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A three-dimensional co-culture model of the aortic valve using magnetic levitation

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ABSTRACT

The aortic valve consists of valvular interstitial cells (VICs) and endothelial cells (VECs). While these cells are understood to work synergistically to maintain leaflet structure and valvular function, few co-culture models of these cell types exist. In this study, aortic valve co-cultures (AVCCs) were assembled using magnetic levitation and cultured for 3 days. Immunohistochemistry and quantitative reverse-transcriptase polymerase chain reaction were used to assess the maintenance of cellular phenotype and function, and the formation of extracellular matrix. AVCCs stained positive for CD31 and α-smooth muscle actin (αSMA), demonstrating that the phenotype was maintained. Functional markers endothelial nitric oxide synthase (eNOS), von Willebrand factor (VWF) and prolyl-4-hydroxylase were present. Extracellular matrix components collagen type I, laminin and fibronectin also stained positive, with reduced gene expression of these proteins in three dimensions compared to two dimensions. Genes for collagen type I, lysyl oxidase and αSMA were expressed less in AVCCs than in 2-D cultures, indicating that VICs are quiescent. Co-localization of CD31 and αSMA in the AVCCs suggests that endothelial–mesenchymal transdifferentiation might be occurring. Differences in VWF and eNOS in VECs cultured in two and three dimensions also suggests that the AVCCs possibly have anti-thrombotic potential. Overall, a co-culture model of the aortic valve was designed, and serves as a basis for future experiments to understand heart valve biology.

1. Introduction

The aortic valve, located between the left ventricle and the aorta, regulates unidirectional blood flow from the heart to the systemic circulation. The valve consists of three leaflets, each of which is comprised of two cell types: valvular interstitial cells (VICs) that populate the interior of the leaflet, and valvular endothelial cells (VECs) that cover the surface of the leaflet. VICs are a heterogeneous group of cells with fibroblast and smooth muscle cell phenotypes that maintains the extracellular matrix (ECM) of the leaflet [1–3]. VECs regulate the transfer of signals in the bloodstream to the valve interior, mediate inflammatory and hemostatic responses, and have a phenotype similar to vascular endothelial cells, but demonstrate major differences in alignment to flow and mechanobiology [4–7]. Together, both VICs and VECs work to maintain the leaflet structure and valve function.

The interactions between these two cell types are critical for normal valve function. The presence of VECs has been shown to keep VICs in a quiescent state, as defined by the low expression of α-smooth muscle actin (αSMA) [8]. The key role that their interaction plays in valve function is also demonstrated by the finding that dysfunction of both cell types appears in calcific aortic valve disease (CAVD) [9]. Injury to the valvular endothelium leads to thrombosis, inflammation and lipid accumulation [10–15]. These factors lead to the activation of VICs, or increased expression of αSMA, matrix remodeling, and their progression towards an osteoblastic phenotype, which ultimately result in calcification and stenosis [16–20].

Despite the well-established notion that both cell types and the interaction between the two are critical to valve maintenance, function and disease, there are few co-culture models of VECs and VICs in the literature. The lack of co-culture models of the aortic valve can partly be attributed to the inability to produce such models using traditional 2-D cell culture techniques, with which the majority of research on valvular cells is conducted. 2-D environments are poor representations of the native 3-D environment

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in which valvular cells reside [21–23]. The inadequacy of 2-D cultures for valvular research is highlighted by the varied results of studies on the effect of statins as treatments for calcific aortic valve disease. Statins in 2-D *in vitro* cultures of VICs significantly reduced calcific nodule size and area, as well as αSMA expression [24–28], yet clinical trials have not shown any improvement in outcome for patients with calcific aortic stenosis who took statins [29–33]. Indeed, studies of the effect of statins on VICs in 3-D collagen gels showed a smaller, conditional reduction in calcification in comparison to 2-D cultures [26]. These results demonstrate the enormous gap in complexity and fidelity between simple 2-D cell culture models and the human body, and necessitate the development of cost-effective, clinically relevant and representative 3-D co-culture models of the aortic valve.

To that end, this study used a magnetic levitation method with magnetic nanoparticles to assemble 3-D co-cultures of VECs and VICs. In this method, cells are incubated with a nanoparticle assembly consisting of poly-L-lysine, magnetic iron oxide (MIO; Fe_3O_4, magnetite) and gold nanoparticles that form a gel via electrostatic interactions [34–37]. The uptake of this gel by cells renders them magnetic and allows for their manipulation, specifically by levitating the cells off the surface into the media, where the cells aggregate and interact to form larger 3-D structures. This method has previously been used to create 3-D cultures of glioblastomas, smooth muscle cells, adipose stem cells and pulmonary cells [37–41]. Magnetically levitated human glioblastoma cells demonstrated greater proliferation and more *in vivo*-like protein expression in comparison to 2-D cultures [37].

In addition, this method has previously been used to create co-cultures. Layered co-cultures of the lung were sequentially assembled using epithelial cells, smooth muscle cells, fibroblasts and endothelial cells within 8 h [41]. After 2 days of culture, ECM (collagen type I, fibronectin, laminin) was formed and organized, and the phenotypes of all four cell types were maintained. After 7 days, epithelial cell function and phenotype were still present [41]. In comparison, previously reported co-culture models of the valve leaflet consisted of collagen-based gels which were embedded and contracted with VICs, and then seeded with VECs on their surfaces in a process that took between 4 and 8 days to assemble [8,42]. As a result, magnetic levitation is an alternative method for rapidly assembling co-culture models.

Based on the success of the magnetically levitated co-culture of the bronchiole, this study used magnetic levitation to create 3-D co-cultures of aortic valve cells. VICs and VECs were sequentially assembled into layered co-cultures. Immunohistochemistry was used to verify the phenotype and function of both VECs and VICs, and assess ECM formation within the co-culture. Unlike previous studies using magnetic levitation, this study is the first to analyze the gene expression profiles of these cultures using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). In addition, the effects of the magnetic nanoparticles and exposure to the magnetic field on cell proliferation were investigated. The expected result of this study was a VIC and VEC co-culture model that maintained cell phenotype and function, and synthesized relevant ECM.

2. Materials and methods

2.1. Cell isolation and culture

Aortic valves were extracted from fresh porcine hearts obtained from a local commercial abattoir (Fisher Ham and Meats, Spring, TX). Aortic VECs and VICs were harvested as previously described [43,44]. Both cell types were cultured in an incubator (37 °C, 5% CO_2, 95% humidity) with changes of medium every other day.

VECs were isolated from the leaflets via digestion using collagenase II (60 U ml^{-1}) and dispase (2 U ml^{-1}) [43]. VECs were seeded on flasks or glass slides coated with 2.5% gelatin in 1:1 H_2O:phosphate-buffered saline (PBS, pH ~7.4) [45], and cultured in specialized medium (EGM-2, Lonza Biosciences, Walkersville, MD) with 2% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). At the first passage, magnetic cell sorting was used to purify the VECs for CD31+ cells (mouse monoclonal anti-CD31 antibody TLD-3A12, Millipore, Billerica, MA) [43]. VECs were used at their third passage.

VICs were isolated from the leaflets with a multistep digestion using collagenase II (~450 U ml^{-1}), hyaluronidase (~50 U ml^{-1}) and collagenase III (~350 U ml^{-1}) [44]. VICs were seeded on uncoated flasks or glass slides in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% bovine growth serum (BGS, Hyclone, Logan, UT), 1% HEPES buffer and 1% P/S. VICs were used at their third passage.

2.2. Magnetic levitation

Magnetic levitation using the Bio-Assembler Kit (Nano3D Biosciences, Houston, TX) was employed to create 3-D cultures (Fig. 1B) [37]. Confluent flasks of cells were treated with a magnetic nanoparticle assembly (8 μl cm^{-2} of cell culture surface area or 50 μl ml^{-1} medium, NanoShuttle (NS), Nano3D Biosciences) for overnight incubation to allow for cell binding to the nanoparticles. NS was fabricated as previously described, by mixing Au nanoparticles prepared by citrate reduction, poly-L-lysine and iron oxide [34,35,37]. Treated cells were then detached with trypsin and resuspended in an ultra-low attachment 24-well plate with 400 μl of medium. A magnetic driver of 24 neodymium magnets (field strength = 50 G) designed for 24-well plates and a plastic lid insert were placed atop the well plate to levitate the cells to the air–liquid interface.

2.3. Cell metabolism

The effects of both the NS and magnetic field on cell metabolism over 8 days were measured using an MTT assay. Briefly, MTT reagent (0.5 mg/ml in medium, thiazolyl blue tetrazolium bromide, Sigma–Aldrich, St Louis, MO) was added to each well. After 3–4 h of incubation, the medium was aspirated to yield the formazan blue crystals at the bottom of the well. Acidified isopropanol (0.1 N HCl in isopropanol) was added to dissolve the formazan blue crystals, and the absorbance of the resulting solution was read in triplicate on a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA) at 570 nm with background subtraction at 690 nm. All measurements were normalized to day 0 results.

To test the effects of adding NS (+NS) or exposure to a magnetic field (+mag) to VIC and VEC proliferation, 25,000 cells were seeded in 24-well plates. For +NS wells, NS was added to the wells (8 μl cm^{-2}) on day 0 for incubation overnight. For +mag wells, neodymium magnets were placed 1 mm underneath each well (field strength = 300 G). Medium was changed at day 1 and every other day after that for the duration of the study. Cell metabolic activities were measured on days 2, 4, 6 and 8 ($n = 9$).

2.4. Co-culture assembly

Magnetic levitation was used to create co-cultures of VICs and VECs (Fig. 2A) [41]. VICs and VECs were incubated overnight with NS, and then levitated into 3-D cultures of 500,000 cells each. After 4 h of levitation, a 0.1875 in. outside diameter Teflon pen housing a neodymium magnetic rod was used to sequentially pick up a 3-D culture of each cell type to assemble the co-culture: first VECs, then VICs. The still attached co-culture was then submerged in 150 μl