Spatial Heterogeneity of Endothelial Phenotypes Correlates With Side-Specific Vulnerability to Calcification in Normal Porcine Aortic Valves

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Abstract**—**Calcific aortic valve sclerosis involves inflammatory processes and occurs preferentially on the aortic side of endothelialized valve leaflets. Although the endothelium is recognized to play critical roles in focal vascular sclerosis, the contributions of valvular endothelial phenotypes to aortic valve sclerosis and side-specific susceptibility to calcification are poorly understood. Using RNA amplification and cDNA microarrays, we identified 584 genes as differentially expressed in situ by the endothelium on the aortic side versus ventricular side of normal adult pig aortic valves. These differential transcriptional profiles, representative of the steady state in vivo, identify globally distinct endothelial phenotypes on opposite sides of the aortic valve. Several over-represented biological classifications with putative relevance to endothelial regulation of valvular homeostasis and aortic-side vulnerability to calcification were identified among the differentially expressed genes. Of note, multiple inhibitors of cardiovascular calcification were significantly less expressed by endothelium on the disease-prone aortic side of the valve, suggesting side-specific permissiveness to calcification. However, coexisting putative protective mechanisms were also expressed. Specifically, enhanced antioxidative gene expression and the lack of differential expression of proinflammatory molecules on the aortic side may protect against inflammation and lesion initiation in the normal valve. These data implicate the endothelium in regulating valvular calcification and suggest that spatial heterogeneity of valvular endothelial phenotypes may contribute to the focal susceptibility for lesion development. **(*Circ Res*. 2005;96:792-799.)**

Key Words: transcriptional profiling ■ microarray analysis ■ calcific aortic sclerosis ■ hemodynamics

Calcific aortic valve sclerosis, characterized by thickening and calcification of the valve leaflets, is a common disease associated with significant morbidity.[1] Until recently, calcific aortic sclerosis was considered a passive degenerative process secondary to aging and the accumulation of mechanical damage to the valve matrix. However, recent studies demonstrate an association between clinical risk factors for atherosclerosis and the development of valvular disease,[2] suggesting a more complex etiology than appreciated previously. Early degenerative lesions in human valves are characterized by increased cellularity, increased extracellular matrix deposition, and the accumulation of oxidized lipoproteins, nonfoam cell and foam cell macrophages, and occasional T cells within the valve interstitium.[3,4] These histological findings resemble early sclerotic lesions of the vasculature, and together with the shared risk factors, suggest that, as in atherosclerosis, the initiation of aortic valvular sclerosis involves chronic inflammatory processes potentiated by systemic factors. In advanced calcific valvular lesions, prominent features include mineralized deposits composed of hydroxyapatite, several bone matrix proteins, and mature osteoblasts and osteoclasts.[5–7] Thus, valvular calcification, rather than being attributable to passive, unregulated precipitation of calcium phosphate, appears to be a highly regulated, active ossification process.

Several theories have been proposed to explain the mechanisms by which cardiovascular calcification occurs.[8] Observations from mouse mutants[9,10] suggest that ectopic mineralization can occur when there is a loss of active inhibition. In healthy cardiovascular tissue, many inhibitors are constitutively expressed, thereby preventing "default" mineralization.[11] The identification of subpopulations of vascular smooth muscle cells,[12,13] adventitial myofibroblasts,[14] and valvular interstitial myofibroblasts[15] that can differentiate to osteoblast/chondroblast-like cells suggests calcification can also occur through induction of bone formation. Among the most potent stimulants of myofibroblast osteogenic differentiation are macrophage-derived inflammatory cytokines, reactive oxygen species (ROS), and lipid oxidation products,[15–18] suggesting a mechanism by which the inflammatory response in early lesions contributes indirectly to mineral formation. How-

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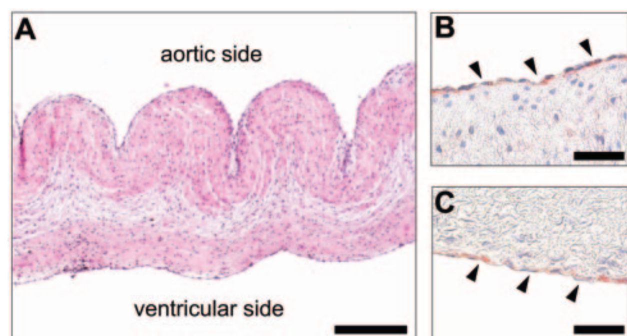

Figure 1. A, Histological section of a normal porcine aortic valve stained with hematoxylin and eosin. B and C, Immunostaining for vWF demonstrated intact endothelium on the aortic (B) and ventricular (C) surfaces of the valves (arrowheads). Bars=200 μm in A and 40 μm in B and C.

ever, particularly in the context of aortic valve sclerosis, these mechanisms are poorly understood.

In the vascular system, the endothelium is an important regulator of physiology and pathology, including atherogenesis. Similarly, the endothelium lining the surface of valve leaflets is presumed to be involved in valve homeostasis and pathology, although the contribution of endothelial phenotypes and dysfunction to valve pathologies at the cellular and molecular level has received little attention. Notably, in humans[3,19] and pigs (supplemental Figure I, available online at http://circres.ahajournals.org), lipid deposits and calcific lesions in the aortic valve occur preferentially in the fibrosa, the layer of the valve immediately beneath the endothelium on the aortic side of the valve. The preferential susceptibility to lesion formation on the aortic rather than ventricular surface of the aortic valve may result from coordinated regulation of gene expression by the respective endothelia, resulting in side-specific endothelial phenotypes that favor or inhibit calcification. Furthermore, during the cardiac cycle, the aortic valve endothelium is subjected to complex fluid dynamics that are distinctly different on either side of the valve.[20] Thus, there is a spatial correlation between the focal nature of calcific lesions and the local hemodynamic environment, similar to that observed for atherosusceptible regions in the large arteries.[21] Local environmental factors, including biomechanical forces and molecular transport, may therefore also contribute to differential endothelial phenotypes that define the sidedness of the valve and the focal susceptibility to calcification.

To investigate phenotypic heterogeneity in valve endothelium and its implications for the focal genesis of valvular disease, differential endothelial gene expression was analyzed on the aortic side versus ventricular side of normal adult pig aortic valves. The resulting expression profiles, representative of the steady state in vivo, reveal that although the aortic side of normal nondiseased valves is permissive to calcification, it is generally protected from inflammatory processes associated with the initiation of sclerosis in the absence of additional risk factors.

Materials and Methods

Aortic valve leaflets were harvested from eight adult male pigs from a local abattoir (Hatfield Industries, Hatfield, PA) immediately after death. Endothelial cells were isolated separately from the aortic and ventricular surfaces of each valve leaflet using a modified Hautchen method.[22] Total RNA from the isolated cells (100 ng≈10 000 cells/side) was linearly amplified by one round of T7-mediated in vitro transcription.[23] cDNA probes were synthesized from 2 μg amplified antisense RNA using indirect labeling (described in the online data supplement, available at http://circres.ahajournals.org). Paired fluorescently labeled cDNA probes from the aortic and ventricular surfaces of the same valve were competitively hybridized to an Agilent Human 1 cDNA microarray containing 12 922 clones (Agilent Technologies).

Arrays (n=8) were scanned at 10-μm resolution, and images were analyzed with Agilent Feature Extraction Software (version A.7.1.1). The intensity ratios on each array were log transformed and normalized via global lowess with the functions provided in the Comprehensive R Archive Network (CRAN) Statistical Microarray Analysis package (sma version 0.5.14; http://cran.r-project.org/; online data supplement). Differential expression analysis was performed with PaGE (see http://www.cbil.upenn.edu/PaGE and the online data supplement). Patterns from Gene Expression (PaGE) is a false discovery rate (FDR)–based method[24] that uses a permutation approach to estimate the FDR. The normalized log ratios were tested with a one-sample T statistic and the FDR was 5%. The relative expression of selected genes was validated by quantitative real-time polymerase chain reaction (QRT-PCR) using paired aortic and ventricular endothelial samples isolated from 10 additional adult male pigs. Statistical significance was tested using the one-sample T statistic and one-tailed permutation P values. The list of differentially expressed genes was analyzed for statistically over-represented biological themes using Expression Analysis Systematic Explorer (EASE).[25] Data were additionally mined with Genespring (Silicon Genetics) for annotation and with literature searches. The complete annotated study is publicly available in a MIAME-compliant frame-

TABLE 1. Comparison of Relative Expression Levels Measured by Microarray Analysis and by QRT-PCR for Selected Genes

	A/V Fold Change†		
Gene*	Microarray	QRT-PCR	P Value‡
CX43	−1.72	−1.33	0.0050
FGFR2	2.14	>3.24‖	0.00083
GPX3	−1.35	−1.92	0.0020
GSTO1	1.40	1.57	0.18
LGALS1	−2.08	−2.17	0.0017
NOS3	1.44	1.29	0.0033
OSTF1	1.24	1.43	0.0025
PECAM1	1.44	1.26	0.037
PTGS1	−1.54	−2.50	0.00030
SELP	1.66	2.05	0.00083
VCAM1	1.37	−1.02	1.00
VWF	1.60	2.61	0.0025
ICAM1	Not differentially expressed	1.00	1.00
SELE	Not differentially expressed	Not detected	–

* CX43 indicates connexin 43; FGFR2, fibroblast growth factor receptor 2; GPX3, glutathione peroxidase 3; GSTO1, glutathione *S*-transferase-Ω1; LGALS1, galectin 1; OSTF, osteoclast-stimulating factor 1; PECAM1, platelet/endothelial cell adhesion molecule; PTGS1, prostaglandin–endoperoxide synthase 1; SELP, selectin P; VCAM1, vascular cell adhesion molecule 1; ICAM, intracellular cell adhesion molecule 1; SELE, selectin E.

†A/V fold change indicates aortic-side to ventricular-side fold change; ‡permutation-based one sample T statistic, one-tailed in direction predicted by microarray; ‖ratio based on 6 of 10 samples because FGFR2 was detected on the aortic side but not on the ventricular side of four samples, resulting in infinite A/V fold changes.

TABLE 2. Prominent Biological Classifications Identified by EASE*

Biological Classification	No. of Genes Differentially Expressed†	#Genes Represented on Array	*P* Value‡
Peripheral nervous system development	4	8	0.00059
Rho protein signal transduction	5	16	0.0015
Eicosanoid biosynthesis	4	10	0.0016
mRNA polyadenylation	3	5	0.0017
Apoptosis	22	202	0.0022
Aspartate family amino acid metabolism	3	6	0.0032
Cell motility	20	201	0.0094
Glutamate channel activity	3	9	0.011
Tumor suppressor	4	17	0.013
Gluconeogenesis	3	10	0.016
Steroid hormone nuclear receptors	5	25	0.022
Actin cytoskeletal organization	6	41	0.026
Transforming growth factor-β signaling	7	47	0.038
Cell adhesion	28	353	0.042
Skeletal development	9	83	0.044

*Shown are the top 10 over-represented classifications containing three or more genes and five other highly represented categories potentially relevant to valvular pathology. (The complete list is available at www.cbil.upenn.edu/RAD/PigValveStudy.)

†Aortic side vs ventricular side; ‡Fisher exact probability.

work through the RNA Abundance Database (http://www.cbil.upenn.edu/RAD/php/displayStudy.php?study_id=1270).[26] Additionally, all supplemental material can be accessed at http://www.cbil.upenn.edu/RAD/PigValveStudy/.

For histological and immunohistochemical staining, fresh porcine aortic valve leaflets were fixed in 10% neutral-buffered formalin, paraffin-embedded, and serial sectioned. Sections were stained with hematoxylin and eosin or for various antigens using standard peroxidase-based detection methods (online data supplement).

Results

Sample Characterization

The gross appearances of the porcine valves used for RNA isolation were normal and consistent with histological examination, which showed no pathology and intact endothelium (Figure 1). Immunostaining of cells isolated by the modified Hautchen technique confirmed their purity[22] (supplemental Figure II). From the freshly isolated endothelial cells, >100 ng of intact (28S:18S ribosomal RNA ratio ≈2) total RNA, representative of the steady state in vivo, was obtained from each side of the valve. We have shown previously that this amount of RNA is sufficient for amplification and identification of differential expression with fidelity and enhanced sensitivity.[27]

Side-Dependent Differential Gene Expression

Differential gene expression by endothelial cells from the aortic side versus ventricular side of normal aortic valves was assessed using PaGE. At 5% FDR, 584 genes were identified as differentially expressed, with higher expression of 285 genes and lower expression of 299 genes on the aortic side of the valve relative to the ventricular side. (A fully annotated list of the differentially expressed genes is available at http://www.cbil.upenn.edu/RAD/PigValveStudy/.) The array predictions were validated by QRT-PCR for 83% of a subset of genes relevant to valvular disease (Table 1), consistent with our previous reports in arterial cells.[27,28]

Analysis of Biological Themes and Pathways

Using EASE, the list of differentially expressed genes was analyzed globally to identify biological themes that were significantly over-represented in the data set (Table 2). The nonrandom coordinated expression of multiple genes in an over-represented biological theme is suggestive of the functional involvement of that pathway or class of genes in side-dependent susceptibility to disease. Included among the most prominent classifications were groups of genes related to the cell cycle and apoptosis, nuclear and metabolic activity, intracellular signaling, cytoskeletal organization, and skeletal development. The gene groupings identified by EASE are detailed at http://www.cbil.upenn.edu/RAD/PigValveStudy/ and, together with the annotated gene list, provide a rich public database. Although an exhaustive analysis of the complete data are beyond the scope of this article, differential expression patterns incorporating several prominent classifications with putative significance to valve pathology were identified.

Aortic-Side Endothelial Gene Expression Is Permissive to Valvular Calcification

Of particular relevance to valvular pathology was the significant over-representation of differentially expressed genes related to skeletal development and vascular calcification (Table 2). Most remarkable was the lower expression on the aortic side of several transcripts for inhibitors of vascular and

TABLE 3. Differentially Expressed Genes Related to Skeletal Development and Vascular Calcification

Lower Expression on Aortic Side				Higher Expression on Aortic Side			
Gene*	Accession No.	A/V Fold Change†	Putative Effect‡	Gene*	Accession No.	A/V Fold Change†	Putative effect‡
TNFRSF11B	U94332	−3.53	+	*BMP4*	NM_001202	1.57	+
NPPC	D90337	−3.12	+	*PTN*	AU120808	1.53	+
CHRD	AF209928	−1.37	+	*HAPLN1*	U43328	1.49	+
PTH	V00597	−1.31	+	*FBN1*	X63556	1.39	+
COL11A1	J04177	−1.44	–	*CHAD*	AF371328	1.37	+
BMP1	NM_006129	−1.52	?	*OSTF1*	BC007459	1.24	?
BMP6	AA426586	−1.29	?				

*CHAD indicates chondroadherin; CHRD, chordin; COL3A1, collagen type III α1; COL11A1, collagen type XI α1; FBN1, fibrillin 1; HAPLN1, hyaluronan and proteoglycan link protein 1; NPPC, natriuretic peptide precursor C (CNP); OSTF1, osteoclast-stimulating factor 1; PTN, pleiotrophin; TNFRSF11B, tumor necrosis factor receptor superfamily member 11b (osteoprotegerin).

†Aortic side to ventricular side fold change by microarray analysis; ‡+procalcific on aortic side;–anticalcific on aortic side; ?unknown; refer to supplemental Table 3.

valvular calcification (Table 3). These included osteoprotegerin (OPG; tumor necrosis factor receptor superfamily; member 11b), C-type natriuretic peptide (CNP), and parathyroid hormone (PTH), each of which has been shown to inhibit cardiovascular calcification (Discussion). Also underexpressed on the aortic side was the transcript for chordin, a secreted protein that inhibits the osteoinductive effects of bone morphogenetic proteins (BMPs) by sequestering them in latent complexes.[29] We immunostained aortic valve sections with an anti-OPG antibody and, consistent with the microarray predictions, noted striking differential expression of the OPG protein, with strong staining in and around the ventricular-side endothelium but little expression on the aortic side (Figure 2A through 2C).

Coupled with the relative lack of expression of inhibitors on the aortic side was increased expression of several transcripts associated with bone formation. Most notable was the higher expression of BMP4 by the endothelium on the aortic side of the valve. Also more highly expressed on the aortic side were transcripts for several proteins associated with the extracellular matrix of bone or cartilage (Table 3), suggesting that the aortic side endothelium may contribute to a matrix environment that is permissive to calcification. Notably, transcripts for several molecules with demonstrated roles in vascular and valvular calcification (eg, matrix gla protein, and transforming growth factor-β1) were not identified as differentially expressed in the normal valve (supplemental Table 2).

Aortic-Side Endothelial Gene Expression Is Antioxidative and Not Inflammatory

The histological similarities between early atherosclerotic vascular lesions and calcific valvular lesions suggest valvular calcification may initiate through inflammation, leukocyte adhesion and invasion, and oxidative processes. Many of these processes are regulated by the endothelium. Notably, relevant transcripts were differentially expressed on opposite sides of normal aortic valves (Tables 2 and 4). However, in the normal valves, the aortic-side expression profile was decidedly noninflammatory. The proinflammatory molecules CCL13 and Duffy blood group were less expressed on the aortic side of the valve. None of the prototypical inflammatory cytokines/chemokines (eg, the interleukins) were differentially expressed. Consistent with the lack of inflammation

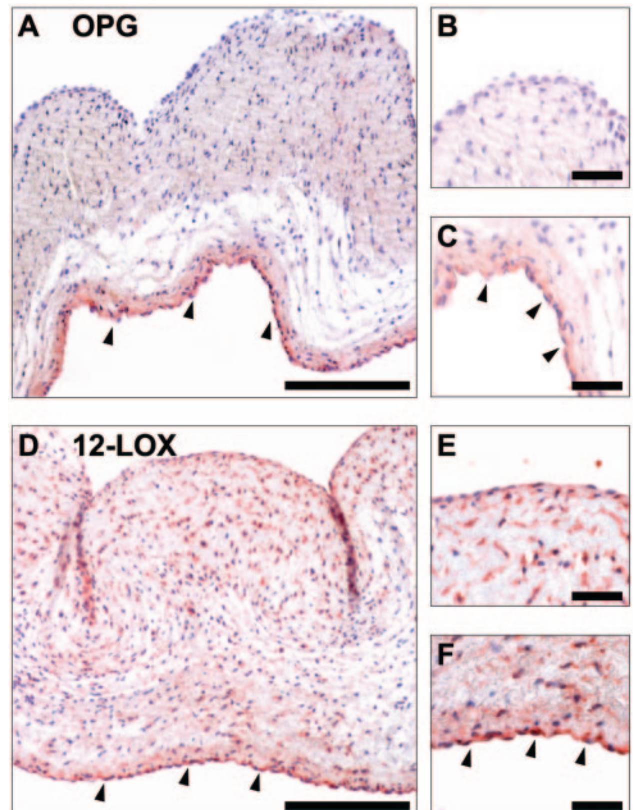

Figure 2. A through C, Immunostaining for OPG revealed striking side-dependent expression (A), with little OPG expression on the aortic side (B) but strong staining on the ventricular side of the valve (C; arrowheads). D through F, 12-lipoxygenase (12-LOX) was also clearly differentially expressed (D), with less endothelial expression on the aortic side (E) of the normal valve than on the ventricular side (F; arrowheads). Strong, heterogenous expression of 12-lipoxygenase was also observed in the interstitial myofibroblasts. Differential endothelial protein expressions illustrated in this figure are consistent with the microarray analyses. Bars=200 μm in A and D and 40 μm in B, C, E, and F.

TABLE 4. Differentially Expressed Genes Related to Inflammation and Oxidation

Lower Expression on Aortic Side				Higher Expression on Aortic Side			
Gene*	Accession No.	A/V Fold Change†	Putative Effect‡	Gene*	Accession No.	A/V Fold Change†	Putative Effect‡
ALOX12	M62982	−4.53	–	*MGST2*	U77604	1.82	–
NPPC	D90337	−3.12	–	*NOS3*	BG741096	1.44	–
LGALS1	BC001693	−2.22	–	*PRDX2*	BC000452	1.28	–
FY	AF030521	−1.97	–	*SELP*	NM_003005	1.66	+
CCL13	U59808	−1.53	–	*vWF*	X04385	1.60	+
OLR1	AB017444	−1.32	–				
GPX3	D00632	−1.36	+				

*ALOX12 indicates arachidonate 12-lipoxygenase; CCL13, chemokine (C-C motif) ligand 13; FY, Duffy blood group; GPX3, glutathione peroxidase 3; LGALS1, galectin 1; MGST2, microsomal glutathione *S*-transferase 2; NPPC, natriuretic peptide precursor C; OLR1, oxidized low-density lipoprotein receptor 1; PRDX2, peroxiredoxin 2; SELP, selectin P.

†Aortic side to ventricular side fold change by microarray analysis; ‡+proinflammatory/oxidative on the aortic side; –anti-inflammatory/oxidative on the aortic side; refer to supplemental Table 3.

on the aortic side, the transcripts for galectin-1 and CNP, both of which are upregulated by proinflammatory mediators,[30,31] were underexpressed. Of the adhesion molecules associated with early inflammatory responses and leukocyte adhesion, only P-selectin and von Willebrand factor (vWF) were differentially expressed. E-selectin, vascular cell adhesion molecule 1, and intracellular adhesion molecule 1 were not differentially expressed, as confirmed by QRT-PCR (Table 1). Although P-selectin and vWF mRNA were more highly expressed on the aortic side (confirmed by QRT-PCR), differential vWF protein expression was not evident by immunostaining (Figure 1B and 1C).

The transcription of genes for many proinflammatory cytokines, chemokines, and adhesion molecules is regulated in vascular endothelial cells by the nuclear factor κB (NF-κB) pathway.[32] Among the most potent activators of the NF-κB pathway are ROS generated by oxidative stress and implicated in the initiation and progression of atherosclerosis.[33] Data mining revealed a strong antioxidative profile in the aortic-side endothelium that may protect against ROS- and NF-κB–mediated inflammation (Table 4). The transcripts for several intracellular antioxidative enzymes, including microsomal glutathione *S*-transferase 2 and peroxiredoxin 2, were more highly expressed on the aortic side of the valve. Glutathione *S*-transferase-Ω1 was also more highly expressed on the aortic side by microarray analysis and by QRT-PCR but was not statistically significant in the QRT-PCR validation because of large intra-animal variability (Table 1). Glutathione peroxidase 3 was underexpressed on the aortic side of the valve, but this antioxidative enzyme acts extracellularly, and therefore its role in maintaining the intracellular endothelial redox state is unclear. Further contributing to the antioxidant profile on the aortic side was higher expression of endothelial NO synthase (eNOS; associated with several beneficial effects including resistance to oxidative stress[34]), and lower expression of the oxidized low-density lipoprotein receptor 1, which binds oxidized lipoproteins to promote ROS-induced activation of NF-κB[35] and the endothelial expression of calcification-related genes.[36] Additionally, arachidonate 12-lipoxygenase expression was significantly lower on the aortic side of the valve. Lipoxygenase enzymes oxidize polyunsaturated fatty acids to synthesize hydroperoxyacids, which are potent pro-oxidant mediators. In endothelial cells, 12/15 lipoxygenase and its products are implicated in mediating atherosclerosis.[37] Consistent with the aortic-side antioxidative transcriptional profile, immunostaining clearly revealed lower expression of the 12-lipoxygenase protein by endothelial cells on the aortic side of the valve relative to the ventricular side (Figure 2D through 2F). Thus, the balance of oxidative transcripts was decisively shifted toward an antioxidative state on the aortic side of the valve, and this protective profile may be responsible for suppressing the expression of proinflammatory genes in the normal valve.

Discussion

Although the endothelium is recognized to play critical roles in the initiation and progression of sclerotic diseases of the vasculature, the contribution of valvular endothelial phenotypes to calcific aortic sclerosis and its focal genesis are poorly understood. Using RNA amplification and transcriptional profiling of aortic-side versus ventricular-side endothelium from normal porcine valves, we identified spatially distinct endothelial phenotypes on opposite sides of the aortic valve in vivo. The differential gene expression profiles suggest that the endothelium on the disease-prone aortic side of the valve is permissive to calcification but is protected in the normal valve against inflammation and lesion initiation by antioxidative mechanisms. These data demonstrate that the endothelium may play critical roles in regulating valvular calcification through inhibitory and stimulatory mechanisms, and the spatial heterogeneity of valvular endothelial phenotypes may contribute to the focal susceptibility for lesion development.

A striking observation from the gene expression data was the relative absence on the aortic side of the valve of OPG, CNP, PTH, and chordin. OPG plays a role in the local and systemic regulation of bone resorption[38] but also appears to

suppress local cardiovascular calcification because mice lacking OPG have calcified arteries,[9] and aortic valve myofibroblasts treated with receptor activator of nuclear factor kappa B ligand (RANKL) in the absence of OPG differentiate to an osteogenic phenotype in vitro.[39] Similarly, CNP- and PTH-related polypeptide inhibit vascular myofibroblast calcification in vitro.[40,41] In vivo, PTH administered intermittently to *LDLR−/−* mice promotes skeletal bone formation while simultaneously suppressing vascular osteogenesis and calcification, in part through direct action of PTH on the osteogenic differentiation of vascular myofibroblasts.[42] Chordin is an antagonist of BMP2 and BMP4 and therefore may regulate osteogenic differentiation indirectly by mitigating the osteoinductive effects of endogenous BMPs. Local release of each of these inhibitors from the ventricular-side endothelium may act in a paracrine manner to inhibit ectopic mineralization or myofibroblast osteogenic differentiation. Conversely, the absence of these inhibitors on the aortic side may, in the presence of a procalcific challenge, permit the phenotypic transition of myofibroblasts to osteoblast-like cells and ectopic mineralization. Previous studies have identified OPG in the interstitium of normal but not sclerotic aortic valves.[39] However, the striking side-dependent expression we observed at the mRNA and protein levels in situ argues strongly for a previously unrecognized role for the endothelium in regulating local calcification processes through paracrine signaling mechanisms. Additionally, OPG is an autocrine antiapoptotic factor for endothelial cells,[43] and therefore, a deficiency of OPG on the aortic side may contribute to endothelial injury as an initiating event in lesion formation.

Also notable was the higher aortic-side expression of BMP4, which, together with the closely related protein BMP2, has been observed in advanced atherosclerotic lesions,[13,44] calcific valvular lesions,[45] and in vascular endothelial cells in human coronary arteries, where BMP4 appears to mediate inflammatory responses.[46] Endothelial-derived BMP2 has been shown to regulate orthotopic bone formation[47] and to regulate vascular myofibroblast osteogenesis in vitro,[48] suggesting endothelial-derived BMPs may have the potential to stimulate valvular calcification. Although BMP4 mRNA was more highly expressed on the aortic side, we did not observe histological evidence of inflammation or calcification, suggesting compensatory mechanisms limit the effects of BMP4 gene expression in the normal valve. Similarly, Sorescu et al[46] only observed BMP4 protein expression in endothelial cells when they were overlying foam cells in inflamed vascular regions.

Side-dependent endothelial phenotypes are likely to be a combination of intrinsic phenotype determined during development and maintained postnatally, and spatially sensitive phenotype determined by local environmental factors, such as hemodynamic characteristics. Chi et al[49] demonstrated intrinsic regional differences in endothelial phenotypes by profiling endothelial cells isolated from various vascular beds and grown in culture for several passages. The different endothelia had distinct and characteristic gene expression profiles, indicating that they displayed intrinsic phenotypic differences even in the absence of tissue-specific microenvironmental stimuli. A similar comparison of cultured vascular and valvular endothelial cells also demonstrated distinct transcriptional profiles.[50] At a higher level of spatial resolution, we have shown in the current and previous studies[28] site-specific heterogeneity of endothelial phenotypes within a single tissue in vivo. In the case of the valvular endothelium, the side-specific phenotypic differences appear to be in part intrinsic because we have observed morphological and functional differences between endothelial cells derived from opposite sides of aortic valves and grown in culture under identical conditions for multiple population doublings (Simmons et al, unpublished data, 2004). Phenotypic differences in adult aortic and ventricular-side valvular endothelial cells may reflect functional differences determined early in development.

However, it is probable that as in the vasculature, local environmental cues regulate the biology of the valvular endothelium. As with atherogenesis, the correlation between the differential blood flow characteristics and the focal nature of valvular calcification suggests that hemodynamic forces may play a critical regulatory role. Although the relationship between flow and vascular endothelial cell transcriptional profiles has been well studied in vitro[51] and recently in vivo,[28] little is known about the response of valvular endothelial cells to hemodynamic forces. It was reported recently that aortic valve endothelial cells are sensitive to laminar shear stress in vitro and, at least in their flow-mediated alignment, respond distinctly from vascular endothelium.[52] We observed differential expression by the valvular endothelium of several genes that are mechanoregulated in vascular endothelial cells (eg, BMP4, CNP, eNOS, and SMAD6, etc). Although the direction of regulation of some of these genes was similar in lesion-prone regions of the valve and the vasculature,[28] this was not always the case. Contributing to the distinct responses may be the unique and complex biomechanical stimuli experienced by the valvular endothelium in vivo, the phenotypic differences between valvular and vascular endothelial cells, or other nonbiomechanical factors. Therefore, although there appears to be a correlative link between hemodynamics, valvular endothelial phenotypes, and susceptibility to calcification, further investigations are required to understand these links and to demonstrate causality.

Acknowledgments

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