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Unique Morphology and Focal Adhesion Development of Valvular Endothelial Cells in Static and Fluid Flow Environments

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Background—The influence of mechanical forces on cell function has been well documented for many different cell types. Endothelial cells native to the aortic valve may play an important role in mediating tissue responses to the complex fluid environment, and may therefore respond to fluid flow in a different manner than more characterized vascular endothelial cells.

Methods and Results—Porcine endothelial cells of aortic and aortic valvular origin were subjected to 20 dynes/cm^2 steady laminar shear stress for up to 48 hours, with static cultures serving as controls. The aortic valve endothelial cells were observed to align perpendicular to flow, in direct contrast to the aortic endothelial cells, which aligned parallel to flow. Focal adhesion complexes reorganized prominently at the ends of the long axis of aligned cells. Valvular endothelial cell alignment was dependent on Rho-kinase signaling, whereas vascular endothelial cell alignment was dependent on both Rho-kinase and phosphatidylinositol 3-kinase signal pathways.

Conclusions—These differences in response to mechanical forces suggest a unique phenotype of valvular endothelial cells not mimicked by vascular endothelial cells, and could have implications for cardiovascular cell biology and cell-source considerations for tissue-engineered valvular substitutes. **(*Arterioscler Thromb Vasc Biol*. 2004;24:1429-1434.)**

Key Words: endothelial ■ morphology ■ heart valves ■ shear stress ■ signal transduction

Valvular heart disease is a serious cardiovascular complication that often results in surgical intervention or even valve replacement. Current treatment options are acceptable for older patients,[1] but inadequate for younger populations.[2] Much research has been done to investigate the mechanisms and treatments of vascular diseases, but little basic research has been conducted to understand valvular cell biology and mechanisms of disease. As vascular biology and tissue engineering advancements continue,[3] heart valve therapy will become a new frontier for bioengineering research.[4] Heart valves perform a critical function in maintaining unidirectional flow through the vasculature, and as such the cells and tissue that constitute the leaflets of the valve may be uniquely suited to endure this demanding environment. Leaflet tissue is organized differently than vascular wall tissue, and evidence has emerged demonstrating that the interstitial cells of the leaflet milieu exhibit a different phenotype in comparison with vascular smooth muscle cells (VSMC).[5] Like blood vessels, the surfaces of valve leaflets are lined with endothelial cells, which are critical in maintaining a nonthrombogenic surface,[6] transport of nutrients, and transduction of mechanical and biochemical signals.[7] Valvular leaflet failure has been traced to endothelial dysfunction and denudation, and much pathology unique to the valve originates with the endothelium.[8,9]

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In vitro research has been instrumental in the discovery of endothelial function in mechanical environments.[10,11] Endothelial cells align parallel to the direction of unidirectional flow, through the reorganization of cytoskeletal filaments and focal adhesion complexes.[12–14] Fluid flow also induces secretion of vasoactive agents such as nitric oxide and prostaglandin to maintain vessel tone,[15,16] and these processes are regulated by complex signaling events.[17–19] Disruption of this signal process, through the oscillation of fluid flow direction[20,21] or signal pathway blockade, inhibits cell alignment, focal adhesion reorganization, and agent release.[22,23] The complete picture of this process is still incomplete, but it is the focus of much research.

Valvular endothelial cells are much less understood. Similar to vascular endothelium, these cells exhibit contact inhibition and grow with cobblestone-like morphology in vitro.[24] Observations of cell morphology in vivo show alignment concomitant with leaflet collagen fibers, but perpendicular to fluid flow.[25] It is currently not known whether valvular

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endothelial cells are phenotypically similar to vascular endothelial cells. Preliminary observations indicate that subpopulations of valvular endothelial cells are capable of transdifferentiating into smooth muscle–like phenotypes.[26] Genes uniquely expressed in valvular endothelium are also critical for appropriate valve development.[27] Heart valve leaflets develop in the embryo by forming protrusions within the heart tube, which are then populated through specially differentiated mesenchymal cells.[28] The endothelium that eventually remains may therefore be distinct from vascular endothelial cells. Indeed, preliminary evidence indicates that valvular endothelial cells exhibit differences in transcriptional profiles in comparison to vascular endothelial cells, and valvular endothelial cells are more proliferative in vitro.[29]

The objective of this study was to quantitatively compare the morphological responses of valvular and vascular endothelial cells to steady laminar fluid flow in vitro, and determine the changes in spatial arrangement of focal adhesion complexes. We report that the valvular endothelial cells align perpendicular to fluid flow, in contrast to vascular endothelial cells, which align parallel. The valvular cell alignment is independent of phosphatidylinositol 3-kinase (PI3K), whereas this signaling pathway is critical for vascular endothelial alignment.

Materials and Methods

Cell Isolation and Culture

Porcine aortic valve endothelial cells (PAVECs) and porcine aortic endothelial cells (PAECs) were isolated from intact porcine hearts through collagenase digestion (please see online Methods, available at http://atvb.ahajournals.org). Cells at passage 5 were used in all experiments. Cell phenotype was confirmed through expression of von Willebrand factor, acetylated low-density lipoprotein (LDL) uptake, and nonexpression of α-smooth muscle actin (please see online Methods).

Shear Stress Experiments

Steady, unidirectional laminar shear stress of 20 dynes/cm^2 was applied to monolayers of PAVECS or PAECS for either 24 or 48 hours,[10] with static cultures serving as controls. Few morphological response differences have been reported with pulsatile versus steady flow,[21] and steady shear therefore can serve as a first approximation of the flow environment for comparison in vitro. On completion of the experiments, the slides were stained for f-actin (rhodamine phalloidin, Molecular Probes #R-415, 1:400) and cell nuclei (Hoechst, Sigma #33258, 1:100) as described.[12] Focal adhesion components were immunofluorescently labeled for β1 integrins (Chemicon #MAB2000, 1:100), focal adhesion accessory molecules (vinculin, Upstate Biotech #05-386, 1:100), and signaling complexes (focal adhesion kinase (FAK), Upstate Biotech #06 to 543, 1:100). The samples were then imaged using laser confocal microscopy. A nondimensional shape index parameter was used to quantify cell elongation.[10] The shape index and cell orientation angle for at least 50 cells per slide were tabulated for analysis. Two factor ANOVA statistics were used with post hoc tests for interactions between conditions. $P<0.05$ was considered significant for these studies. Quantification of total focal adhesion protein levels was conducted using Western blotting (please see online Methods).

Signal Pathway Inhibition

Monolayers of PAVECS or PAECS were incubated with either Rho-kinase inhibitor (Y-27632, Calbiochem #688000, 5 μmol/L), PI3K inhibitor (Wortmannin, Calbiochem #681675, 1 μmol/L), or calpain inhibitor I (CI-1, Roche #1086090, 20 μmol/L) for 30 minutes before flow and during 24 hours of flow (20 dynes/cm^2). Static cultures and vehicle-only incubation cultures served as controls. Inhibitor concentrations were determined from the literature and confirmed through serial dilution response assays.

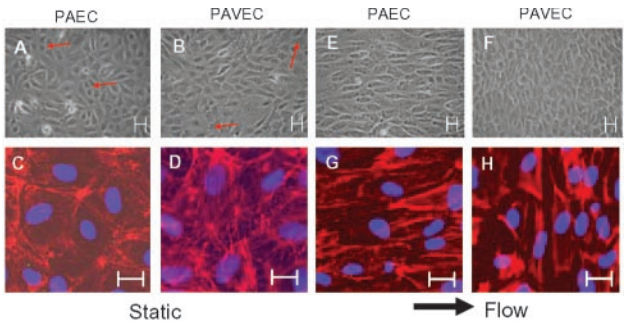

Figure 1. Images of PAECs (A, C, E, and G) and PAVECs (B, D, F, and H) in static and steady fluid flow environments. A through D, static culture; E through H, after 48 hours of 20 dynes/cm^2 steady laminar shear stress. Top panels are phase microscopy images, and bottom panels are laser confocal microscopy images. Cells are stained for f-actin (red) and cell nuclei (blue). Flow direction is horizontal left to right. Scale bar=50 μm. J and K, cell alignment regressions at static culture condition.

Results

Valvular Endothelial Cells Are Similar to Vascular Endothelial Cells in Static Culture

PAVECs grow similar to vascular endothelial cells in static culture (Figure 1A through 1D), but some filamentous extensions appear to extend from the cytoplasm of one PAVEC and overlap the border of another cell (arrows). This morphology has also been reported with endocardial endothelial cells,[30] perhaps indicating that valvular endothelial cells share some characteristics with those cells. Quantification of cell shape and angle of orientation for PAECs and PAVECs in static culture (Figure III, available online at http://atvb.ahajournals.org) shows that both cell types are randomly oriented, with the angle of orientation ranging from 0° to 90°. There is no correlation with shape index. The mean orientation angle for the PAECs in static culture is 41±21° degrees, whereas it is 40±25° degrees for PAVECs under static conditions (not significant). PAECs in static conditions have a shape index of 0.84±0.07, whereas for PAVECs it is 0.78±0.08. This lower shape index is caused by the more stellate pattern the valvular cell presents.

Valvular Endothelial Cells Align Differently Under Steady Flow

As shown in Figure 1E through 1H, PAECs align parallel to steady flow, as has been well documented. Porcine valvular endothelial cells, in contrast, align perpendicular to steady laminar flow in vitro. Biochemical staining shows that cytoplasmic actin filaments also align perpendicular to the flow direction. Quantification of these morphological changes demonstrates the extent of the alignment caused by shear stress in both cell types. The shape index versus orientation data from the two cell populations clearly diverge after 48 hours of steady flow (Figure II, available online at http://atvb.ahajournals.org). The PAVECs clearly align toward 90°, perpendicular to the fluid flow direction, whereas

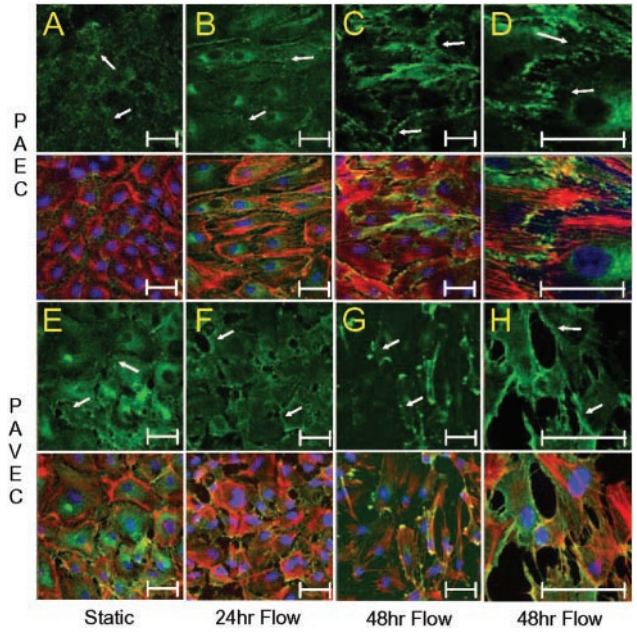

Figure 2. β1 integrin expression of PAECs and PAVECs; cells stained for b1 integrin (green), f-actin (red), and cell nuclei (blue). Top portion of panels are β1 integrin expression only. Scale bar=50 μm. Flow direction left to right.

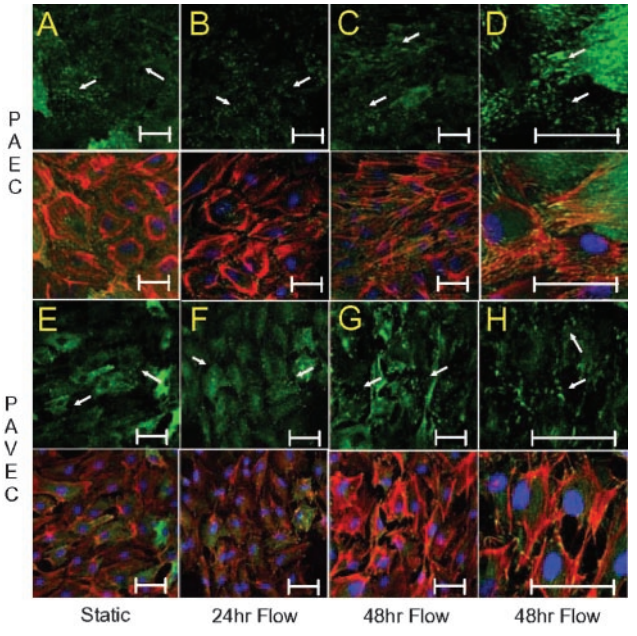

Figure 3. Vinculin expression of PAECs and PAVECs; cells stained for vinculin (green), f-actin (red), and cell nuclei (blue). Top portion of panels are vinculin expression only. Scale bar=50 μm. Flow direction left to right.

the PAECs align parallel to flow (0°). The cells that are more elongated are also more oriented toward their preferred direction. The cell populations at 24 hours of flow begin to diverge in a similar manner, but the trends are not as pronounced as at 48 hours (Figure II). With laminar shear stress, PAECs orient parallel to the flow direction, and the orientation is also more pronounced with time (Figure III). After 24 hours, the average angle of deviation from the flow direction for PAECs reduces to 25±22°, and further decreases to 11±8° by 48 hours, denoting nearly complete parallel orientation. PAVECs, in contrast, progress toward a near perpendicular orientation. After 24 hours of flow, the orientation angle is 60±22.42°, which further increases to 76±13° after 48 hours of flow. The cell shape index also changes with the application of steady flow (Figure III). Decreasing with time under flow, indicating that the cells are becoming more elongated. After 24 hours, the shape index for PAECs is 0.68±0.17, whereas for the PAVECs it is 0.67±0.12. After 48 hours of flow, these values reduce further to 0.51±0.15 and 0.64±0.14, respectively. These results indicate that the elongation of the PAECs continued beyond the 24 hour period, whereas the elongation of the PAVECs stabilized after 24 hours of flow.

Different Patterns of Focal Adhesion Develop Under Flow

The development and reorganization of focal adhesions paralleled changes in morphology for both cell types, resulting in different patterns between the two cell types. The spatial arrangement of focal adhesion components, represented by the clustering of β1 integrins (Figure 2, arrows), accessory molecules (vinculin, Figure 3, arrows), and signaling molecules (FAK, Figure 4, arrows), was concentrated at the upstream and downstream portions of flow in PAECs. The arrangement of focal adhesions in static cultures of PAECs was more homogeneous, with some tendency to concentrate at the cell periphery. All adhesion components were colocalized with f-actin termini, indicating that they are directly involved in supporting the changing cytoskeletal tension under flow. The reorganization of adhesions in PAVECs under flow is not concentrated at the upstream and downstream portions of the flow, but rather at the ends of the

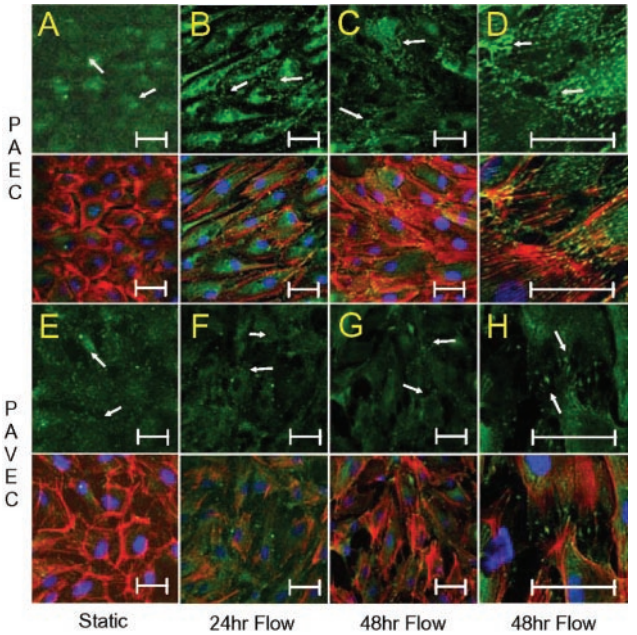

Figure 4. FAK expression of PAECs and PAVECs; cells stained for FAK (green), f-actin (red), and cell nuclei (blue). Top portion of panels are FAK expression only. Scale bar=50 μm. Flow direction left to right.

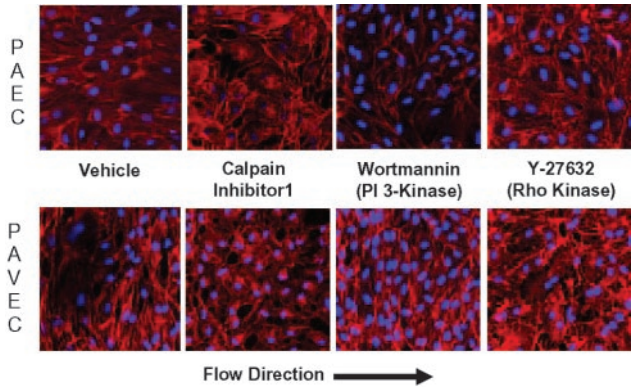

Figure 5. Effects of inhibitors on morphological response to flow; top row, PAECs; bottom row, PAVECs. Cells were exposed to 20 dynes/cm^2 steady laminar shear stress for 24 hours while in the presence of either Rho-kinase inhibitor (Y-27632, 5 μmol/L), PI3K inhibitor (Wortmannin, 1 μmol/L), or calpain inhibitor (calpain inhibitor I, 20 μmol/L). Shown are phase contrast microscopy images with confocal microscopy inserts. Cells were stained for f-actin (red) and cell nuclei (blue). Flow direction is horizontal left to right. Scale bar=50 μm.

long axis of the aligned cells. Focal adhesions of PAVECs in static culture are somewhat similar to PAECs, with a tendency to concentrate near the periphery of the cell and also within the cellular extensions. Vinculin and FAK colocalize with f-actin termini, along with β1 integrins, suggesting mechanical connectivity. Interestingly, there was significant presence of β1 integrin without vinculin or FAK at the upstream portion of PAVECs under flow, which was incompletely connected to f-actin (arrows). This suggests that there may be a partitioning of mechanical signal in PAVECs that is different in PAECs. There are most likely other focal adhesion components involved in the reorganization process than those examined in this study, which may account for this. Western blotting indicated no significant differences in total focal adhesion protein levels between either cell type or flow condition, confirming that the arrangement of focal adhesion proteins, and not the total amount of adhesion proteins, is important for the morphological differences seen between these two cell types. (Figure VII, available online at http://atvb.ahajournals.org).

Signal Pathway Involvement in the Regulation of Cell Morphology Under Flow

Figure 5 shows the changes in morphology of PAVECs and PAECs in static and flow environments when specific signal kinases or proteases are inhibited. The cytoskeletal reorganization of PAECs under flow is blocked by the inhibition of Rho-kinase, PI3K, and calpain. The alignment of PAVECs under flow was also disrupted through the inhibition of Rho-kinase and calpain, but not PI3K. No differences in cell orientation were observed in static cultures. Additional studies were conducted to investigate the action of calpain inhibitor I (CI-1) on focal adhesion pattern development as represented by vinculin localization. CI-1 disrupted focal adhesion reorganization under flow, and adhesions remained diffuse and peripheral (Figure 6). The disruption was similar in both cell types, suggesting that calpain acts similarly in both cells to enable focal adhesion reorganization.

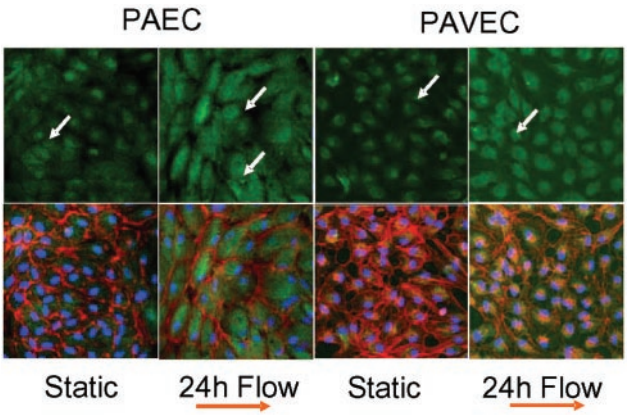

Figure 6. Effects of calpain inhibitor I on focal adhesion reorganization as determined by vinculin arrangement; cells stained for vinculin (green), f-actin (red), and cell nuclei (blue). Scale bar=50 μm.

Discussion

This study demonstrates that valvular endothelial cells align perpendicular to fluid flow in vitro, in contrast to vascular endothelial cells, and that this alignment is mediated by the reorganization of focal adhesions within the cell. Furthermore, the signaling pathways that mediate these responses are different for the two cell types. Vascular endothelial alignment is calpain-, Rho-kinase–, and PI3K-dependent, whereas valvular endothelial cell alignment is calpain- and Rho-kinase–dependent, but PI3K-independent. To our knowledge, the only other cell type that aligns perpendicular to the direction of fluid flow is the VSMC.[31] Paranya et al[26] found that some populations of ovine and human valve endothelial cells could be irreversibly induced to produce α-smooth muscle actin (α-SMA) through incubation with transforming growth factor (TGF)β-1. The most dramatic results were found with clonal expansions of individual cells that may have been more progenitor-like. However, they also found populations of valvular endothelial cells that stained positive for both CD-31 and α-SMA in vivo, possibly indicating a differentiating phenotype. It is also known that VSMCs secrete paracrine factors, such as TGFβ-1 in the presence of fluid flow.[32] These studies suggest that it may be possible that valvular endothelial cells differentiate in the presence of fluid flow to a more smooth muscle–like phenotype, and therefore align perpendicular to flow and express α-SMA. Additional monolayer studies were conducted to determine whether the PAVECs were transdifferentiating into a more smooth muscle–like (or valvular interstitial cell–like) phenotype. No expression of α-SMA was detected in static culture, or after 48 hours of flow (Figure IV, available online at http://atvb.ahajournals.org), suggesting that these cells do not transdifferentiate under flow. There may be other markers, whose expressions describe an intermediate phenotype,[33] and it may be possible that these cells may be encouraged through flow to exhibit the beginning stages of altered differentiation not observed in this study. It is also important to note that these experiments were conducted with early passage endothelial cells isolated from both sides of the aortic leaflets. There may be some differences between the endothelium

from the aortic and ventricular sides of the leaflets, as has been observed with the interstitial cells of the mitral leaflet.[34] Such differences may be flow-regulated, as seen in the vasculature. The complex fluid dynamics of the aortic valve may suggest a large plurality of different endothelial morphologies in vivo depending on location on the leaflet surface, not just between aortic and ventricular sides.[35] It would be difficult, however, to determine such differences through the types of studies presented here, as relatively large cell populations are required to achieve confidence in the results. Indeed, higher passage valvular endothelial cells (greater than P7) do not appear to exhibit this alignment tendency and progress to a more nonaligned or even parallel-aligned state (data not shown). Cells expanded in vitro from a very small initial population of isolated cells may therefore not respond similarly to a defined flow environment.

Focal adhesion patterns are important indicators of cell function, as they provide sensory and response instruments for cells in contact with extracellular matrix. The predominant integrins for interaction with collagen involve $\beta 1$ components, the integrin examined in these studies. Focal adhesions are centers for adhesion, migration, and signaling in response to mechanical forces such as shear flow. The differences in patterns observed between PAECs and PAVECs suggest differences in sensing of and adaptation to the external environment between the cell types. The resulting changes are consistent with in vivo observations, which strongly suggest that there may be differential mechanisms controlling responses to fluid flow.

To investigate these mechanisms further, molecules involved in signal pathways were independently inhibited to assess their effects on these two cell types. Important signal pathways that originate at cytoplasmic integrin terminals are Rho-kinase and PI3K. These cell types have established roles in regulating the actin cytoskeleton, cell growth, and migration, and are critical to the morphological changes in vascular endothelial cells. Rho-kinase, PI3K, and calpain are involved in a myriad of cytoskeletal functions. Rho-kinase (p160ROCK or ROCK1) is a downstream target of Rho, which is part of the Ras family of small GTPases.[36] Expression of constitutively active Rho inhibits parallel alignment of vascular endothelial cells, as does inhibition of the Rho-kinase.[37,38] Rho-kinase is also important for biphasic migration of sparse endothelial cells in short-term response to flow.[39] Y-27632 is a potent cell-permeable inhibitor of Rho-kinase by competing with ATP for the binding catalytic site.[40] PI3K is critical for the modulation of endothelial production of nitric oxide in response to shear stress.[41] PI3K has been reported not to influence cell elongation in response to steady shear in sparse cultures,[39] but its effects on morphology in confluent cultures under long term (24 hour) shear has not been studied. Wortmannin is a fungal metabolite that irreversibly blocks the catalytic activity of PI3K. In addition to kinases, calpain I is a cysteine protease that has substrates for proteins in focal adhesion components such as talin[42,43]and phosphorylated FAK.[44] It thus may be important in the reorganization of focal adhesion complexes and their initiation of signal cascades. Calpain inhibitor I has been shown to inhibit the morphological reorganization of lung epithelial cells after exposure to tetradecanoylphorbol-13-acetate.[45] The results of our study demonstrate that calpain function, and therefore the reorganization of focal adhesion complexes, is critical for cellular rearrangement in response to shear stress. Rho-kinase inhibition of endothelial reorganization with shear is confirmed in aortic endothelial cells and now established with valvular endothelial cells. The differences in morphological response between the two cell types under shear with the inhibition of PI3K suggests a phenotypic difference between the two cell types.

Coupled with the differences in focal adhesion patterns, valvular endothelial cells may compartmentalize mechanical signals in a different manner than vascular endothelial cells. This may be related to the differences in the native mechanical environment of the two cell types. Aortic valve leaflets experience up to 40% strains at a strain rate of 25/s, both much greater than the vessel wall.[46,47] The circumferential alignment of endothelial cells observed on leaflet surfaces also corresponds to circumferential alignment of collagen fibers. The confirmation of the endothelial alignment in vitro without the confounding effects of underlying matrix alignment, coupled with the different signal pathways that have been implicated in the alignment process of the two endothelial types, strongly suggests differences in the behavior of these two cell types, and elucidating these differences will require further study.

These results highlight considerations for appropriate cell sourcing of tissue-engineered valvular substitutes. Using nonvalvular endothelial cells, at least for an aortic valve substitute, may sacrifice important functional behavior. It is currently unknown if the dissimilar responses of vascular and valvular endothelial cells to fluid flow has any effect on overall leaflet function, but different responses may imply altered paracrine signaling to surrounding cells.[32] Assuming that appropriate cell behavior is essential for the successful tissue-engineering of substitutes, a better understanding of valvular cell behavior is critical to the development of living replacement heart valves.

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