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## Aortic Valve

### Turning Over a New Leaf(let) in Endothelial Phenotypic Heterogeneity

Peter F. Davies, Anthony G. Passerini, Craig A. Simmons

**A**ortic valve diseases are debilitating cardiovascular disorders associated with significant morbidity and mortality. Although there continue to be major efforts to improve the longevity of replacement valves and to improve tissue engineered substitutes,<sup>1</sup> the underlying mechanisms that may be responsible for the initiation and development of valve pathology have received less attention than have other sclerosing cardiovascular diseases such as atherosclerosis. The endothelium lining of the cardiovascular system plays an important regulatory role in vascular physiology and pathology. In similar fashion, the surfaces of valve leaflets are presumed to be generally protected (eg, anticoagulant) and regulated (eg, permeability) by the endothelium. The functional properties of endothelium or its presence/absence are associated with a variety of valve pathologies,<sup>2</sup> and systemic endothelial dysfunction is linked to aortic valve calcification.<sup>3</sup> However, only recently have cell and molecular studies focused on the characterization of valve endothelial phenotypes with the idea that some aspects of phenotypic change or dysfunction may contribute to valve pathologies, a situation analogous to atherosclerosis.

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During the cardiac cycle, the aortic valve endothelium is subjected to complex fluid dynamics that are distinctly different on each side of the valve. As has been described for many years, arterial endothelial alignment *in vivo* generally follows the measured or predicted shear stress direction,<sup>4</sup> and endothelial cells *in vitro* align with the dominant direction of the applied shear stress.<sup>5</sup> The responses, which are reversible,<sup>4,6</sup> represent endothelial structural remodeling in response to hemodynamic shear stress. It might be expected that endothelial cells isolated from aortic valves and grown in tissue culture will behave in a similar manner as arterial endothelium. However, in this issue of *Arteriosclerosis, Thrombosis, and Vascular Biology*, Butcher et al<sup>7</sup> demonstrate that aortic valve endothelium in culture exhibits a distinctly different response to directional flow than that shown by cultured arterial endothelium isolated from nearby

regions of the aorta. The study was inspired by the observations made by Deck et al<sup>8</sup> that endothelium on the ventricular aortic valve surface tends to align circumferentially with the free edge of the leaflets rather than in the direction of predicted maximum shear stress induced by systolic flow. The new studies show that over a 48 hour period, cultured aortic valve endothelial cells align *perpendicular* to, rather than *parallel* to, the flow direction. The alignment differences were associated with differences in signaling pathways. Although arterial endothelial cell alignment was dependent on both Rho-kinase and phosphatidylinositol 3-kinase (PI3K) pathways, valve endothelium appeared not to require activation of PI3K to align perpendicular to the flow. These findings are relevant to valve pathology and to the development of replacement tissues, as well as to the broader issues of endothelial phenotypic heterogeneity (Figure).

In normal mammals, endothelial phenotype appears to be determined by a combination of intrinsic programmed gene expression<sup>9</sup> and local epigenetic or environmental factors. Of the latter, the influence of the local hemodynamic environment on phenotype *in vitro*<sup>10,11</sup> and *in vivo*<sup>12,13</sup> has clearly been demonstrated. Endothelial orientation is also subject to “contact guidance” by the underlying substrate *in situ*.<sup>14</sup> Contact guidance is consistent with the physical continuity of extracellular matrix (ECM), focal adhesions, and cytoskeletal elements, connections that are important in the determination of cell morphology and cell differentiation. Cytoskeletal organization and focal adhesion biology are highly regulated by Rho-kinase and PI3K signaling pathways,<sup>15</sup> which were noted to be different for valve versus arterial endothelium.<sup>7</sup> Some memory of orientation may have been carried over to the cultured valve endothelial cells to direct the synthesis of oriented ECM in the experiments conducted by Butcher et al. However, if this were the case, the valve cells required the reintroduction of flow to activate such memory. Whatever the explanations for the valve cell alignment responses, the study demonstrates interesting phenotypic differences for cultured valve endothelium that have implications for endothelial biology in general as well as for cell source considerations and engineered phenotypes for tissue engineered valve substitutes. The useful applications of this finding, however, will best be realized by investigating differential endothelial phenotypes in greater detail and at improved spatial resolution within the valve structure itself (see below).

Global endothelial gene expression analysis is a powerful tool to investigate spatial heterogeneity of endothelial phenotypes in general. Farivar et al<sup>16</sup> noted significant differences in transcriptionally active genes expressed by arterial and valve endothelial cells grown in primary culture. Although there was common expression of 55 activated genes (of 847

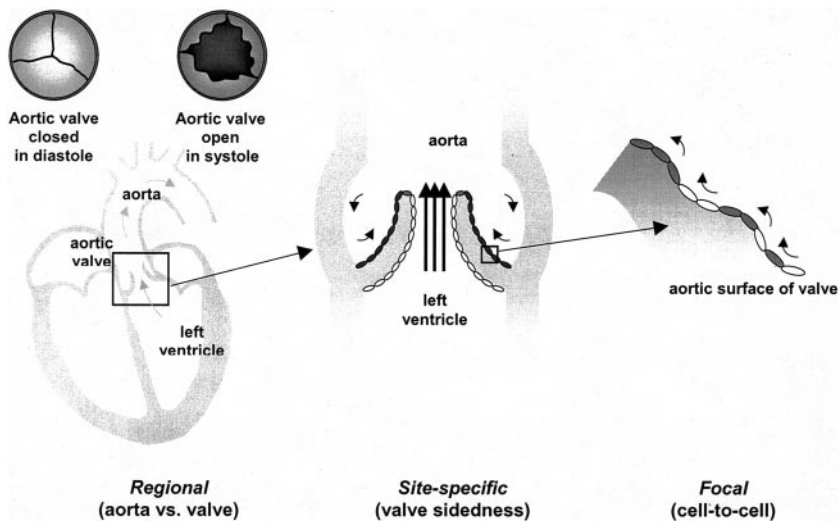
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Endothelial heterogeneity scaled for valve vs artery, aortic side vs ventricular side of the aortic valve, and cell-to-cell variation within the monolayer of each side.

interrogated), an additional 48 active genes were expressed by only one or the other cell type. Phenotypic memory in culture was also recently noted by Chi et al<sup>9</sup> in endothelial cells isolated from many different vascular locations and then cultured for up to 5 passages before transcriptional profiling. Remarkably, hierarchical clustering revealed patterns of gene expression that reflected some of the known properties of the cells in situ; arterial versus venous markers, microvascular characteristics, and even characteristics of vascular bed symmetry were identified. Intrinsic differential phenotypic properties of endothelium therefore appear to be established during development, then presumably reinforced, enhanced, and retained by cues present in the local environment. The extent to which the intrinsic differentiated phenotype is further regulated by local conditions, such as hemodynamic/biomechanical stimuli, can now be investigated globally at the level of gene expression and in a more limited way for candidate protein expression and activity.

Refinements of genomics techniques permit transcriptional profiling of small regions of endothelium reflective of the steady state in vivo.<sup>17</sup> This has recently been used to address regional phenotypic differences as a function of hemodynamic characteristics in both arteries<sup>13</sup> and heart valves.<sup>18</sup> Passerini et al<sup>13</sup> profiled endothelial cells isolated directly from arterial regions of disturbed and undisturbed flow in normal adult pigs to better define gene expression changes that underlie a predisposition to atherogenesis in regions of complex blood flow. The coexistence of pro- and anti-inflammatory gene expression patterns in the same atherosusceptible locations was noted. This balance of opposing mechanisms suggests that endothelial cells were primed for an atherogenic inflammatory response but kept in check by compensatory mechanisms in the absence of additional risk factors. The studies identified arterial hemodynamics as a contributor to regional endothelial heterogeneity via multiple pathways and as a subtle risk factor for atherosusceptibility. A similar approach is currently being applied to differential phenotyping of heart valve endothelium.<sup>18</sup> In this emerging work, the principal objective is to refine the spatial genomics to comparisons of each side of the valve leaflet because

differential susceptibility to calcific sclerosis is site-specific.<sup>19</sup> Calcification occurs preferentially in the aortic valve on the side that is exposed to more highly disturbed blood flow, suggestive of a role for hemodynamics in valvular calcification. Thus there are hemodynamic similarities to the disturbed flow characteristics that spatially correlate with regions of atherosusceptibility in large arteries.<sup>13,20</sup> In paired comparisons of aortic side versus ventricular side endothelium, Simmons et al (manuscript in preparation) observed differential transcriptional profiles across multiple pathways, including prominent representation of proliferative, apoptotic, and calcification pathways. These early data suggest differential endothelial phenotypes that define the sidedness of the aortic valve, a significant improvement in spatial resolution for this tissue (Figure, middle). The approach also addresses the complex mechanisms associated with aortic-side susceptibility of the valve to sclerosis and calcification. Furthermore, it should be possible to contrast the in situ and intrinsic (cultured) phenotypes, because valve endothelium from each side can be cultured for comparison of gene expression profiles from the same tissues in vitro and in situ. A higher level of spatial resolution would be obtained by profiling single or small groupings of endothelial cells.<sup>21</sup> There is ample evidence of cell-to-cell heterogeneity of endothelial morphology<sup>22</sup> and transport properties<sup>23</sup> throughout the aortic valve suggesting that phenotypic differences might be resolved at the single cell level (Figure, right).

In summary, the provocative observations noted by Butcher et al in culture reveal interesting differences in the responses to flow of the valve endothelium compared with the aortic endothelium. However, they likely represent merely the tip of the iceberg regarding valve endothelial phenotypes, and in situ endothelial heterogeneity in general.<sup>8,20</sup> More detailed investigation of both valve and vascular bed endothelium is required by global phenotyping at the highest spatial resolution possible.

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