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EXPERIMENTALLY INDUCED DISEASE

Development and Evaluation of a Tissue-Engineered Fibrin-based Canine Mitral Valve Three-dimensional Cell Culture System

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Summary

Myxomatous mitral valve disease is the most common cardiac disease of the dog, but examination of the associated cellular and molecular events has relied on the use of cadaveric valve tissue, in which functional studies cannot be undertaken. The aim of this study was to develop a three-dimensional (3D) cell co-culture model as an experimental platform to examine disease pathogenesis. Mitral valve interstitial (VIC) and endothelial (VEC) cells were cultured from normal and diseased canine (VIC only) valves. VICs were embedded in a fibrin-based hydrogel matrix and one surface was lined with VECs. The 3D static cultures (constructs) were examined qualitatively and semiquantitatively by light microscopy, immunofluorescence microscopy and protein immunoblotting. Some constructs were manipulated and the endothelium damaged, and the response examined. The construct gross morphology and histology demonstrated native tissue-like features and comparable expression patterns of cellular (α-smooth muscle actin [SMA] and embryonic smooth muscle myosin heavy chain [SMemb]) and extracellular matrix associated markers (matrix metalloproteinase [MMP]-1 and MMP-3), reminiscent of diseased valves. There were no differences between constructs containing normal valve VICs and VECs (type 1) and those containing diseased valve VICs and normal valve VECs (type 2). Mechanical manipulation and endothelial damage (type 3) tended to decrease α-SMA and SMemb expression, suggesting reversal of VIC activation, but with retention of $SMemb^+$ cells adjacent to the wounded endothelium consistent with response to injury. Fibrin-based 3D mitral valve constructs can be produced using primary cell cultures derived from canine mitral valves, and show a phenotype reminiscent of diseased valves. The constructs demonstrate a response to endothelial damage indicating their utility as experimental platforms.

Keywords: myxomatous mitral valve disease; tissue engineering; valve endothelial cell; valve interstitial cell

Introduction

Myxomatous mitral valve disease (MMVD) is the most common cardiac disease in dogs (Whitney, 1974; Buchanan, 1977; Beardow and Buchanan, 1993; Connell *et al.*, 2012). Most studies, to date, have examined the cellular and molecular features of the disease using native valve tissue collected at the time of necropsy examination. However, tissue availability can be variable and unpredictable, and there can be heterogeneity in samples obtained. There is, therefore, a need for cell culture models to

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provide consistent and reproducible experimental platforms for studies that are not readily achievable using valve tissue alone.

In MMVD, affected leaflets become thickened, distorted and less transparent. Valve endothelium denuding is common and there is evidence of endothelial-to-mesenchymal transition (Han *et al.*, 2010, 2013; Lu *et al.*, 2015). Extracellular matrix (ECM) changes can be severe, but are located mainly in the distal third of the leaflet up to the free edge, and include collagen loss, production of fibrillar collagen that fails to mature, and increased proteoglycan and glycosaminoglycan synthesis (Cole *et al.*, 1984; Tamura *et al.*, 1995; Gupta *et al.*, 2009; Aupperle *et al.*, 2009a; Hadian *et al.*, 2010; Han *et al.*, 2010).

The pathogenesis of MMVD is only partially understood, but may in part be a result of valve leaflet maladaptive responses to long-term shear stress damage of the valve endothelium, triggering subendothelial valve interstitial cell (VIC) activation and ECM remodelling (Pedersen and Haggstrom, 2000; Gotlieb *et al.*, 2002; Corcoran *et al.*, 2004). VICs, the major source of valve matrix, change from a quiescent interstitial phenotype to an activated myofibroblast and then congregate in the subendothelium (Stein *et al.*, 1989; Mow and Pedersen, 1999; Corcoran *et al.*, 2004; Barth *et al.*, 2005; Black *et al.*, 2005; Han *et al.*, 2008). Activated VICs are characterized by increased expression of alpha smooth muscle actin (α-SMA) and/or the embryonic form of non-muscle myosin heavy chain (SMemb) and have heightened wound repair and ECM remodelling capability (Tamura *et al.*, 2000; Rabkin *et al.*, 2001; Gotlieb *et al.*, 2002; Black *et al.*, 2005; Disatian *et al.*, 2008; Han *et al.*, 2008). Similar effects have been seen with endothelial damage in an ovine mitral valve organ culture model, suggesting that endothelial changes likely contribute to MMVD pathogenesis (Lester *et al.*, 1992, 1993).

The capability to investigate complex interactions between VICs and valvular endothelial cells (VECs) and the effect of VECs on VIC function, and by extension valve remodelling, is limited using native valve tissue. Tissue engineering (TE) has been used widely to model cardiovascular diseases and to examine cell behaviour, function and pathophysiological responses, and should be applicable to MMVD (Butcher *et al.*, 2004; Butcher and Nerem, 2006; Gupta *et al.*, 2008a, b). In that context, three-dimensional (3D) cultures and TE models are superior to traditional two-dimensional (2D) cultures as they more resemble the *in vivo* arrangement of cell distribution and orientation, cell signalling and communication, and ECM production and organisation (Mueller-Klieser, 1997).

The aims of the present study, therefore, were to use primary canine mitral valve cells, derived from both normal and diseased dogs, to develop a fibrin-based VEC/VIC 3D co-culture system (construct), and to partially assess its utility as a platform to investigate the pathogenesis of MMVD, by mechanical manipulation and endothelial damage.

Materials and Methods

Cell Isolation, Culture and Characterization

Canine mitral VECs and VICs were isolated and cultured as described previously (Liu *et al.*, 2015). VECs were derived from healthy mitral valves only, while VICs were derived from both healthy and diseased valves (Whitney grade 1 and 2; mild to moderate disease; Whitney, 1974). Dogs ranged in age from 1.5 to 5 years, with one elderly (i.e. >7 years) dog, from which VICs only were harvested. VECs were harvested at passage 2 or 3 and VICs at passage 4 to 7. All harvesting of valve tissues and cells was undertaken with full owner permission and with ethical approval from the Veterinary Ethics Research Committee of the Royal (Dick) School of Veterinary Studies, the University of Edinburgh. Constructs were then produced using cells from different dogs. Cell phenotypes were confirmed by immunofluorescence microscopy examining for expression of the general mesenchymal marker vimentin and the VEC-specific marker CD31 (platelet and endothelial cell adhesion molecule [PECAM]-1). Each construct was divided into quarters for immunofluorescene microscopy and western blotting (WB), with the remainder archived for future studies.

Native Mitral Valve Tissue

Mitral valves were examined to demonstrate patterns of marker distribution for healthy and diseased valves. Sections were obtained from archived valves as previously reported (Lu *et al.*, 2016).

Fabrication of Fibrin-based 3D Mitral Valve Constructs

Constructs were developed based on previously described techniques with minor modification (Flanagan *et al.*, 2006; Cholewinski *et al.*, 2009). Each construct was fabricated in one well of a sterile 24-well culture plate by mixing 175 µl VIC suspension in pH 7.4 Tris buffered saline (TBS) (1×10^6 cells/gel), 37.5 µl 40 IU/ml thrombin (Sigma, Poole, UK) 37.5 µl 50 mM $CaCl_2$ (BDH, Poole, UK) and 250 µl of 10 mg/ml bovine plasma fibrinogen (Sigma). The hydrogel was left to polymerize at 37°C in 5% CO_2 in an incubator for 40–60 min. VECs (1.8×10^4/ml) were re-