Valvular endothelial cells and the mechanoregulation of valvular pathology

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Endothelial cells are critical mediators of haemodynamic forces and as such are important foci for initiation of vascular pathology. Valvular leaflets are also lined with endothelial cells, though a similar role in mechanosensing has not been demonstrated. Recent evidence has shown that valvular endothelial cells respond morphologically to shear stress, and several studies have implicated valvular endothelial dysfunction in the pathogenesis of disease. This review seeks to combine what is known about vascular and valvular haemodynamics, endothelial response to mechanical stimuli and the pathogenesis of valvular diseases to form a hypothesis as to how mechanical stimuli can initiate valvular endothelial dysfunction and disease progression. From this analysis, it appears that inflow surface-related bacterial/thrombotic vegetative endocarditis is a high shear-driven endothelial denudation phenomenon, while the outflow surface with its related calcific/atherosclerotic degeneration is a low/oscillatory shear-driven endothelial activation phenomenon. Further understanding of these mechanisms may help lead to earlier diagnostic tools and therapeutic strategies.

Keywords: shear stress; strain; gene; calcification; vegetations

1. INTRODUCTION

Heart valve disease is a significant contributor to cardiovascular deficiencies and a strong predictor of mortality (Trenouth *et al.* 1976; Schwarz *et al.* 1979; Lund *et al.* 1997; Mehta *et al.* 2001). Valvular disease in Western nations is predominantly of a degenerative (senile, calcific) nature (Passik *et al.* 1987; Dare *et al.* 1993), while in developing nations, rheumatic/bacterial heart disease dominates (Akinkugbe *et al.* 1991; da Silva & Pereira 1997). In addition, congenital heart defects, which affect 1% of all live births, can result in predispositions to these pathologies. Unfortunately, most valvular pathologies are diagnosed by echocardiography, which identifies structural deficiencies at an advanced state of disease progression (Baxley 1994; Heper & Yorukoglu 2002), often requiring surgical valve replacement with a non-living substitute valve. These procedures have greatly enhanced the length and quality of life in these patients, but patients eventually suffer from similar degenerative (calcific) and/or endocarditis (bacterial/thrombotic) events (Borkon *et al.* 1987; Oxenham *et al.* 2003).

Advances in vascular biology have contributed greatly to the discovery of vascular disease pathogenesis, but similar exploration of valvular pathology has lagged far behind. Research over the last 30 years has demonstrated that the most prevalent vascular disease, atherosclerosis, is initiated at specific regions of

the vasculature identified by certain haemodynamic patterns (Baxley 1994; Heper & Yorukoglu 2002). Heart valves, particularly the aortic valve, experience the greatest and most complex stresses within the cardiovascular system, and we are now beginning only to appreciate the complex interactions between the native valvular cells that enable the leaflets to open and close 100 000 times a day for a lifetime (Schneider & Deck 1981; Deck *et al.* 1988).

Recent evidence is beginning to suggest that there may be very important roles for mechanics in the stimulation of valvular diseases. Certain valvular lesions develop preferentially on one side of the leaflet or the other, and these regions are exposed to vastly different mechanical forces. Endothelial cells line all blood contacting surfaces, including heart valve leaflets and large arteries. Endothelial cells have the ability to sense and respond to different mechanical and haemodynamic environments, and the ability to rapidly alter their behaviour with changes in these stimuli. Recent evidence has implicated systemic endothelial dysfunction in the initiation of pathology (Poggianti *et al.* 2003), but to date there has been little mechanistic connection to the haemodynamic environment. Some of our recent *in vitro* data suggest that valvular endothelial cells are in fact distinct from vascular endothelial cells in their response to haemodynamics, which suggests that these cells are uniquely designed to function on valvular leaflets (Butcher *et al.* 2004). It is therefore probable that local haemodynamics plays an important role in valvular endothelial biology and dysfunction.

The purpose of this review therefore is to combine what is known about valvular and vascular mechanics with the knowledge of valvular disease pathogenesis,

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paying particular attention to the role of valvular endothelium, and formulate a hypothesis as to how mechanics and haemodynamics could be acting on the valvular endothelium to both maintain normal function and initiate dysfunction. The review will conclude with an assessment of how this insight can be applied to future diagnostic and therapeutic agents.

2. DIFFERENCES IN HAEMODYNAMIC ENVIRONMENT OF HEART VALVES AND LARGE ARTERIES

The blood flow patterns in the vasculature are determined by the complex interrelationship between cardiac function, the viscous nature of blood, the mechanical properties of the vessel walls and local geometry. Flow in the ascending aorta is just barely laminar (mean Reynolds number ~ 2000 , peak ~ 4500) and highly pulsatile (Womersley number ~ 15), but both these numbers gradually decrease further down the aorta (Caro *et al.* 1974).

Wall shear stress, considered the most important haemodynamic parameter for endothelial cells, defined as the gradient of flow velocity multiplied by viscosity, can be determined analytically for a Newtonian fluid and straight tubular arteries from the following equation:

$$\tau_w = \frac{4\mu Q}{\pi R^3}.$$

Here, Q is the volumetric flow rate, μ the viscosity, and R the vessel radius (Poiseuille flow). The mean wall shear stress in a large artery is believed to be within a remarkably small range (10–20 dynes cm^{-2}). Actual wall shear stresses are much more difficult to measure, especially at the level of the valve. Local changes in geometry can dramatically increase or reduce shear stresses, notably apparent at stenoses (increase) and bifurcations (reduce). Shear stresses on the surface of valve leaflets are much harder to measure. Reports of peak wall shear stress vary from 20 dynes cm^{-2} to over 1000 dynes cm^{-2} (Walburn & Stein 1984; Woo & Yoganathan 1985; Nandy & Tarbell 1988; Einav *et al.* 1990; Weston *et al.* 1999). Part of the reason for the differences in these measurements can be explained by the differences in how far downstream of the valve the measurements were made, what type of flow in which the valve was functioning and whether the valve was natural or bioprosthetic. It is known that haemolysis occurs at shear stresses in excess of approximately 400 dynes cm^{-2} (Paul *et al.* 2003), and conservative analytical solutions in steady flow suggest a lower bound of 20 dynes cm^{-2} for peak shear stress. Therefore, it is probable that the peak shear stress on the inflow surface of valve leaflets are in the range of 100–200 dynes cm^{-2} . The inflow surface experiences these extremely high shear stresses only for a very brief period of the cardiac cycle, and it is unclear how significant this peak in shear stress is. If the blood flow velocity through the valve is averaged across the cardiac cycle (aka steady flow), then the resulting shear stress is much less, approximately 20 dynes cm^{-2} (Weston *et al.* 1999). It is important to note that the velocity gradients are very different between the two sides of a leaflet. While the inflow surface experiences a strong pulsatile unidirectional shear stress, the outflow surface

experiences a much lower recirculating shear stress. The exact measurement of these shear stress profiles will require more advanced measurement techniques and rigorous fluid–structure coupled computational models, the foundation of which has already been laid (Aluri & Chandran 2001; Leuprecht *et al.* 2002).

In addition to the wall shear stress, there are other components to the mechanical environment of the valve leaflet. These include normal pressure stress and mostly planar tissue strains. Ventricular and aortic pressure waves are different in that the aorta remains pressurized at all times, while ventricular pressure reduces to near zero in diastole. This creates different pressure profiles for valvular leaflets and large arteries. While transaortic pressures range from 80 to 120 mm Hg, transvalvular pressure ranges from approximately 0 to 80 mm Hg. Maximum pressure stress in the aorta occurs in systole, whereas in leaflets it is during diastole, making tissue strains out of phase between these two locations. Strain in both tissues is a function of both this pressure pulse and the motions of adjacent structures. Measurements of the motions of aortic segments *in vivo* have determined that vascular tissues experience pulsatile circumferential strains of the order of 10%, with minimal axial and radial strains (Holzapfel & Weizsacker 1998; Wedding *et al.* 2002; Draney *et al.* 2004; Stalhand *et al.* 2004). Valvular leaflets, in contrast, are strained in a more complicated biaxial pattern. Peak circumferential strains are approximately 10%, while radial strains are as high as 40% (Thubrikar *et al.* 1980, 1986a). These strains are also imposed at very high rates (200% s^{-1}) in valvular leaflets (Brewer *et al.* 1977). In addition to planar strains, leaflets are also bent to very high radii of curvature during opening, which results in an additional compressive strain in the fibrosa and tensile strain in the ventricularis (Smith *et al.* 2000). Thus, the endothelium on the two sides of the leaflet experiences a very different general strain pattern. It is also known that both valvular and vascular tissues have residual strains in unloaded conditions, both of the order of 10% (Vaishnav & Vossoughi 1987; Vesely *et al.* 1993). It is not yet known whether these residual tissue strains correspond to states of strain in the cells. The surface strains on both arteries and valve leaflets are not homogeneous, but vary between leaflet and around the aortic circumference (Dagum *et al.* 1999). Indeed, local changes in geometry will again have an important effect on the magnitude of strains felt by the endothelial cells. Thubrikar and colleagues also demonstrated that the aortic sinus plays an important role in shielding stress accumulation in valve leaflets and will also have different effects on the ventricularis and fibrosa (Thubrikar *et al.* 1986b).

3. RESPONSE OF ENDOTHELIAL CELLS TO MECHANICAL ENVIRONMENT

(a) Morphology

Endothelial cell shape varies spatially throughout the vascular tree, even between adjacent cells, and much work over the last 30 years has demonstrated a strong correlation between endothelial cell shape and the mechanical environment. *In vivo*, vascular endothelial

cells resemble elongated ellipsoids, with the long axis of the cells aligned parallel to the direction of flow (Wong *et al.* 1983). At sites of bifurcations and high curvatures, vascular endothelial cells appear to be larger and more rounded (Langille & Adamson 1981; Kim *et al.* 1989). *In vitro* experimentation has shown a remarkable consistency of endothelial shape change to different patterns of shear stress for a variety of arterial and venous locations, while both pulsatile and steady laminar shear stresses result in elongated highly aligned cells. For a purely oscillatory or turbulent shear stress, the monolayer is composed of rounded cell bodies with no discernable alignment (Helmlinger *et al.* 1991). In relation to underlying cells and matrix, vascular endothelial alignment is parallel to underlying matrix orientation, but switches to parallel to the flow direction once shear stress is applied regardless of matrix orientation (Imberti *et al.* 2002).

Scanning electron microscopy of valvular endothelial cell alignment shows, however, a remarkably different pattern. Valvular endothelial cells appear to be aligned with their long axis parallel to the circumferentially aligned underlying tissue, and thus perpendicular to the direction of flow (Deck 1986). Both the ventricular and aortic surfaces are aligned in this manner, but in some cases, the middle portion of the aortic surface contains rounded cells without discernable alignment (Inai *et al.* 2004). Recent *in vitro* evidence indicates that valvular endothelial cells align perpendicular to flow even without the presence of an aligned substrate (Butcher & Nerem 2006). Similar to vascular endothelial cells, this response was dependent on changes in cytoskeletal actin filaments and resulted in prominent stress fibres along the long axis of the cell (Butcher *et al.* 2004).

Endothelial cells also respond morphologically to substrate strain patterns. *In vitro* experiments have shown that vascular endothelial cells align with their major axis perpendicular to the principal axis of stretch (Terracio *et al.* 1988; Sipkema *et al.* 2003; Moretti *et al.* 2004). Equibiaxial strain of vascular endothelial cells results in a rounded endothelial cell shape with a tent-like cytoskeletal actin structure (Gorfien *et al.* 1990). To date, no *in vitro* data exist on the morphological response of valvular endothelial cells to substrate strain.

Interestingly, the combination of tissue strain and fluid shear stress may result in the endothelial cell morphologies observed *in vivo*. Since blood vessels experience mainly a circumferential tissue strain, vascular endothelial cells would align perpendicular to this stimulus, which is also parallel to the direction of blood flow. In valvular endothelial cells, however, the principal strain direction is radial, and cells would align perpendicular to this, or circumferentially. This alignment would be perpendicular to the direction of flow. The *in vivo* situation is much more complex than this, with matrix strains and fluid shear stresses often out of phase with each other. While almost all *in vitro* endothelial cell morphology studies involved only one mechanical stimulus, the interplay between strain and shear stress in endothelial biology may be as important as their effects individually (Wang *et al.* 2001), and the fact that this interplay may unify the seemingly divergent morphological responses seen by these two different types of endothelial cells warrants further study.

(b) Gene and protein expression

In addition to morphological changes, endothelial cells respond to both mechanical strain and fluid shear stress by the modulation of gene and protein expression. The regulation of gene and protein expression in endothelial cells is dependent on the type, magnitude and duration of mechanical stimulus and, to a lesser extent, the vascular location of the cells of interest. The now widespread use of microarray technology has caused a dramatic increase in information in this field. While the expression of thousands of genes can be assayed at once, the differences in microarray platform, cell types, mechanical stimuli, duration and statistical analysis make integration of this information truly difficult. Endothelial cells modulate many genes and proteins in reacting to a change from residing in a static culture environment to one with flow, and these transient expression changes may be of little relevance to *in vivo* endothelial biology. Therefore, only phenotype modulation to a more 'chronic' duration of shear stress will be discussed in this review. Using morphology as an indicator, vascular endothelial alignment is largely completed by 24 h, while valvular endothelial alignment is completed by 48 h. An analysis of several microarray studies using vascular endothelial cells isolated from different regions reveals many genes modified by similar mechanical stimuli. Those genes for which confirmational data (Western, RT-PCR) exist are summarized in table 1. It appears that the effects of pulsatile and steady laminar shear are similar in terms of genes changed, while oscillatory and disturbed (turbulent) flows modulate gene expression similarly. Cyclic stretch, however, modulates some genes in a manner similar to unidirectional flow and others like disturbed flow. Unidirectional shear stress downregulates adhesion proteins (VCAM-1 and PECAM-1) and chemokines (IL-1 β and IL-8) and upregulates redox proteins (CYP1A1, CYP1B1 and Cu/ZN-SOD) and growth factors (TGF β and VEGF). Oscillatory or turbulent shear stress has the opposite effect, upregulating adhesion proteins and chemokines. Steady and pulsatile shear stresses therefore seem to promote an atheroprotective phenotype, while oscillatory or turbulent shear stress promotes an atherogenic phenotype. However, the effects of mechanical strain are much more complicated, indicating both atheroprotective- and atherogenic-like responses.

To begin to ascertain similarities and differences between vascular and valvular endothelial cell response to shear stress, we performed a cDNA microarray analysis of cultured porcine aortic and aortic valve endothelial cells exposed to 20 dynes cm⁻² steady laminar shear stress for 48 h, with static cultures serving as controls (figure 1). This shear stress level was determined based on the mean shear stress across the aortic valve averaged across the cardiac cycle as determined by Weston *et al.* (1999).

Based on our morphological studies, valvular and vascular endothelial cells complete their morphological alignment to flow by 48 h, and thus the gene profile is more likely to represent a more 'chronic' shear response (Butcher *et al.* 2004). Although not perfectly mimicking the native wall shear stress flow condition, this profile can serve as a reasonable approximation.

Table 1. Endothelial protein expression in response to chronic shear stress profiles. (see table 3 in the electronic supplementary material for references and full gene names)

category	steady	pulsatile	oscillatory	turbulent	strain
<i>adhesion</i>					
ICAM-1	down	down	up	up	up
VCAM-1	down	down	up	up	up
E-selectin	down	?	up	up	up
MCP-1	down	down	up	?	up
PECAM (CD31)	up	up	?	?	?
<i>coagulation</i>					
tissue factor	down	down	up	?	up
t-PA	up	up	?	n.s.	up
thrombomodulin	down	down	n.s.	down	
PAI-1	down	down	?	n.s.	up
TF pathway inhibitor	up	up	down	?	?
TSP-1	down	down	up	up	up
<i>redox</i>					
CYP1A1	up	up	?	?	up
CYP1B1	up	up	?	?	up
Cu/Zn-SOD	up	up	n.s.	n.s.	?
NADPH	n.s.	up	up	?	up
<i>growth</i>					
bFGF	n.s.	n.s.	n.s.	n.s.	n.s.
TGF β 1	up	up	up	up	?
VEGF	up	up	down	?	up
<i>vasoactivity</i>					
eNOS	up	up	n.s.	n.s.	up
ET-1	down	down	up	down	up
prostacyclin	n.s.	n.s.	?	?	up
<i>extracellular matrix</i>					
BMP-4	down	down	up	?	?
MMP-9	n.s.	up	up	?	up
collagen IV	up	up			up
MMP-2	n.s.	up	?	?	up
<i>chemokine</i>					
IL-8	down	down	up	up	up
IL-13	down	down	up	up	up
<i>other</i>					
Kruppel-like lung factor	up	up	?	?	?
Cxn 43	up	up	down	down	up
CxN 37	up	up	down	down	n.s.

The microarray results were analysed statistically (Butcher *et al.* 2004) and clustered according to similarity in expression patterns. Some results of this analysis are shown in figure 2. Eleven different groups of genes were found to have similar expression levels across the shear stress and cell type conditions. Surprisingly, when the individual arrays were clustered according to similar gene expression profiles, all of the arrays representing a particular condition were grouped together, suggesting a repeatable and unique gene expression profile. It appears that shear stress condition was more of a distinguishing factor than cell type, but several expression groups show potentially important differences in regulation. The expressions of several genes regulated by shear stress for both vascular and valvular endothelial cells are given in table 2.

(c) *Mechanotransduction*

The responses of endothelial cells to different mechanical stimuli (shear stress, strain and combinations) are

Figure 1. Valvular and vascular endothelial cell morphology in static and fluid flow environments. Insets depict F-actin filament organization (light grey) counterstained for cell nuclei (dark grey) at 40 $\times$. Phase contrast images at 20 $\times$ magnification. PAEC, porcine aortic endothelial cells; PAVEC, porcine aortic valve endothelial cells.

Figure 2. Heatmap rendering of the clustered microarray data of valvular and vascular endothelial cells in static and fluid flow environments. Eleven different groups with unique expression profiles are identified A–K. Normalized log-transformed mean values for each cluster are plotted across conditions.

Table 2. Gene expression in valvular and vascular endothelial cells in response to steady laminar shear stress. (U, upregulation; D, downregulation; s.d., standard deviation)

gene	aortic valve EC			aortic EC		
	fold	s.d.	<i>p</i> value	fold	s.d.	<i>p</i> value
<i>VCAM-1</i>	D 1.25	0.18	0.003	D 1.06	0.15	0.732
<i>Cu/Zn SOD</i>	U 1.70	0.17	0.007	U 1.44	0.14	<0.001
<i>CYP1A1</i>	U 8.38	1.27	<0.001	U 9.33	2.87	<0.001
<i>eNOS</i>	U 1.18	0.22	0.06	U 1.14	0.18	0.162
<i>Cx36</i>	D 1.25	0.09	0.07	D 1.29	0.12	0.17
<i>BMP-4</i>	D 2.05	0.02	<0.001	D 2.07	0.91	<0.001

the results of complex signal cascades that initiate within the first seconds of stimulations. While mechanotransduction pathways are numerous and probably redundant, specific endothelial responses to shear stress can be traced to specific pathways. Endothelial cells can sense shear stress through several ways: phosphorylation of receptors that are recruited and/or clustered on the flow surface (Takahashi *et al.* 1997); changes in focal adhesion organization on the basal surface (Shyy & Chien 2002); and by changes in local strain patterns within individual cells (Helmke *et al.* 2001). While numerous reviews exist detailing vascular endothelial mechanotransduction to shear stress, it is unclear whether these pathways are similarly followed in valvular endothelial cells. We used focal adhesion arrangement and morphological alignment as an

indicator of response, and compared the response of valvular and vascular endothelial cells with shear stress in the presence of inhibitors to prominent signal pathways (Butcher *et al.* 2004). Both valvular and vascular endothelial morphological alignment is preceded by reorganization of focal adhesion proteins, including β 1 integrin, vinculin and focal adhesion kinase (figure 3, arrows), yet the pattern of reorganization is somewhat different between the two types of cells. Plaques in both cells are localized prominently at the ends of the long axis of the cells, but this orientation is parallel to flow in vascular endothelium and perpendicular in valvular endothelial cells.

We found that morphological alignment is dependent on the action of Rho-kinase, phosphoinositide-3 kinase (PI-3 kinase) and calpain in vascular endothelial

Figure 3. Mechanotransduction differences between valvular and vascular endothelial cells. Valvular endothelial cells align perpendicular to flow independent of PI-3 kinase, which is required for vascular endothelial cell alignment. Alignment of both endothelial cells is dependent on focal adhesion rearrangement. Modified from Butcher *et al.* (2004).

cells. Valvular endothelial cells require Rho-kinase and calpain, but not PI-3 kinase to align perpendicular to the direction of flow (figure 3). Disruption of the aforementioned pathways also inhibited focal adhesion reorganization. These results suggest that valvular endothelial cells share some, but not all mechanotransduction pathways with vascular endothelial cells. Other researchers have shown the importance of Rho-kinase in mediating vascular endothelial cell polarization, but not PI 3-kinase (Wojciak-Stothard & Ridley 2003). PI 3-kinase, on the other hand, is critical to the production of nitric oxide (Zeng & Quon 1996; Wojciak-Stothard & Ridley 2003). Calpain is a cysteine protease important for the breakdown and reorganization of focal adhesion proteins, particularly focal adhesion kinase.

4. PHYSIOLOGICAL ROLES OF VALVULAR ENDOTHELIAL CELLS

Endothelial cells perform many functions critical for blood and tissue homeostasis. Since all blood vessels and heart valves are lined with a surface layer of endothelial cells, it is probable that several functions are conserved regardless of location along the vascular tree. Physiological roles of endothelial cells can be broadly separated into three categories: regulation of coagulation; regulation of underlying cells/tissue; and transmittance/clearance of agents from the blood. Each of these topics will be discussed in context of valvular endothelial cell function.

(a) Regulation of coagulation

Endothelial cells are the primary means of controlling coagulative responses to ensure free-flowing blood in normal function and clotting only during injury. The coagulation regulation pathway is truly complex, with a myriad of factors, catalysts and antagonists, allowing clotting kinetics to be precisely regulated. Both valvular and vascular endothelial cells express von Willebrand factor (vWF; Manduteanu *et al.* 1988), secrete tissue

factor (TF; Drake & Pang 1989) and seem to modulate expression of coagulative factors in response to shear stress (table 2). Valvular endothelial cells, like vascular EC, are normally non-thrombogenic, but secrete TF when stimulated by inflammatory cytokines or lipopolysaccharide. Valvular interstitial cells, on the other hand, express high levels of TF constitutively (Drake & Pang 1989). It is not clear whether important differences exist in the coagulative states of valvular and vascular cells *in vivo*, but a study by Welters *et al.* (1998) showed that some coagulation factors are upregulated during coronary artery bypass graft surgeries compared with during aortic valve repair, suggesting that ECs from these vessels are in a more hypercoagulative state. Platelet adhesion is enhanced at higher shear rates, and vWF has been shown to unfold in the presence of high shear stress, promoting cleavage by ADAMTS13 and subsequent adhesion of platelets and fibrin (Tsai 2003). As discussed earlier, the inflow surfaces of valve leaflets, particularly the aortic valve, experience very high shear stresses, which may enhance coagulation if there is endothelial denudation. Valvular endothelial cells may be conditioned for survival in a higher shear environment *in vivo* in comparison with vascular endothelial cells, and may therefore resist denudation and the secretion of coagulative factors.

(b) Regulation of underlying cells

Both valvular and vascular endothelial cells have a basal lamina separating them from underlying cells (Manduteanu *et al.* 1988), but the composition of the underlying cells is very different between the two tissues. Much is known about vascular medial wall cells, which are almost exclusively smooth muscle in nature in arteries, expressing contractile proteins such as α -smooth muscle actin, calponin and myosin heavy chain (Stegemann & Nerem 2003; Stegemann *et al.* 2004). Valvular interstitial cells, on the other hand, are much more fibroblastic in nature, expressing only a small amount of these markers in a subendothelial

section of the ventricularis *in vivo* (Cimini *et al.* 2003). The subendothelial matrix composition is also different between vascular and valvular tissues. While vascular tissues contain a lamellar network of circumferentially aligned elastin and smooth muscle cells for much of the medial wall, valvular tissues contain a somewhat lamellar elastin–myofibroblast network, but only on the ventricularis. The fibrosa contains large collagenous bundles that are mostly circumferentially aligned (Vesely 1998). It is well known that the vascular endothelium regulates blood vessel lumen size by secretion of vasoactive agents such as nitric oxide (dilator) and endothelin (constrictor). Studies using fistulas and vein grafts have documented the ability of vascular endothelial cells to sense fluid flow and secrete agents in order to maintain a specific range of wall shear stress (Cambria *et al.* 1994). A similar role for valvular endothelial cells has yet to be demonstrated *in vivo*. Valvular endothelial cells and excised leaflets respond to vasoactive agents by alterations in calcium currents and the production of prostaglandin (Laskey *et al.* 1994) and can contract and dilate *in vitro* (Pompilio *et al.* 1998; Bowen *et al.* 2004). These contractions can be inhibited by blocking specific agonist receptors, suggesting innate capability and vasoactive feedback mechanisms. Interstitial cells have been shown to contract and relax in culture when stimulated with these agents (Messier *et al.* 1994) *in vitro*, suggesting that valvular interstitium has the capability to modulate tissue dimensions. Different regions of the aortic valve are more responsive to these agents than others, suggesting the potential for a complex signal regulation. However, a connection to mechanical or fluid dynamic stimuli is still controversial. *In vitro* experiments similar to those conducted with vascular endothelial cells may help answer this question.

In addition to vasoactivity, vascular endothelial cells have been shown to promote a quiescent smooth muscle cell phenotype by inhibiting cell proliferation and matrix synthesis, and maintenance of smooth muscle markers. *In vitro* and *in vivo* evidence has demonstrated that this regulation is optimized when endothelial cells are subjected to unidirectional laminar shear stress. Conversely, valvular interstitial cells are considered quiescent when not expressing smooth muscle markers (Rabkin *et al.* 2001) and evidence suggests that matrix synthesis and remodelling occurs at an increased constitutive rate in aortic valve leaflets (Schneider & Deck 1981). Valvular endothelial denudation does stimulate interstitial cell proliferation in organ cultures similar to what is seen in vascular endothelial denudation (Lester *et al.* 1992). We recently conducted a study assessing the effects of steady laminar shear stress in valvular endothelial–interstitial cell interactions in a tissue-engineered valvular leaflet model (Butcher & Nerem 2006). We report that valvular endothelial cells inhibit interstitial cell proliferation, while increasing matrix synthesis, and that these effects are enhanced with shear stress. These data suggest that mechanical factors play a role in the valvular endothelial cell regulation of interstitial cell biology.

(c) *Transmittance/clearance of agents from the blood*

In addition to coagulative factors, nutrients and molecular stimulants circulate within the blood, and another major role of endothelial cells is the interpretation and response to these agents. Experiments have determined that the diffusion limits through tissues is approximately 250 µm, which is within the range of small blood vessels and most of the valve leaflet. It appears that hypoxic conditions stimulate angiogenesis in both vessels and leaflets, but almost no blood vessels are present in normal valve leaflets (Tompkins *et al.* 1989). Endothelial cells are also responsible for clearing circulating lipids from the bloodstream, namely low-density lipoproteins (LDL). Experiments by Tompkins *et al.* demonstrated that valvular endothelium has an increased permeability than vascular endothelium, but their permeability to LDL seems to be much lower, suggesting that LDL concentration in aortic valve leaflets is near saturated (Tompkins *et al.* 1989). These results suggest that valvular endothelial cells may participate differently than vascular endothelial cells in these processes.

5. MECHANICAL BASIS FOR VALVULAR PATHOLOGY

The primary concern for researchers studying endothelial biology is to determine mechanisms of and treatment for pathologies affecting these tissues. Atherosclerosis and the associated coronary artery disease is the leading cause of death in the Western world (AHA 2001). Research over the last 30 years has determined that atherosclerosis is an inflammatory disease that is localized to regions of turbulent and/or oscillatory shear stress, namely arterial branches, inner curvatures and sinuses. *In vitro* research has implicated endothelial dysfunction as a principal initiator. Increased oxidative stress stimulates endothelial activation characterized by the expression of monocyte adhesion receptors (VCAM-1, ICAM-1 and E-selectin), and this results in the recruitment of circulating monocytes and leukocytes. These cells then cross the endothelial monolayer, transdifferentiate into macrophages and foam cells and attempt to ingest accumulating lipids. These cells also secrete cytokines that stimulate smooth muscle cell differentiation, matrix synthesis and proliferation. Advanced lesions are also associated with necrotic cell cores, angiogenesis and unstable fibrous caps that can spontaneously rupture, leading to thromboemboli and local tissue infarction. Vascular calcification is also correlated with atherosclerosis, especially in older patients (Allison & Wright 2005) and patients with hypercalcaemia or hyperphosphataemia (Nishizawa *et al.* 2004).

Valvular pathology can be primarily separated into two classes: sclerotic degeneration and vegetative endocarditis. Sclerotic degeneration bears a striking similarity to atherosclerosis. Valvular endothelial cells in diseased valves appear activated, with increased expression of VCAM-1, ICAM-1 and E-selectin (Müller *et al.* 2000). Infiltration of foam cells also appears to be concomitant with endothelial defenestration (Sarphie 1985). Vegetative endocarditis is somewhat unique to valvular leaflets

Figure 4. Schematic illustrating the potential roles for mechanical forces in the initiation of valvular pathologies. The inflow surface experiences high-magnitude pulsatile shear stresses and is the predominant location for thrombotic/bacterial endocarditis (A–G). The outflow surface experiences much lower, oscillatory shear stresses and is the predominant location for sclerotic/calcific lesion formation (1–6).

and can be either bacterial or non-bacterial in nature. Generally, endothelial dysfunction and focal defenestration causes upregulation of coagulation factors, which stimulates platelet adhesion and synthesis of fibrin. Circulating bacteria can preferentially adhere to these regions (Allen *et al.* 2002) and proliferate under the protection of additional clot formation (Sullam *et al.* 1985).

What has not been investigated mechanistically is the role of mechanical forces in stimulating these events in valvular tissue. Since mechanical factors are strong initiators of the inflammatory cascade in vascular endothelium, it is probable that valvular endothelial cells can also be stimulated by abnormal haemodynamics and tissue mechanics. Based on the differences in haemodynamic environment between the surfaces of the valve leaflet and observations of pathogenesis on these surfaces, the following hypothesis is proposed for the role of mechanical forces in the development of valvular pathology. This hypothesis is discussed below and diagrammed in figure 4 and split into two portions: inflow and outflow surfaces. Although the aortic valve will be considered as the model tissue for simplicity, much of the pathology is observed similarly in the mitral valve.

(a) *Inflow surface and endocarditis*

Investigation of endocarditis on valve leaflets shows a strong preference to the inflow surfaces of valves (Harasaki *et al.* 1978; Corcoran *et al.* 2004), where

high pulsatile shear stresses dominate. Several stages, labelled A to G in figure 4 and now described, depict the progression of this pathology in relation to the aforementioned mechanical environment and endothelial response. (A) Normal blood flow in this region is strongly unidirectional, pulsatile, and just barely laminar. Any increase in pressure or viscosity, as would be the case in hypertensive or hyperlipidemic conditions, could very easily push the flow into the turbulent regime. (B) This transition would dramatically increase already high shear stresses in the vicinity of the leaflet. These shear stresses could cause either injury/denudation of the valvular endothelial cells and/or platelet rupture. (C) Platelets and platelet particles could then adhere to the valve inflow surface, especially in regions of denudation (Allen *et al.* 2002; Leask *et al.* 2003 for references). Studies show that platelet adhesion is actually enhanced at higher shear rates, which would most probably occur at the leaflet-free edge for their proximity to maximum bloodstream velocities. Constitutively secreted and matrix-associated TF and vWF would then be released to the bloodstream, further activating the coagulation cascade. (D) The surrounding endothelium will become activated in the presence of the platelets and coagulation, upregulating receptors for circulating cells (VCAM-1, ICAM-1 and E-selectin). (E) In the case of bacterial endocarditis, circulating bacteria adhere very well to the fibrin–platelet matrix. Bacterial survival is promoted through endothelially derived coagulation factor stimulation of additional clot formation, which would protect the growing

bacterial vegetations. (F) The micro-organisms would then begin to breakdown the underlying matrix through secretion of matrix metalloproteinases (Edep *et al.* 2000; Soini *et al.* 2001), causing localized tissue damage and cell death. (G) Eventually, the growing vegetations would cover most of the leaflet surface, and the disorganized fibrous healing associated with cell-mediated remodelling (Moreillon *et al.* 2002) would cause leaflet stiffening and thickening, leading to cell death from diffusion limitations and/or angiogenic responses. The patient would experience increasing stenosis from the constricting annulus and regurgitation from the inability of vegetated leaflets to coapt. Non-bacterial thrombotic endocarditis (NBTE) works in a similar way, except bacterial lesions are not present. In this case, the thrombotic vegetations would increase with the clot, adding mass to the leaflets, altering coaptation and stimulating pathological tissue remodelling, which would result in a similar conclusion.

(b) Outflow surface and degenerative sclerosis

The progression of outflow-related pathology is next described and diagrammed in figure 4 through stages labelled 1 to 6. (1) The outflow surface of valves experiences a much lower magnitude, recirculating flow regime, similar to branching regions in the aorta known to cause vascular sclerosis. Porat *et al.* mapped the location of the *Tie-1* promoter in the cardiovascular system and found that it was colocalized with bifurcations and inner curvatures, and strikingly only on the outflow surfaces of the valves (Porat *et al.* 2004). (2) Valvular endothelial cells on the outflow surface of valves sometimes appear larger and more rounded (Sarphe 1982, 1985), similar to the endothelial morphology in atherogenic vascular regions. Low shear oscillatory blood flow on the fibrosa surfaces would result from poor drainage of blood into the coronary ostia, as would be the case if there is increased resistance from these vessels due to stenotic occlusion. (3) Valvular endothelial cells become activated in response to this flow pattern, upregulating receptors for monocytes, leukocytes and T-cells (VCAM-1, ICAM-1 and E-selectin; Ghaisas *et al.* 2000). This activation could be preceded by increases in oxidative stress as mediated by inflammatory agents such as BMP-4, as is the case with vascular endothelial cells in oscillatory shear. (4) Adherent leukocytes also secrete inflammatory cytokines such as IL-1 β and INF γ , which induce endothelial cell apoptosis, interstitial cell proliferation and secretion of MMP-1 and MMP-2 (Kaden *et al.* 2003) and these begin to degrade the collagen and elastin network. In hypercholesteremic rabbits, the non-flow surface contains many endothelial fenestrations and caveolae, promoting cell infiltration (Sarphe 1982). Increase in oxidative factors further promotes cell apoptosis and oxidation of LDL (Prasad & Bhalodkar 2004). Endothelial denudation also stimulates interstitial cell proliferation and matrix synthesis concomitant with a wound response. This thickening in turn reduces the ability of valve leaflets to be fed through diffusion, resulting in hypoxia-induced cell necrosis and/or angiogenesis (Chalajour *et al.* 2004). (5) The resulting necrotic cell fragments can then be nucleating sites for calcification nodules

(Müller *et al.* 2000). Calcification appears to be localized to regions of endothelial denudation (Müller *et al.* 2000), and is further propagated by the expression of BMP-2 and TGF β 1 from interstitial cells and macrophages (Mohler *et al.* 1999). TGF β 1 can also induce endothelial transdifferentiation to an activated mesenchymal phenotype as evidenced by the expression of α -smooth muscle actin. An activated interstitial cell phenotype may contribute to tissue contraction and stiffening. TGF β 1-induced expression of RANKL may also enhance calcification and stimulate pathological angiogenesis. (6) Each of these positive feedback loops contributes to irreversible tissue thickening and stiffening, concomitant with the upregulation of angiogenic factors (VEGF) in regions of hypoxia, causing increasingly pronounced stenosis and/or regurgitation.

In addition to haemodynamic forces, alterations in tissue strains can induce pathological endothelial activation. As shown in table 2, vascular endothelial cells respond to stretch by expressing both atherogenic and atheroprotective proteins. It is currently unknown what types of proteins valvular endothelial cells express in response to stretch. Types of strain, magnitude, frequency and duration have all been shown to be independent stimulators of cell function. Mechanical strain can also interact with fluid shear stress synergistically. Endothelial cells subjected to uniaxial stretch and fluid shear stress respond differently when these stimuli are applied at different phases (Dancu *et al.* 2004). If they are in phase with each other, cells respond by increasing production of eNOS and COX-2 and decreasing ET-1, thus exhibiting an atheroprotective phenotype. If these stimuli are 180° out of phase, the opposite response occurs. Peak fluid shear stress and tissue strains are commonly out of phase in valvular leaflets, but it is unclear if valvular cells respond similarly to different patterns of stimuli.

6. ROLES FOR TISSUE ENGINEERING

The importance of appropriate valvular endothelial cell phenotype is also of critical importance in many if not all valvular replacement strategies. Clinical results involving pulmonary autograft (Ross procedure) have demonstrated long-term effectiveness in adults as well as children, representing the gold standard for future valve replacements (Affonso da Costa *et al.* 1998; Oury *et al.* 1998b). These valves contain native tissue structure and appropriate cell phenotypes, which are undoubtedly responsible for their success. Although the pulmonary circulation is considerably less severe than the aortic circulation after birth, the complex haemodynamics of the aortic root may be proportionally similar to the pulmonary root. Thus, it is probable that pulmonary valve endothelium functions similarly to aortic valve endothelium to interpret the mechanical and haemodynamic signals and stimulate interstitial cell remodelling of pulmonary tissue into aortic tissue (Oury *et al.* 1998a).

The increased demand for the Ross procedure coupled with the relative lack of experienced surgeons capable of performing these surgeries necessitates the development of other useful options for valvular

replacements, particularly for the majority of patients living in the developing world. Current mechanical and bioprosthetic valves can give 15–20 years of adequate function in adults if tailored to specific patient demographics (Hammermeister *et al.* 2000), but performance is dramatically reduced in growing children, who require several reoperations to accommodate larger valve diameters (Turrentine *et al.* 2001). Thus, a great need still exists for better replacement options.

Tissue engineering has great potential to extend the functional life of both mechanical and bioprosthetic valves, as well as to fabricate implantable valves from living tissue *in vitro*. Mechanical valves suffer from bleeding events that are not completely controllable through anticoagulation therapy, not to mention the limitations of activity placed on the patients. Bioprosthetic valves are less thrombogenic, but eventually fail from structural degradation (Hammermeister *et al.* 2000). In both cases, tissue engineering can be used to modify the surfaces of these valves to accommodate endothelial cell adhesion and function, and some work has been initiated to this end (Fischlein & Fasol 1996; Jansson *et al.* 2001). Recent work with tissue-engineered living valves has shown proof of concept by 20 weeks of function in the sheep pulmonary circulation with apparent replication of native cell phenotypes and trivial to mild regurgitation (Hoerstrup *et al.* 2000). Similar valves have begun to be implanted in humans in Europe and longer-term results of these studies are awaited (Dohmen *et al.* 2002).

With this rapid progress, comes concern that long-term integration may be sacrificed for short-term feasibility. Current living valve replacements use cells isolated from available autologous vascular sources or mesenchymal stem cells. What is becoming clear from this review is that not all endothelial cells are alike, and therefore not all may function similarly in similar mechanical environments. Although stem cells have the potential to be differentiated into native valvular phenotypes, it is still unclear what those valvular phenotypes are or what stimuli are capable of initiating this differentiation. Results have shown that *in vitro* conditioning is beneficial in preparing living conduits for implantation, suggesting that a complex combination of stimuli are probably responsible (Hoerstrup *et al.* 2002).

Tissue engineering can also help advance our understanding of these issues in a laboratory setting. Engineered leaflet models containing isolated native valvular cells in defined matrices can be exposed to well-controlled mechanical and/or biochemical stimuli to ascertain their effects. In this way, native valvular cell interactions and biomechanical responses can be ascertained, which will serve as benchmarks for the differentiation of more usable cell sources such as available autologous sources, circulating progenitors cells and stem cells. Results can be interpreted in a positive feedback loop to reduce the need for more expensive animal implantations, potentially reducing the time required to generate successful valvular substitutes. In addition, mechanisms of valvular cell pathology initiation and progression can be studied more effectively in these controlled environments, potentially contributing to earlier diagnosis, prediction and treatment options.

In summary, the mechanical environment imposed by the haemodynamics of the aortic valve is much more complex than that of large arteries and varies dramatically along the surfaces of the valve. These stimuli may lead to different types of pathology on the two different sides of the valve. Further investigation of the role of mechanics in the initiation of these diseases may enable the discovery of key regulating molecules, the expression of which could be selectively targeted through pharmacological intervention before gross structural deterioration occurs.

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