et al., 1984; Peterson et al., 1985). It is associated with significant mortality and morbidity. The occurrence of this disease increases with age so that about 4% of the elderly over 75 years old have severe AS. It is the most common reason for aortic valve replacement (Davies et al., 1996; Subramanian et al., 1984; Peterson et al., 1985; Lindroos et al., 1993). Mild to moderate AS produces no symptoms, but it often has other pathological association such as increased coronary risks (Davies et al., 1991; Otto et al., 1995). However, severe AS is associated with very significant morbidity and mortality, and often requires aortic valve replacement (Peterson et al., 1985; Lindroos et al., 1993).

Before 1970 most cases of AS were thought to be caused by rheumatic involvement, but this is now a rare cause of aortic valve stenosis. Rheumatic involvement of aortic valve is almost always accompanied by rheumatic mitral involvement (Carabello et al., 1997). Over the past 30 years, dramatic changes of AS have occurred. In developed countries, the number of patients with rheumatic AS has decreased significantly, and the main types of this disease are now calcific bicuspid and tricuspid AS. For the patients under 60 years old, congenital bicuspid aortic valve is the most common etiology of AS, on the other hand, for patients over 60 years old, degenerative tricuspid valve is more common (Davies et al., 1996; Subramanian et al., 1984; Peterson et al., 1985).

1.2.3 Treatment

Medical treatment for patients with mild to moderate or asymptomatic aortic stenosis includes regular follow-up with prophylaxis against infective endocarditis. The progression rate of aortic stenosis is relatively unpredictable but the average decrease rate of valve area was estimated to be approximately 0.12 cm^2 per year, although half of patients showed no progression over 3-9 years (Bonow et al., 1998).

Symptomatic aortic stenosis is usually considered a surgical condition and has become the most common reason for valve replacement in the developed world (Davies et al., 1996, Subramanian et al., 1984; Peterson et al., 1985; Lindroos et al.,

1993). The effect of aortic valve replacement on natural history of severe aortic stenosis is significant. Postoperative survival after valve replacement for aortic stenosis exceeds 75% at 15 years in most patients with severe AS, but very elderly patients are not included. The left ventricular ejection fraction always increases after aortic valve replacement (Peterson et al., 1985).

To date, no medical therapy has been shown to alter the natural history of calcific aortic stenosis. However, researchers have demonstrated some similarities between coronary heart disease and calcific aortic stenosis (Deustscher et al., 1984; Aronow et al., 1987; Mohler et al., 1991; Mautner et al., 1993; Lindroos et al., 1994; Gotoh et al., 1995; Stewart et al., 1997; Wilmshurst et al., 1997; Otto et al., 1999; Novaro et al., 2001). HMG-coenzyme A reductase inhibitors (statins) have been demonstrated to slow the progression of atherosclerotic coronary artery disease (Steinberg et al., 2004). As a result, statins might slow the progression of calcific aortic stenosis in theory. In an observational study, Novaro et al (2001) compared the rate of progression of aortic stenosis in patients who received statins and those who did not receive statins. In this study, all these patients with mild to moderate aortic stenosis, statins use was a significant independent factor of a lesser increase in peak gradient and of a smaller decrease in valve area (Novaro et al., 2001). In addition, three retrospective studies have produced similar results (Aronow et al., 2001; Pohle et al., 2001; Bellamy et al., 2002). However, the recently published prospective SALTIRE study showed that high dose statins use showed no benefit on decreasing rate of disease progression (Cowell et al., 2005).

1.2.4 Current and future impacts

To date, no pharmacological therapies have been demonstrated to improve outcomes in symptomatic patients. Given the apparent similarities between aortic valve stenosis and atherosclerosis, future studies on therapy for calcific aortic valve disease now include pharmacotherapies traditionally reserved for atherosclerosis, which may slow disease progression. However, studies supporting similarities between calcific aortic valve disease and atherosclerosis have produced circumstantial evidence without clear evidences of definite pathways. In addition, because many recent studies have focused on clarifying the similarities between calcific aortic valve disease and atherosclerosis,

no studies explaining the observed discrepancies have been demonstrated. Until definite pathways are identified and proven pharmacological interventions are demonstrated, the treatment of calcific aortic valve disease should be guided by regular clinical follow-up and surgical valve replacement. In patients with symptomatic aortic stenosis who were not candidates for aortic valve replacement, pharmacological therapy was tailored to adjunctive treatments for congestive heart failure, cardiac arrhythmias, and hypertension (Khot et al., 2003; Chocklingam et al., 2004).

1.3 Risk factors for aortic stenosis

Historically, calcific aortic stenosis, especially in structurally tricuspid valves, was only thought to be associated with aging and "wear and tear" of the aortic valve (Pomerance, 1967; Pomerance, 1972).

Over the past decades, a growing understanding of the risk factors for calcific aortic stenosis have led to new insights into how it develops. Researchers have found histologic similarities between the lesion of aortic stenosis and atheromatous coronary artery disease (Otto et al., 1994; O'Brien et al., 1996). In addition, researchers have established an association between traditional atherosclerotic and other risk factors and the development of calcific aortic valve disease (Deustscher et al., 1984; Aronow et al., 1987; Mohler et al., 1991; Mautner et al., 1993; Lindroos et al., 1994; Gotoh et al., 1995; Stewart et al., 1997; Wilmshurst et al., 1997; Otto et al., 1999; Novaro et al., 2001).

1.3.1 Risk factors for both aortic stenosis and atherosclerosis

Recent epidemiological studies have suggested some clinical evidence that pathogenetic mechanisms of atherogenesis and aortic stenosis may overlap.

A summary of reported studies is provided in table 1.3-1

Author	Study Design	n	Mean Age (yrs)	Positive Risk Factors
Deutscher et al. (1983) (41)	Case control	54 cases	NA	Hypercholesterolemia, diabetes
		359 controls	NA	
Hoagland et al. (1985) (42)	Case control	105 cases (41 BAV)	66	None
		439 controls		
Aronow et al. (1987) (43)*	Hospital survey	571	82	Hypertension, hypercholesterolemia, diabetes, low HDL cholesterol
Mohler et al. (1991) (44)	Retrospective	39 BAV	62	Race, gender, low triglyceride, smoking
		30 degenerative	72	
Lindroos et al. (1994) (45)*	Prospective	501	> 75	Hypertension, low BMI
Boon et al. (1997) (46)	Retrospective echo database	515 cases	67	Age, hypertension, hypercholesterolemia
		562 controls		
Stewart et al. (1997) (47)*	Prospective	5,201	73	Gender, age, hypertension, lipoprotein (a), LDL cholesterol
Wilmshurst et al. (1997) (48)	Prospective case control	20 cases (6 BAV)	66	Cholesterol
		20 controls		
Chan et al. (2001) (49)	Prospective case control	48 cases (all BAV)	56	Cholesterol, history of hypertension
		52 controls (all BAV)		
Aronow et al. (2001) (50)	Retrospective	180 cases	82	Gender, smoking, hypertension, diabetes, cholesterol, not on statin
Chui et al. (2001) (51)	Retrospective case control	43 cases (18 BAV)	66	Cholesterol
		40 controls		
Peltier et al. (2003) (52)	Prospective case control	220 cases	68	Hypertension, smoking, high BMI, cholesterol
		220 controls		

Table 1.3-1. Clinical trials for risk factors associated with aortic valve stenosis, (Adapted from Chan KL, Is aortic valve stenosis a preventable disease? J Am Coll Cardiol 42(4): 593-9, 2003.)

The largest prospective study in this regard was Cardiovascular Health Study (Stewart et al., 1997). This population-based study of the elderly has demonstrated positive risk factors of aortic stenosis including age, male gender, history of hypertension, higher levels of lipoprotein (a) and LDL cholesterol. The two early case-control studies showed contradictory results (Destscher et al., 1983; Hoagland et al., 1985). In both studies, the subjects were largely drawn from patients referred for cardiac catherization, most of the subjects had a high prevalence of atherosclerotic risk factors. The studies by Aronow et al (Aronow et al., 1987; Aronow et al., 2001) examined patients over 80 years old, and the prospective studies by Lindroos et al (1994) studied patients more than 75 years of age. They both demonstrated that traditional atherosclerotic risk factors, such as hypertension and hypercholesterolemia, have been shown to be associated with progression of aortic stenosis. In a retrospective study, Mohler et al (1991) compared risk factors for aortic stenosis in bicuspid versus tricuspid aortic valves in surgical patients who had had aortic valve replacement. Race, male gender, and higher triglyceride levels were greatly related to bicuspid aortic stenosis, while male gender and regular smoking were major risk factors for degenerative tricuspid aortic stenosis. As a result, there is an interaction between atherogenic factors and probability of development of aortic stenosis irrespective of underlying aortic valve structure.

1.3.2 Risk factors associated especially with aortic stenosis

Although many studies have showed that atherosclerotic risk factors, particularly hypercholesterolemia, were associated with calcific aortic stenosis, only about half of the patients with aortic stenosis had coronary artery disease, and a minority of patients with coronary disease had aortic stenosis (Otto and O'Brien, 2001). This study suggests that while there are some common risk factors for both coronary artery disease and aortic valve stenosis, there are some additional risk factors.

1.3.2.1 Renal dysfunction

Renal failure has long been known to have an increased risk of heart disease and cardiac death (London and Parfrey, 1997). A number of studies have shown that aortic valve stenosis occurs with increased frequency in patients with renal failure and uremia as compared to the general population (Maher et al., 1981; Maher et al., 1987). In addition, rapid progression of aortic valve stenosis in patients on renal dialysis has

also been demonstrated in several studies (Schonenberger et al., 2004; Perkovic et al., 2003).

1.3.2.2 Metabolic bone disease

Metabolic bone diseases, such as Paget's disease and secondary hyperparathyroidism, which have high rates of bone remodelling and abnormal serum calcium level, especially hypercalcemia, have been reported to be associated with aortic valve stenosis (Strickberger et al., 1987; Stefenelli et al., 1993). To date, no mechanism(s) have been postulated for the putative association between aortic stenosis and either renal failure or metabolic bone diseases.

1.3.2.3 Genetic aspects associated with aortic valve stenosis

Recently, researchers have identified potential genetic markers for the development of calcific aortic stenosis. In a case-control study, patients with aortic stenosis had higher incidence of B allele of the gene encoding the vitamin D receptor than of b allele. This polymorphism suggested that the B allele might make patients more susceptible to progression of aortic valve calcification (Ortlepp et al., 2001). In addition, other genetic polymorphisms of interleukin-10, connective tissue growth factor, and chemokine receptor-5 may affect the degree of valvular calcification (Ortlepp et al., 2004). Other studies of apolipoprotein polymorphisms have also provided a possible genetic component related to valvular calcification and stenosis (Avakian et al., 2001; Novaro et al., 2003). In a word, these findings may suggest that genetic polymorphisms may be related to pathogenesis of aortic valve stenosis.

1.3.3 Impact of risk factors on progression rates of aortic stenosis

Aortic valve stenosis is a variably progressive disease. Once mild stenosis is present, the valve area decreases by an average of 0.1 cm^2 per year in this condition, and the transvalvular pressure gradient increases by 6 to 8 mm Hg per year (Otto et al., 1989; Brener et al., 1995; Otto et al., 1997). Nevertheless, disease progression rates in individual patients vary widely.

According to all the studies that have been performed, several factors associated with rapid progression of aortic valve stenosis have been demonstrated, including age (Peter et al., 1993; Nassimiha et al., 2001; Novaro et al., 2001), male sex (Aronow et al., 2001), hypertension (Aronow et al., 2001), diabetes mellitus (Aronow et al., 2001), dyslipidemia (Mohler et al., 1991; Aronow et al., 2001; Palta et al., 2000; Nassimiha et al., 2001), regular smoking (Mohler et al., 1991; Aronow et al., 2001; Palta et al., 2000; Nassimiha et al., 2001; Ngo et al., 2001), elevated serum calcium (Palta et al., 2000), and elevated serum creatinine (Palta et al., 2000).

1.4 Histological changes associated with aortic "sclerosis"/"stenosis"

1.4.1 Early changes

The conventional view was that AS was a "degenerative" process, with the valve damage being correlated with "wear and tear" (Davies et al., 1996; Thubrikar et al., 1986). Hydrodynamic studies have shown that there is flow disturbance at the aortic side of the aortic cusps. A high mechanical stress occurred at the flexion area of the aortic cusps near the attachment to the aorta root and the line of coaptation (Thubrikar et al., 1986). Aortic valve endothelium at areas of high mechanical stress demonstrated subtle changes consistent with mild damage and became more susceptible to lipid deposition and infiltration by macrophages. It has been postulated that this process was accelerated in bicuspid aortic valve because the abnormal cusps and raphe are more prone to greater mechanical stress (Davies et al., 1996).

In the early stage of the disease, macroscopically, sclerotic aortic valve has areas of irregular fibrous thickening and calcification on the aortic side. Microscopically, all diseased aortic valve cusps have disruption of the endothelium on the aortic side, which is similar to what occurs in coronary artery disease (Ross, 1999). In addition, calcified lesions of aortic valves and coronary arteries both contain lipid and inflammatory cells. Once the endothelium is disrupted, possibly by mechanical stress and other factors, the disease progresses to different stages of aortic sclerosis and stenosis. However, regardless of any stage, aortic valve lesions demonstrate an overlying disrupted basement membrane with subendothelial accumulation of

intracellular and extracellular lipids and lipoproteins (Otto et al., 1994; O'Brien et al., 1996). A chronic inflammatory infiltrate is also present in the lesions of aortic valve, which is made up of foam cell, non-foam cells, macrophages and T lymphocytes (Otto et al., 1994). Additionally, neoangiogenesis has been identified in calcific aortic valve tissue (Strickberger et al., 1987). All these changes can be found in the lesions of atherosclerotic coronary arteries, which suggested that the early aortic valve lesion is an inflammatory process accelerated at least in part by atherosclerotic risk factors.

1.4.2 Process of calcification/ossification

Active calcification is prominent early in the disease process and is a major factor in the leaflet stiffness of severe stenosis. With aortic sclerosis, microscopic areas of calcification co-localize in areas of lipoprotein accumulation and inflammatory cell infiltration. Oxidized low density lipoprotein (LDL) stimulates valvular fibroblasts to release matrix vesicles, a nidus for early calcification (Mohler et al., 1999). Not surprisingly, it has been shown that macrophages express osteopontin, a protein needed in bone formation, with the degree of mRNA expression of osteopontin related to the degree and location of valvular calcification (O'Brien et al., 1995; Mohler et al., 1997). In addition, a subset of valvular myofibroblasts is an osteoblast phenotype and has been associated with development of calcific nodule formation (Mohler et al., 2001; Rajamannan et al., 2003). An increased rate of calcific nodule formation by these myofibroblasts has been induced in vitro by exposure to oxidized lipids and transforming growth factor-β1 (Mohler et al., 1999).

As the disease progresses, active bone formation is demonstrated. In an evaluation of some human aortic valves removed for aortic valve replacement, most valves had evidence of dystrophic calcification, and a few aortic valves contained lamellar or endochondral bone tissue with hematopoietic marrow and evidences of bone remodeling (Mohler et al., 2001). Within the specimens that contained bone tissue, bone morphogenic protein-2 and –4 that promote osteogenesis has been present (Mohler et al., 2001; Kaden et al., 2004). Furthermore, these findings are constitent with the concept that the majority of determinants of calcification/ossification may be relevant to the pathogenesis of aortic stenosis.

1.5 The valvular endothelium as a modulator of AS development

1.5.1 Physiology of vascular endothelium

The endothelium is a continuous layer of cells that separates blood from the vessel wall; it manifests a variety of phenotypes that vary between different vascular beds. The endothelium controls many functions, including maintenance of blood circulation and fluidity as well as regulation of vascular tone, coagulation, and inflammatory response (Beherndt and Ganz, 2002; Cannon, 1998; Furchgott and Zawadzki, 1980). The vascular endothelium responds to flow and shear forces via a pathway that leads to phosphorylation of endothelial nitric oxide synthase (eNOS), which produces nitric oxide (NO) and leads to vasodilation (Scotland et al., 2002; Dimmeler et al., 1999). This response allows conduit arteries to accommodate increases in flow and controls change in shear (Brouet et al., 2001). Regulation of eNOS occurs through attachment to a protein (e.g. caveolin) and other phosphorylation reactions (Fontana et al., 2002; Harrison et al., 1997). In addition, the endothelium limits local thrombosis by producing tissue plasminogen activator, maintaining a negatively charged surface, and secreting heparans and thrombomodulin (Beherndt and Ganz, 2002). Interactions between endothelium and platelets are critical to inhibition of platelet aggregation and thrombus formation (Salvimini et al., 1993).

1.5.2 Normal aortic valve endothelium (potential physiology)

The aortic valve of the adult hearts is a highly specialized trileaflet structure that derives from the endocardial cushions of the fetal heart (Eisenberg and Markwald, 1995). In normal condition, aortic valve consists of three principal layers: the ventricularis at the inflow surface, the spongiosa in the centre, and the fibrosa at the outflow surface. The extracellular matrix components of these layers have been well characterized: the ventricularis contains collagen and is rich in elastin, the spongiosa is primarily composed of glycosaminoglycans with some collagen, and the fibrosa contains densely packed collagen fibers aligned in parallel with the free edge of valve cusp (Schoen and Levy, 1999).

Aortic valve leaflets are composed of an outer layer of endothelial cells (ECs) that cover interstitial mesenchymal cells located throughout the leaflet. Both ECs and

interstitial cells of aortic valve have been isolated and cultured in vitro. ECs from human cardiac aortic valve appear spindle-shaped in culture, but like all ECs, they express inducible intercellular adhesion molecule-1 and E-selectin (Simon et al., 1993).

It might be expected that the valvular endothelium, like the vascular endothelium, will play an important physiological role. In support of this, it has been demonstrated that normal aortic valve endothelium can release nitric oxide (NO) and prostacyclin (PGI_2) (Pompilio et al., 1998). However the role of these autacoids in valvular physiology is relatively unknown, compared to their well-defined role in the vasculature. Endothelium-derived nitric oxide (NO) is the most potent endogenous vasodilator. NO exerts its effect largely via stimulation of soluble guanylate cyclase to produce cyclic GMP (Furchgott and Zawadzki, 1980; Ignarro et al., 1984; Murad, 1996). Downstream effects of cGMP involve activation of a family of enzymes, protein kinases G (PKG) (Pfeifer et al, 1998), and subsequent reduction in intracellular calcium. The mechanism of this effect is incompletely understood but involves reduction in formation of inositol 1,4,5-trisphosphate (IP_3) (Ruth et al., 1993), a signalling molecule responsible for the release of intracellular calcium in response to vasoconstrictor agents. In addition release of calcium from the sarcoplasmic reticulum by IP_3 is inhibited by phosphorylation of IP_3 receptor associated cGMP kinase substrate (IRAG) (Schlossmann et al, 2000). Moreover uptake of calcium by sarcoplasmic reticulum is stimulated by phosphorylation of phospholamban thereby activating sarcoplasmic reticulum calcium ATPase (SERCA) (Cornwell et al, 1991). In addition PKG may lead to disruption of smooth muscle cross bridge formation by phosphorylation of thin filament binding proteins such as vasodilatory-stimulated phosphoprotein (VASP) (Reinhard et al., 1995). Accordingly NO is a critical modulator of blood flow and blood pressure (Johnstone et al., 1993). It is released by the endothelium in response to shear stress and plays an important role in flow-mediated vasodilation. Endothelial release of NO physiologically opposes the vasoconstrictor effects of norepinephrine, endothelin, angiotensin II, and serotonin (Cooke and Dzau, 1991; Vallance et al., 1997). Prostacyclin (PGI_2) is also a potential endogenous vasodilator, its production is via cyclooxygenase and prostacyclin synthase. As a result, endothelium-dependent relaxations can be explained by endothelial release of NO and PGI_2 (Furchgott and Vanhoutte, 1989).

On the other hand, it has been demonstrated that endothelial cells can produce potent vasocontrictor peptides, which were termed "endothelin" (ET) (Yanagisawa et al., 1988). In addition, researchers have also shown that stimulation of nitric oxide production inhibits the expression and production of endothelin (Boulanger and lü scher, 1990; Lüscher et al., 1992). This effect is not only limited to NO: the release of prostacyclin inhibits the production of endothelin by activating adenylate cyclase (Nakashima and Vanhoutte, 1993).

1.5.3 Endothelial dysfunction and ADMA

ADMA (asymmetric dimethylarginine) is an endogenous inhibitor of nitric oxide synthase, which is derived from the catabolism of proteins containing methylated arginine residues. These proteins are mostly found in the nucleus and may be involved in RNA processing and transcriptional control (Najbauer et al., 1993). There are 2 types of enzymes that methylate arginine residues. These enzymes are protein arginine methyltransferase types I and II (PRMT I and PRMT II) (Ghosh et al., 1988; Tang et al., 2000). PRMT type I forms ADMA and NMA, whereas PRMT type II forms symmetric dimethylarginine (SDMA) and NMA. ADMA inhibits endogenous NOS, SDMA does not inhibit NOS. There are a number of type I PRMTs, which are special for different proteins (Najbauer et al., 1993). By contrast, myelin basic protein is the only known substrate for type II PRMT. When these proteins undergo hydrolysis, their methylated arginine residues are released. Methylated arginines are excreted in the urine (Kakimoto and Akazawa, 1970). This phenomenon explains the increase in plasma ADMA levels in patients with renal failure. Methylated arginines may also be metabolized. However, the major metabolic pathway for NMA and ADMA is the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (Ogawa et al., 1987). Two isoforms of DDAH are known, I and II. Either or both isoforms have been found in every cell type examined. DDAH I is typically found in tissues expressing neuronal NOS, while DDAH II dominates in tissues containing the endothelial isoform of NOS (Leiper et al., 1999). DDAH is a labile enzyme, which may be partially inactivated by oxidative stress (Leiper et al., 1999).

In the past, Vallance et al (1997) firstly demonstrated that endogenous ADMA

antagonized endothelium-dependent vasodilation. Significant elevation of plasma ADMA was found in patients with renal failure. In addition, plasma from patients with renal failure induced vasoconstriction of vascular rings in vitro. Administration of L-arginine to patients with renal failure also restored endothelial function (Hand et al., 1998). In addition, plasma ADMA concentrations have been found to be elevated in patients with atherosclerosis, as well as in the setting of risk factors for cardiovascular diseases (Boger et al., 1997; Boger et al., 1998). Hypercholesterolemic animals and humans manifest impaired endothelium-dependent vasodilation. In these individuals, plasma ADMA levels are better correlated with endothelial dysfunction than are LDL cholesterol levels. Furthermore, the endothelial vasodilator dysfunction associated with elevated plasma ADMA level is reversed by L-arginine administration, consistent with the notion that ADMA is a competitive inhibitor (Piatti et al., 2001). It has been well known that aortic valve stenosis has a lot of similarities with atherosclerosis, and progression of atherosclerosis is closely associated with endothelial dysfunction. Recently, researchers have demonstrated that aortic sclerosis, the early stage of aortic valve stenosis, is associated with endothelial dysfunction (Poggianti et al., 2003). These findings raise the possibility that the plasma ADMA in patients with aortic sclerosis/stenosis may be elevated.

1.5.4 Potential for interaction between endothelium and interstitial cells in the aortic valve

Aortic valve leaflets are composed of an outer layer of endothelial cells (ECs) that cover interstitial mesenchymal cells located throughout the leaflet. Both ECs and interstitial cells of aortic valve have been isolated and cultured in vitro. ECs from human cardiac aortic valve appear spindle-shaped in culture, but like all ECs, they express inducible intercellular adhesion molecule-1 and E-selectin (Simon et al., 1993). The interstitial cells, although not well recognized, have been reported to share some characteristics with both fibroblasts and smooth muscle cells (Filip et al., 1986). Endothelial-derived relaxing factors, prostacyclin and NO have been shown to inhibit platelet aggregation in healthy vessels (Grylewski et al., 1988). Healthy endothelium has been demonstrated to continually release these antiaggregants intraluminally, but the release is particularly increased in the vicinity of aggregating platelets in response to plasma thrombin and other aggregation stimulation factors (Vane et al., 1990). This effect is considered to limit the growth of platelet plug of the area of vessel damage.

Interactions between endothelium and platelets are critical to the occurrence on inhibition of platelet aggregation and thrombus formation (Salvimini et al., 1993). In addition, aortic valve endothelium is different from vascular endothelium. Hydrodynamic studies have shown that there is flow disturbance at the aortic side of the aortic cusps. A high mechanical stress occurs at the flexion area of the aortic cusps near the attachment to the aorta root and the line of coaptation (Thubrikar et al., 1986). AS, irrespective of the presence and/or absence of coronary artery disease, is associated with platelet hyperaggregability and hyporesponsiveness to antiaggregatory effects of NO donor. Treatment with the prophylactic antianginal agent perhexiline improves platelet responsiveness to NO (Chirkov et al., 2002).

Furthermore, transforming growth factor-β1 (TGF-β1) has been shown to induce transdifferentiation of adult valve endothelial cells (Paranya et al., 2001). The transdifferentiated endothelial cells upregulate matrix metalloproteinase expression, and this subsequently enable cell migration into the valve interstitium (Kaden et al., 2004). Additionally, it has been shown that prolonged exposure of aortic valve interstitial cells to TGF-β1 leads to calcific nodule formation and apoptosis (Jian et al., 2003). However, it is still unclear whether the interstitial cells have interactions with endothelial cells. Abluminal interactions of endothelial layers have been studied most extensively in the case of the endocardial endothelium. Endothelin released from endothelium has been demonstrated to affect myocardial contractibility (Mohan et al., 1995). Therefore, there is clear evidence that endothelial cells release paracrine hormones such as NO, ET-1 and PGI_2 which can potentially affect the function of adjacent tissue. However direct evidence for an interaction between endothelium derived agents and valvular interstitial cells is lacking. There is some circumstantial evidence that NO may play a role in AS. Firstly, there is evidence that AS is associated with endothelial dysfunction (Poggianti et al., 2003) . Secondly, bicuspid aortic valve which is accompanied by premature calcification is a characteristic of eNOS deficiency in knock-out mice (Lee et al., 2000). Thirdly, during the couse of the studies carried out in this thesis, Rajamannan et al. (2005) have reported that atorvastatin (statins) inhibits calcification and enhances nitric oxide synthase production in the aortic valve in the hypercholesterolaemic rabbit model. In this

model, serum cholesterol and aortic valve calcification were greatly increased in the cholesterol fed compared with control rabbits . Atorvastatin inhibited calcification in the aortic valve of rabbits fed with cholesterol. eNOS protein concentrations were unchanged in the control and cholesterol groups. On the other hand, they were increased in the atorvastatin treated group. Serum nitrite concentrations were also decreased in the hypercholesterolaemic animals and increased in the cholesterol-fed group treated with atorvastatin (Rajamannan et al., 2005). Thus the protective effect of atorvastatin in this model could be due to the associated elevation in NO. However, a direct effect of NO on the aortic valve calcification has never been tested.

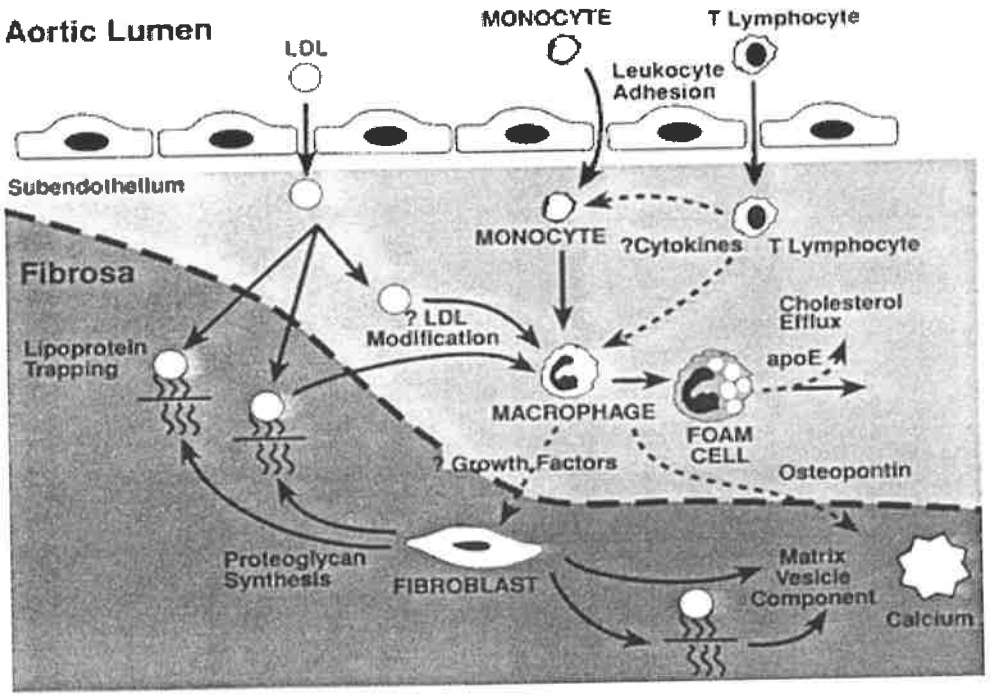


Figure 1.6-1. Schematic diagram of some possible pathways of pathogenesis of aortic valve stenosis. (Adapted from O'Brien KD et al: Apolipoproteins B, (a), and E Accumulate in the Morphologically Early Lesion of 'Degenerative' Valvular Aortic Stenosis. Arterioscler Thromb Vasc Biol 16:523-32, 1996.)

Figure 1.6-1 summarizes possible pathways of pathogenesis of aortic valve stenosis.

1.6 Hypotheses about pathogenesis of aortic valve stenosis

1.6.1 Role of mechanical stress

Hemodynamic studies have demonstrated that there is flow disturbance and near stagnation at the aortic side of aortic valve cusps in the patients with aortic valve stenosis. Higher mechanical stress occurs at the flexion area and the line of coaptation. As a result, aortic endothelium at the area of higher mechanical stress demonstrates subtle changes consistent with mild damage and becomes more prone to lipid deposition and infiltration by macrophages and T-lymphocytes.

This process has been postulated as the initiating event for aortic valve calcification and fibrosis, as also seems to happen in the development of atherosclerosis in coronary arterial walls (Thubrikar et al., 1985). Because of the abnormal mechanical stress, lipid deposition tends to be localized to the aortic surface of flexion areas, which are under higher mechanical stress and lower shear stress. The abnormal mechanical forces may be more evident in bicuspid aortic valve due to abnormal cusps and raphe, which will result in a greater degree of lipid deposition in earlier stages (O'Brien et al., 1996). In addition, some studies have examined the mechanical stress in the aortic bioprosthetic valves. In these in vitro models, it is postulated that mechanical stresses initiate calcification by damaging the structural integrity of the valve leaflet. Therefore, calcification of bioprostheses can theoretically be inhibited by reducing functional stresses through the modification of design and tissue properties to duplicate those of the natural aortic valve (Thubrikar et al., 1983).

1.6.2 Role of lipids

The study by O'Brien et al (1996) provides evidence that lipoprotein deposition is a key component of the pathogenesis of aortic valve stenosis (O'Brien et al., 1996). Possibly due to the abnormal mechanical stress, lipid deposition is localized to the sclerotic/stenotic aortic valve, which is similar to what tends to happen in the process of atherosclerosis. In addition, oxidized low density lipoprotein (LDL) has been found in nonrheumatic stenotic aortic valves (Olsson et al., 1999). This study has suggested that the process following lipid deposition is oxidized modification of these

lipoproteins, and after more extensive modification the oxidized particles are taken up by macrophages to form foam cells and non-foam cells (Otto et al., 1994; Olsson et al., 1999). Oxidized LDL has been shown to have prothrombotic effects, as well as to be toxic to other types of cells, including endothelial cells and aortic valve myofibroblasts. It has also been suggested that aortic valve fibroblasts, due to exposure to oxidized LDL, can release matrix vesciles and other cytoxic factors to form calcific nodule (Olsson et al., 1994; O'Brien et al., 1995; Mohler et al., 1997). In addition, statin treatment has been demonstrated to be an effective medical treatment in decreaseing serum cholesterol. In recent years, Rajamannan et al have demonstrated high cholesterol diet would accelerate aortic valve calcification in an animal model, which could be inhibited by statins treatment (Rajamannan et al., 2001; Rajamannan et al., 2002; Rajamanan et al., 2003; Rajamannan et al., 2004; Rajamannan et al., 2005). These studies have suggested that hypercholesterolemia may be involved in the pathogenesis of aortic valve stenosis. These findings in the early lesions of calcific aortic valve are therefore closely similar to the process in the early lesions of atherosclerosis (Otto et al., 1994).

On the other hand, Drolet et al (2003) have found that vitamin D_2 may play a significant role in the development of AS in rabbits fed with high cholesterol diet, whereas hypercholesterolaemia per se in their study did not induce calcification.. Furthermore a recent controlled trial of high dose atorvastatin treatment failed to demonstrate any benefit of statin therapy on progression of aortic valve stenosis (SALTIRE investigators, 2005).

1.6.3 Role of inflammation

1.6.3.1 Oxidative stress and myofibroblasts

As stated above, it has been suggested that aortic valve myofibroblasts, upon exposure to oxidized LDL, release matrix vesicles that form the nodule (Olsson et al., 1994; O'Brien et al., 1995; Mohler et al., 1997). In addition, myofibroblasts in stenotic valves appear to be different from myofibroblasts in normal valves in that they secrete alpha-actin and desmin in addition to vimentin (Olsson et al., 1994). The majority of these cells also express histocompatibility antigens (HLA-DR molecules) usually associated with immunocompetent cells (O'Brien et al., 1995). In addition, myofibroblasts may be capable of releasing cytokines, including osteopontin, and possibly play an important role in the development of fibrosis and calcification, which

are found commonly in AS valves. Furthermore, endochondral bone formation may occur (Olsson et al., 1994).

1.6.3.2 Oxidative stress and macrophage infiltration

Inflammation is a prominent early feature of aortic valve calcification. T lymphocytes and macrophages have been identified in the calcified valves, even in the early stage of aortic valve stenosis (Otto et al., 1994; O'Brien et al., 1996). They co-localize near the calcified nodules. T lymphocytes are present in the subendothelial and fibrosa layer of stenotic aortic valves (Olsson et al., 1994; Otto et al., 1994). After they are activated, they can release several cytokines, including transforming growth factor (TGF)-β1, which is a potent activator of extracellular matrix formation. Immunohistochemical studies of human calcific aortic valves have found higher levels of TGF-β1 in calcified cusps than noncalcified cusps (Jian et al., 2003). Adhesion molecules, such as vascular cell adhesion molecule 1, which is not normally expressed in normal aortic valve endothelium, are found in diseased aortic valves. Monocytes have been shown to adhere to these molecules, migrate into the subendothelial space of the valve due to the response to chemoattractant molecules and differentiate into macrophages (Olsson et al., 1994).

1.6.4 Calcifying valve cells

Recent studies have indicated that cells that reside in the aortic valve may undergo trans-differentiation and participate in the calcific process. The origin of these cells remains unclear, but they are likely to be a population of interstitial cells. Vascular smooth muscle cells are important in fibrosis and calcification of atherosclerotic plaques in coronary arteries. The aortic valve contain cells in interstitial layer, called myofibroblasts, which are similar to vascular smooth muscle cells in nature, they presumably secrete collagen and other extracellular matrix proteins to maintain the interstitial intergrity of the valve leaflet (Filip et al., 1986). These cells undergo a phenotypic transformation to become osteoblast-like cells that spontaneously form calcific nodules (Mohler et al., 1999; Parhami et al., 1997). Their activities are modulated by hydroxycholesterol and some growth factors including TGF-β1 (Mohler et al., 1999; Parhami et al., 1997; Watson et al., 1994).

Fibroblasts in aortic valve stenosis are different from normal vascular fibroblasts in the vessel because they secrete alpha-actin, desmin and vimentin (Olsson et al., 1994). Under stimulation, the valvular interstitial cells produce proteins instrumental to valve calcification. These factors include osteopontin, osteonectin, and alkaline phosphatase (Olsson et al., 1994). Most of the valvular interstitial cells also express histocompatibility antigens usually correlated with immunocompetent cells (O'Brien et al., 1995).

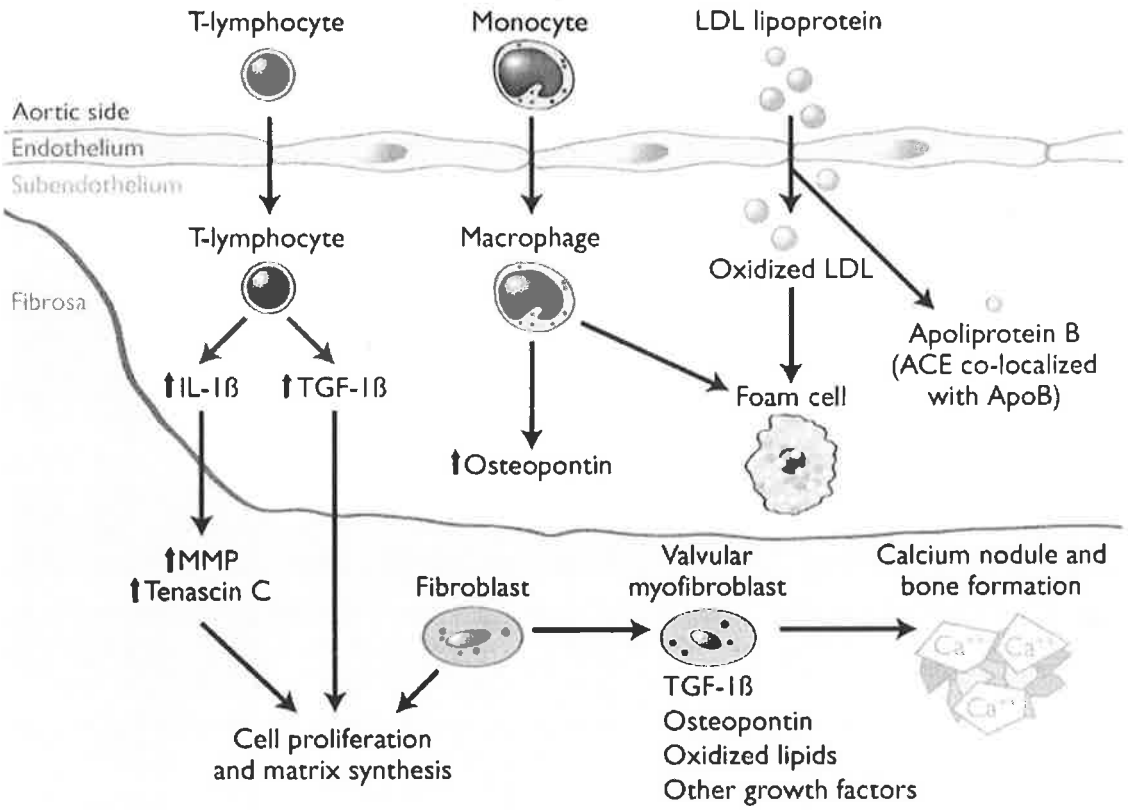


Figure 1.7-1. Schematic diagram for potential molecular factors involved in calcific aortic valve stenosis. T lymphocytes and macrophages infiltrate endothelium and release cytokines that act on valvular fibroblasts to promote cellular proliferation and extracellular matrix remodelling. A subset of valvular fibroblasts within fibrosa layer differentiates into myofibroblasts that possess characteristics of smooth muscle cells. LDL that is taken into the subendothelial layer is modified oxidatively and taken up by macrophages to become foam cells. ACE is co-localized with apolipoprotein B (ApoB) and leads to generation of angiotensin II (Ang II), which acts on angiotensin I receptors (AT-1R), expressed on valvular myofibroblasts. In addition, a subset of

valvular myofibroblasts differentiates into osteoblast phenotype, promoting calcium nodule and bone formation. IL indicates interleukin; TGF-β1, transforming growth factor-beta1; and MMP, matrix metalloproteinases. (Adapted from Freeman RV and Otto CM: Spectrum of Calcific Aortic Valve Disease: Pathogenesis, Disease Progression, and Treatment Strategies. Circulation 111:3316-3326, 2005.)

1.7 Biochemical pathogenesis of aortic valve calcification

1.7.1 TGF-β1

1.7.1.1 Extent of TGF-β1

Transforming growth factor (TGF) β family was first described by DeLarco and Todardo in 1978. It was termed sacroma growth factor following its isolation from sacroma virus-transformed mouse cells. The TGFβ family has been demonstrated to be a potent regulator of extracellular matrix synthesis, cell cycle progression, apoptosis, and migration (Pasche, 2001; Blobe et al., 2000). Recently, in vitro and in vivo studies have demonstrated that TGF-β1 isoform is important in vascular development, atherogenesis, neointima proliferation, and vessel remodelling.

The TGFβ family belongs to a superfamily of over 25 diverse dimeric extracellular polypeptides of 110-140 amino acids, including bone morphogenetic proteins, activins, and inhibins (Massague et al., 1994). TGFβ1 is also expressed in endothelial and smooth muscle cells (SMC).

TGFβ-type peptides are synthesized from large precursor molecules containing an N-terminal propeptide region, termed the latency-associated peptide (LAP). The LAP undergoes intracellular peptidase cleavage at the C terminal to generate a 25 kD mature TGFβ domain. The domain noncovalently binds to the cleaved 75-80 kD latency associated protein to form a dimeric complex known as small latent complex. Inside the cell, most small latent complexes bind to the latent TGFβ binding protein family to form an inactive larger latent complex. The majority of secreted larger latent complexes bind to extracellular matrix molecules in the pericellular space, via

covalent association with the N-terminal region of the LTBP molecules, where it is stored in an inactive state (Saharinen et al., 1999; Sinha et al., 1998).

The activation of TGFβ requires strict regulation. Because newly secreted TGFβ cannot bind to their receptors when associated with LAP, a main regulator of TGFß bioavailability is the dissociation of mature TGFβ from the latent complex. Once there is need for active TGFβ, the proteolytic cleavage of LTBP releases a truncated form of the molecule. As a result, it is able to bind to its receptors and to intergrins on the cell surface, leading to a conformational change that eventually releases the active TGFβ molecule (Saharinen et al., 1999; Sinha et al., 1998; Munger at al., 1999; Annes et al., 2004).

To date, three discrete, high-affinity receptors for TGFβ, designated Ⅰ, Ⅱ, and Ⅲ have been described, although TGFβ only directly binds to the latter two. Type Ⅲ receptor is the most abundant type. Type Ⅰ and Ⅱ receptors are transmembrane molecules, and TGFβ receptor Ⅱ binds to TGFβ, either directly or through association with TGFβ receptor Ⅲ. The TGFβ/ TGFβ receptor Ⅱ structure then recruits and binds TGFβ receptor to form a heteromeric complex, which can produce an intracellular signal.

Serine-threonine protein kinases in the intracellular domains of TGFβ receptor Ⅰ and Ⅱ initiate signal transduction events, mainly by a sequential phosphorylation cascade of the Smad family of proteins (Attisano et al., 1994; Wrana et al., 1994; Ikedo et al., 2003; Goumans and Mummery, 2000; Piek et al., 1999; Zwijsen et al., 2000).

1.7.1.2 Effects of TGF-β1 on vascular cell monoculture

The dominant effects of TGF-β1 on endothelial cells are the inhibition of migration, restriction of cell cycle progression and induction of apoptosis, all of which are dose-dependent effects (Heimark et al., 1986). In three-dimensional culture, TGF-β1 at low concentrations was associated with the invasion of endothelial cells into collagen gels, but TGF-β1 became inhibitory at high concentrations (Muller et al., 1987). Secondly, similarly, TGF-β1 had a dose-dependent effect on smooth muscle cell proliferation,

being stimulatory at lower concentrations and inhibitory at higher concentrations (Majack et al., 1987; Battegay et al., 1990). In addition, it exerted a major effect upon extracellular matrix deposition, with exogenous or transfected TGF-β1 leading to increased synthesis of collagen Types Ⅰ, Ⅲ, Ⅴ, Ⅵ and Ⅷ, elastin and fibrillin-1 (Murray et al., 2003; Verrecechia and Mauviel, 2002). In contrast to endothelial cells, the addition of TGF-β1 inhibited apoptosis of smooth muscle cells (Pollman et al., 1999).

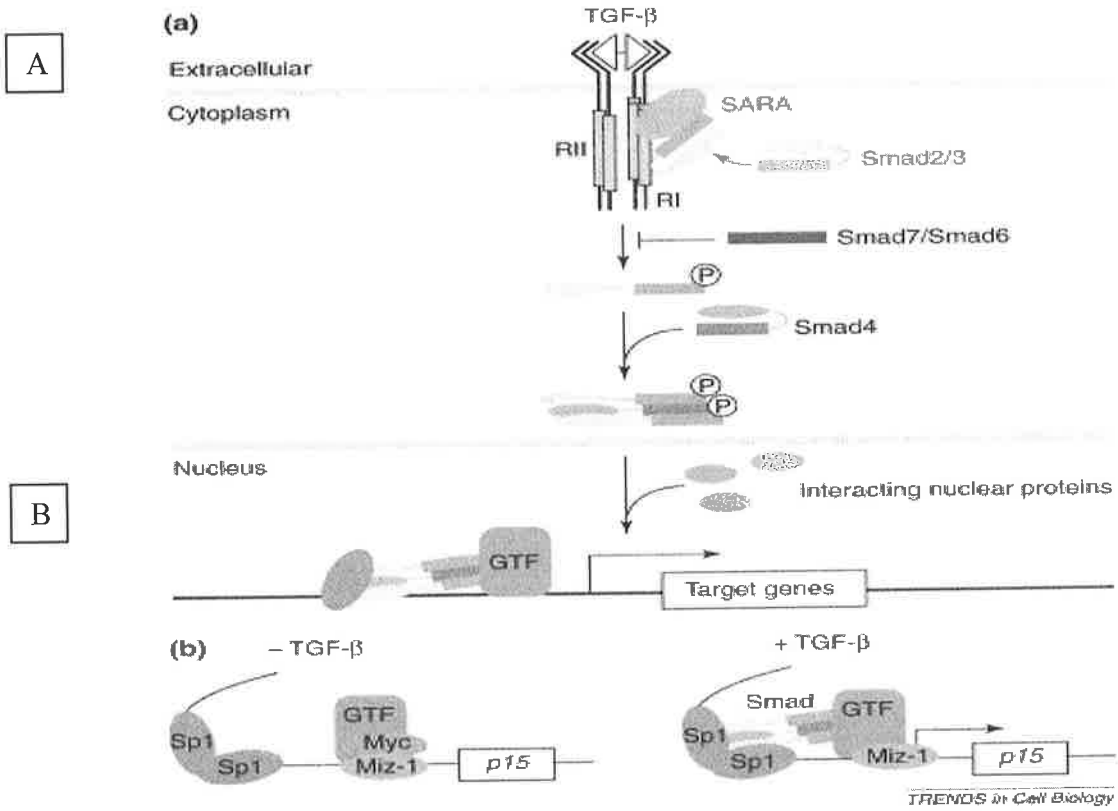


Figure 1.7-2. **The TGF-β signaling pathway (A)** All three transforming growth factor β (TGF-β) isoforms, TGF-β1, TGF-β2 and TGF-β3, bind to the same type receptor, Type II. Localization of TGF-β at the cell surface through binding with two cell-surface proteoglycans, stimulates ligand binding to the receptor complexes. Ligand binding to this receptor leads to phosphorylation and activation of Type I receptor. Subsequently, the receptor-associated Smads 2 and/or 3 are phosphorylated by Type I receptor, and released from the hetero-oligomeric receptor complex. Phosphorylated Smad2 and/or 3 bind in a heterotrimeric complex with Smad4, which subsequently accumulates in the nucleus. In addition, Smad6 and Smad7 are

inhibitory Smads that block Smad2/3 binding to Type I receptor, or Smad2/3 oligomerization with Smad4. **(B)** TGF-β-mediated induction of the cyclin-dependent kinase (CDK) inhibitor $p15^{Ink4B}$ leads to growth arrest. This process is mediated by Smad-mediated transcriptional activation and is the initiating event in the growth-inhibitory effects of TGF-β. Then, Myc dissociates from its partner Miz-1 in response to TGF- **β**, leading to DNA to activate transcription (Adapted from Akhurst RJ and Derynck R: TGF-**β** signaling in cancer – a double-edged sword. Trends in Cell Biology 111:S44-51, 2001.)

1.7.1.3 Effects of TGF-β1 on aortic valvular cell monoculture

In the past, researchers firstly created in vitro aortic valve cell culture from human and canine aortic valve interstitial cells (Mohler et al., 1999) and they found that a population of valvular interstitial cells spontaneously formed distinct calcified nodules containing hydroxyapatite within three weeks in canine and within six weeks in human aortic valve fibroblasts. The nodules contained an inner ring of dead cells surrounded by an outer ring of living cells. Cells associated with nodules had osteoblast-like characteristics and stained positively for extracellular bone matrix proteins. Incubating canine cells with potential calcifying stimuli tested the stimulus for calcification. The rate of nodule formation was increased with the addition of TGF-β1, 25-hydroxycholesterol and bone morphogenetic protein 2 compared to control over 25 days (Mohler et al., 1999).

Recently, researchers have used immunohistochemistry to quantitate TGF-β1 and its associated peptide and receptors in the cusps of sheep aortic valve. Immunohistochemistry studies revealed that calcific aortic stenosis cusps characteristically contained higher levels of TGF-β1 within the extracellular matrix than noncalcified cusps. Noncalcified normal valves demonstrated only focal intracellular TGF-β1. Addition of TGF-β1 to sheep aortic valve interstitial cell cultures led to a series of events, including cellular migration, aggregation, formation of apoptotic-alkaline phosphatase enriched nodules, and calcification of these nodules. The time course of these events in the sheep aortic valve fibroblast culture system was rapid, with nodule formation with apoptosis by 3 days, and calcification after 7 days. This study has therefore demonstrated that TGF-β1 is characteristically present within

calcific aortic stenosis cusps, and mediates the calcification of aortic valve interstitial cells in culture through mechanisms involving apoptosis (Jian et al. 2003). Taken together, TGF-β1 has been implicated in calcification process of aortic valve stenosis.

1.7.1.4 Interaction between TGF-β1 and other molecular factors

It is well known that renin-angiotensin system (RAS) and TGF-β1 play an important role in cardiac remodelling. There is some evidence to show that cardiac fibrosis and dysfunction are regulated by a network involving RAS and TGF-β1. Angiotensin II stimulates the expression of TGF-β1 in cardiac myocytes and fibroblasts. TGF-β1 induces cardiac fibroblast proliferation and myocyte hypertrophy via autocrine/paracrine pathways, thereby mediating cardiac remodelling induced by angiotensin II (Wenzel et al., 2001; Gray et al., 1998; Kupfahl et al., 2000).

In addition, a recent study has shown that TGF-β1 produces myocyte fibrosis in vivo, this effect is not only produced by the stimulation of matrix protein formation, mediated by interaction between matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinases (TIMP), but also by enhanced inhibition identified by increased TIMP levels, which suggest that TGF-β1 effect on cardiac fibrosis is associated with the MMP/TIMP system (Seeland et al., 2002).

1.7.2 Vitamin D and (VDUP)-1

Recently, Drolet et al (2003) have firstly produced an experimental animal model of acquired aortic valve stenosis in rabbits fed with a high-cholesterol diet and supplements of vitamin D_2. Control animals displayed no abnormalities of the aortic valve. Despite important increases in blood total cholesterol levels, animals fed with cholesterol-enriched diet did not develop any significant functional aortic valve abnormality over 12 weeks. However, most of the animals fed with cholesterol-enriched diet plus vitamin D_2 developed a significant decrease in aortic valve area and significant increases in transvalvular gradients. These findings suggest that vitamin D_2 may play a significant role in the development of AS at least in this species (Drolet et al., 2003).

In addition, recent studies have shown that thioredoxin (TRX) is a key regulator of cellular redox balance. Vitamin D_3–upregulated protein (VDUP)-1 is its endogenous inhibitor of TRX and therefore induces intracellular redox stress (Yamanaka et al., 2000). Recently, researchers have demonstrated an effect of VDUP-1 in human aortic smooth muscle cells. VDUP-1 had marked antiproliferative effects in smooth muscle cells through the suppression of TRX activity, suggesting that the regulation of VDUP-1 is a critical molecular switch in the transduction of pro-oxidant mitogenic signals. These data also demonstrated that activation of the TRX reductase plays a pivotal role in the redox-dependent proliferation of smooth muscle cells (Schulze et al., 2002). Both findings may suggest that Vitamin D both directly, and indirectly via VDUP-1, may be involved in aortic valve calcification.

1.7.3 Matrix metalloproteinases (MMPs)

1.7.3.1 Extent of MMPs

MMPs were firstly discovered in 1962. Since then over 66 MMPs have been cloned and sequenced (Benjamin et al., 2001).

Matrix metalloproteinases (MMPs) are a family of zinc ion-dependent endopeptidases capable of cleaving components of extracellular matrix (Falk, 1999). Most of them are synthesized and secreted as inactive proenzymes. The majority of MMPs include a propeptide domain with a unique and highly conserved cysteine-containing sequence ('cysteine switch') that is able to bind zinc in the catalytic domain, thereby making the enzyme inactive (Nagase and Woessner, 1999). Proteolytic disruption of the cysteine-zinc ion bond and removal of the propeptide domain activates the catalytic domain. Calcium ions are also required for expression of enzyme activity. The C-terminal hemopexin-like domain has been shown to play a role in substrate binding (Nagase and Woessner, 1999).

MMPs can be categorized into several groups: collagenases, gelatinases, stromelysins, matrilysins, metalloelastases, and membrane-type matrix metalloproteinases (MT-MMPs). The collagenases include MMP-1, MMP-8, MMP-13. This group is thought to initiate cleavage of triple helical collagens Ⅰ, Ⅱ, and Ⅲ. Gelatinases are

composed of the 72-kDa MMP-2 and the 92 kDa MMP-9 (sukhova et al., 1999). These enzymes are known to cleave native type Ⅳ, Ⅴ, Ⅶ, and Ⅹ collagens and elastins, as well as the products of collagens types Ⅰ, Ⅱ, and Ⅲ after proteolysis by collagenases (Hahn et al., 1999). The stromelysin group is composed of MMP-3, MMP-10 and MMP-11. Their substrates include proteoglycan core protein, laminin, fibronectin, elastin, as well as nonhelical regions of collagens (Murphy et al., 1993). In addition, these MMPs have higher affinities for other extracellular matrix molecules (Pei and Weiss, 1995). The sole member of the matrilysin group is MMP-7, which has greater activity than the other MMPs against versicans, which is a chondroitin sulfate proteoglycan that is particularly abundant in atherosclerotic plaque and it can degrade other common stromelysin substrates (Lijnen et al., 1999; Jormsjo et al., 2001). MMP-12 is a 22-kD MMP against elastin. In addition, MMP-12 is able to degrade other components of the extracellular matrix (Jormsjo et al., 2000). The MT-MMPs are similar in structure to the soluble MMPs because they contain the propeptide region with the conserved cysteine switch, the zinc-catalytic domain, and the hemopexin-like domain near their terminus (Pei and Weiss, 1996).

1.7.3.2 Regulation and activation of MMPs

MMP activity is regulated at three levels: gene transcription, posttranslational activation of zymogens, and interaction of secreted MMPs with inhibitors (Galis and Khatri, 2002). It is thought that for most MMPs (excluding MMP-2), the key step to regulation is at the level of transcription. MMP gene expression is regulated through the interaction of transcription factors, and co-activators and co-repressor proteins with *cis*-acting elements in the promoter region of MMP genes. The mechanism by which gene transcription is mediated is thought to be through a prostaglandin E_2 (PGE_2)-cAMP dependent pathway. G-protein has also associated with this pathway (Corcoran et al., 1994). Transcriptional activation can be stimulated by a lot of cytokines, hormones, and growth factors. Other factors such as heat shock protein 60, and *Chlamydia pneumoniae* may regulate MMPs (Siwik et al., 2000). However, not all MMPs react similarly to the same stimulus and the impact of various factors can be cell-specific. For example, TGF-β1 has been shown to inhibit MMP-12 expression of human peripheral blood macrophages, although in human monocytes it increases expression of MMP-2 and MMP-9. Similarily, stromal cell-derived factor has been

reported to reduce MMP-9 expression in monocytes of patients with unstable angina. However, in human megakaryocytes it increases expression and release of MMP-9 (Lane et al., 2000). In addition, MMP gene transcription can be modified by promoter region sequence variants (Price et al., 2001).

Activation of latent zymogens can occur intracellularly, at the cell surface by MT-MMPs, in the extracellular space through activation of other proteases, or even by previously activated MMPs through a process called stepwise activation (Santavicca et al., 1996). A variety of proteases, especially plasmin, are thought to be the major contributors in the initiation of extracellular stepwise activation via cysteine switch (Zucker et al., 1995). In addition, MMP activity is regulated by tissue-specific inhibitors, of which there are four known tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3, TIMP-4). The TIMPs are secreted by a variety of cell lines including smooth muscle cells and macrophages. Their activity can be increased by platelet-derived growth factor and TGF-β1 and either increased or decreased by different interleukins (Fabunmi et al., 1998).

1.7.3.3 MMPs and atherosclerosis

There is evidence that MMPs influence the process of atherosclerotic lesion formation. Firstly, MMP activity may contribute to the pathogenesis of atherosclerosis by facilitating migration of vascular smooth muscle cells through the internal elastic lamina into the intimal space, where they proliferate and contribute to plaque formation (Mtairag et al., 2001; Davies and Thomas, 1985; Ravn and Falk, 1999). Secondly, MMPs activity may decrease plaque volume by degrading extracellular matrix in the intima (Ardans et al., 2001). In addition, some studies have demonstrated that MMPs may facilitate positive remodelling of the artery wall through digestion of the external elastic lamina. As a result, it can minimize luminal encroachment of accumulating plaque (Schoenhagen et al., 2002; Pasterkamp et al., 2000).

1.7.3.4 MMPs and aortic valve stenosis

Recent studies have demonstrated a possible role of MMPs in the pathogenesis of atherosclerosis (Pauly et al., 1994; Brown et al., 1995); by analogy, this raises the possibility of a role in the pathogenesis of aortic valve stenosis.

Recently, Edep et al (2000) have found that an inflammatory infiltrate composed of macrophages and lymphocytes was present in nonrheumatic aortic stenosis, compared to normal aortic valve. The increased expression of MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), and MMP-3 (stromelysin) was demonstrated in aortic valves of patients with severe aortic stenosis, together with unique expression of MMP-9 (gelatinase B) in nonrheumatic aortic valves, implying that MMP activity may be involved in the pathogenesis of aortic valve stenosis (Edep et al., 2000). Jian et al (2001) have found that tenascin, which has been shown to be an extracellular glycoprotein found in developing bone and atherosclerotic plaque, and matrix metalloproteinase-2 (MMP-2) were coordinated and interdependent in cultured aortic valve cusps. These findings have demonstrated that MMP-2 and tenascin were correlated with calcification of aortic valve stenosis (Jian et al., 2001). In addition, MMP expression could be stimulated by various factors such as pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) or interleukin (IL)-1β (Galis et al., 1994; Galis et al., 1995). Kaden et al (2003 and 2005) have demonstrated that TNFα and IL-1β increased cell proliferation assessed by bromodeoxyuridine incorporation, and induced a time-dependent increase in the expression of MMP-1 and MMP-2 by western blotting and zymography. In addition, immunohistochemistry studies have shown greater expression of TNF-α and IL-1β in stenotic aortic valves, compared to control valves, which indicated that matrix remodelling in calcific aortic stenosis involved the expression and activation of MMPs (Kaden et al., 2003; Kaden et al., 2005).

1.7.4 RANKL (receptor activator of nuclear factor kappa B ligand)

1.7.4.1 System summary: RANKL

Recently, a new cytokine pathway of the tumor necrosis factor superfamily has been demonstrated to be involved in the regulation of bone resorption and vascular calcification. This cytokine system composes of the transmembrane protein "receptor activator of nuclear factor kappa B (RANK), its ligand (RANKL), and the soluble receptor osteoprotegerin (OPG). Animal studies have shown that the RANKL–RANK system is a major regulator of osteoclast differentiation and activation (Nakagawa et

al., 1998), and that RANKL is an important modulator of osteoclastogenesis and lymphocyte development (Kong et al., 1999). Osteoprotegerin is a soluble decoy receptor that binds to RANKL. As a result, it inhibits the interaction between RANKL and RANK. OPG is expressed at high concentrations by a variety of tissues and cell types including arterial smooth muscle cells and endothelial cells, but RANKL and RANK are not expressed in vascular tissue under physiologic conditions (Simonet et al., 1997; Min et al., 2000).

Deletion of the OPG gene leads to severe aortic calcification, and to an expression of RANKL and RANK in the calcified areas of aorta, suggesting that RANKL might promote vascular calcification and that OPG may have a protective role (Bucay et al., 1998). In human atherosclerotic lesions, expression of RANKL and OPG was associated with areas of calcification (Dhore et al., 2001). In addition, mice deficient for OPG, developed vascular calcification and showed an increased expression of RANKL in calcified areas. Although transgenic delivery of OPG from birth could prevent de novo vascular calcification, administration of OPG later in life did not reverse existing calcific lesions (Min et al., 2000). In addition, the bone matrix protein, osteopontin, is thought to inhibit vascular calcification and increase the expression of OPG in endothelial cells by binding with $_{v3}$ integrin. OPG also has been shown to inhibit arterial calcification induced by warfarin or vitamin D, suggesting a potential interdependence of the OPG cytokine system with these mechanisms of arterial calcification (Price et al., 2001). These data therefore suggest that RANKL might stimulate vascular calcification, and that OPG might tend to limit this effect.

1.7.4.2 RANKL possible relevance in aortic valve stenosis

Recently, researchers have demonstrated that RANKL and OPG are also expressed in human aortic valves (Kaden et al., 2004). Utilizing immunohistochemistry, marked expression of RANKL was found in calcified valves while only scattered positive cells were shown in normal valves. OPG was strongly expressed in normal aortic valves, and despite a significant increase in cellularity, the total amount of OPG was significantly lower in stenotic than in normal valves. The increase of the total cell number in stenotic valves was partly due to infiltration of leukocytes, suggesting an increase in the number of human aortic valve myofibroblasts since leukocytes and myofibroblasts were the only cell types so far described in aortic valves.

As a result, the lower proportion of OPG-positive cells in stenotic valves was possibly due to a decrease in OPG expression. Additionally, areas within stenotic valves exhibiting focal calcification contained less cells positive for OPG than areas without calcification. These immunohistochemistry data were in agreement with previous reports demonstrating that RANKL was not present in normal vascular tissue while OPG was expressed constitutively in many tissues including arterial smooth muscle cells and endothelial cells (Kaden et al., 2004).

In an established cultured cellular model, stimulation with RANKL led to a significant elevation in matrix calcification, nodule formation (Kaden et al., 2004). In addition, RANKL has been shown to promote matrix calcification and to induce an osteogenic phenotype in human aortic valve myofibroblasts cultured under mineralizing conditions as indicated by a significant increase in alkaline phosphatase (ALP) activity, and increased synthesis of the bone-type ALP isoenzyme and of osteocalcin. Furthermore, RANKL increases DNA binding of Cbfa-1. Thus, the results suggested that the RANKL/OPG cytokine system might regulate valvular calcification in calcific AS (Kaden et al., 2004).

It has also been reported that the soluble form of RANKL has sequence homology to other cytokines of the tumor necrosis superfamily, such as TNFα etc. (Chinnaiyan et al., 1996). In addition, TNFα has been demonstrated to be associated with calcification of aortic valve stenosis (Kaden et al., 2004). Recently, Kaden et al (2005) have found that in the cultured human aortic valve myofibrolasts model, RANKL increased cell proliferation compared to control. MMP-1 was detectable time-dependently in media with RANKL-stimulated cells, but absent in media from control cells. MMP-1 activity was increased by RANKL, which was measured by collagenase activity assay. Zymography showed an increase in active MMP-2 in RANKL-stimulated cells. These data support an involvement of MMPs in the extracellular matrix remodelling during calcification of aortic valve stenosis. RANKL might be a regulator of this process (Kaden et al., 2005).

1.7.5 Angiotensin II

1.7.5.1 Renin-angiotensin system (RAS): summary

The RAS has been considered to be a circulating system involved in the regulation of blood pressure and salt and fluid homeostasis (Reid et al., 1978). As described above, the kidney releases renin and its inactive precursor prorenin into the circulation. Angiotensinogen derived from liver is subsequently cleaved by renin in blood to form Ang I (angiotensin I). ACE (angiotensin converting enzyme), located on the luminal side of the vascular endothelium, finally converts Ang I to the active Ang II (angiotensin II), which promotes vasoconstriction and aldosterone release. The components of the RAS are synthesized in many tissues, and tissue Ang II levels may be controlled independently. Additionally, renin, angiotensinogen, ACE and angiotensin receptors are present in the heart, suggesting that Ang II synthesized locally within the myocardium may be an autocrine/paracrine regulator of cardiac function and structure (Baker et al., 1992; Dzau and Re, 1994). In addition, the local, tissue-based production of Ang II by cardiac muscle and the vascular wall is likely to be a more important source of Ang II. In addition, it has been recently found that in normal and diseased vessels, Ang II can also be formed via chymase, a neutral endopeptidase produced abundantly by vascular cells. However, this enzyme is different from ACE and is not affected by ACE inhibitors (Arakawa and Urata, 2000).

1.7.5.2 RAS and pathogenesis of atherosclerosis

Recently, it has been demonstrated that ACE, Ang II, and Ang II receptors are present in human atherosclerotic lesions. In addition, these components of the renin–angiotensin system are produced by all cellular components of the vessel: macrophages, smooth muscle cells, endothelial cells, and fibroblasts (Pratt, 1999). As a result, local production of Ang II within the arterial wall is important in the normal regulation of arterial tone and is potentially critical to the pathogenesis of atherosclerosis.

Furthermore, Ang II may be an important regulator of multiple processes critically implicated in vascular pathophysiology. Ang II has been shown to regulate the growth and migration of smooth muscle cells and fibroblasts, apoptosis of endothelial cells,

and differentiation of monocytes into macrophages. In addition, Ang II can induce potent inflammatory responses in vascular cells by stimulating the release of inflammatory factors and cytokines. Ang II can also alter the extracellular matrix remodeling via activation of matrix metalloproteinases and induce a procoagulant state (Baker et al., 1992; Dzau and Re, 1994).

1.7.5.3 RAS and pathogenesis of aortic valve stenosis

The renin-angiotensin system controls blood pressure and fluid and electrolyte balances through coordinated effects on blood vessels, kidneys, and the heart, however, if it is unregulated, it may have proatherothrombtic effects. Angiotensin-converting enzyme (ACE), Ang II, and bradykinin receptor binding were observed in aortic valve of Sprague-Dawley rats. In addition, high-density angiotensin-converting enzyme binding was present in the sites of fibrosis and inhibited by lisinopril (ACE inhibitor) in these animals (Pratt, 1999). As described above, it has been suggested that RAS is associated with the pathogenesis of atherosclerosis. Ang II, the final product of RAS system, has been implicated in different proinflammatory effects that potentially contribute to the atherosclerotic process. Because of the similarities between atherosclerosis and aortic valve stenosis, RAS may be crtically involved in the pathogenesis of aortic valve stenosis. Recently, researchers have found that ACE is distributed extracellularly in the lesions of aortic valve sclerosis and stenosis, co-localized with apoliprotein B (O'Brien et al., 1995). In addition, Ang II is co-localized with ACE in the lesions of aortic valve stenosis. The angiotensin- I receptor, a main receptor for Ang II, was also found in stenotic aortic valve lesions. These results suggested that RAS might be involved in the pathogenesis of aortic valve stenosis. In addition, recently, Helske et al (2004) demonstrated Ang II-producing enzyme systems were present in normal and stenotic aortic valves. Compared to control valves, stenotic valve showed a significant increase in both messenger ribonucleic acid (mRNA) and protein expression of ACE, which has shown to be co-localized with macrophages. Additionally, the expression of angiotensin- I receptor protein and chymase mRNA and protein was upregulated, and mast cells number was significantly increased in stenotic valves than in normal valves. The mast cells were also found in the calcified areas, in contrast to control valves (Helske et al., 2004). These findings have shown that ACE and chymase, two Ang II-forming enzymes,

were locally expressed in stenotic aortic valves, and owing to infiltration of macrophages and mast cells, were further upregulated in stenotic valves. These novel findings, implicating chronic inflammation and an increased expression of local Ang II-forming systems, suggested that therapeutic interventions aiming at inhibiting these processes might slow the progression of aortic stenosis. However, a recent observational study has suggested that ACE inhibitors did not appear to slow progression of aortic valve stenosis (Rosenhek et al., 2004). On the other hand, a retrospective study has shown a significant association between ACE inhibitor use and a lower rate of aortic valve calcification accumulation (O'Brien et al., 2005).

1.7.6 Oxidized low density lipoprotein (Ox-LDL) is associated with pathogenesis of aortic valve stenosis

As described above, hypercholesteromia is an independent risk factor for aortic stenosis and atherosclerosis. Elevated plasma low-density lipoprotein (LDL)-cholesterol is also a key risk factor for the development of atherosclerosis and hypercholesterolemia (Thomas et al., 1966). LDL is a complex of a large –molecular-mass protein, apoliprotein B, neutral and polar lipids, and lipophilic antioxidants, mainly vitamin E and beta-carotene. Oxidative modification of LDL by free oxygen radicals leads to fragmentation of apoliprotein B. As a result, the particles lose their affinity for the LDL receptor and bind to scavenger receptor, resulting in foam cell formation (Cathcart et al., 1985; Morel et al., 1984; Sparrow et al., 1989; Witztum and Steinberg, 1991). Recent studies indicate that oxidized low-density lipoprotein (Ox-LDL) has several proatherogenic effects, such as induction of inflammation molecules, cytokines by macrophages and smooth muscle proliferation (Mehta et al., 1995; Liao et al., 1997; Minuz et al., 1995). In addition, oxidized LDL has greater effects on local vascular homeostasis and endothelial cell cytotoxicity in addition to stimulating migration and mitogenesis of macrophages and smooth muscle cells (Chisolm et al., 2000; Li et al., 1999). Therefore, ox-LDL has been utilized as a marker of oxidative modification of proteins in atherosclerosis. Recently, Mehrabi et al (2000) has demonstrated that Ox-LDL is present in aortic valve leaflets and vascular endothelium. Valvular ox-LDL was significantly correlated with Ox-LDL in the intimal and medial layer of coronary arteries from the same patients (Mehrabi et al., 2000). Taken together, it can be suggested that oxidative stress induced by Ox-LDL may be

associated with the pathogenesis of aortic valve stenosis.

1.8 Summary of major molecular factors previously implicated in the pathogenesis of aortic valve stenosis

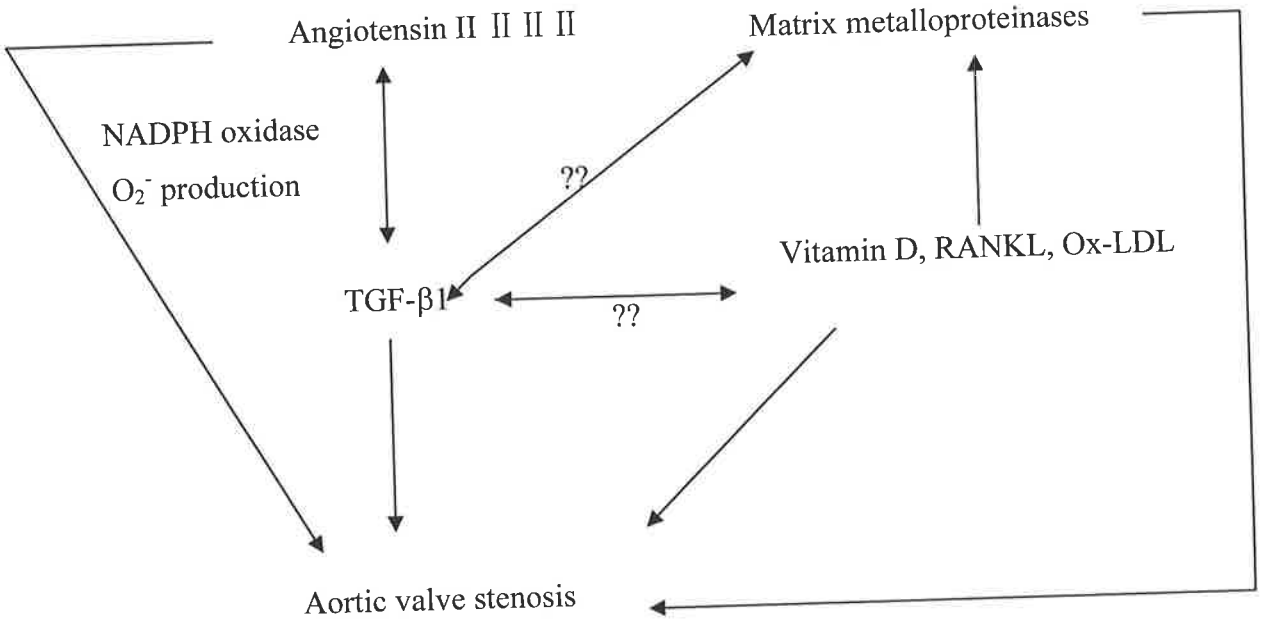


Figure 1.8-1 Summary of all possible molecular factors associated with aortic valve stenosis. From the figure, there is an interaction between angiotensin II and TGF-β1, and also TGF-β1 can stimulate matrix metalloproteinase formation. However, the relationship between TGF-β1 and other molecular factors (e.g. Vitamin D, RANKL and Ox-LDL) remains unknown. However, all these molecular factors have been implicated in the pathogenesis of aortic valve stenosis.

As described above, TGF-β1 seems to be a central stimulator of aortic valve calcification.

1.9 Hypotheses

1. Endothelial factors, such as nitric oxide (NO) can inhibit aortic valve calcification.

2. Oxidative stress is associated with pathogenesis of aortic valve stenosis.

1.10 Aims of the current studies

Aortic stenosis (AS) is characterized by accelerated aortic valve calcification, but the pathogenesis of this process is poorly understood. The aim of this study was to evaluate the potential impact of NO supplementation on valve matrix calcification in a tissue culture model, and the interaction between superoxide anion release and the process. The experiments were divided into three components:

1. A tissue culture model was established to mimic aortic valve calcification. Calcification stimulator, such as TGF-β1, was utilized to stimulate aortic valve calcification, and conditions optimal for the TGF-β1 effects were examined.

2. The effects of the "direct" nitric oxide (NO) donor, DETA-NONOate on TGF-β1 response were determined at various concentrations of DETA-NONOate. The biochemical mechanism(s) underlying the observed DETA-NONOate response were also explored.

3. In order to examine the role of oxidative stress, the superoxide scavenger, 4-hydroxy-TEMPO (TEMPOL), and superoxide dismutase (SOD and PEG-SOD) were utilized. In addition, dihydroethidium (DHE) staining was utilized to quantitate in situ superoxide release.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Primary cell culture preparation

Digest solution

Collagenase, lima Bean trypsin inhibitor and fatty acid free BSA (Bovine Serum Albumin) were purchased from Invitrogen Corp, UK. Solutions were made in [Ca^{2+} and Mg^{2+}] free Hanks Balanced Salt Solution at least 30 minutes prior to use. The solutions were sterilized using 0.2μm filter (Pall corporation, USA).

Media Material

Fetal Calf Serum (FCS)

Purchased from Invitrogen Corp, UK. Stock solutions were stored at -20°C.

Low glucose Dulbecco Modified Eagle Media (DMEM)

Purchased from Invitrogen Corp, UK. Stocks were stored in a fridge

Penicillin/streptomycin

Purchased from Invitrogen Corp, UK. Stock solutions were stored at -20°C.

Sodium hydrogen carbonate

Purchased from Merck Pty Ltd, Australia. Stocks were stored at room temperature.

Media preparation

DMEM, thawed penicillin/streptomycin, FCS and sodium hydrogen carbonate were added into a 2L bottle and agitated at moderate speed. Sterilization of the media was carried out in a sterile hood (Gelman Science, Australia) utilizing a 0.2 μm filter (Pall Corporation, USA). After sterilization, the media was stored in the fridge.

2.1.2 Passage of the cell culture

Digestion solution

Trypsin and ethylenediamine tetra-acetic acid (EDTA) were purchased from Invitrogen Corp, UK. Stock solutions were made in [Ca^{2+} and Mg^{2+}] free Hanks Balanced Salt solution and the pH value of the solution was adjusted to 7.4. Sterilization of the solution was carried out in a sterile hood (Gelman Science, Australia) utilizing a 0.2 μm filter (Pall Corporation, USA). After sterilization, the stock solutions were stored at -20°C.

2.1.3 Calcification assay

Porcine Transforming Growth Factor-β1 (Porcine TGF-β1)

Purchased from R&D systems Inc, USA. Stock solution was made in 5 mM HCL containing 9.1% bovine serum albumin (BSA) and sterilized in a sterile hood (Gelman Science, Australia) utilizing a 0.2 μm filter (Pall Corporation, USA). After sterilization, the stock solution was aliquoted and stored at -80°C. Dilutions of TGF-β1 were made in low serum (0.67% FCS) DMEM.

(Z)-1-[N- (2-aminoethyl)-N- (2-ammonioethyl) amio] diazen-1-ium-1, 2-diolate (DETA-NONOate)

Purchased from Cayman Chemical Co, UK. Stock solution was made in 0.01M NaOH (pH 10) and sterilized in a sterile hood (Gelman Science, Australia) utilizing a 0.2 μm filter (Pall Corporation, USA). After sterilization, the stock solution was aliquoted and stored at -80°C. Dilutions of DETA-NONOate were made in low serum (0.67% FCS) DMEM.

4-hydroxy-TEMPO (TEMPOL)

Purchased from Sigma Chemical Co, Germany. Stock solution was made in Milli Q water and sterilized in a sterile hood (Gelman Science, Australia) utilizing a 0.2 μm filter (Pall Corporation, USA). After sterilization, the stock solution was stored at -20°C. Dilutions of TEMPOL were made in low serum (0.67% FCS) DMEM.

Nω-nitro-L-argininie Methyl Ester (L-NAME)

Purchased from Sigma Chemical Co, Germany. Stock solution was made in Milli Q water and sterilized in a sterile hood (Gelman Science, Australia) utilizing a 0.2 μm filter (Pall Corporation, USA). After sterilization, the stock solution was stored at -20°C. Dilutions of L-NAME were made in low serum (0.67% FCS) DMEM.

8-bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP)

Purchased from Alexis Corp, Switzerland. Stock solution was made in Milli Q water and sterilized in a sterile hood (Gelman Science, Australia) utilizing a 0.2 μm filter (Pall Corporation, USA). After sterilization, the stock solution was stored at -20°C. Dilutions of 8-Br-cGMP were made in low serum (0.67% FCS) DMEM.

Superoxide dimutase (SOD)

Purchased from Sigma Chemical Co, Germany. Stock solution was made in Milli Q water and sterilized in a sterile hood (Gelman Science, Australia) utilizing a 0.2 μm filter (Pall Corporation, USA). After sterilization, the stock solution was stored at -20°C. Dilutions of SOD were made in low serum (0.67% FCS) DMEM.

Superoxide dismutase-polyethylene glycol (PEG-SOD)

Purchased from Sigma Chemical Co, Germany. Stock solution was made in Milli Q water and sterilized in a sterile hood (Gelman Science, Australia) utilizing a 0.2 μm filter (Pall Corporation, USA). After sterilization, the stock solution was stored at -20°C. Dilutions of PEG-SOD were made in low serum (0.67% FCS) DMEM.

2.1.4 Dihydroethidium (DHE) staining

Dihydroethidium (DHE)

Purchased from Sigma Chemical Co, Germany. Stocks were placed in -20°C and kept under nitrogen gas.

Diethyldithiocarbamic acid (DETCA)

Purchased from Sigma Chemical Co, Germany. Stocks were placed in -20°C.

Krebs-Hepes Buffer

NaCl (100mM), KCl (4.8mM), $CaCl_2$ (2.5mM), $MgSO_4$ (0.5mM), K_2HPO_4 (1mM), $NaHCO_3$ (25mM), and Na-HEPES (25mM) were purchased from Merck Pty Ltd, Australia. Glucose (11mM) was purchased from Amersham Pty Ltd, Australia. The pH value of stock solution was adjusted to 7.4. Stock solution was made in distilled water and stocked in fridge.

As described above (See Section 2.1.3), stock solution and dilutions of porcine TGF-β1, DETA-NONOate and TEMPOL were made in the same way as described in Section 2.1.3

2.2 Methods

2.2.1 Establishment of primary cell culture

Healthy pigs were housed in the Queen Elizabeth Hospital Animal House and killed under anaesthesia, according to methods approved by the Queen Elizabeth Hospital&University of Adelaide Animal Ethnics Committee. The first purpose was to establish primary aortic valve endothelium and myofibroblast cell cultures. Pigs were killed under intravascular ketamine/xylizine maintained with 2% Hacolane/oxygen. The whole hearts were removed quickly and placed in the sterile physiological saline solution on ice. After these procedures, the following procedures were carried out in a sterile lamina flow carbinet (Gelman Science, Australia). Instruments were sterilized with 70% ethanol followed by flaming. The sterilized instruments were used to remove the aortic valves quickly. This was followed by a wash in [Ca^{2+} and Mg^{2+}] free Hanks balanced solution to remove blood in the valves. The aortic valves were rewashed with the same buffer, and subsequently added into a sterile tube (Greiner Bio-one, Germany) containing 20mls fresh digest solution. Digestion was placed in a waterbath (37°C) with agitation at moderate speed for about 50 minutes. After agitation, the digest media containing endothelial cells were removed. Remaining aortic valve leaflets were removed and transferred to low glucose DMEM. A sterile scalpel blade was used to gently scrap leaflets to remove leftover endothelium. The leaflets were subsequently washed in low glucose DMEM,

and transferred to a new dish of low glucose DMEM. Another sterile scalpel blade was used to chop the membranes into pieces (approximate 0.5mm x 0.5mm). The pieces were subsequently placed in sterile tissue culture flasks (Greiner Bio-one, Germany) and 10mls media was added into each flask. These flasks were then placed in an incubator (5% CO_2 in air, 37°C, 95% relative humidity). For endothelium, the digest media containing endothelial cells (as described above) were placed on ice and centrifuged in a centrifuge (Sigma, Germany) under the speed of 350 g at 4°C. After centrifugation, the supernatant was removed from the tube. Aortic valve endothelium was separated by magnetic beads coupled with antibody CD31 (Dynabeads CD31). Endothelial cells were covalently bound to tosyl-activated magnetic polydisperse polymer particles (Dynabeads) and then the aortic valve endothelium-coated beads were collected utilizing a magnetic particle concentrator (MPC). The leftover pellets were resuspended in PBS/0.1%BSA (bovine serum albumin), followed by adding 25 μl washed Dynabeads (Dynal biotech, Norway). The pellets were subsequently incubated at 2-8°C for 15-30 minutes with gentle tilting and rotation. After incubation, at least two folds volume of PBS/0.1%BSA was added to the pellets followed by placing them in Dynal magnetic particle concentrator (MPC) for 2 minutes. Suprenatant was discarded and pellets were subsequently gently resuspended in the same volume of fresh PBS/0.1%BSA as the volume discarded. As described above, the procedure was repeated for 5-6 times to make sure fibroblast contamination could be removed. The cells were then transferred to sterile tissue culture flasks coated with gel and placed in an incubator.

2.2.2 Passaging cells (P2-P4)

When cells in sterile tissue culture flasks (Griener Bio-one, Germany) became 95% confluent, they were passaged. Firstly, media was removed from sterile tissue culture flasks and replaced by trypsin/EDTA digest solutions. The flasks were subsequently placed in an incubator (Forma Science, USA) for 5 minutes. After incubation, each flask was checked under the field of inverting microscope (Olympus, Japan) to make sure the cells had completely lifted. Media was then added into each flask and mixed completely. After complete mixture, the media were collected into a sterile tube (Greiner Bio-one, Germany), followed by centrifugation (110 g, 4°C). The

supernatant was then removed. Another 20 mls fresh media was added and subsequently followed by resuspension of the cells utilizing a sterile pipette. After resuspension, a counting chamber (Neubauer, Germany) was used to count the cell number. Lastly, cells were distributed into sterile tissue culture flasks according to the cell density needed and the flasks were placed in an incubator (Gelman Science, Australia).

2.2.3 Cell counts

For our experiments, a counting chamber (Neubauer, Germany) was used to determine cell concentration. Before utilizing the counting chamber, the mirror-like polished surface and the coverslip were cleaned with lens paper. After cleaning the surface and coverslip, the coverslip was subsequently placed over the counting surface prior to adding cell suspension. Enough liquid was introduced so that the mirrored face was fully covered. The charged counting chamber was then placed on the optical microscope (Olympus, Japan) and the counting grid was brought into focus. Under the microscope field, the grid was divided into 9 large squares, each square had a surface area of one square mm, and the depth of the chamber was 0.1mm. In the central square, it is composed of 25 small squares and the area of each small square is 1/25 square mm. Aortic valve fibroblasts and endothelial cells were counted in the central squares of two grids in the counting chamber. In addition, the counts of both sides had to be similar, or the procedure had to be repeated. In conclusion, final cell concentration was mean cell counts of two sides $x10^4$ per ml.

2.2.4 Calcification assay

Firstly, we passaged myofibroblasts and distributed cells into 6-well plate (Becton Dickson, USA) with cell density of $0.5x10^6$ in each well (Mohler et al., 1999). The plates were subsequently placed in the incubator (Note: media for incubation was DMEM with 10% FCS). When cells in each well had become 90% confluence, we began chemical supplementations to cell culture. In order to decrease cell growth, we added low serum (0.67% FCS) DMEM to replenish high serum (10% FCS) DMEM during chemical supplementations. Dilutions of TGF-β1, DETA-NONOate, TEMPOL

and L-NAME were made up in low serum DMEM. Final concentration of TGF-β1 was 5ng/ml as described by Mohler et al.(1999). A full concentration response curve for TGF-β1 in porcine cells is shown as an appendix in chapter 6.4. DETA-NONOate was used at 1~100μM (the concentration more than 100μM will be toxic to cell culture, data not shown), while TEMPOL and L-NAME were both present at100μM.. In general, inhibitor agents were used at established maximally effective concentrations unless these proved toxic when present for the extended duration of the experiment. In the latter case maximally tolerated concentrations were used. After chemical supplementations, all the plates were placed in an incubator. In general, our purpose was to examine DETA-NONOate, TEMPOL and L-NAME effects on nodule formation in the presence and absence of TGF-β1. For calcification assay, an inverting microscope (Olympus, Japan) was utilized to count calcific nodule number in each well. To remove subjectivity, blinded counting was carried out, which meant that one observer didn't know the details of chemical supplementations in cell culture. Due to the half-life of DETA-NONOate, DETA-NONOate was replenished every 24 hours to keep consistent nitric oxide in the media, while other chemicals were changed 2 times a week. Furthermore, experiments were conducted in triplicate in at least 3 cultures at cell passages 2-4. All experiments were conducted over a period of 10-14 days and an observer blinded to treatment counted nodules alternate days. It should be noted that the TGF-β1 concentration used in our experiment is quoted from Mohler's study (Mohler et al., 1999). In addition, my colleague Dr Kumaril has done the study of the relationship of TGF-β1 concentration response in maximal nodule formation in porcine aortic valve fibroblasts (Figure 6.1 Chapter 6).

2.2.5 Dihydroethidium (DHE) staining (in situ superoxide detection)

Myofibroblasts were passaged into 4-well or 8-well chamber slides (Sybron, USA) utilizing high serum (10% FCS) DMEM. Once cells in the wells have become 90% confluence, 10% serum DMEM was replaced by low serum (0.67%) DMEM. The cells were subsequently incubated with DETA-NONOate (20μM), or TEMPOL (100μM) in the presence and absence of TGF-β1. After several days' incubation, media were removed from the chamber slides and replaced with Krebs-Hepes solution containing DETCA (3mM) and DHE (5μM). The chamber slides were subsequently placed in an incubator (37°C) for 45 minutes. Due to DHE sensitivity to light, all the

chamber slides were protected from light utilizing aluminium foil. DHE is oxidized on reaction with superoxide anion to ethidium bromide, which binds to DNA and emits red fluorescence (Miller et al., 1998). The red fluorescence was detected through a 590-nm long pass filter using a fluorescence microscope (Nikon, Japan), and was digitally photographed using a CCD camera (Nikon, Japan) mounted to the microscope. On each treatment group, 4 regions of confluent myofibroblasts were chosen. The intensity of red fluorescence was analyzed using Video Pro 32 color image analysis software (Leading edge, Australia). Confluent regions were counted by an observer blinded to the treatment protocol.

2.3 Statistical methods

i) Statistical analysis of calcification assay: Effects of drug treatments on nodule formation were expressed as percentages of mean peak nodule counts (blinded counts) relative to paired control wells in the presence and/or absence of TGF-β1. Treatments were performed in triplicate and each experiment was repeated for at least three cell cultures. Data were represented as mean±SE (n=3). Statistical comparison utilised one-way ANOVA with Dunnett's post hoc test for multiple comparisons.

ii) Statistical analysis of DHE staining: Effects of drug treatments on in situ superoxide release were expressed as percentages of mean fluorescence intensity relative to paired control in the presence of TGF-β1. Treatments were performed in duplicate and each experiment was repeated for at least three cell cultures. Data were represented as mean±SE (n=3), and we used paired t-test or one-way ANOVA with Dunnett's post hoc test for multiple comparisons. Statistical significance in all cases was defined as $p<0.05$.

Note: For treatment experiments (e.g. DETA-NONOate, L-NAME, TEMPOL and 8-Br-cGMP), multiple treatments were performed in every experiment. As a result, in order to compare to TGF-β1 control group, one way ANOVA followed by Dunnett's

test was utilized. However, in the next chapter for clarity, these results were graphed separately.

On the other hand, for the single comparison between control and TGF-β1 treated groups, paired t-test was utilized.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Effects of TGF-β1 (5ng/ml) on calcific nodule formation in porcine aortic valve cell cultures

3.1.1 Effects of media concentration of fetal calf serum (FCS) on TGF-β1 induced nodule formation

In the presence of TGF-β1, when porcine aortic valve fibroblasts were cultured for up to 7 days, the nodule counts were significantly increased in 0.67% serum containing media and peak nodule count was 232±3. However, cells cultured with 10% serum containing media had almost no nodule formation irrespective of the presence or absence of TGF-β1. Furthermore, there was no spontaneous nodule formation in the cells cultured with 10% serum containing media (Figure 3.1-1). These results therefore showed that cells cultured with 0.67% serum containing media responded to TGF-β1 with incremental nodule formation. The basis for lack of nodule formation in cells cultured with higher concentrations of serum (such as 10% serum) was not explored, nor were effects of higher concentrations of TGF-β1 examined in cells cultured in 10% serum media.

3.1.2 Heterogenous effects of TGF-β1 (5ng/ml) on calcific nodule formation in porcine aortic valve cell cultures

Although we elected to utilize stimulation of nodule formation by TGF-β1 in the presence of 0.67% serum containing media (see above), this approach did not prevent considerable differences in both time course and peak extent of responses to TGF-β1 in different cell cultures (Figure 3.1-2 and table 3.1-1).

Specifically, the five cultures depicted in figure 3.1-2 vary markedly as regards: (1) presence/absence of "spontaneous" nodule formation, (2) numbers of calcified nodules in the presence of TGF-β1, (3) time to "peak" nodule response to TGF-β1 (Table 3.1-1). However, although the total numbers of nodules formed varied in different experiments, TGF-β1 has been shown to definitely stimulate nodule

formation. Given that the total number of nodules formed in response to TGF-β1 varied considerably, the absolute reduction by further treatments on nodule formation would also vary markedly. For this reason, effects of treatments were expressed as percentage of TGF-β1 control.

Different passages of one porcine aortic valve cell cultures were associated with different degrees of stimulation in the presence and absence of TGF-β1 (Table 3.1-2), but TGF-β1 has been shown to increase nodule formation in all the passages relative to controls. For our experiments, passage 4 of aortic valve matrix cell culture was utilized due to the high rates of calcification. Nodule formation occurred in both the absence and presence of TGF-β1, but remained relatively low in the absence of TGF-β1. Because the greatest difference between control and treated cells was seen after 4 passages, this condition was used in subsequent experiments.

As described above, figure 3.1-3 summarizes the effects of TGF-β1 on peak nodule formation in porcine aortic valve cell cultures (passage 4) relative to controls. In control groups, the mean peak nodule count was 58.3±37.5 (n=5; Figure 3.1-3). When treated with TGF-β1 (5ng/ml) (Mohler et al., 1999), peak nodule count was 210.2±66.3 (n=5, $p<0.05$; Figure 3.1-3).

3.2 Histology of porcine aortic valve interstitial cells and calcific nodule

Examination of nodules during cell growth phases revealed variable clarity of distinction of the border zone of nodules from that of surrounding tissue: hence the need for blinded counting. However, at the end of TGF-β1-stimulated phases, detailed examination of histology of the tissue was undertaken in several ways: (a) The presence of calcification within nodules could be demonstrated. The nodule contained an inner ring of dead cells surrounded by an outer ring of living cells. Cells associated with nodules had calcific characteristics and stained positively for Alizarin Red S (See figure 3.2-1A). (b) Individual cells could be demonstrated on immunohistochemical staining to contain smooth muscle α-actin (Figure 3.2-1B) and vimentin (Figure 3.2-

1C). These findings therefore established that a high proportion of cells in TGF-β1 treated cultures exhibited characteristics of myofibroblasts.

3.3 Effects of DETA-NONOate (1-100μM) and 8-Br-cGMP (1mM) on TGF-β1 induced nodule formation in porcine aortic valve cell cultures

The direct nitric oxide (NO) donor, DETA-NONOate (1-100μM) (the concentration more than 100μM will be toxic to cell culture, data not shown) was utilized to examine NO effects on calcific nodule formation in porcine aortic valve cell cultures in the presence of TGF-β1 (5ng/ml) (Mohler et al., 1999), DETA-NONOate co-incubation inhibited TGF-β1 induced nodule formation in a concentration-related manner, with threshold effects occurring at 1μM, and 50% suppression at approximate 10μM DETA-NONOate (Figure 3.3-1A and 1B). The fitted concentration-response curve for DETA-NONOate is shown in figure 3.3-2.

Furthermore, in order to test the downstream product of NO, cGMP effects on nodule formation in porcine aortic valve cell cultures in the presence of TGF-β1, the analogue 8-Br-cGMP was utilized. From figure 3.3-3, the mean data showed that 8-Br-cGMP co-incubation (1mM) inhibited nodule formation by 90% ($p<0.01$, ANOVA; Figure 3.3-3).

3.4 Effects of L-NAME on TGF-β1 induced nodule formation in porcine aortic valve cell cultures

As described above, our experiments have demonstrated that exogenous nitric oxide (NO) inhibited calcific nodule formation in the presence of TGF-β1. In order to examine whether endogenous NO in porcine aortic valve fibroblasts might have some effect on nodule formation in the presence of TGF-β1, the non-specific nitric oxide synthase (NOS) inhibitor, L-NAME was used. In a representative study, the peak nodule count of cells treated with TGF-β1 (5ng/ml) (Mohler et al., 1999) and L-

NAME was 13.5±4.0, while the mean peak nodule count was 14.6±5.0 in the cells treated with TGF-β1 (figure 3.4-1A). In order to demonstrate reproducibility, all the experiments were repeated for at least three times. As is shown in figure 3.4-1B, L-NAME (100μM) did not significantly modify TGF-β1 induced nodule formation. These experiments, however, were performed only in the presence of TGF-β1 stimulation, and it therefore remained possible that interaction with L-NAME might have been revealed in the absence of extensive TGF-β1 "background" stimulation of calcification.

In order to determine whether L-NAME might increase nodule formation under circumstances in which basal rather than potentially maximally stimulated nodule formation applied, three experiments were performed in which L-NAME (100μM) was added to cultures in the absence of TGF-β1. Two of these cultures demonstrated no spontaneous nodule formation; the results of the third culture are shown in figure 3.4-2. From these limited data, it appears possible that L-NAME might increase nodule formation in the absence of background TGF-β1 stimulation.

3.5 Effects of TEMPOL (100μM), PEG-SOD (100U/ml) and SOD (500U/ml) on TGF-β1 induced nodule formation in porcine aortic valve cell cultures

Our secondary hypothesis is that oxidative stress is associated with TGF-β1 induced calcification of porcine aortic valve fibroblasts. In order to examine the relationship between superoxide anion release and TGF-β1 induced nodule formation, TEMPOL (100μM), PEG-SOD (100U/ml) and SOD (500U/ml) were added into the tissue culture in the presence of TGF-β1. The data therefore suggest that TEMPOL and PEG-SOD inhibits TGF-β1 induced nodule formation. The mean data suggest that this concentration of TEMPOL and PEG-SOD suppressed nodule formation by about 70% and 50% respectively ($p<0.01$, one way ANOVA with Dunnett's test; Figure 3.5-1).

3.6 Effects of DETA-NONOate (20μM) and TEMPOL (100μM) on intracellular superoxide anion release

In previous experiments, we have demonstrated that NO donor DETA-NONOate and superoxide scavenger TEMPOL and PEG-SOD can inhibit nodule formation in porcine aortic valve fibroblasts in the presence of TGF-β1. As a result, these data might suggest that superoxide anion release induced by TGF-β1 contributes to calcific nodule formation and NO might have an inhibitory role in this process. In order to test this hypothesis, dihydroethidium (DHE) fluorescent images were acquired from cultures treated without any added drugs (control), with TGF-β1, with TGF-β1 (5ng/ml)/DETA-NONOate (20μM), and with TGF-β1 (5ng/ml)/TEMPOL (100μM). Individual representative results are shown in figure 3.6-1. As seen from figure 3.6-1C, DHE fluorescence was markedly increased in the presence of TGF-β1, but decreased towards control values when DETA-NONOate (Panel E) or TEMPOL (Panel F) were also present.

Mean quantitative data, shown on figure 3.6-2A and 2B, confirm these changes. TGF-β1 approximately doubled DHE staining ($p<0.05$; Panel A) while both DETA-NONOate and TEMPOL approximately halved DHE staining ($p<0.01$ and $p<0.05$ respectively; Panel B). All the images were taken by identical sensitivity setting (confluent regions of cells), and the images were analyzed by Video Pro 32 color image analysis system (Leading edge, Australia).

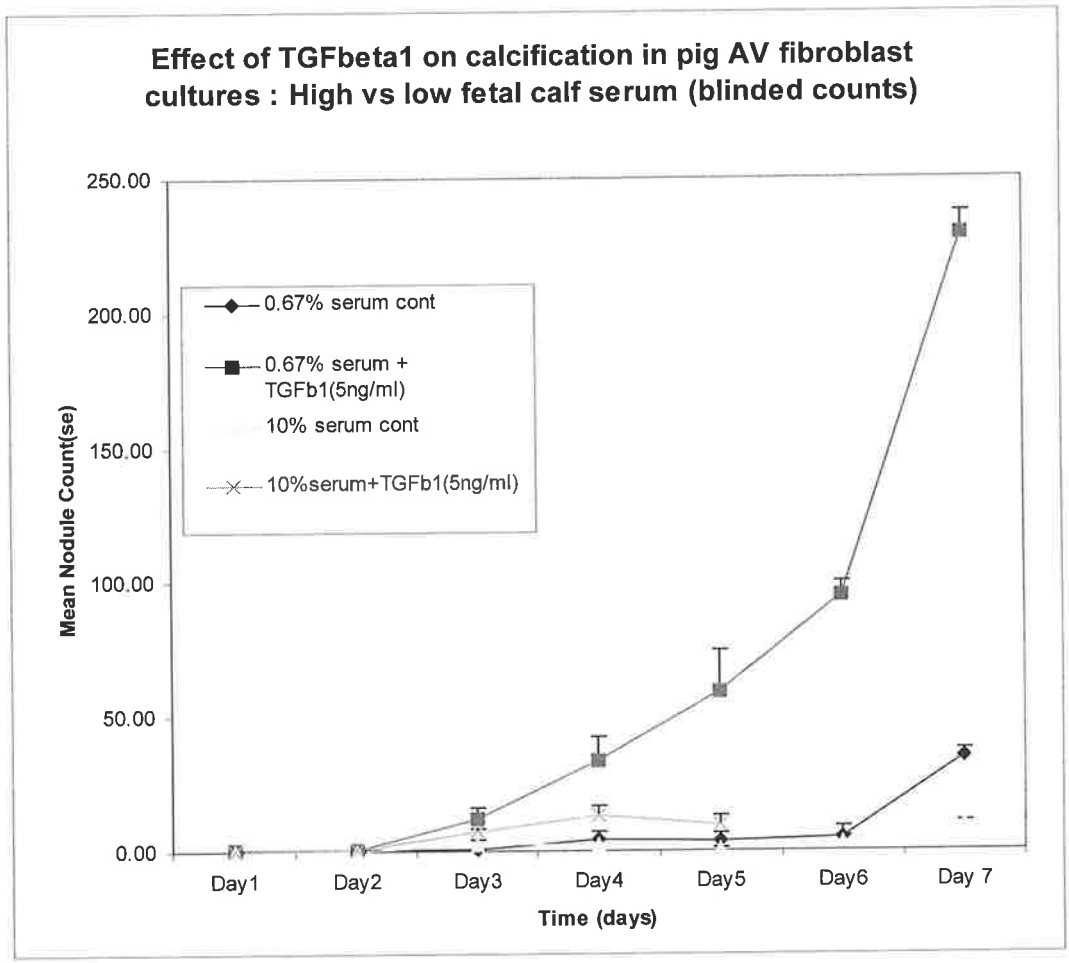
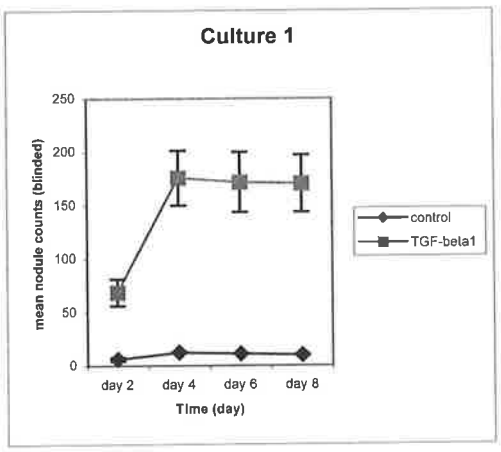
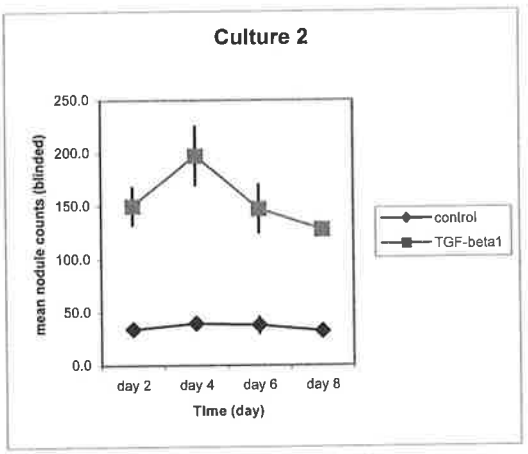


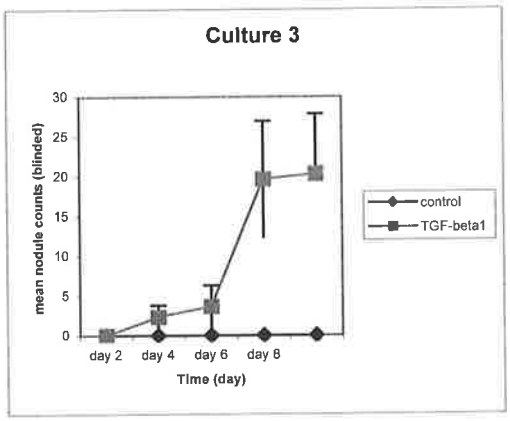
Figure 3.1-1 Effects of 0.67% FCS and 10% FCS media on nodule formation in the absence/presence of TGF-β1. Treatments were performed in triplicate, and error bars represent standard errors. In order to remove subjectivity, blinded counting was used in this experiment.



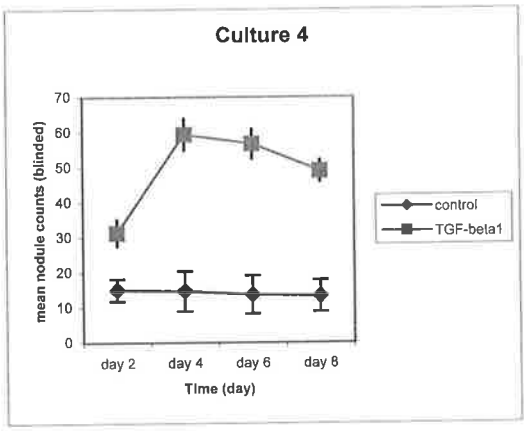
A



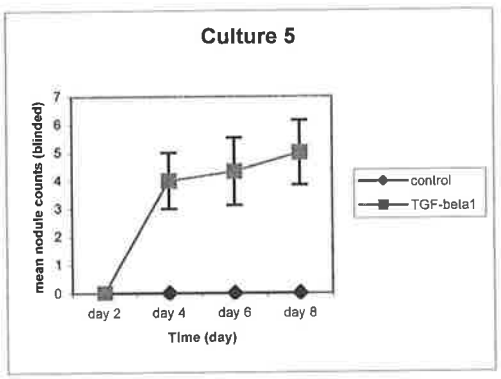
B



C



D



E

Figure 3.1-2 Relative rates of calcific nodule formation against time in the absence and presence of TGF-β1 in five different tissue cultures. All the treatments were conducted in triplicate and all the cells were in passage 4. Data were expressed as mean ± SE.

Parameter	Median	Range
Number of spontaneous nodules (n=5)	8	0-34
Number of nodules with TGF-β1 (n=5)	62	0-201
Time to peak response (days) (n=5)	6	4-8

Table 3.1-1 Summary of data from figure 3.1-2

Passage number	Control Peak nodule count	TGF-β1 (5ng/ml) Peak nodule count
2	0	59.6
3	12	110
4	12.2	176.3

Table 3.1-2. Implications of number of passages on nodule formation in the absence and presence of TGF-β1 on multiple examinations of culture material from a single pig. Data were mean nodule counts from triplicate wells. All data were obtained by investigators blinded to treatment.

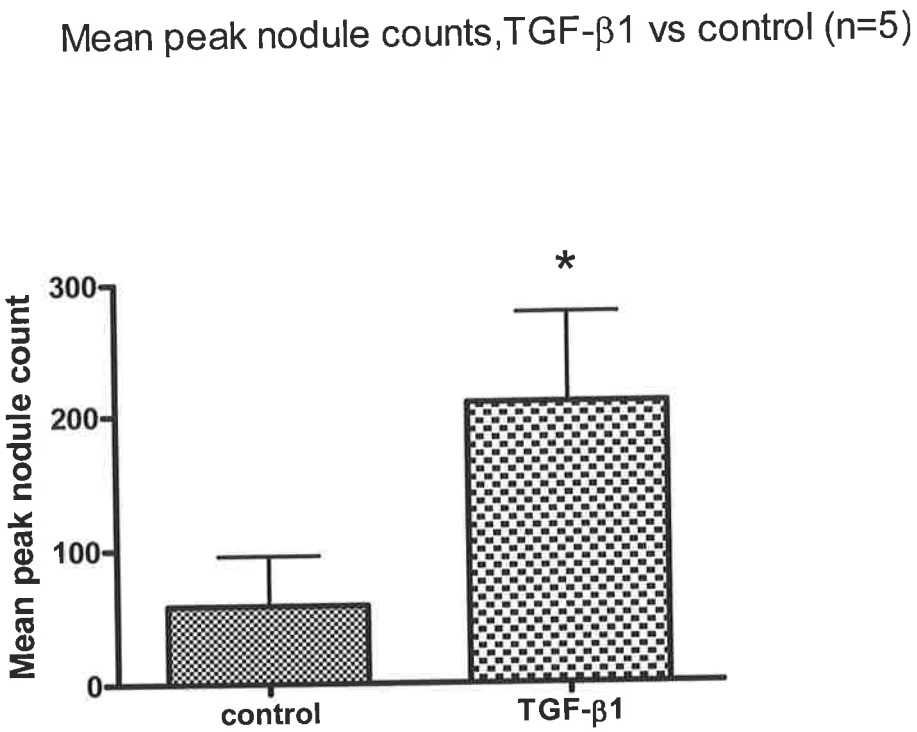
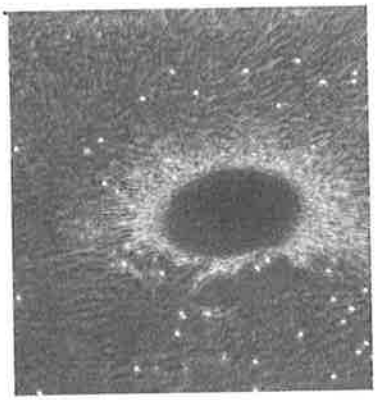
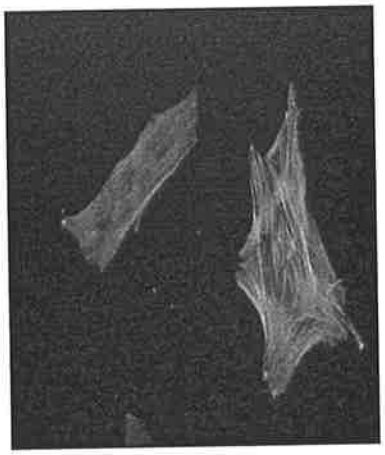


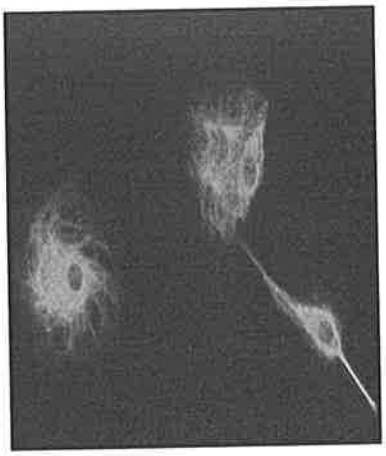
Figure 3.1-3. The effects of TGF-β1 on peak nodule count (blinded) in porcine aortic valve fibroblasts (passage 4) relative to control vehicles. Data was represented as mean±SE. n=5 cultures (treatments in triplicate/culture). * $p<0.05$ vs control (paired t-test).



A. Calcific nodule in porcine aortic valve fibroblasts



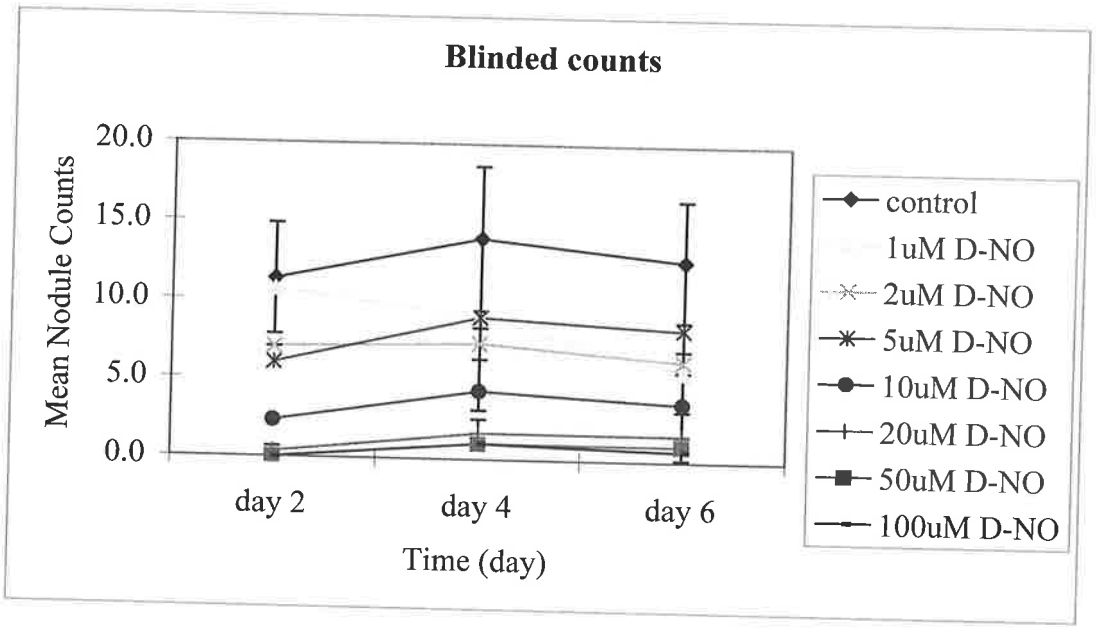
B. Anti-smooth muscle α-actin staining



C. Anti vimentin staining

Figure 3.2-1. Characteristics of nodule formation in tissue cultures of porcine aortic valve matrix cells. Fluorescence histochemistry of porcine aortic valve fibroblasts in tissue culture utilizing phase contrast microscopy: A. Alizarin Red S staining of calcific nodule formation. Original magnification: x 40. B. Anti-smooth muscle α-actin staining of aortic valve interstitial cells. C. Anti vimentin staining of aortic valve interstitial cells.

A



B

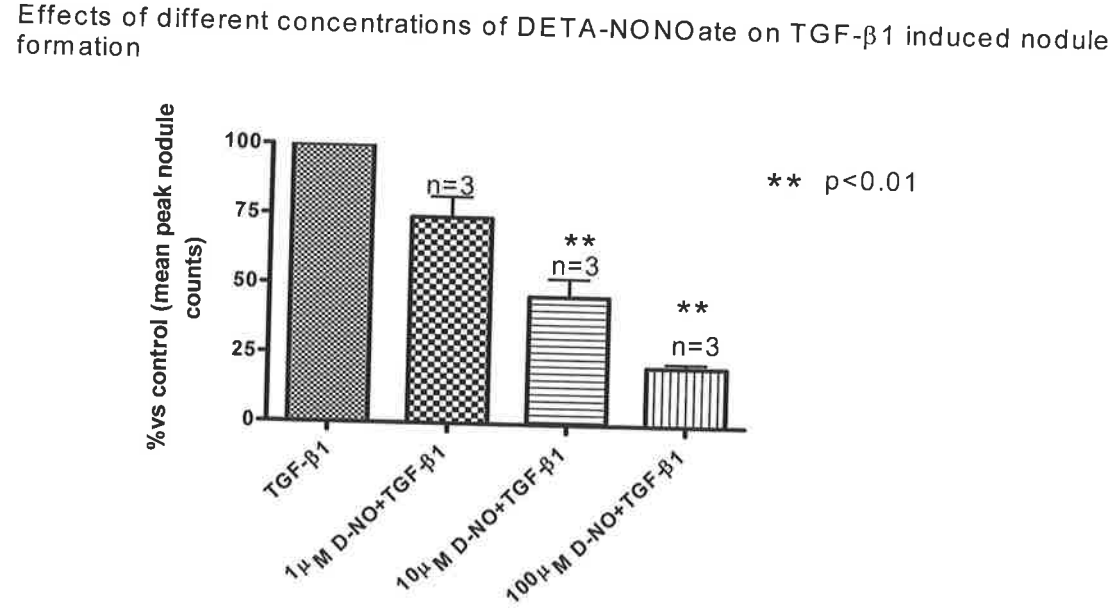


Figure 3.3-1 Suppression of nodule formation in TGF-β1 stimulated cell culture by increasing concentrations of DETA-NONOate.

Panel A: Individual data (triplicate measurements) from a single passage 4 cell culture.

Panel B: Mean data was from three experiments and suppression was assessed statistically by one-way ANOVA followed by Dunnett's test (n=3 cultures).

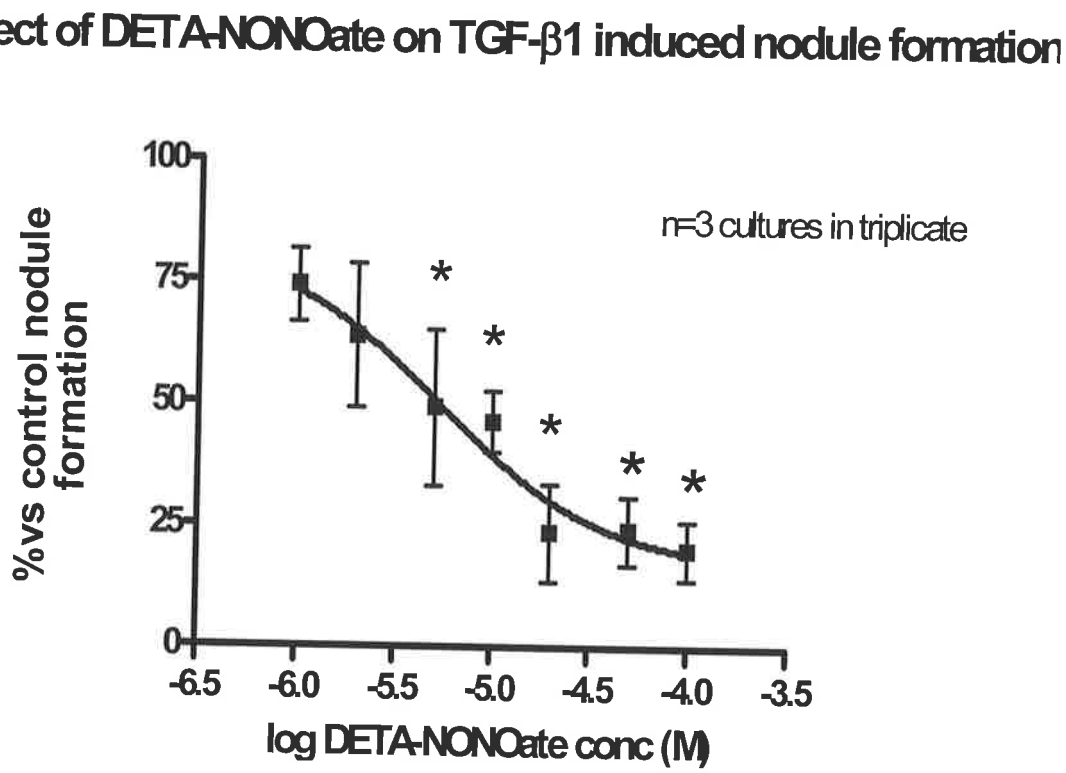


Figure 3.3-2 Fitted concentration-response curve: Effects of DETA-NONOate (1-100μM) on calcific nodule formation in the presence of TGF-β1. Data were fitted using Graphad software (R^2=0.62, EC_{50}=5.3μM (0.035-78.7μM)). Error bars represent standard errors. (One way ANOVA, $p<0.0001$ followed by Dunnett's test, * $p<0.05$ vs TGF-β1 control).

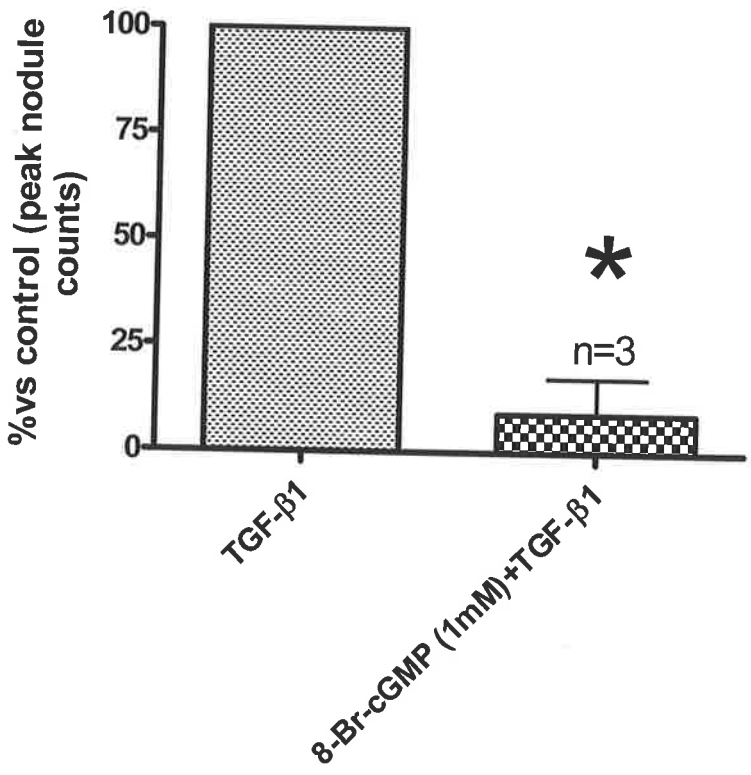
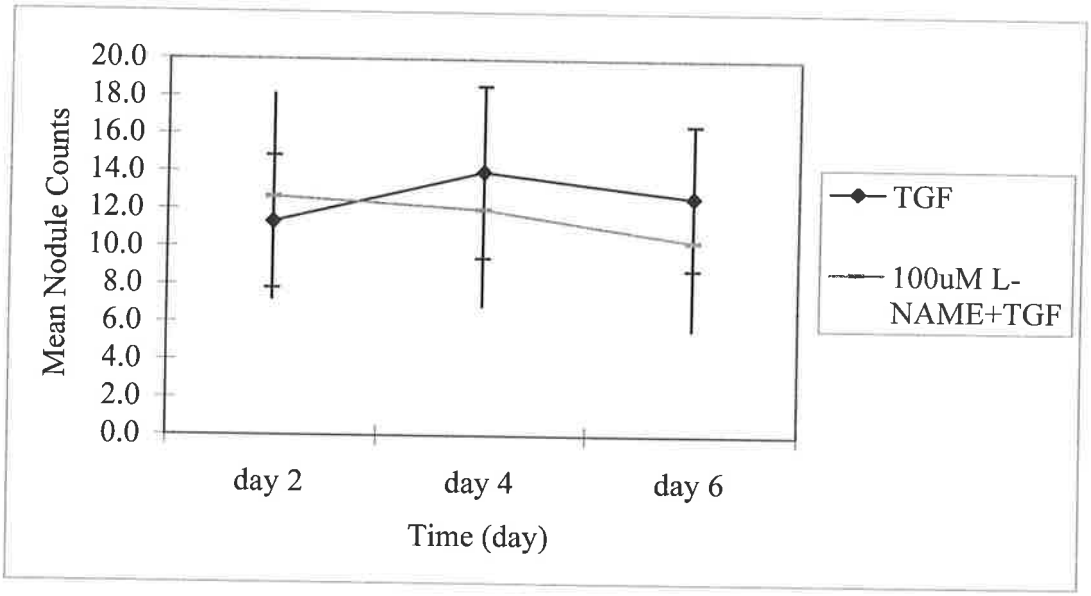


Figure 3.3-3 Effects of 8-Br-cGMP (1mM) on TGF-β1 induced nodule formation. All experiments were conducted for three times, n=3 cultures (triplicate treatments/culture). The data was represented as mean ± SE. Error bars represent standard errors. * $p<0.01$ vs TGF-β1 control (one way ANOVA).

A



B

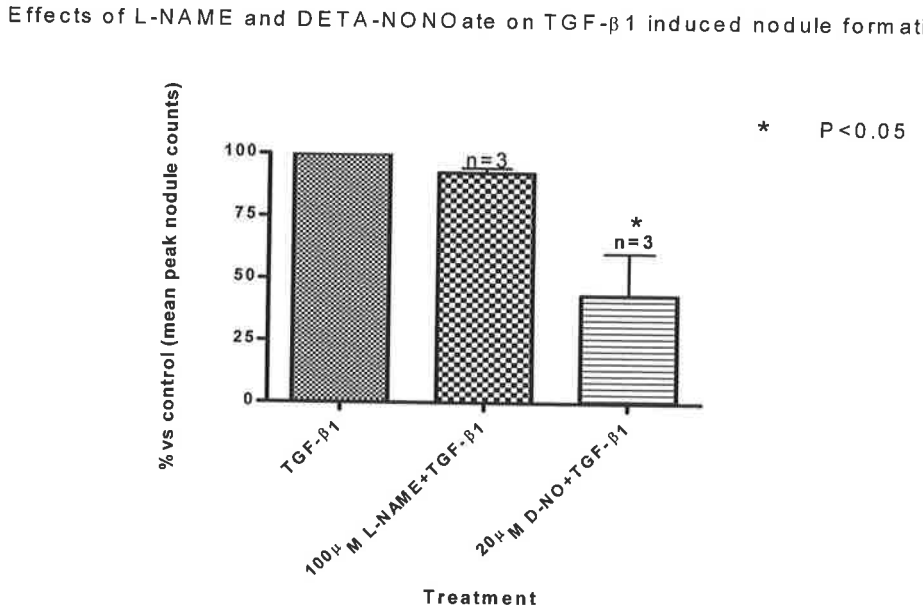


Figure 3.4-1

Panel A. Representative results of L-NAME (100μM) effect on TGF-β1 induced nodule formation in porcine aortic valve fibroblasts. The treatments were performed in triplicate and all the cells were in passage 4. Data were expressed as mean ± SE (n=3 cultures). Error bars represent standard errors. For reproducibility, the same experiments were repeated for at least three times.

Panel B. Grouped data: Effects of L-NAME (100μM) and DETA-NONOate (20μM) on TGF-β1 induced nodule formation. All experiments were conducted in three separate cultures. The data were represented as mean±SE (n=3 cultures). Error bars represent standard errors. One way ANOVA with Dunnett's test, * $p<0.05$ vs TGF-β1 control.

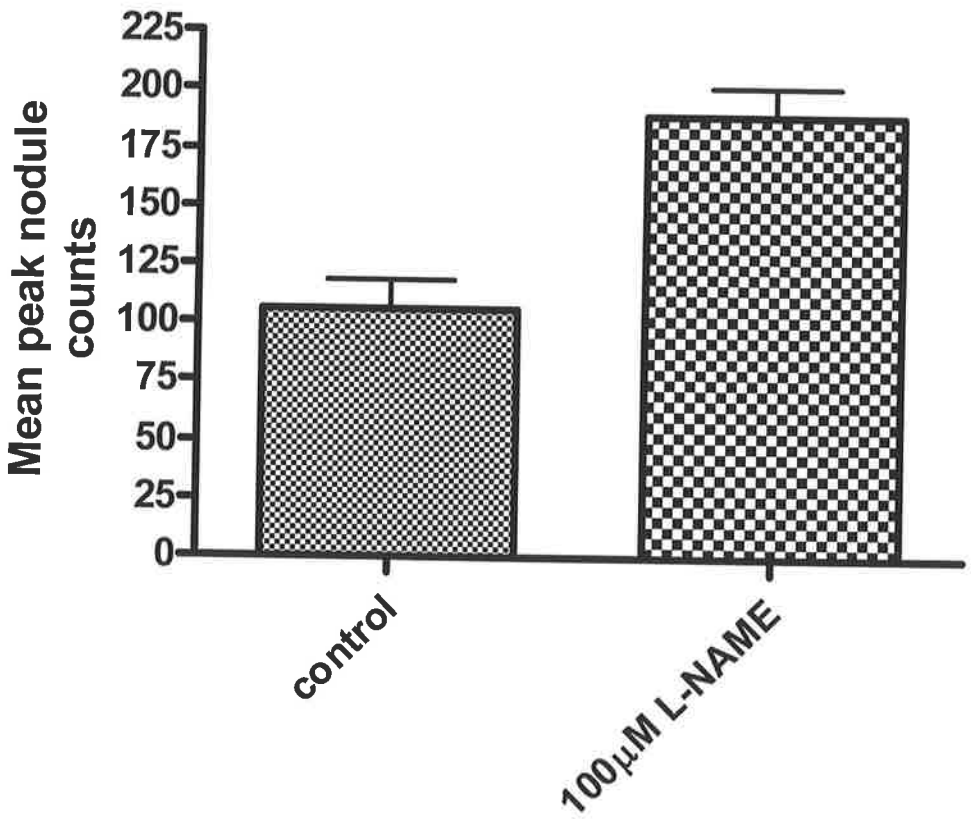


Figure 3.4-2 Effects of L-NAME (100μM) on spontaneous nodule formation in a single cell culture. The experiment was performed in triplicate. All counts were performed in blinded mode. Error bars represent standard errors.

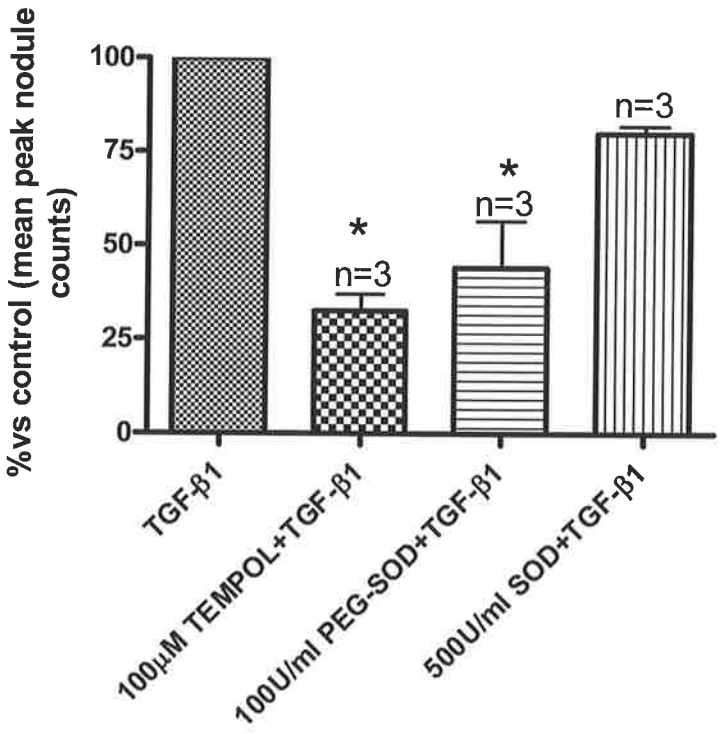
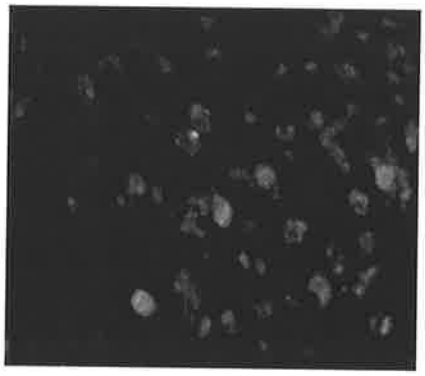
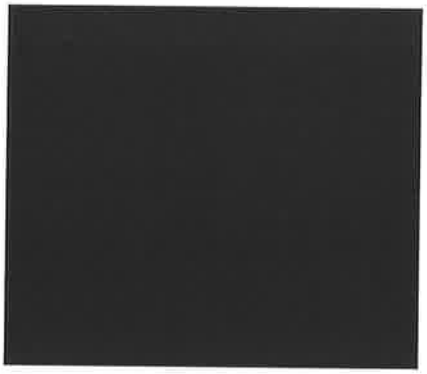


Figure 3.5-1

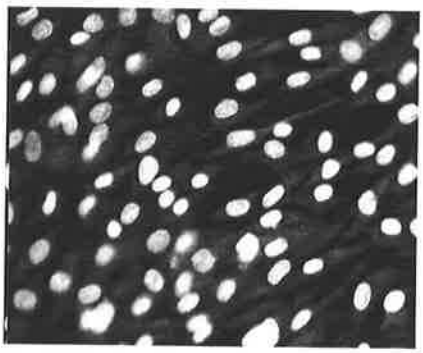
Grouped data: Effects of TEMPOL (100μM), PEG-SOD (100U/ml) ans SOD (500U/ml) on TGF-β1 induced nodule formation. The data was represented as mean ± SE (n=3 cultures). Error bars represent standard errors. * $p<0.01$ vs TGF-β1 control (one way ANOVA with Dunnett's test).



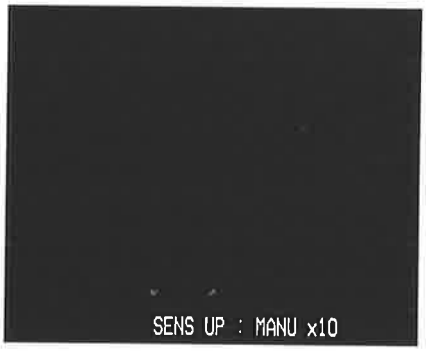
A. Control



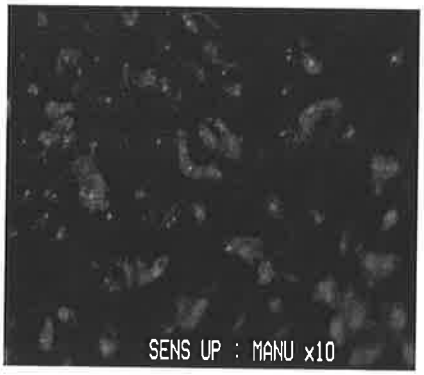
B. Control without DHE



C. TGF-β1 (5ng/ml)



D. TGF-β1 without DHE

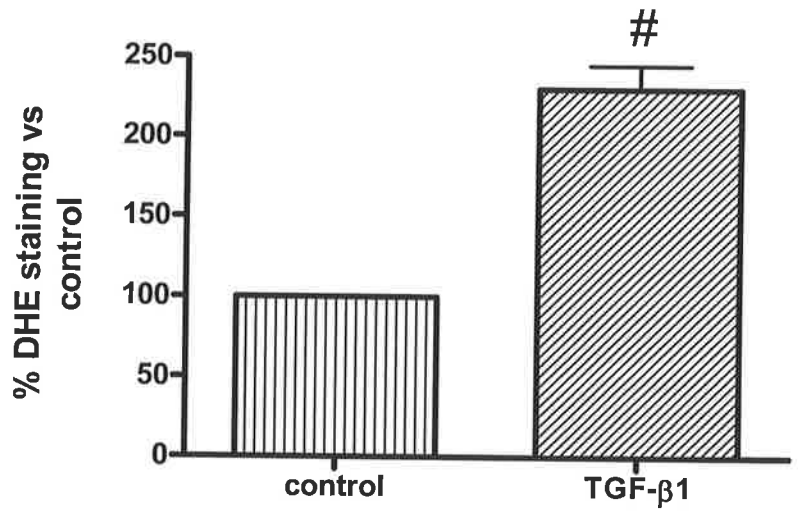


E. TGF-β1+DETA-NONOate (20μM)

F. TGF-β1+TEMPOL (100μM)

Figure 3.6-1 In situ detection of superoxide in porcine aortic valve fibroblasts. Red fluorescence stained with/without superoxide-sensitive dye DHE were obtained from control (A and B) and different treatment groups (C-F) after onset of nodule formation in TGF-β1-treated aortic valve fibroblasts. All images were acquired at identical setting (confluent regions of cells).

A



B

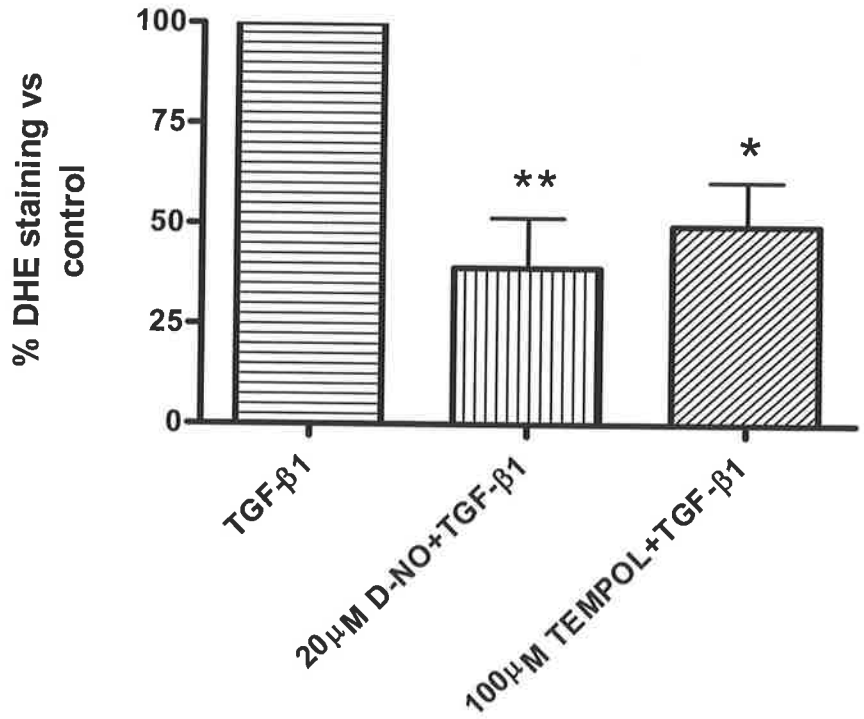


Figure 3.6-2. Effects of TGF-β1 (Panel A) and its interaction with DETA-NONOate and TEMPOL (Panel B) on DHE staining: group data is the mean of three experiments. n=3 cultures (duplicate treatments/culture) # $p<0.05$ (paired t-test), * $p<0.05$ (one way ANOVA followed by Dunnett's test), ** $p<0.01$ (one way ANOVA followed by Dunnett's test).

3.7 Discussion

The major conclusions to be drawn from this series of experiments are:

(1) TGF-β1 stimulates the development of calcific nodules in porcine aortic valve matrix cells in culture and intracellular superoxide over the same time course, with maximal effect on passage 4 cells.

(2) The direct nitric oxide (NO) donor DETA-NONOate inhibits TGF-β1 effects in a concentration-dependent manner.

(3) DETA-NONOate also inhibits TGF-β1 induced increases in intracellular superoxide anion.

(4) The superoxide scavenger TEMPOL reduces TGF-β1 induced nodule formation and intracellular superoxide anion and PEG-SOD inhibits TGF-β1 induced nodule formation.

Therefore, these data suggest that superoxide anion release induced by TGF-β1 contributes to calcific nodule formation, and that NO can limit this process. It remains to be determined whether these effects of NO are relevant to the impact of valvular endothelial dysfunction and/or to a potential therapeutic role for NO donors in prevention of AS.

3.7.1 TGF-β1 effect on porcine aortic valve matrix cells

TGF-β1 is a member of the same gene super family as the bone morphogenic protein (Bonewald and Dallas, 1994). In addition, it has been demonstrated to have osteogenic activity (Miyazono et al., 2001) and it is known to be pro-apoptotic in many cell types (Hishikawa et al., 1999; Pollman et al., 1999; Mattey et al., 1997). Furthermore, TGF-β1 has been demonstrated previously to potentiate calcification in cell cultures of vascular smooth muscle (Watson et al., 1994) and Mohler et al have demonstrated that TGF-β1 induced calcification in primary culture of canine and

human aortic valve interstitial cells (Mohler et al., 1999) in a time-dependent manner. More recently, Jian et al (2003) have shown that addition of TGF-β1 to sheep aortic valve fibroblasts culture led to a series of events including cellular migration, aggregation, apoptosis and calcific nodule formation.

Furhtermore, TGF-β1 has been found to be present in calcific aortic valve cusps, compared to non-stenotic aortic valves (Jian et al., 2003). In the present study, our results have demonstrated that nodule formation of porcine aortic valve matrix cells increased with duration of exposures to porcine TGF-β1, compared to controls (Figure 3.1-2). In addition, in some but not all cell cultures, we observed spontaneous nodule formation could occur in the absence of exogenous TGF-β1 (Figure 3.1-2A, B and D).

For our analysis, peak nodule counts were utilized as a measurement of maximal effect of TGF-β1. Furthermore, in order to test reproducibility, we performed different cell cultures and different passages of cell cultures. Variability was demonstrated in different porcine cell cultures and different passages of the same porcine cell culture (Table 3.1-1 and Table 3.1-2). However, TGF-β1 has been shown to be an inducing factor for calcific nodule formation in all porcine aortic valve cultures. Additionally, in our experiments, it has been demonstrated that different media in porcine aortic valve matrix cells had different effects on nodule formation. 10% FCS (Fetal Calf Serum) media is widely used in the cell culture (Werner and Kissel, 1995). In our experiments, the aortic valve fibroblasts incubated with 10% FCS media had almost no nodule formation after several days' exposure to TGF-β1. On the other hand, at the same time, the cells incubated with low serum (0.67% FCS) media had consistent and increasing nodule formation in the presence of TGF-β1 (Figure 3.1-1). These results suggested that low serum media was an ideal media for nodule formation in the presence of TGF-β1. In conclusion, our results demonstrated that TGF-β1 induced calcific nodule formation in porcine matrix cells, irrespective of culture and passage number, provided the serum content of media was low.

3.7.2 NO inhibitory effects on TGF-β1 induced nodule formation

Recently, it has been demonstrated that normal aortic valve endothelium can release nitric oxide (NO) and prostacyclin (PGI_2) (Pompilio et al., 1998). Endothelium-derived nitric oxide (NO) is the most potent endogenous vasodilator known, exerting its effect largely via stimulation of soluble guanylate cyclase to produce cyclic GMP (Furchgott and Zawadzki, 1980; Ignarro et al., 1984; Murad, 1996). NO is a critical modulator of blood flow and blood pressure (Johnstone et al., 1993). It is released by the endothelium in response to shear stress and plays an important role in flow-mediated vasodilation. NO released by endothelium inhibits the vasoconstrictor effects of norepinephrine, endothelin, angiotensin II, and serotonin (Cooke and Dzau, 1991; Vallance et al., 1997). Recently, aortic valve sclerosis, which is the early phase of aortic valve stenosis, has been reported to be associated with systemic endothelial dysfunction (Poggianti et al., 2002). In addition, hemodynamic studies have demonstrated that there is flow disturbance and near stagnation at the aortic side of aortic valve cusps in the patients with aortic valve stenosis. Higher mechanical stress occurs at the flexion area and the line of coaptation. As a result, aortic endothelium at the area of higher mechanical stress demonstrates subtle changes consistent with mild damage and becomes more susceptible to lipid deposition and infiltration by macrophages and T-lymphocytes. This process has been demonstrated as an early event of aortic valve calcification and fibrosis and is in many respects analogous with the development of atherosclerosis in coronary arterial walls (Thubrikar et al., 1985).

As described above, our fundamental hypothesis is that endothelial dysfunction is related to aortic valve calcification, in that endothelial factors (e.g. NO) can inhibit aortic valve calcification. In the present study, DETA-NONOate (in concentration from 1μM to 100μM) significantly inhibited TGF-β1 induced nodule formation. In addition, our results have shown that different doses of DETA-NONOate (1-100μM) had different effects on TGF-β1 induced calcification (figure 3.3-2A and 2B). However, the concentrations of NO released by different concentrations of DETA-NONOate are not known. Therefore, the precise NO dose-response relationship on TGF-β1 induced nodule formation remains uncertain. This makes it difficult to determine the relationship between the concentration-response curve of DETA-

NONOate for inhibition of nodule formation and the potential "endogenous" effects of NO, although it is clear that the concentrations of NO released from DETA-NONOate would considerably exceed endogenous release rates.

The biological effects of NO can be classified as those mediated by activation of soluble guanylyl cyclase (sGC) and those mediated "directly" by NO (sGC-independent effects) (Lucas et al., 2000). In general, sGC-independent effects of NO are mediated by S-nitrosation reactions of NO with metals, such as iron and copper (Stubauer et al., 1999; Rae et al., 1999).

In addition, nitrosation of proteins have been implicated widely as a signal transduction modality. A study has revealed that overexpression of thioredoxin (TRX) in endothelial cells resulted in an overall increase in intracellular S-nitrosation content in protein fractions that was reduced by inhibition with NOS inhibitors. In addition, Gu et al have shown that MMP-9 is activated by S-nitrosation in vitro utilizing a MMP-9 construct complete with the pro-peptide and catalytic domains but lacking the hemopexin domain to reduce interfering effects of tissue inhibitors of MMPs (Gu et al., 2002). Recently, it has been demonstrated that the NO/cGMP/PKG pathway interferes with the TGF-β/Smad2 signaling pathway in endothelial cells by mediating the proteasomal degradation of activated Smad (Saura et al., 2005).

In order to examine one possible mechanism(s) of NO inhibitory effects on TGF-β1 induced nodule formation, the analogue of the downstream product of NO, cGMP was utilized. In the present study, 8-Br-cGMP (1mM) significantly inhibited TGF-β1 induced nodule formation (Figure 3.3-3). These findings may suggest that NO inhibits TGF-β1 induced nodule formation possibly via the production of cGMP. However, cGMP is not only formed by stimulation of sGC by nitric oxide (NO) but also by particulate guanylyl cyclase (pGC) by natriuretic peptides (Lucas et al., 2000). Given cGMP inhibitory effects on nodule formation in the presence of TGF-β1, the implications of natriuretic peptides needs to be investigated.

Furthermore, in order to examine effects of endogenous NO release on TGF-β1 induced nodule formation in porcine aortic valve matrix cells, a non-selective nitric

oxide synthase inhibitor (L-NAME) was utilized. In preliminary studies in our laboratory, it has been shown that substance P (3nM)-evoked NO release in porcine aortic valve fibroblasts has been significantly inhibited by addition of L-NAME (data not shown). These findings suggested that endogenous NO can be produced by porcine aortic valve matrix cells, and thus can be inhibited by L-NAME (100μM) supplementation. In the present study, our results have shown that L-NAME (100μM) had almost no effect on TGF-β1 induced nodule formation (Figure 3.4-1). This certainly is insufficient to exclude a "physiological" interaction between NO and nodule formation. First, it would be required to investigate interactions between L-NAME and TGF-β1 under conditions of known submaximal stimulus towards nodule formation. Second, it is possible that the effects of NO on nodule formation may be exerted via stimulated, rather than basal NO release: For this purpose it would be appropriate to examine interactions between TGF-β1 (low concentrations) and L-NAME in the presence of a stimulator of NO release, such as substance P.

3.7.3 Oxidative stress associated with TGF-β1 induced nodule formation

In cell cultures, exposure of cardiac fibroblasts to superoxide anions stimulates their proliferation by increasing the production of TGF-β1 (Li et al., 1999). Additionally, TGF-β1 has been shown to induce an increase in NADPH-driven reactive oxygen species production in human cardiac fibroblasts and lung fibroblasts (Cucoranu et al., 2005; Thannickal et al., 1995). In the current experiments, incubations of cultured cells with TGF-β1 induced increases in intracellular superoxide anion release (Figure 3,6-2A). However, it was not clear from those experiments whether the increase in superoxide anion release was a "primary" effect of TGF-β1, or whether it might be a nonspecific effect observed in association with calcific nodule formation irrespective of the stimulus for nodule formation. Experiments of the effects of TGF-β1 might have partially clarified this issue. However, it was elected to utilize an approach of examining the impact of inhibitor of superoxide anion generation.

In order to evaluate the relationship between oxidative stress and TGF-β1 induced nodule formation, free superoxide scavenger (TEMPOL) and superoxide dismutase (SOD and PEG-SOD) were utilized in the cell culture. TEMPOL is an antioxidant that works as a superoxide dimutase mimic (Samuni et al., 1990), directly reacts with both

carbon-centered and peroxy radicals (chateauneuf and Tauber, 1987), and prevents the reduction of hydrogen peroxide to the hydroxyl radicals (Samuni et al., 1991). In addition, as shown in figure 3.7-1, PEG-SOD is superoxide dimutase, which has been shown to make superoxide anion convert to hydrogen peroxide (Koppenol et al., 1992). In our study, TEMPOL has been shown to inhibit inracellular superoxide anion and calcific nodule formation in the presence of TGF-β1 (Figure 3.5-1 and Figure 3.6-2B) and PEG-SOD inhibits calcific nodule formation in the presence of TGF-β1 (Figure 3.5-1). PEG-SOD with cell permeability had significant effects while SOD without cell permeability did not have sigibificant effect (Figure 3.5-1). These findings suggest that intracellular oxidative stress is associated with TGF-β1 induced nodule formation in aortic valve cell cultures. However, as shown in figure 3.7-1, superoxide anion are normally kept low due to conversion to hydrogen peroxide via superoxide dismutase, and hydrogen peroxide can subsequently be converted to water and oxygen via catalase (Koppenol et al., 1992). Our recent experiments have only shown that PEG-SOD and TEMPOL can inhibit calcific nodule formation induced by TGF-β1. The effects of hydrogen peroxide remain unknown and could be explored by experiments using PEG-catalase.

On the other hand, our results have demonstrated that NO decreased intracellular superoxide release in the presence of TGF-β1 and inhibited TGF-β1 induced calcific nodule formation, identified by in situ superoxide detection and blinded nodule counting (Figure 3.3-2 and Figure 3.6-2B). Currently the foremost mechanism for the loss of bioavailable NO is thought to be the reaction of NO with superoxide. In normal physiology superoxide is detoxified by the enzyme superoxide dismutase (SOD) to H_2O_2 and eventually water, therefore preventing its interaction with NO. However, if the levels of superoxide increase sufficiently, NO is able to outcompete SOD for superoxide (Figure 3.7-1). This reaction has the dual effect of scavenging NO and thereby reducing its availability, but also of producing the potent oxidant peroxynitrite (Koppenol et al., 1992). Our findings may suggest that NO inhibitory effects on TGF-β1 induced nodule formation may be due to reaction of NO with superoxide, but the definite pathway remains unclear.

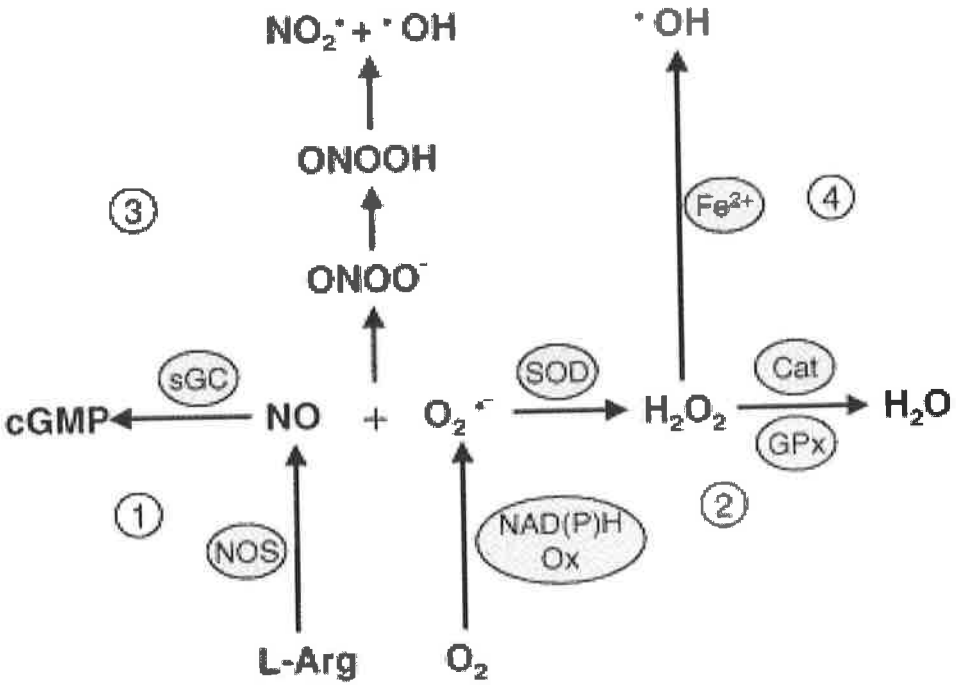


Figure 3.7-1. The formation of peroxynitrite by the reaction of nitric oxide and superoxide anions. Cells have the capacity to produce NO and superoxide, the reactants required for the production of peroxynitrite, in both extra- and intracellular compartments. Under normal physiological conditions, NO produced from l-arginine diffuses to its target soluble guanylyl cyclase (sGC) (1), while the levels of superoxide anions are kept low by conversion to hydrogen peroxide (H_2O_2) and then water (2). However, in the levels of superoxide anions increase such that the interaction with NO becomes the favoured reaction (3) leading to the production of peroxynitrite and the potent nitrogen dioxide and hydroxyl radicals. Under certain conditions hydroxyl radicals can been formed from hydrogen peroxide through the Fenton reaction (4). (Adapted from Naseem KM: The role of nitric oxide in cardiovascular diseases. Mol Aspects Med. 26(1-2): 33-65, 2005.)

3.7.4 Limitations of the experiments

Although our experiments have demonstrated that NO inhibited calcific nodule formation and in situ superoxide release in the presence of TGF-β1, there are still some limitations. Firstly, we only demonstrated that NO inhibits TGF-β1 induced calcific nodule formation and in situ superoxide release. However, it has been demonstrated that normal aortic valve endothelium can release nitric oxide (NO) and prostacyclin (PGI_2) (Pompilio et al., 1998). Furthermore, NO and PGI_2 have been demonstrated to be important in regulating vasorelaxation. In our experiments, we have only used NO donor in the aortic valve cell cultures, while the effect of PGI_2 on aortic valve calcification is still unknown. Secondly, the NO donor was directly added into the aortic valve cell culture. The normal aortic valve leaflets are composed of an outer layer of endothelial cells that cover interstitial mesenchymal cells located throughout the leaflet (Filip et al., 1986). In our experiments, NO has been shown to inhibit TGF-β1 induced calcification. However, in current experiments, NO was from NO donor, DETA-NONOate, which is not directly from physiological aortic valve endothelium. As a result, the interaction between aortic valve endothelium and matrix cells has not been investigated directly. Thirdly, although our experiments have shown that NO inhibited calcific nodule formation and in situ superoxide release in the presence of TGF-β1, further experiments are needed to clarify whether it is due to the formation of peroxynitrite via reaction with superoxide anion or the production of downstream product-cGMP. Last but not least, although we have shown that exogenous NO can inhibit calcification and in situ superoxide anion in porcine aortic valve matrix cells, the clinical relevance of NO donor use in the patients with aortic stenosis needs to be examined. In particular, it remains to be determined whether these effects of NO are relevant to the impact of valvular endothelial dysfunction and/or to a potential therapeutic role for NO donors in the prevention of AS.

CHAPTER 4

Conclusions

AND

Future studies

4.1 Conclusions

The major conclusions to be drawn from this series of experiments are:

(1) TGF-β1 stimulates the development of calcific nodules in porcine aortic valve matrix cells in culture and intracellular superoxide over the same time course, with maximal effect on passage 4 cells.

(2) The direct nitric oxide (NO) donor DETA-NONOate inhibits TGF-β1 effects in a concentration-dependent manner.

(3) DETA-NONOate also inhibits TGF-β1 induced increases in intracellular superoxide anion.

(4) The superoxide scavenger TEMPOL inhibits TGF-β1 induced nodule formation and intracellular superoxide anion and PEG-SOD reduces TGF-β1 induced nodule formation.

In conclusion, this is the first in vitro tissue culture model to demonstrate that NO can inhibit TGF-β1 induced calcification and in situ superoxide release in vitro.

4.2 Future studies

The main implications of these findings are multiple and each may lead to relevant future experiments, these are:

4.2.1 Role of TGF-β1

As in chapter 1, TGF-β1 has been shown to be a major stimulus for aortic valve calcification (Mohler et al., 1999; Jian et al., 2003). In our study, TGF-β1 was also demonstrated to stimulate calcification in aortic valve cell culture. However, the definite mechanism(s) of TGF-β1 induced calcification is unknown. To date, three discrete, high-affinity receptors for TGFβ, designated Ⅰ, Ⅱ, and Ⅲ have been described, although TGFβ only directly binds to the latter two. Serine-threonine

protein kinases in the intracellular domains of TGFß receptor I and II initiate signal transduction events, mainly by a sequential phosphorylation cascade of the Smad family of proteins (Attisano et al., 1994; Wrana et al., 1994; Ikedo et al., 2003; Goumans and Mummery, 2000; Piek et al., 1999; Zwijsen et al., 2000). As shown in figure 1.7-2 (TGF-β1 signaling pathways), ligand binding to Type II receptor leads to phosphorylation and activation of Type I receptor. Subsequently, the receptor-associated Smads 2 and/or 3 are phosphorylated by Type I receptor, and released from the hetero-oligomeric receptor complex. Phosphorylated Smad2 and/or 3 bind in a heterotrimeric complex with Smad4, which subsequently accumulates in the nucleus. In addition, Smad6 and Smad7 are inhibitory Smads that block Smad2/3 binding to Type I receptor, or Smad2/3 oligomerization with Smad4. Ultimately, in order to test TGF-β1 possible signaling pathways, this could be addressed in (a) animal models-eg with knockout of selective receptors of TGF-β1, and/or of generation of downstream effectors of TGF-β1 (e.g. Smad siRNA etc). (b) Human studies-eg with clinical availability of TGF-β1 antagonists.

In addition, it has been shown that TGF-β1 is modulated positively by other molecular factors (e.g. angiotensin II). Angiotensin II has been shown to stimulate the expression of TGF-β1 in cardiac myocytes and fibroblasts. TGF-β1 induces cardiac fibroblast proliferation and myocyte hypertrophy via autocrine/paracrine pathways, thereby mediating cardiac remodelling induced by angiotensin II (Wenzel et al., 2001; Gray et al., 1998; Kupfahl et al., 2000). Whether these events occur in aortic valve fibroblasts is unknown. However, it has been demonstrated that angiostensin converting enzyme (ACE) and TGF-β1 were significantly increased in the patients with aortic stenosis (Filitz et al., 2001). Ultimately, the interaction between angiotensin and TGF-β1 needs to be investigated in aortic valve cell cultures.

4.2.2 Pathophysiological modulation by NO

Aortic valve leaflets are composed of an outer layer of endothelial cells that cover interstitial mesenchymal cells located throughout the leaflet (Simon et al., 1993). It has been known that aortic valve and vascular endothelium can release NO (Scotland et al., 2002; Pompilio et al., 1998). Recently, it has been demonstrated that normal aortic valve endothelium can release nitric oxide (NO) and prostacyclin (PGI_2)

(Pompilio et al., 1998). NO is a critical modulator of blood flow and blood pressure (Johnstone et al., 1993). It is released by the endothelium in response to shear stress and plays an important role in flow-mediated vasodilation (Cooke and Dzau, 1991; Vallance et al., 1997). Prostacyclin (PGI_2) is also a potential endogenous vasodilator, its production is via cyclooxygenase and prostacyclin synthase. As a result, endothelium-dependent relaxations can be explained largely by endothelial release of NO and PGI_2 (Furchgott and Vanhoutte, 1989). On the other hand, it has been demonstrated that endothelial cells can produce endothelin (ET) that induces both constrictor and hypertrophic effects (Yanagisawa et al., 1988). Ultimately, the potential contribution of such endogenous factors to "calcfic"/"anti-calcific" events could be addressed: (i) Tissue culture model-eg Prostacyclin and endothelin effects on TGF-β1 induced aortic valve calcification. (ii) Measurement of endothelin and prostacyclin production released by aortic valve matrix cells and endothelial cells.
The potential that genesis of aortic stenosis may reflect inadequate NO release in response to shear stress is also of interest. Furthermore, aortic valve endothelium is different from vascular endothelium. Hydrodynamic studies have shown that there is flow disturbance at the aortic side of the aortic cusps. A high mechanical stress occurs at the flexion area of the aortic cusps near the attachment to the aorta root and the line of coaptation (Thubrikar et al., 1986). Aortic valve endothelium at areas of high mechanical stress demonstrates subtle changes consistent with mild damage and became more susceptible to lipid deposition and infiltration by macrophages. It has been postulated that this process is accelerated in bicuspid aortic valve because the abnormal cusps and raphe are more prone to greater mechanical stress (Davies et al., 1996). However, our tissue culture model does not replicate this physiological/pathological condition of aortic valve. Further studies need to be done to mimic these conditions.

In addition, in the current experiments, NO was from NO donor, DETA-NONOate, which is not directly from physiological aortic valve endothelium. It has been known that aortic valve leaflets are composed of an outer layer of endothelial cells that cover interstitial mesenchymal cells located throughout the leaflet (Simon et al., 1993). Ultimately, this could be addressed by co-culture of aortic valve endothelial cells and interstitial cells. This coculture model should be important in examining the pathogenesis of aortic valve stenosis.

4.2.3 NO-TGF-β1 interactions

In our study, our data suggest that superoxide anion release induced by TGF-β1 contributes to calcific nodule formation, and that NO can limit this process. However, the definite mechanism(s) of NO inhibitory effects remain unclear. Ultimately, two possible experiments could address these questions. (a) It has been known that the formation of cyclic GMP (cGMP) is only the first step the cascade of signalling mechanisms induced by NO. This process is via soluble guanylyl cyclase (sGC), then cGMP can induce the activation of a specific protein kinase, also known as protein kinase G (PKG), which leads to phosphorylation of serine or threonine residues on other proteins and thereby modify their activities (Koppenol et al., 1992). As a result, the tissue culture model with downstream effectors of NO (e.g. cGMP analogue and/or specific inhibitors of PKG phosphorylation etc) should be examined. (b) Our studies have shown that NO can inhibit TGF-β1 induced calcific nodule formation and intracellular superoxide release. NO has been demonstrated to react with superoxide anion to form peroxynitrite (Koppenol et al., 1992). Therefore, the effects of peroxynitrite on aortic valve calcification need to be examined. This could be tested in the cell culture model by measuring peroxynitrite concentrations or those of its produts, nitrotyrosine in cell culture.

4.2.4 Role of superoxide anion

Some pharmacological agents have been shown to prevent loss of NO in the development of atherosclerosis (e.g. statins/ACEI/organic nitrate). Given the similarities between atherosclerosis and aortic valve stenosis, these agents may be potential medical therapies for prevention of aortic valve stenosis. Further clinical and tissue culture studies needs to be performed. Even in coculture of aortic valve interstitial cells and endothelial cells, these active drugs (e.g. ACEI, statins etc) can be utilized to investigate whether they have direct effects on aortic valve calcification, independent of whole body effects on blood pressure, lipids etc. It must ultimately be emphasized that any cell culture "model" of aortic stenosis may bear only indirect relationships to the clinical disease state. From a clinical point of view, therefore, the current conclusions about the role of NO and superoxide anion in the development of calcific nodules should be regarded as "hypothesis-generating". The final form of experimentation to contemplate would therefore involve correlations between

interventions which might favourably alter availability of NO and clearance of superoxide anion (e.g. ACEI, statins etc) and effects on clinical progression of aortic stenosis.

CHAPTER 5

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CHAPTER 6

APPENDICES

6.1 Abbreviations

ACE	angiotensin converting enzyme
ACEI	angiotensin converting enzyme inhibitor
ADMA	asymmetric dimethylarginine
ALP	alkaline phosphatase
Ang I	angiotensin I
Ang II	angiotensin II
Apo B	apoliprotein B
AS	aortic valve stenosis
AT-1R	angotesin II receptor type one
BAV	bicuspid aortic valve
BSA	bovine serum albumin
cGMP	guanosine 3′,5′-cyclic monophosphate
DETA-NONOate	(Z)-1-[N- (2-aminoethyl)-N- (2-ammonioethyl) amio] diazen-1-ium-1, 2-diolate
DETCA	diethyldithiocarbamic acid
DHE	dihydroethidium
DMEM	dulbecco modified eagle media
EC	endothelial cells
EDTA	ethylenediamine tetra-acetic acid
eNOS	endothelial nitric oxide synthase
ET	endothelin
FCS	fetal calf serum
H_2O_2	hydrogen peroxide
IL	interleukin
LAP	latency-associated peptide
LDL	low density lipoprotein
L-NAME	Nω-nitro-L-argininie Methyl Ester
ml	millimetre
mM	millimoles
mRNA	messenger ribonucleic acid
MMPs	matrix metalloproteinases

MT-MMPs	membrane-type matrix metalloproteinases
MPC	magnetic particle concentrator
$NaHCO_3$	sodium hydrogen carbonate
NO	nitric oxide
NOS	nitric oxide synthase
OPG	osteoprotegerin
Ox-LDL	oxidized low density lipoprotein
PBS	phosphate-buffered saline
PEG-SOD	superoxide dismutase-polyethylene glycol
pGC	particulate guanylyl cyclase
PGE_2	prostaglandin E2
PGI_2	prostacyclin
PKG	protein kinase G
PRMT	protein arginine methyltransferase
RANKL	receptor activator of nuclear factor B ligand
RNA	ribonucleic acid
rpm	revolutions per minute
ROS	reactive oxygen species
SDMA	symmetric dimethylarginine
SE	standard errors
sGC	soluble guanylyl cyclase
SMC	smooth muscle cells
SOD	superoxide dismutase
TAV	tricuspid aortic valve
TEMPOL	2,2,6,6-tetramethyl-4-piperidinol-N-oxyl
TIMP	tissue inhibitors of metalloproteinases
TNF-α	tumor necrosis factor-α
TGF-β1	transforming growth factor-β1
TRX	thioredoxin
VDUP	vitamin D3–upregulated protein
μM	micromoles
8-Br-cGMP	8-bromo-guanosine 3′,5′-cyclic monophosphate

6.2 Instruments

Primary cell culture preparation

Instruments	Manufacturers
Sterile hood	Gelman Science, Australia
Optical microscope	Olympus, Japan
4K-15 laboratory centrifuge	Sigma, Germany
350 g, 4°C for aortic valve endothelium, 110 g, 4°C for aortic valve fibroblasts	
Waterbath	Paton Industries, Australia
IX-70 electronic microscope	Olympus, Japan
Cellstar 250mls tissue culture flask	Greiner Bio-one, Germany
Cellstar 50mls tissue culture flask	Greiner Bio-one, Germany
Sterile serological 10 ml pipette	Sarstedt, France
Cellstar PP-test sterile 50 ml tubes	Greiner Bio-one, Germany
STERI-CULT 200 incubator	Forma Scientific, USA
pH value meter	Orion Research, USA
Dynabeads CD31 Endothelial Cells	Dynal Biotech, Norway
Nuova stir plate	Thermolyne Coporation, USA

Media preparation

Glements ward suction unit	Glements, Australia
0.2µm syrindge driven filter unit	Pall Corporation, USA
Sterile hood	Gelman Science, Australia

Passaging aortic valve myofibroblasts

Cellstar 250mls tissue culture flask	Greiner Bio-one, Germany
Cellstar PP-test sterile 50 ml tubes	Greiner Bio-one, Germany
Sterile serological 10 ml pipette	Sarstedt, France
4K-15 laboratory centrifuge	Sigma, Germany
Tiefe 100mm counting chamber	Neubauer, Germany

Optical microscope	Olympus, Japan
Sterile hood	Gelman Science, Australia

Calcification assay

Six well sterile tissue culture plate	Becton Dickson, USA
0.2 μm syringe driven Filter Unit	Millex, Ireland
IX-70 electronic microscope	Olympus, Japan
STERI-CULT 200 incubator	Forma scientific, USA
Eppendoff pipette	Eppendoff, Germany
Vortex mixer	Ratek, Australia
Sterile hood	Gelman science, Australia
10ml medical syringe	Becton Dickson, Singapore

Dihydroethidium (DHE) Staining

Nikon T-300 Electronic telescope	Nikon, Japan
LAB-TEK chamber slide	Sybron, USA
Super high pressure mercury lamp	Nikon, Japan
CCD camera and camera control unit	Panasonic, Japan
HP-72 table PC	HP, USA
Video Pro 32 color image analysis system	Leading edge, Australia

6.3 Single cell staining of porcine aortic valve interstitial cells

Plating of the cells

For our experiments, ethanol was utilized to rinse both sides of coverslips and the coverslips were subsequently placed in 6-well plates, followed by sterilization under ultraviolet light for at least 15 minutes. After sterilization of pates, aortic valve interstitial cells were seeded in 6-well plates 7 days prior to staining.

Staining procedures

After incubation, the wells were subsequently washed with cold phosphate-buffered saline (PBS) to remove media and TGF-β1, followed by adding 1ml of 4% paraformaldehyde to each well. After incubation for 20 minutes at room temperature, paraformaldehyde was removed and cells were washed with cold PBS. Then 1ml 0.2% Triton-PBS was added into each well and cells were incubated for 10 minutes in –20°C. After incubation, the wells were treated with 0.05% Tween/3% BSA-PBS for 20 minutes at room temperature and subsequently washed with cold PBS.

Furthermore, the dilutions of mouse monoclonal anti –α smooth muscle actin (Sigma, Germany) and mouse monoclonal anti-vimentin (Sigma, Germany) were made up in advance (1:28 anti-actin, 1:100 anti-vimentin). After incubation with Tween/BSA-PBS, the antibody dilutions were added onto the coverslips and then the coverslips were incubated with antibodies for 1 hour at room temperature, followed by washing coverslips with cold PBS. The second biotinylated anti-mouse antibody solutions (1:500 dilution) were subsequently added onto the coverslips and the coverslips were washed with cold PBS. After these procedures, Avidin D fluorestein (1: 200 dilution) was added into the coverslips followed by incubation for 1 hour at room temperature. The coverslips were subsequently washed with cold PBS. After washing, the fine-tipped forceps were utilized to remove the coverlips and the coverslips were subsequently placed on microscope slides, followed by adding Dako fluorescent mounting medium into the microscope slides. The slides were then viewed under the fluorescent microscope (Nikon, Japan) and digitally photographed by a CCD camera (Nikon, Japan) mounted to the fluorescent microscope. The images were semi-

quantitatively analyzed using Video Pro 32 color image analysis software (Leading edge, Australia).

Note:

1. Control of PBS/BSA (test of autofluorescence).
2. Nonspecific binding of the first antibody was tested by using control isotype matched mouse IgG at the same dilution as the first antibody.

Alizarin Red-S Staining for Calcification

Porcine aortic valve interstitial cells were plated in triplicate at a density of 500000 cells in tissue culture plates and placed into a 37-degree incubator. Once cells have become 95% confluent, TGF-β1 (5ng/ml) was added, and the cultures were incubated until definite nodule had formed. The medium was then removed, and the culture was gently rinsed with phosphate-buffered saline (PBS) and fixed for 10 minutes in 10% formalin. After fixation, the cultures were rinsed with 10% formalin and stained for calcium with 2% Alizarin Red-S for 10 minutes at room temperature. After staining, the calcific nodules stained by Alizarin Red-S were detected by an electronic microscope (Olympus, Japan) and photographed by using a camera (Olympus, Japan).

6.4 Concentration response curve for TGF-β1 induced nodule formation.

The following concentration response curve was generated by Dr Kumaril Mishra (Cardiology Unit, The Queen Elizabeth Hospital, South Australia).

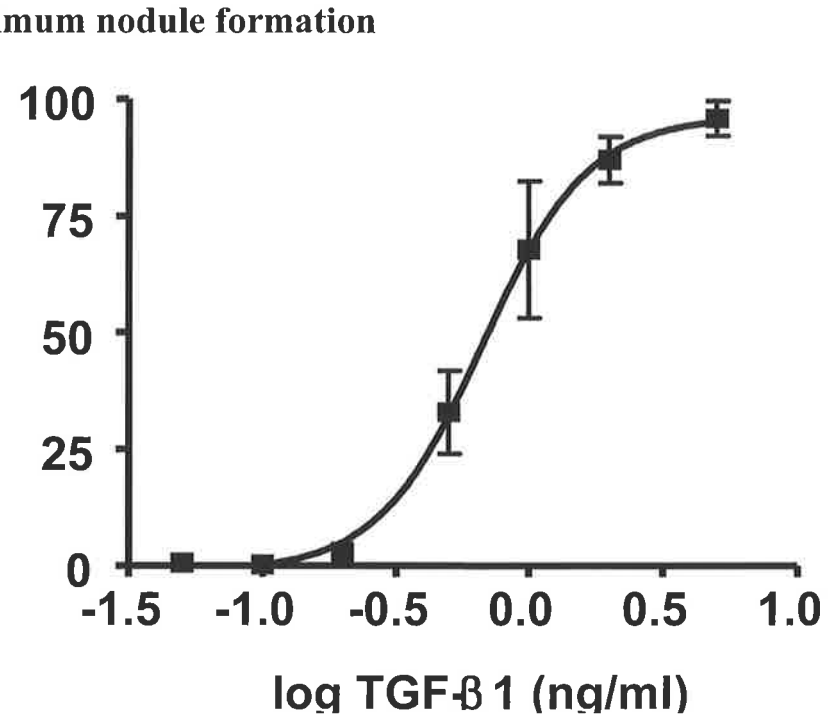

Figure 6.1 Effects of increasing concentrations of TGF-β1 on % maximum nodule formation in porcine aortic valve interstitial cells. Data are shown as mean and standard error from 4 cultures.