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Original Article

Evaluation of a porcine model of early aortic valve sclerosis

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ABSTRACT

Background: Calcific aortic valve disease (CAVD) is associated with significant cardiovascular morbidity. While late-stage CAVD is well-described, *early* pathobiological processes are poorly understood due to the lack of animal models that faithfully replicate early human disease. Here we evaluated a hypercholesterolemic porcine model of early diet-induced aortic valve sclerosis.
Methods: Yorkshire swine were fed either a standard or high-fat/high-cholesterol diet for 2 or 5 months. Right coronary aortic valve leaflets were excised and analyzed (immuno)histochemically.
Results: Early human-like proteoglycan-rich onlays formed between the endothelial layer and elastic lamina in the fibrosa layer of valve leaflets, with accelerated formation associated with hypercholesterolemia ($P<.05$). Lipid deposition was more abundant in hypercholesterolemic swine ($P<.001$), but was present in a minority (28%) of onlays. No myofibroblasts, MAC387-positive macrophages, or fascin-positive dendritic cells were detected in 2-month onlays, with only scarce myofibroblasts present at 5 months. Cells that expressed osteochondral markers Sox9 and Msx2 were preferentially found in dense proteoglycan-rich onlays ($P<.05$) and with hypercholesterolemia ($P<.05$). Features of more advanced human CAVD, including calcification, were not observed in this necessarily short study.
Conclusions: Early aortic valve sclerosis in hypercholesterolemic swine is characterized by the formation of proteoglycan-rich onlays in the fibrosa, which can occur prior to significant lipid accumulation, inflammatory cell infiltration, or myofibroblast activation. These characteristics mimic those of early human aortic valve disease, and thus the porcine model has utility for the study of early valve sclerosis.

1. Introduction

The most common valvular disease in the United States [1] and Europe [2] is calcific aortic valve disease (CAVD). CAVD [3] encompasses both sclerosis, which is present in more than 25% of those over 65 years of age [4,5] and is characterized by leaflet thickening without left ventricular outflow obstruction, and stenosis, which is present in more than 2% of those over 65 years and is characterized by stiffened leaflets, obstructed flow, and compromised cardiac function [4,6]. While the majority of patients with sclerosis do not develop stenosis and the association between sclerosis and stenosis is unclear, the consequences of both are significant: sclerosis is associated with a 50% increased risk of cardiovascular death and myocardial infarction [7] and the prognosis for patients with stenosis is very poor (20–60% mortality) [8,9].

In CAVD, the valvular extracellular matrix (ECM) structure and arrangement becomes disorganized, an active pathobiological process that is mediated by pathways similar to those involved in valve development and bone and cartilage metabolism [10–13]. While many features of late-stage human CAVD are well-described, there is little understanding of early sclerosis. The few reports describing early human valvular lesions suggest similarities to early atherosclerotic lesions [14–17], but more than 60% of patients with CAVD do not have clinically significant coronary atherosclerosis [18], suggesting divergent processes.

Early human sclerotic lesions occur preferentially within the disease-prone fibrosa layer. ECM proteins, predominantly proteoglycans [19], deposit between the endothelial layer, which has a disrupted basement membrane, and the displaced, fragmented, and reduplicated elastic lamina, thereby creating a lesion onlay [14,15,17,20]. Lipoproteins can be observed in the subendothelial region of the lesion (~25% of valves) [14,17], co-localized with proteoglycans [16,19]. Macrophages are sometimes present (~20–60% of valves), typically at the surface of the lesions [14,15], and often as aggregates ranging from a few to a substantial number of cells [14] with the occasional presence of foam cells [14,21] and T lymphocytes

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[14]. α-Smooth muscle actin (α-SMA) positive cells, associated with late CAVD, are only occasionally found within early lesions (~22% of valves) [14]. Little else is known about the cellular phenotypes in early disease, and in particular the expression of osteochondral proteins that are observed in late disease [22–25]. Mineralization is generally absent in lesions in young (20–40 year old) adults [15], and is variably observed in the earliest lesions studied to date from older patients (~5–77% of valves, depending on the extent of disease) [14,15,17], co-localized with the deeper portions of the lipoprotein deposit [14]. The layered appearance of early lesions suggests an active disease process with a sequential progression [14].

The study of early CAVD is hampered not only by the difficulty in obtaining human tissue samples with comparable extents of early disease, but also by the lack of well-characterized animal models. Mice and rabbits are often used to study valvular disease, but they do not develop focal human-type lesions (reviewed in [26]). Swine are excellent models for atherosclerosis studies as they: (1) share similar vascular anatomy, lipid profile, lipoprotein metabolism, and genome with humans; and (2) produce human-type atherosclerotic lesions on high-fat/high-cholesterol diets and naturally with age (reviewed in [26]). Swine have recently been used to study CAVD and display the potential for developing valvular lesions [27,28]. The presence of valvular lesions in swine and their utility in atherosclerosis research suggest that they may be an important model for studying the initiation and progression of CAVD. However, description of disease characteristics in this model has been largely limited to the endothelium [27,28] and not validated against findings in human disease.

We hypothesized that early changes in the aortic valve ECM and cellular phenotypes in a diet-induced hypercholesterolemic porcine model would mimic characteristics of very early human sclerotic lesions. To test this, we characterized early changes in the aortic valve ECM and cellular phenotypes in this porcine model and compared the findings to those reported for early human sclerosis. We found that the porcine model does faithfully mimic characteristics of very early human sclerotic lesions and thus enables new insights into early disease progression. However, slow disease development and the practical husbandry challenges of rapid growth and the large size of Yorkshire swine generally precludes this model from longer longitudinal studies to verify and investigate more advanced CAVD.

2. Methods

2.1. Animal model

Twenty-four male Yorkshire barrows were divided into four groups (six animals per group): (1) 2-month control barrows (start weight: 59–65 kg; age: ~3.6 months) fed a standard grower corn/soybean based diet containing 15% protein, 75% carbohydrate, and 10% fat (kcal%) at 3.51 kcal/g for 2 months (65 days); (2) 2-month high-fat/high-cholesterol (HF/HC) diet experimental barrows (start weight: 59–65 kg; age: ~3.6 months) fed the grower diet supplemented with an additional 12% lard and 1.5% cholesterol and containing 15% protein, 53% carbohydrate, and 32% fat at 4.02 kcal/g for 2 months (65 days); (3) 5-month control barrows (start weight: 14–16 kg; average age: ~2.0 months) initially fed a standard corn/soybean based starter III diet, due to their young age, containing 19% protein, 71% carbohydrate, and 10% fat at 3.51 kcal/g until ~40 kg followed by the grower diet used for group 1 up until 5 months (155 days); (4) 5-month HF/HC diet experimental barrows (start weight: 14–16 kg; age: ~2.0 months) initially fed the starter III diet supplemented with an additional 12% lard and 1.5% cholesterol and containing 19% protein, 49% carbohydrate, and 32% fat at 4.01 kcal/g until ~40 kg followed by the grower diet from group 2 up until 5 months (155 days) (Supplemental Table 1). The experimental diet was fed at 87% of the mass of the control diet to provide isocaloric intake. Feed was adjusted weekly based on body weight to achieve a growth rate of ~0.75 kg/day (actual average 0.88 kg/day) for the 2-month trial and ~0.70 kg/day (actual average 0.62 kg/day) for the 5-month trial. Swine weighing 106–126 kg were sacrificed by electrical stunning and bleed out, according to standard abattoir practices. Blood samples were taken by venipuncture from the ear or orbital sinus at baseline and biweekly or monthly thereafter for the measurement of serum total cholesterol, high-density lipoproteins, and triglycerides. The protocol was approved by the University of Guelph (#09R057) and University of Toronto (#20007312) Animal Care Committees. All animals were housed individually and were treated in accordance with the recommendations of the Guide for the Care of Laboratory Animals published by the United States National Institute of Health [29].

2.2. Porcine aortic valve tissue

Twenty-four right coronary leaflets (6 per group) were harvested within 2 hrs of sacrifice and frozen in 10% dimethyl sulfoxide (DMSO) in RPMI medium in a 1°C freezing container and stored at −180°C for 6–7 months. Only right coronary leaflets were examined in this study for consistency and because of their larger size; other leaflets may demonstrate different disease patterns. At the time of analysis, leaflets were thawed for 5 min in a 37 °C bath and DMSO was removed with three PBS with Ca^{2+} and Mg^{2+} 10 min doublings on ice. Leaflets were marked with 11–14 black marks (100–250 μm diameter) with calibrated toothpicks and Tissue Marking Dye (TBS; Durham, NC, USA) within the belly and coaptation line (Supplemental Fig. 1) to identify points for histological examination and mechanical testing (not reported here). They were then fixed in 10% neutral buffered formalin for 48 hours at room temperature and stored in 70% ethanol at 4°C for 1–2 months. Samples were segmented so that each marked location and the center transverse section could be embedded individually (Supplemental Fig. 1). Due to technical difficulties only a transverse section through the midline of the leaflet was processed for one 2-month control leaflet. The samples were embedded in paraffin and sectioned in the radial direction through the black dye locations or to provide full transverse center sections according to routine procedure at 5 μm thickness.

2.3. Histological analysis

For each leaflet, all marked locations (2 months: $n=139$; 5 months: $n=148$) and center sections ($n=24$) were sectioned and stained with Movat's pentachrome (MP) stain (Electron Microscopy Sciences, Hatfield, PA, USA) to identify ECM components. Center sections and six locations from each leaflet (2 months: $n=66$; 5 months: $n=72$), representing the range of ECM conditions seen within each leaflet according to MP staining, were selected for further immunohistochemical analysis.

2.4. Immunohistochemical analysis

The presence, extent, and pattern of cellular components and lipoprotein deposition within the valve leaflets were detected using the following immunohistochemical antibodies: (1) lipoprotein deposition with anti-apolipoprotein B (ApoB; sheep polyclonal, 3.6 μg/ml, AHP214, AbDSerotec, Raleigh, NC, USA); (2) macrophages with anti-macrophage (mouse monoclonal, 20 μg/ml, MAC387, ab22506, Abcam, Cambridge, MA, USA); (3) dendritic cells with anti-fascin (mouse monoclonal, 10 μg/ml, 55 K2, ab78487, Abcam); (4) myofibroblasts with anti-α-smooth muscle actin (α-SMA; mouse monoclonal, 4 μg/ml, 1A4, ab7817, Abcam) [30]; and (5) osteochondrogenic cells with anti-RUNT-related transcription factor 2 (Runx2; rabbit polyclonal, 40 μg/ml, M-70, sc-10758, Santa Cruz Biotchnology, Santa Cruz, CA, USA), anti-Msh homobox 2 (Msx2; goat polyclonal, 28.6 μg/ml, T-20, sc-17731, Santa Cruz), and anti-SRY-box 9 (Sox9; rabbit polyclonal, 3 μg/ml, ab26414, Abcam). The immunohistochemical protocol was as follows: Sections were melted for 30 min at 60 °C and deparaffinized with xylene and