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Review Article

In vitro models of aortic valve calcification: solidifying a system

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ABSTRACT

Calcific aortic valve disease (CAVD) affects 25% of people over 65, and the late-stage stenotic state can only be treated with total valve replacement, requiring 85,000 surgeries annually in the US alone (University of Maryland Medical Center, 2013, http://umm.edu/programs/services/heart-center-programs/cardiothoracic-surgery/valve-surgery/facts). As CAVD is an age-related disease, many of the affected patients are unable to undergo the open-chest surgery that is its only current cure. This challenge motivates the elucidation of the mechanisms involved in calcification, with the eventual goal of alternative preventative and therapeutic strategies. There is no sufficient animal model of CAVD, so we turn to potential *in vitro* models. In general, *in vitro* models have the advantages of shortened experiment time and better control over multiple variables compared to *in vivo* models. As with all models, the hypothesis being tested dictates the most important characteristics of the *in vivo* physiology to recapitulate. Here, we collate the relevant pieces of designing and evaluating aortic valve calcification so that investigators can more effectively draw significant conclusions from their results.

1. Introduction

Aortic stenosis, which has an estimated prevalence of 2% in patients between 70 and 80 years of age, is most often caused by calcific aortic stenosis, the late-stage presentation of calcific aortic valve disease (CAVD; note all acronyms and abbreviations used in this article can be found in Table 1) [1]. Prevalence of any aortic valve calcification was investigated in a randomized trial, and, for those aged 75-76, the prevalence was 48%; this further increased in the 80–81 and 85–86 year-old cohorts [2]. The incidence of this age-related disease is expected to grow dramatically as the US population over 65 nearly doubles over the next 25 years [3]. Calcific aortic valve stenosis is the main indication for the 85,000 valve replacements performed annually in the US and necessitates open chest surgery [4], it is currently the only cure [5]. If the biological mechanism of valvular calcification was better understood, we could create more targeted, non-invasive therapeutics; a comprehensive review of CAVD therapeutic targets can be found in Hutcheson et al. [6]. To elucidate the important mechanisms that regulate the progression of CAVD, we first need to design models that recapitulate the *in vivo* human process.

In vivo models offer the complexity found in the human and can prevent overlooking an important variable. However, this complexity comes at the expense of confounding factors, especially because the experiments are performed in animals significantly different from humans. For example, leporine models must be fed very high cholesterol diets to induce the advanced disease observed in humans [7] or vitamin D2 to generate calcification [8]. Murine models require dietary and or genetic modification as well [9–11] to induce calcification; *Ldlr* −/− mice must be fed a high-cholesterol diet and while *Apoe* −/− mice develop hypercholesterolemia over time [12] it is unclear whether it progresses through the same mechanism as the human disease [13]. A full review of animal models of CAVD can be found in Sider et al. [13]. Since *in vitro* models allow better isolation and manipulation of variables and the *in vivo* models are far from perfect, we focus on *in vitro* models and their usefulness.

Once believed to be a passive process, aortic valve calcification is now thought to be an active process mediated largely by aortic valve interstitial cells (AVICs) [14]. AVICs are a heterogeneous population of fibroblast-like cells present in all three layers of the aortic valve and important in the structural maintenance of the valve, especially in maintenance of the extracellular matrix (ECM) [15,16]. Progression of CAVD is marked by the formation of calcific nodules (CNs), which are cellular aggregates characterized in humans by a mixture of calcium phosphate phases [17]. Two well-established hypothetical mechanisms of CN formation exist: 1) transforming growth factor β1 (TGF-β1) mediates activation of myofibroblasts, causing calcification via apoptotic mechanisms [18], and 2) a population of myofibroblasts spontaneously transdifferentiate into osteoblast-like cells and these cells regulate mineralization (Fig. 1) [19,20]. In a study of human valves, 83% of the group demonstrated evidence of

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dystrophic calcification and 13% of those valves had mature lamellar bone and evidence of active bone remodeling [21]. It is unclear whether these processes occur simultaneously or sequentially [22]. Recent progress and the need for a robust *in vitro* system with which we can probe and clarify the mechanism of aortic valve calcification motivate this review.

2. Defining aortic valve interstitial cells

Human aortic valves consist of three layers: 1) the fibrosa faces the aorta and is composed mostly of type I fibrillar collagen arranged circumferentially in parallel bundles in a matrix of elastin, 2) the spongiosa is the middle layer composed of glycosaminoglycans that act as shock absorbers for the valve, and 3) the ventricularis faces the left ventricle and is primarily composed of elastin fibers oriented radially [23]. AVICs are present throughout the leaflets and are a heterogeneous population of myofibroblasts, fibroblasts, and smooth muscle like cells. Aortic valve endothelial cells (AVECs) sheath the surface of the leaflets and are oriented circumferentially and form single cell monolayers, expressing Von Willebrand factor and nitric oxide (NO) [24–26]. Circulating cells have recently been implicated in the progression of calcification as well; elevated levels of endothelial progenitor cells with an osteoblastic phenotype and osteogenic precursor cells have been associated with severe and early heterotopic ossification, respectively [27,28]. Early stages of CAVD develop lesions similar to atherosclerotic lesions, which suggests a role for inflammatory cells and biochemical signals [29,30]. Elevated levels of macrophages and T-lymphocytes have been found in human calcified aortic valves [21,31–33]. These cell populations all contribute to CAVD progression, but it is likely that they influence AVIC behavior through the secretion of factors.

Table 1
Acronyms and abbreviations

αSMA	Alpha smooth muscle actin
AFM	Atomic force microscopy
ALP	Alkaline phosphatase
AVEC	Aortic valve endothelial cell
AVIC	Aortic VIC
aVIC	Activated VIC
BMP2	Bone morphogenic protein 2
BMP4	Bone morphogenic protein 4
β-catenin	Intracellular transducer of Wnt pathway
CAVD	Calcific aortic valve disease
CN	Calcific nodule
CNP	C-type natriuretic peptide
ECM	Extracellular matrix
EDS	Energy-dispersive X-ray spectroscopy
ELISA	Enzyme-linked immunosorbent assay
EMT	Endothelial to mesenchymal transformation
eNOS	Endothelial NO synthase
ESEM	Environmental SEM
FGF-2	Fibroblast growth factor 2
IHC	Immunohistochemistry
IL1-β	Interleukin 1-β
IL6	Interleukin 6
LRP5	Low density lipoprotein receptor-related protein 5
MMP	Matrix metalloproteinase
Msx2	Msh homeobox 2
NF-κB	Nuclear factor kappa-B
NO	Nitric oxide
Notch1	Notch homolog 1
obVIC	Osteoblastic VIC
Osteocalcin/BGLAP	Bone gamma-carboxyglutamic acid-containing protein
oxLDL	Oxidized low-density lipoprotein
PParγ	Peroxisome proliferator-activated receptor gamma
pVIC	Progenitor VIC
qVIC	Quiescent VIC
RANKL	Receptor activator of NF-κB ligand
RT-PCR	Reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
Runx2/CBFα1	Runt-related transcription factor 2/core-binding factor subunit alpha-1
SEM	Scanning electron microscopy
Smad	Intracellular transducer of TGF-β pathway
Sox9	Transcription factor Sox9 of the SoxE family
TEM	Transmission electron microscopy
TGF-β1	Transforming growth factor β1
TIMP	Tissue inhibitor of metalloproteinase
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor alpha
VIC	Valve interstitial cell
Wnt3a	Signaling protein of the Wnt family
$5HT_{2B}$	5-hydroxytryptamine receptor 2B

As the AVIC population is heterogeneous, we should consider the characteristics of various subpopulations. Recently, AVICs were categorized into five groups based on their phenotypic behavior: embryonic progenitor endothelial/mesenchymal cells, quiescent VICs (qVICs), activated VICs (aVICs), progenitor VICs (pVICs), and osteoblastic VICs (obVICs) [34]. We will refer to these subtypes for ease of discussion. Embryonic progenitors are usually present in the cardiac cushions and give rise to qVICs via endothelial to mesenchymal transformation (EMT). While these are very important in valve development, there is also evidence that these progenitors participate in adult valve repair. qVICs are responsible for maintaining physiological valve structure and function. The exact activity of these cells is undefined, but they are believed to regulate low-level matrix degradation and synthesis and inhibition of angiogenesis. pVICs are considered valve stem cells and they are likely responsible for VIC proliferation in response to tissue injury. pVICs may originate from AVECs that undergo an EMT-like process [34–36]. These EMT-related events are likely directly mediated by the mechanical forces present in the valve. In a recent study using chick explanted atrioventricular canals, EMT was found to occur preferentially in higher regions of strain [37]. This developmental process is likely recapitulated in an unregulated fashion during CAVD progression. This suggests that as the valve stiffens, more AVECs are transformed into pVICs and qVICs, allowing subsequent activation.

aVICs are qVICs that have become myofibroblasts characterized by alpha smooth muscle actin (αSMA) and increased contraction [34]. This activation occurs under pathological injury cues or abnormal mechanical stress via cytokines and growth factors produced by activated AVECs and macrophages. aVICs are associated with increased ECM secretion and degradation, matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) expression, proliferation and migration, and secretion of cytokines including TGF-β1. If apoptotic pathways become abnormal, aVICs can lead to calcification; this is referred to as the dystrophic pathway. obVICs are VICs that have undergone osteoblastic differentiation and promote calcification *in vitro*. This differentiation is induced by the addition of organic phosphate to culture media and subsequent calcification depends on the upregulation of alkaline phosphatase (ALP) activity. Adding bone morphogenic protein 2 (BMP2) and 25-hydroxycholesterol increased the rate of CN formation, as did TGF-β1, which induced calcification via an apoptotic mechanism [38]. BMP2 has been shown to be higher in stenotic human aortic valves [39] and upregulates osteogenic pathways involving Msx2 and Wnt signaling [40] and Runt-related transcription factor 2/core-binding factor subunit alpha-1 (Runx2/CBFα1) [41]. It is likely that AVECs are regulating aVIC or obVIC function and that, given the presence *in vivo* of both BMP2 and TGF-β1, a combination of osteogenic and dystrophic pathways is occurring. Therefore, we are most concerned with the transitions to and behavior of aVICs and obVICs.

AVIC–AVEC co-culture systems have provided some insight into the complex regulation of AVICs. When porcine AVICs were cultured with the same number of AVECs in a 3-dimensional (3D) model, they demonstrated decreased αSMA, cell number, and increased total protein and sulfated glycosaminoglycan content compared to AVICs cultured alone in the 3D collagen gel [42]. Given osteogenic differentiation media, AVICs in 3D collagen hydrogels showed much higher levels of