Calcification of Porcine Aortic Valvular Interstitial Cells

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Introduction

Calcific aortic stenosis (AS), stiffening of aortic valve leaflets and narrowing of the aortic orifice, is the most common heart valve disease in developed countries [1]. The aortic valve is located between the aorta and the left ventricle and consists of 3 leaflets. Each leaflet contains outer endothelial layers and interstitial cells interspersed in the matrix between the endothelial layers. Hardening of the leaflets is caused by calcification and in some cases, bone formation within the interstitium of the leaflets.

The endothelial cells on the ventricular side of the leaflets are exposed to a generally unidirectional high velocity laminar shear flow whereas the flow pattern on the aortic side is more irregular and disturbed. Interestingly, calcification occurs preferentially within the interstitium closer to the aortic side of the leaflet and is likely mediated by valve interstitial cells (VICs) [2, 3]. In a recent paper published by Simmons et al [4], it was found that inhibitors of calcification such as osteoprotegerin (OPG) were less expressed by the endothelial cells on the disease-prone aortic side. This could be caused by the different flow patterns seen on the two sides of the leaflet.

The calcification process itself is thought to be an active process initiated by endothelial dysfunction, which causes up-regulation of certain growth factors and cytokines that trigger calcification by VICs, as shown in Fig 1 [5].

![Fig 1. Hypothesized calcification process in the aortic valve [5].](image-url)
Research Objectives

The research objective for this summer project is to further investigate various aspects of this calcification process, such as the influence of signaling factors produced by endothelial cells (EC). Because calcification is believed to be initiated by endothelial dysfunction, we hypothesize that the EC produce paracrine signals that inhibit calcification and thus protect the valve. In addition, because the VICs are more prone to calcification on the aortic side, we hypothesize that EC subjected to a shear flow produce more inhibitory signals than EC in a static or low shear environment.

Cell Isolation and Culture

Aortic valve leaflets were dissected from healthy pig hearts obtained from a local slaughterhouse. EC were scraped off using a scalpel blade and cultured in a M199 & 10% fetal bovine serum (FBS) & 1% antibiotics medium. The leaflets denuded of the endothelial layers were then digested in a collagenase solution (600 U/mL) to isolate the VICs. VICs were cultured in a DMEM & 10% FBS & 1% antibiotics medium. EC were observed to be cobblestone-like in morphology whereas interstitial cells were more elongated and spindle-shaped. These findings are consistent with the literature. Early passaged cells were used for all experiments.

Experimental Methods & Results

In order to verify our hypotheses on the role of EC in regulating calcification by VICs, it was first necessary to establish a cell culture model for calcification in VICs alone. The main purpose of my summer work thus far was focused on driving the VICs towards an osteogenic lineage to induce bone mineralization.

In the first attempt, VICs were cultured with an osteogenic supplemented (OS) media composed of normal interstitial cell culture media plus 10 mmol/L β-glycerophosphate (βGP), which serves as a trigger to induce bone mineralization. At confluence, VIC were cultured in this media for 14 days in tissue culture-treated 12-well plates and were compared to control cells grown without βGP for bone nodule formation, alkaline phosphatase (ALP; an essential enzyme for bone mineralization) staining and calcium deposits visualized by von Kossa staining. Bone nodules are aggregates of cells commonly found in vitro in calcifying interstitial cells or bone cells. After 14 days, the OS cell cultures were expected to exhibit increased numbers of bone nodules and more intense ALP and calcium staining compared to control. Unfortunately, there was no visible difference observed between the two cell cultures for all three parameters. This led to the conclusion that βGP alone might not be sufficient to induced calcification in 2 weeks.

The second attempt at inducing calcification was more promising. The cells were cultured in 24-well collagen-coated plates for a week. They were cultured only for a week to avoid over-confluence and overlapping cell layers. Collagen synthesis occurs during normal bone formation, so it was predicted that collagen might affect the VICs to
accelerate the calcification process. In addition to βGP, 50 ng/mL RANKL was also added to the OS media. This was based on a published paper by Kaden et al [6], where they observed significantly increased nodule formation, ALP activity and calcium levels with the addition of RANKL to VICs (see Fig 2). Results for our experiment showed that there was increased ALP staining after 7 days in culture, compared to day 0 controls. While the VICs in OS medium appeared to have slightly increased ALP staining compared with the day 7 control cells, the data were inconclusive. We are currently in the process of re-assessing and modifying this experiment.

**Fig 2.** The effects of RANKL on (A, B) nodule formation, (C) mineralization, and (D) ALP activity by valve interstitial cells from the experiment by Kaden et al. [6].

**Future Plans**

I am continuing this research project for my 4th year undergraduate thesis. Two major future experiments planned are the conditioned media experiment and the co-culture experiment, both of which will involve EC.

**Conditioned media experiment:**

Currently, a flow chamber has been set up (by Samir Raza in the Simmons Group) to imitate the flow pattern seen by the EC on the ventricular side of the valve. EC are sheared in this flow chamber in a similar way to what occurs in vivo. In this experiment, I plan to culture VICs with conditioned media taken from the flow chamber. The conditioned media might contain certain paracrine factors produced by the EC under shear stress that will inhibit calcification in the VIC culture. However, there are several drawbacks to this experiment. First, the signaling proteins produced by the EC might
have a short half-life so that by the time the conditioned media is transferred to the VIC culture, most of the proteins would have already degraded. Secondly, direct cell-to-cell contact between the EC and VICs might be required for this signaling pathway.

Co-culture experiment:

The idea for a co-culture experiment is based on a previous paper on calcifying vascular cells (CVC) by Shin et al [7]. In their experiment, EC were plated onto a porous collagen-coated membrane. The membrane was then flipped over and CVC were plated on the other side (see Fig 3a). It was found that mineralization by the CVC decreased when the EC layer was present (see Fig 3b), which is consistent with our hypothesis for valvular cells. My co-culture experiment will follow a similar procedure except that valvular endothelial and interstitial cells will be used as opposed to vascular cells. With this set-up using the porous collagen-coated membrane, direct cell-to-cell contact is possible and since both cells will be cultured together, there is no need to worry about the short half-life of EC signaling proteins. However, this experiment will be performed under static conditions and will need to be modified to accommodate any shear stress effects.

Fig. 3a (top) 3b (bottom): Endothelial-calcifying vascular cell co-culture experiments from Shin et al. [7].

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References


