



Review Article

The aortic valve microenvironment and its role in calcific aortic valve disease

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Abstract

In calcific aortic valve disease, fibrotic and calcific lesions form focally in the fibrosa layer of the valve leaflets. Layer-specific pathosusceptibility suggests that the fibrosa microenvironment is permissive to pathological development. The cellular microenvironment in the aortic valve is defined by a variety of biomechanical-, biochemical-, and extracellular-mediated factors, some of which are unique to the fibrosa. Growing evidence supports the role of these microenvironmental cues in the local regulation of side-specific valve cell phenotypes and focal pathological alterations, revealing new insights into the cellular and molecular processes that contribute to calcific aortic valve disease. © 2010 Elsevier Inc. All rights reserved.

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1. Introduction

Calcific aortic valve disease (CAVD) encompasses a spectrum of disease from early alterations in valve cell phenotypes to sclerotic thickening and matrix remodeling of the valve leaflets to stenosis and leaflet calcification [1]. Traditionally viewed as a degenerative process resulting from “wear and tear” with aging, CAVD is now recognized to be an actively regulated disease. However, the cellular events and molecular mechanisms that drive CAVD, particularly the early stages of the disease when intervention may be more effective, have yet to be fully defined.

Although CAVD is associated epidemiologically with systemic risk factors [2], sclerotic valve lesions form focally and preferentially in the fibrosa, the interstitial layer on the outflow side of the leaflet closest to the aorta [1,3]. Side-specific pathosusceptibility suggests that factors local to the fibrosa promote, or at least permit, pathological development. Thus, clues to cellular and molecular regulatory processes in CAVD may come from an improved understanding of the unique aspects of the fibrosa microenvironment and their relationship to valve pathobiology.

Here we discuss the factors that define the cellular microenvironment in the aortic valve and, in particular, the characteristics that distinguish the pathosusceptible fibrosa from the other layers (Fig. 1). We review recent and emerging studies that demonstrate that fibrosa-side cells are phenotypically distinct from cells on the opposite side of the leaflet and experience unique mechanical forces, biochemical stimuli, and extracellular matrix (ECM) cues that regulate valve cell (patho)biology. Importantly, these factors interact, making cellular response to microenvironmental cues context-dependent. Despite the complexity that this introduces, spatial correlations of microenvironmental cues

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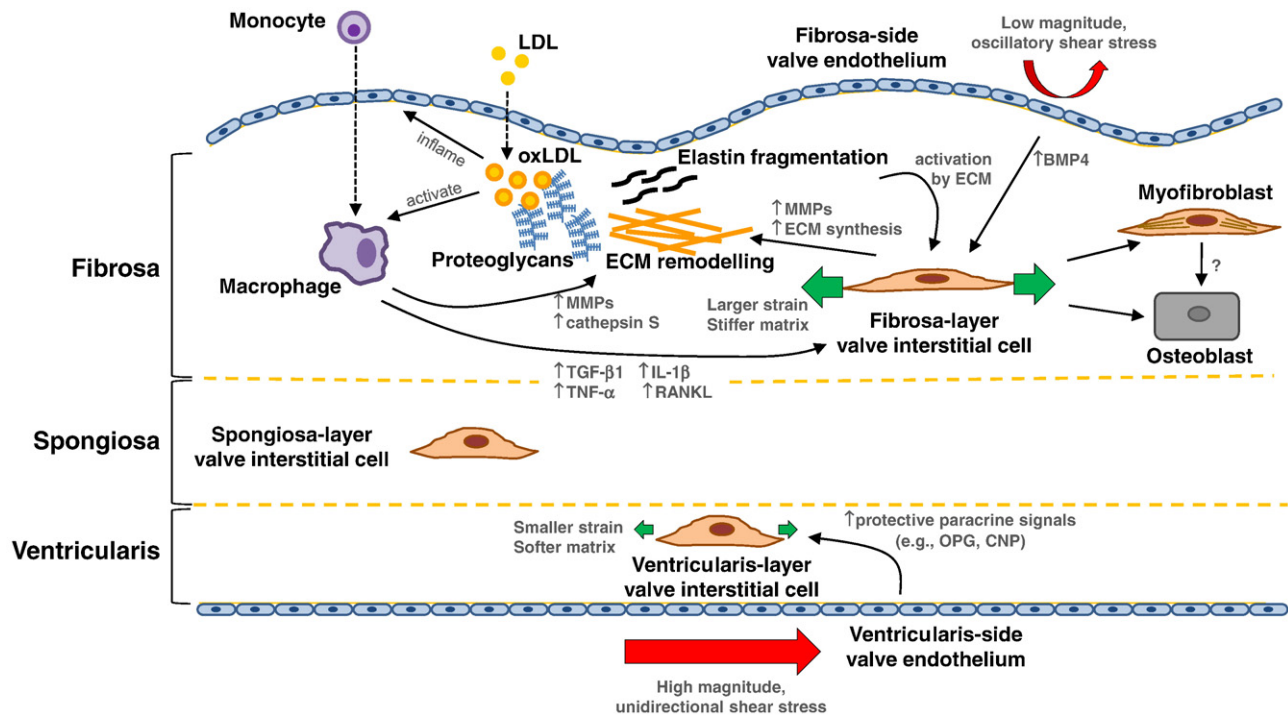


Fig. 1. Microenvironmental factors that potentially play a role in pathological differentiation of VICs and lesion formation in the fibrosa layer of the aortic valve. The fibrosa is the preferred site for low-density lipoprotein (LDL) accumulation, possibly due to trapping in the proteoglycans that accumulate in early valve sclerosis. Oxidatively modified LDL (oxLDL) can inflame the endothelium to attract and bind monocytes, and activate macrophages, which are found primarily in the fibrosa. Activated macrophages produce enzymes like MMPs that mediate ECM remodeling and cathepsin S that fragments elastin, and cytokines like TGF-β1, TNF-α, IL-1β, and RANKL, all of which can potentiate VIC pathological differentiation to myofibroblast and/or osteoblast lineages. Valvular interstitial cell differentiation to osteoblasts may occur via a transitional myofibroblast state, but this has not been demonstrated conclusively. Extracellular matrix remodeling and degradation products can affect VIC activation and differentiation. Valvular interstitial cells, particularly those that are activated, synthesize and remodel the ECM, and produce cytokines, like TGF-β1, that can act in an autocrine/paracrine manner (not shown). Hemodynamic shear stresses caused by disturbed flow on the fibrosa side of the valve may be responsible for the local VECs producing pathological paracrine factors, like BMP4. In contrast, ventricularis-side VECs, which experience high magnitude and unidirectional shear stress, produce paracrine factors like OPG and CNP, which may inhibit pathological differentiation of VICs locally. The VIC response to biochemical and ECM cues is modulated by the local mechanical environment; in particular, the larger strains and stiffer matrix in the fibrosa (large green arrows) may provide a microenvironment that is mechanically permissive for cytokine-induced pathological development. Additionally, VICs and VECs both exhibit layer/side-specific phenotypic differences and respond differentially to some microenvironmental and systemic cues (see the text for details). The spongiosa also contains VICs, but less is known about its microenvironment and the phenotypic characteristics of the cells in this layer.

with pathological outcomes have driven discovery of several key regulators of CAVD, as summarized below.

2. Spatial heterogeneity in valve cell populations

The focal nature of CAVD and the susceptibility for lesions to occur in the fibrosa may be influenced in part by the characteristics of the cells that populate the fibrosa layer. The primary cell types in the aortic valve are valvular endothelial cells (VECs) and valvular interstitial cells (VICs). Recent evidence suggests that both of these populations are heterogeneous and exhibit striking side-dependent phenotypic differences that have the potential to contribute to the focal nature of CAVD.

Valvular endothelial cells form a monolayer that lines the surface of the valve leaflets, separating the circulation from the valve interstitium. As in the vasculature, the endothelium is involved in valve homeostasis and pathology through its principal functions of the maintenance of anticoagulant properties, the regulation of leaflet permeability, and the

initiation and regulation of inflammation and associated pathological consequences. Additionally, the valvular endothelium regulates VIC function through paracrine signals, such as controlling VIC contractility and leaflet mechanics [4]. Of note, VECs from opposite sides of normal porcine leaflets display globally distinct gene expression profiles [5], indicating side-dependent endothelial phenotypes. As discussed below, side-dependent VEC phenotypes may be regulated in part by the distinct hemodynamic environments on opposite sides of the valve. This spatial heterogeneity may contribute to the susceptibility of the fibrosa to lesion formation through differential regulation of permeability, adhesiveness to inflammatory cells, and paracrine signaling to local VICs and circulating cells. As examples of the latter, the fibrosa side endothelium displays a “calcification permissive” transcriptional profile characterized by increased expression of bone morphogenetic protein 4 (BMP4) and decreased expression of multiple inhibitors of fibrosis and calcification, including osteoprotegerin (OPG), C-type natriuretic peptide (CNP), and chordin [5]. In the normal valve, this apparent

vulnerability is balanced by an enhanced antioxidative transcriptional profile on the fibrosa side, which may protect against inflammation and lesion initiation. Side-specific endothelial phenotypic heterogeneity was also evident in swine challenged with a hypercholesterolemic (HC) diet for 2 weeks or 6 months [6]. Unexpectedly, systemic insult resulted in induction and persistence of a *protective* endothelial phenotype on the pathosusceptible aortic side, including downregulation of tumor necrosis factor (TNF)- α and nuclear factor (NF)- κ B pathways. Notably, the aortic side endothelium was more responsive to the HC diet than the adjacent ventricular side endothelium, as measured by side-specific global gene expression; differential sensitivity between the two populations to a systemic stimulus may explain in part side-specific pathosusceptibility.

The other main valvular cell type is the VICs, a heterogeneous population of fibroblasts, with a small population (\sim 1–5%) of myofibroblasts and smooth muscle cells [7–11]. Within the VIC population is a subpopulation of mesenchymal progenitor cells with multilineage differentiation potential [12]. These progenitors are likely the primary source of the pathological cells — primarily myofibroblasts and osteoblasts — that are most often observed in diseased valves. Myofibroblasts comprise up to 30% of the total VIC population in sclerotic leaflets [8,9]. Osteoblast-like cells that express bone sialoprotein, osteocalcin, alkaline phosphatase, and runt-related transcription factor 2 (Runx2) are found in human calcified aortic valves, associated with ectopic bone and cartilage tissue [13,14]. It is conceivable that the tendency for myofibroblasts and osteoblasts to appear in the fibrosa may reflect differential plasticity of fibrosa-layer VICs vs. those from the other layers. Emerging evidence from our lab suggests that this is the case, at least for myofibroblasts (Likhitanichkul and Simmons, unpublished data). Counterintuitively, ventricularis-layer VICs have *greater* myofibroblast differentiation potential than fibrosa-layer VICs in response to profibrotic mechanical and biochemical stimuli *in vitro*. This suggests that fibrosa-layer VICs are inherently less sensitive to profibrotic signals; perhaps this serves as a homeostatic mechanism to counter the many pathological microenvironmental challenges VICs face in the fibrosa (outlined below). Further investigation of this hypothesis and spatially heterogeneous VIC populations is required to establish the implications for valve homeostasis and disease.

3. Local mechanical stimuli

Owing to its location between the left ventricle and aortic root, the aortic valve experiences hemodynamic shear stresses, pressure loads, and flexural deformations that are unlike those experienced by any other tissue in the body. Aortic valve mechanics have been well studied, particularly in the context of the design and failure analysis of mechanical and bioprosthetic valves [15,16]. Native aortic valve pathologies

have also been linked to mechanical factors, primarily based on observations that calcific lesions correlate spatially with regions that experience disturbed hemodynamic flow [5] and high bending stresses [17]. Until recently, it was presumed that these biomechanical forces instigate disease solely by damaging the endothelium [18] or the interstitial matrix [19]. While “wear and tear” may contribute to valve disease, it is increasingly clear that mechanical forces actively regulate valve cell phenotypes and function to contribute to valve homeostasis and pathobiology (reviewed in [20]). Notably, the hemodynamic and biomechanical forces experienced by valve cells differ on opposite sides of the valve leaflets. As discussed below, this suggests that side-specific pathosusceptibility may result in the fibrosa being a mechanically permissive environment for pathological development.

Perhaps the clearest biomechanical difference between the ventricularis and the fibrosa is the local patterns of blood flow. During systole, the ventricular side of the aortic valve leaflets is subjected to unidirectional laminar fluid flow that exerts shear stresses on the endothelium of up to 80 dyne/cm² [21]. In contrast, the fibrosa side endothelium on the “back side” of the leaflet experiences disturbed, oscillatory flow with shear stress magnitudes ranging from -8 to $+10$ dyne/cm². This led us to postulate that local hemodynamic factors contribute to differential endothelial phenotypes that define the sidedness of the valve and the focal susceptibility to calcification [5,22]. Consistent with this hypothesis, oxidative, inflammatory, and chondrogenic/osteogenic gene expression profiles are upregulated in VECs grown under static conditions (mimicking the fibrosa side) vs. steady shear stress conditions (mimicking the ventricularis side) *in vitro* [23]. Similarly, side-specific endothelial phenotypes *in vivo* may be determined in part by the local hemodynamic environment, although causality has yet to be demonstrated [5]. Intriguingly, altered hemodynamics caused increased expression of endothelial adhesion molecules (vascular cell adhesion molecule-1 and intercellular cell adhesion molecule-1) in a transforming growth factor (TGF)- β 1- and BMP4-dependent manner in VECs on the fibrosa side of porcine aortic valves, but not in VECs on the ventricularis side [24]. Thus, side-specific endothelial phenotypes appear to have differential sensitivity to mechanical factors, perhaps explaining in part fibrosa susceptibility to CAVD when hemodynamic forces are altered in hypertensive patients and in those with bicuspid valves.

While VICs are shielded from shear stresses, they are subjected to mechanical deformation through interactions with their ECM, which itself deforms throughout the cardiac cycle as the valve leaflets open and close. Pathological stretch of aortic valve cusps increases VIC expression of remodeling enzymes, proinflammatory proteins, and pathological phenotypic markers [25–27]; these changes occur predominantly in the fibrosa, perhaps reflecting differences in cellular level strains in the different layers, even when leaflets are subjected to nominally uniform stretch. In support of this idea, VIC deformation is the greatest in the fibrosa in leaflets subjected to diastolic transvalvular pressures [28].

Layer-dependent differences in VIC strains could result from differences in the mechanical properties, structure, and residual strain in each of the layers. Indeed, mechanical testing of separated fibrosa and ventricularis layer tissue confirmed that the fibrosa is stiffer than the ventricularis [29,30]; we used a micropipette aspiration technique to obtain a similar result in *intact* leaflets [31]. We also observed considerable heterogeneity in the focal stiffness within both layers, with distinctly stiff and soft regions in the fibrosa and ventricularis, respectively. The inherent stiffness of the ECM is an important factor in the VIC microenvironment, as it defines not only how externally applied strains are transduced from the ECM to the cell, but also the resistance the ECM offers to cell-generated tractional forces. As with mesenchymal stem cells from other sources [32], ECM stiffness regulates a wide range of VIC functions. For example, we showed that VICs preferentially differentiate to osteoblasts and form bone nodules on type I collagen-coated matrices that mimic the stiffer regions of the normal fibrosa [33]. Valvular interstitial cells differentiate to myofibroblasts on even stiffer matrices (that mimic sclerotic tissue) [33,34], but not on softer matrices that mimic the ventricularis [35]. Similarly, VICs differentiated to myofibroblasts on fibrin-modified stiff tissue culture-treated polystyrene but not on fibrin-modified soft polyethylene glycol hydrogels [36].

The collective implication of these studies is that the greater mechanical strain and stiffer ECM experienced by VICs in the fibrosa layer promote pathological development locally. However, it is unlikely that these mechanical cues are solely responsible for driving VIC pathological differentiation; rather, the local mechanical environment likely regulates VIC fate by modulating cellular response to other microenvironmental stimuli, including cytokines and growth factors. For example, TGF- β 1 induction of VIC myofibroblast differentiation occurs only on substrates that are at least as stiff as high-stiffness regions of the normal fibrosa, through a mechanism that involves Wnt/ β -catenin signaling [35]. Thus, the preferential occurrence of myofibroblasts and osteoblasts in the fibrosa layer in CAVD may be due to focal regions in the fibrosa that provide a local mechanical environment that permits or even is required for VIC responsiveness to other pathological stimuli.

4. Local biochemical stimuli

Early CAVD involves lipoprotein deposition [3] and macrophage and T-lymphocyte infiltration [1] in the fibrosa. Oxidized low-density lipoproteins and cytokines produced by activated macrophages and lymphocytes contribute to a local biochemical milieu in the fibrosa that promotes inflammation, oxidative stress, matrix remodeling, VIC pathological differentiation, and fibrosis and calcification (reviewed in [37]).

Many of these factors mediate CAVD in part through pathways involving the activation of TGF- β 1 and BMPs.

Compared to normal human aortic valves, there is abundant expression of TGF- β 1 in stenotic valves, accompanied by a moderate decrease in TGF- β 1 receptors RI and RII [38]. Transforming growth factor- β 1 induces VIC myofibroblast differentiation [39] via Smad-dependent pathways [40], stimulates matrix metalloproteinase (MMP)-2 and MMP-9 expression [41], and increases contractility [33], which can lead to apoptosis-dependent calcification *in vitro* [33,38]. Bone morphogenetic proteins are also upregulated in calcified aortic valves [42]. *In vitro*, BMP2, BMP4, and BMP7 promote calcification by VICs [13,43].

As implied in the previous sections, paracrine factors expressed on a specific side of the leaflet may contribute to the local biochemical milieu to *protect* against pathological development. For example, we identified greater expression of OPG, CNP, and chordin on the disease-protected ventricular side of normal leaflets [5]. Each of these secreted proteins is putatively protective: OPG suppresses cardiovascular calcification [44], and in its absence, receptor activator of NF- κ B ligand (RANKL) is able to bind RANK on VICs to cause elevated MMP-1 and MMP-2 activities [45], DNA binding activity of Runx2, bone-related matrix protein expression, and calcification *in vitro* [46]; CNP inhibits myofibroblast and osteogenic differentiation of VICs *in vitro* [47]; and chordin is a BMP antagonist.

Importantly, and as discussed in the previous section, the effects of some cytokines and growth factors are modulated by the mechanical environment [33,35]. Thus, cytokine signaling in CAVD is context-dependent, with valve mechanics contributing substantially to the microenvironmental context. The implication, therefore, is that the susceptibility of the fibrosa to lesion formation results from it being both a mechanically permissive *and* biochemically rich environment for pathological development. An additional implication of mechanical context-dependent cytokine signaling is that the effects observed in VICs grown on stiff tissue culture polystyrene may not be physiologically relevant or apply to cells grown on softer substrates that mimic valve tissue. The inability of TGF- β 1 to induce myofibroblast differentiation on soft, ventricularis-like substrates is an excellent example [33–35].

5. Local ECM cues

Beyond its structural role, the ECM provides biochemical and, as discussed above, mechanical cues to adherent cells. Alterations in ECM composition [48] and mechanics [49] are characteristic of sclerotic diseases. In CAVD, the fibrosa is particularly prone to remodeling, including disruption and disorganization of collagen bundles [50], fragmentation and stratification of elastin fibers, and increased proteoglycan deposition [48]. Extracellular matrix remodeling is mediated by MMPs, many of which are upregulated and/or have increased activity in calcified aortic valves [50,51], and cathepsin S, a potent elastase that is associated with valvular calcification in a mouse model of chronic renal disease [52].

Macrophages produce MMPs [51] and cathepsin S [52], explaining in part the localization of ECM remodeling to the fibrosa. Macrophages also produce proinflammatory cytokines that stimulate the synthesis and activation of MMP-1 and MMP-2 by VICs, including interleukin (IL)-1 β [53] and TNF- α [54].

Alterations in composition and structure of the fibrosa ECM have the potential to affect VICs indirectly or directly. For example, an indirect consequence of increased proteoglycans in the fibrosa is the retention of lipoproteins and production of oxidized lipid by-products, which may induce inflammation and calcification by VICs locally. Direct effects include the promotion of myofibroblast differentiation and calcification by elastin degradation products [55], and the induction of phospholipid transfer protein expression in VICs by biglycan, potentially resulting in lipid retention, altered lipid metabolism, and inflammation [56]. Further, VIC myofibroblast differentiation and calcification in vitro have been shown to be dependent on ECM composition [57].

Extracellular matrix proteins related to bone, including osteocalcin and osteonectin, are also often expressed (presumably by VIC-derived osteoblasts) in calcified regions of the fibrosa [13], where they bind calcium to promote mineralization. In the vasculature, mineralization is critically regulated by noncollagenous proteins that inhibit mineralization (e.g., matrix gla protein [58]), but little is known about the role of mineralization inhibitors in the valve. An intriguing, but as of yet untested, hypothesis is that the susceptibility of the fibrosa to calcification stems in part from differences in calcium-binding and/or mineralization-inhibiting proteins in the individual layers.

6. Conclusion

The microenvironment of the fibrosa layer of the aortic valve presents biomechanical, biochemical, and ECM cues to VECs and VICs that are distinct from those present in the other layers of the valve. These microenvironmental cues contribute to regulation of valve cell phenotypes locally to prevent or promote valve pathology. Investigations based on spatial correlations between pathological alterations and local microenvironmental cues have provided new insights into the cellular and molecular factors that contribute to CAVD and the complex, nonlinear interactions between these cues. As tools, techniques, and model systems are developed to characterize the fibrosa microenvironment and valve biology with greater spatiotemporal resolution and with improved “systems-level” understanding, additional insights into the molecular regulators of CAVD are expected to emerge and ultimately be translated clinically.

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