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Growth and maturation of heart valves leads to changes in endothelial cell distribution, impaired function, decreased metabolism and reduced cell proliferation

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ABSTRACT

Risk factors of heart valve disease are well defined and prolonged exposure throughout life leads to degeneration and dysfunction in up to 33% of the population. While aortic valve replacement remains the most common need for cardiovascular surgery particularly in those aged over 65, the underlying mechanisms of progressive deterioration are unknown. In other cardiovascular systems, a decline in endothelial cell integrity and function play a major role in promoting pathological changes, and while similar mechanisms have been speculated in the valves, studies to support this are lacking. The goal of this study was to examine age-related changes in valve endothelial cell (VEC) distribution, morphology, function and transcriptomes during critical stages of valve development (embryonic), growth (postnatal (PN)), maintenance (young adult) and aging (aging adult). Using a combination of in vivo mouse, and in vitro porcine assays we show that VEC function including, nitric oxide bioavailability, metabolism, endothelial-to-mesenchymal potential, membrane self-repair and proliferation decline with age. In addition, density of VEC distribution along the endothelium decreases and this is associated with changes in morphology, decreased cell-cell interactions, and increased permeability. These changes are supported by RNA-seq analysis showing that focal adhesion-, cell cycle-, and oxidative phosphorylation-associated biological processes are negatively impacted by aging. Furthermore, by performing high-throughput analysis we are able to report the differential and common transcriptomes of VECs at each time point that can provide insights into the mechanisms underlying age-related dysfunction. These studies suggest that maturation of heart valves over time is a multifactorial process and this study has identified several key parameters that may contribute to impairment of the valve to maintain critical structure-function relationships; leading to degeneration and disease.

1. Introduction

The mature heart valve leaflets are highly organized structures that open and close over 100,000 times a day to regulate unidirectional blood flow through the heart. Movement is largely facilitated by three highly organized layers of extracellular matrix (ECM) that provide all the necessary biomechanics to respond to changes in the hemodynamic environment during the cardiac cycle. In contrast, disruption to ECM organization is often associated with insufficiency that can lead to progressive heart failure [1]. Homeostasis of the valve ECM is mediated by a heterogeneous population of fibroblast-like valve interstitial cells (VICs), and previous studies have shown that VIC function is regulated by a single layer of valve endothelial cells (VECs) that line the surface of the leaflets [2–7]. In addition to regulating VIC behavior, the valve endothelium serves as a physical barrier between the blood and the inner valve tissue; thereby preventing excess infiltration of circulating factors associated with risk and inflammatory cells [2,8]. A murine wire injury model suggests that physical denudation of the aortic valve endothelium is sufficient to induce disease [9], consistent with histological findings reporting loss of endothelial cells in diseased human valves [10–12]. In addition, VEC-specific disruption of essential signaling pathways can alter ECM organization and lead to dysfunction in mice [3,4,

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13–16]. Therefore, integrity and function of the valve endothelium appear to be essential for maintaining structure-function relationships throughout life.

Aging is a significant risk factor of heart valve insufficiency, affecting up to 13.2% of people over the age of 75 [17]. Age-related disorders of the vascular system are attributed to progressive endothelial cell dysfunction and pathological landmarks and mechanisms of this process have been used as early predictors of cardiovascular disease and targeted for therapeutic treatments respectively [18–21]. These studies have largely been focused on the association of vascular endothelial cell dysfunction with impaired endothelial nitric oxide (NO) synthesis. Similar to vascular endothelial cells, VECs also require NO to maintain function as reduced bioavailability of endothelium-derived NO leads to morphological defects at birth and dysfunction in adults [4,16,22–25]. While the requirement for NO is conserved, previous studies have noted several differences between vascular and valvular endothelial cells largely in their molecular and phenotypic response to biomechanical stress [26]. Therefore it is not clear if determinants of age-related endothelial cell dysfunction in vascular disease can account for failure of the valve to maintain structure-function relationships later in life.

To address this deficit, we investigated VEC histology, function and molecular profiles at key stages of valve development, growth, maturation, and aging. Our findings demonstrate that maintenance of the valve is a multifactorial process, and aging is associated with changes in VEC density, decreased function, reduced proliferation, and diverse molecular profiles. These findings highlight the potential mechanisms that may be abrogated in later stages of life, and potentially diseased valves that lead to impaired homeostasis.

2. Methods

2.1. Mice

Tie2GFP (Tg(TIE2GFP)287Sato/J) and wild type *C57BL/6J* were obtained from Jackson Labs. Mice aged to E14.5 (embryonic) post-natal day 1–3 (PN) 4 months old (young adult), and >12 months old (aging adult) were used for all studies unless otherwise stated in the text. All animal procedures were approved and performed in accordance with IACUC and institutional guidelines provided by The Research Institute at Nationwide Children's Hospital.

2.1.1. Isolation of VECs from Tie2GFP mice

Murine VECs were isolated from E14.5, PND2–3, 4 month-old and 12–15 month-old *Tie2GFP* mice as previously described by our lab [27]. Briefly, valvular tissue from both semilunar and atrioventricular valves was dissected and dissociated using collagenase IV for 7 min at 37 °C. The supernatant, containing the dissociated cells was collected and kept on ice. This process was repeated nine times in order to collect an enriched population of endothelial cells. Isolated cells were pelleted and resuspended in HBSS containing EDTA and DNaseI (RNase-free) and subjected to flow cytometry to collect the GFP+ endothelial cells as described below. All samples collected consisted of multiple (1–3 litters or mice) biological replicates pooled together to yield between 8000 (E14.5)–60,000 (12–15 months) GFP+ cells and approximately 10 ng mRNA.

2.2. Histology

2.2.1. Bright-field and immunofluorescence

Whole embryos and whole hearts from embryonic, PN, young adult, and aging adult *Tie2GFP* or *C57BL/6J* mice were dissected and fixed overnight in 4% PFA/1xPBS at 4 °C and subsequently processed for paraffin or cryo embedding. Paraffin tissue sections were cut at 7 μm and subjected to Pentachrome staining according to the manufacturer (American MasterTech). Cryo-embedded tissue sections were cut at 7 μm and stored −20° before immunofluorescent staining. Briefly, cryo sections were blocked for 1 h (1%BSA, 1% cold water fish skin gelatin, 0.1% Tween-20/PBS) followed by incubation with CD31 (BD Biosciences #553370 rat anti-mouse 1:1000) or CD45 (R&D Systems AF114 rabbit 1:200) diluted in 1:1 Block/1xPBS overnight. On the next day, slides were incubated with goat-anti-rat-488 or goat-anti-rabbit-568 Alexa-Fluor secondary antibody for 1 h at room temperature, mounted with Vectashield containing DAPI, and imaged on an Olympus BX51 microscope. Quantification of CD45+ cells was reported as a percentage of total DAPI+ cells in aortic valves from E14.5, PN, young and aging adult *C57BL/6J* mice. Statistical significance was determined using the Student's *t*-test between each time point for n = 3.

2.2.2. Quantification of VEC cell density

VEC density was quantified from at least 5 aortic valve sections taken from 3 biological replicates stained with Toluidine blue. Aortic valve cusps were divided into proximal (area between annulus and hinge region), mid (area between hinge and distal tip) and distal (tip region denoted by increase in cross sectional area of the leaflet) regions based on morphology. For quantification, the number of endothelial cells on the cusp surface spanning the proximal, mid, and distal regions of young and aging adult aortic valves were counted and divided by the endothelial surface distance (μm) measured by ImageJ software. The number of VECs per 50 μm of the valve surface was reported for each region. Significance was determined using the Student's *t*-test between distal, mid, and proximal regions or between young and aging adult regions.

2.2.3. Transmission electron microscopy

Whole hearts from embryonic, PN, young adult, and aging adult *C57BL/6J* mice were placed in 2.5% glutaraldehyde in Millonig's PO_4 with glucose for 24–48 h at 4 °C and then transferred into Millonig's Buffer prior to aortic valve dissection. Dissected aortic valves were post fixed in 1% osmium tetroxide for 2 h to overnight, rinsed in Millonig's PO_4 buffer, and dehydrated in graded ethanol series and cleared in propylene oxide. Tissue was then infiltrated in 50–50 Epon/Araldite–propylene oxide for 4 h followed by infiltration in full Epon/Araldite overnight. Samples were embedded in fresh Epon/Araldite and placed in a 60 °C oven for 24–48 h. Blocks were trimmed and 500 nm thick sections were cut and collected onto slides, which were stained with Toluidine blue and assessed for areas of interest. Selected blocks were cut at 60 nm on a Leica Ultracut UCT ultramicrotome. Sections were collected onto CuPd grids and stained with uranyl acetate and lead citrate before viewing on a Hitachi H 7650 TEM. Observations were concluded from imaging three independent aortic valve samples from each reported time point.

2.3. Culturing porcine aortic valve endothelial cells

Young and aging porcine aortic valve endothelial cells (pAVECs) were isolated from AoV cusps from 6 to 7 month-old juvenile pigs (young) or >2 year-old adult pigs (aging) (Animal Technologies) respectively as previously described [28]. Cells were cultured in DMEM containing 10% L-glutamine, 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (pen/strep), and 50 U/ml heparin sodium salt (Sigma). All experiments were carried out between passages 2–5 and aging and young pAVECs were always compared at the same passage number (p2–3).

2.4. DAR-4M AM staining

2.4.1. Cultured pAVECs

Confluent young and old pAVECs were washed with PBS and treated with 10 μM DAR-4M AM (CalBioChem 251765) for 30 min at 37 °C. Cells were then rinsed, mounted in Vectashield containing DAPI (Vector), and imaged in the Texas Red channel. Alternatively, cultures were treated with an NO donor, 250 μM DETA NONOate (Cayman Chemicals 82120), under the same culture conditions, as a positive control. For