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Culture and characterisation of canine mitral valve interstitial and endothelial cells

M.-M. Liu [a], T.C. Flanagan [b], C.-C. Lu [a], A.T. French [c], D.J. Argyle [a], B.M. Corcoran [a,*]

[a] Royal (Dick) School of Veterinary Studies, The Roslin Institute, the University of Edinburgh, Easter Bush, Roslin, Mid-Lothian, Scotland EH25 9RG, United Kingdom
[b] School of Medicine & Medical Science, Health Sciences Centre, University College Dublin, Belfield, Dublin 4, Ireland
[c] University of Glasgow School of Veterinary Medicine, Bearsden Road, Glasgow, Scotland G61 1QH, United Kingdom

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ABSTRACT

Valve interstitial cells (VICs) have an important role in the aetiopathogenesis of myxomatous mitral valve disease (MMVD) in the dog. Furthermore, there is evidence that valve endothelial cells (VECs) also contribute to disease development. In addition to examining native valve tissue to understand MMVD, another strategy is to separately examine VIC and VEC biology under in vitro culture conditions. The aim of this study was to isolate and characterise canine mitral VICs and VECs from normal dog valves using a combination of morphology, immunohistochemistry and reverse transcription PCR (RT-PCR).

Canine mitral VECs and VICs were isolated and cultured in vitro. The two cell populations exhibited different morphologies and growth patterns. VECs, but not VICs, expressed the endothelial markers, platelet endothelial cell adhesion molecule (PECAM-1 or CD31) and acetylated low density lipoprotein (Dil-Ac-LDL). Both VECs and VICs expressed vimentin and embryonic non-smooth muscle myosin heavy chain (SMemb), an activated mesenchymal cell marker. The myofibroblast marker, alpha smooth muscle actin (α-SMA), was detected at the mRNA level in both VEC and VIC cultures, but only at the protein level in VIC cultures. The morphological heterogeneity and expression of non-endothelial phenotypic markers in VEC cultures suggested that a mixture of cell types was present, which might be due to cell contamination and/or endothelial–mesenchymal transition (EndoMT). The use of a specific endothelial culture medium for primary VEC cultures enhanced the endothelial properties of the cells and reduced α-SMA and SMemb expression.

Introduction

Myxomatous mitral valve disease (MMVD) is characterised by extensive changes to the valve extracellular matrix (ECM), resulting in valve distortion and mechanical instability, and leading to valve insufficiency and mitral regurgitation (Black et al., 2005; Hadian et al., 2007; Aupperle et al., 2009, 2010; Han et al., 2010, 2013b). The mechanisms that lead to valve degeneration are not fully understood, but are presumed to involve changes in valve interstitial cell (VIC) and endothelial cell (VEC) phenotype and function (Corcoran et al., 2004; Disatian et al., 2008; Han et al., 2008, 2013a). Endothelial changes are characterised by detachment, denuding and apoptosis with splitting of the basement membrane and increased expression of basement membrane proteins, including laminin (Aupperle et al., 2009; Han et al., 2013a). VICs show an activated myofibroblast phenotype; their numbers decline in the overtly myxomatous areas, and they proliferate close to the valve surface, most noticeably where there has been endothelial damage (Disatian et al., 2008; Han et al., 2008, 2013a).

VICs are the cells contributing to ECM production and remodelling. Their synthetic activity is driven mainly by the transforming growth factor (TGF)-β cytokine superfamily, but is also thought to be modified by signals from endothelial cells (Olsen et al., 2003; Disatian et al., 2010; Aupperle and Disatian, 2012; Orton et al., 2012; Han et al., 2013a). This VEC–VIC interaction has led to the hypothesis that VEC activation is fundamental to valve matrix homeostasis and that MMVD may be driven by mechanisms involving VEC damage leading to aberrant matrix remodelling.

Future examination of MMVD will require a greater understanding of VEC–VIC interactions and one approach is to use in vitro models. Canine mitral VICs have been previously isolated and cultured using a primary explant technique which favours culturing migratory cells only, but there was limited phenotypic characterisation, and there are no reports of successful cultures of canine VECs (Heaney et al., 2009). The aims of the present study, therefore, were (1) to develop robust methods for the culture and phenotypic characterisation of canine mitral VICs and VECs based on a reported protocol for porcine aortic valve cell isolation (Gould and Butcher,

* Corresponding author. Tel.: +44 131 651 9100.
E-mail address: Brendan.Corcoran@ed.ac.uk (B.M. Corcoran).

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2010), and (2) to examine canine mitral valve cell biology under these culture conditions. Furthermore, as it is recognised to be difficult to maintain endothelial cell phenotype in VEC cultures, different culture protocols were evaluated.

Materials and methods

Cell isolation and culture

Cells were isolated from 12 healthy canine mitral valves showing no MMVD evidence on gross morphological inspection. The dogs were of different breeds, aged 2–5 years and with equal sex distribution. All dogs were euthanased for reasons other than cardiac disease. Samples were collected with full owner consent and sampling conformed to institutional and national ethical guidelines.

Mitral valves were removed within minutes of euthanasia under sterile conditions, rinsed in cold sterile phosphate buffered saline (PBS) and placed on ice until transferred to a tissue culture laminar hood. Excess annular tissue and chordae tendineae were removed, and samples were placed in 35-mm Petri dishes and incubated with 5 mL pre-warmed collagenase solution (600 U/mL) in 5% CO_2 for 10 min at 37 °C. VECs were removed by gently rotating a dry sterile swab over the surface of the leaflet and occasionally dabbing the swab into the collagenase solution. The collagenase solution was aspirated and the suspended cells pelleted by centrifuging at 1000 *g* for 5 min at room temperature. The supernatant was aspirated and the cells were resuspended in advanced DMEM/F-12 medium (Life Technologies) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine (AF-12). Cells were centrifuged, re-suspended again and seeded on culture flasks or plates pre-coated with 2% gelatin and incubated at 37 °C in 5% CO_2. After VEC collection, tissue was placed in a 15 mL conical tube with 10 mL collagenase solution (600 U/mL) and incubated for 18 h at 37 °C in 5% CO_2. After digestion, the tissue was homogenised in the collagenase solution. The released cells were then seeded onto flasks and cultured as described for VECs in AF-12 medium.

Media changes occurred at 2-day intervals for both populations. At confluence, cells were passaged by either trypsinisation (0.05%–1% trypsin /EDTA) or by using 1× TrypLE Express (Life Technologies). In general, VECs were expanded up to passage 3 and VICs were cultured up to passage 8.

Comparison of two culture media for canine mitral VECs

To evaluate the effect of specific endothelial culture medium on primary VEC properties, a canine endothelial basal medium kit (Cn 211k-500, Cell Application; EBM) was compared with the AF-12 medium for VECs. VEC preparations (at passages 1–2) from different dogs ($n = 3$) were used for the medium comparison experiment. Prior to the medium switch, cells were stored in freezing medium (70% AF-12 medium/20% FBS/10% dimethylsulfoxide (DMSO; Invitrogen)) and cryopreserved in a –150 °C freezer or in a liquid nitrogen freezer. Cells were revived in a 37 °C water bath and were washed with pre-warmed AF-12 medium by centrifuging. After removal of the supernatant, the cells were re-suspended in canine EBM and seeded onto culture plates pre-coated with 2% gelatin and incubated at 37 °C in 5% CO_2. Control cultures were maintained in AF-12 for the same VEC preparation. VEC cultures in EBM and AF-12 were harvested at confluence and evaluated for endothelial and mesenchymal marker expression.

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was isolated from both cell types using the RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. Extracted RNA was treated with RNase-free DNase I and concentration of RNA was measured using NanoDrop (Thermal Scientific). RNA was diluted using nuclease-free water (Qiagen) and was denatured at 65 °C for 5 min, then placed immediately on ice. Complementary DNA was synthesised using an Omniscript Reverse Transcription Kit (Qiagen) at 40 °C for 1 h.

The following genes (Table 1) were selected to characterise the VECs and VICs: *CD31* and *von Willebrand Factor* (*vWF*) (endothelial markers); *alpha-smooth muscle actin* (*α-SMA*), *embryonic form of non-smooth muscle myosin* (*SMemb*) and *transgelin* (*SM22*) (activated VIC (aVIC) markers); *vimentin* (mesenchymal origin cells). *GAPDH* was used as a housekeeping gene. The primers of *vWF-1*, *α-SMA* and *GAPDH* were kind gifts (Hannah Hodgkiss-Geere, the University of Edinburgh), and the *vWF3* primer sequence was derived from Fulton et al. (2000). All other primers were originally designed using Primer 3 Input v.4.0 and nucleotide sequences of the above markers were obtained from the NCBI Gene Bank or Ensembl databases.

PCR amplification used the Gotaq PCR Core System (Promega). Initial denaturation at 95 °C for 5 min was followed by 30 cycles of 95 °C for 1 min, 58–61 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were analysed using a standard 2% agarose gel electrophoresis. Molecular Imager Gel Doc system (Bio-Rad) was used to visualise the results after electrophoresis.

Immunocytochemistry

The cell suspension (200 µL) was added to duplicate BD Falcon Culture slides, pre-coated with 2% gelatin for VECs, at a density of 1×10^4 cell/well and incubated in 5% CO_2 at 37 °C for 24–48 h. Slides were directly fixed with acetone for 10 min at –20 °C, washed twice in PBS, incubated for 1 h in blocking buffer (10% goat serum/0.1% Tween 20/PBS) and then overnight in a humidity chamber at 4 °C with primary antibodies against CD31, vimentin, SMemb and α-SMA (Table 2). Slides were washed twice with PBS and incubated with 100 µL goat anti-rabbit IgG or goat anti-mouse IgG secondary antibody for 1 h at room temperature in a dark humid chamber. The slides were then washed three times in PBS, 4′,6-diamidino-2-phenylindole (DAPI) mounted (Vectashield Mounting Medium, Vector), and examined using a Leitz fluorescence microscope (Leica).

Table 1
Primer sequences for the PCR characterisation of valve endothelial cells (VECs) and valve interstitial cells (VICs).

Gene	Primer sequence (5′–3′)	Product size
CD31	AATCCCAAATTCCACGTCAG GAATGGAGCACCACAGGTTT	346 bp
vWF1	CTGGGAGAAGAGAGTCACGG GTGGATGGAGTACACGGCTT	235 bp
vWF2	GGCTGTACCTGGATGAGAGG GACAGGACAGGCTCCTTTTG	228 bp
vWF3 (Fulton et al., 2000)	AATATAGGGCCCCGGCTCACTCAA ACATCCCCGGGCCTCTTCTCATTC	512 bp
SM22	AAGAACGGCGTGATTCTGAG CGGTAGTGCCCATCATTCTT	269 bp
α-SMA	GGGGATGGGACAAAAGGACA GCCACGTAGCAGAGCTTCTCCTTGA	525 bp
SMemb	AGAAGCGAGCTGGAAAACTG TCTTGCTCTGTCCGATTCTG	252 bp
Vimentin	GGAGCAGCAGAACAAGATCC AGACGTGCCAAAGAAGCATT	282 bp
GAPDH	CATCAACGGGAAGTCCATCT GTGGAAGCAGGGATGATGTT	428 bp

DiI-acetylated-low density lipoprotein (DiI-Ac-LDL) labelling

Acetylated low density lipoprotein conjugated with the fluorescence dye 1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) was used to detect endothelial cells. The cell culture protocol prior to labelling was the same as that described for immunocytochemistry. DiI-Ac-LDL (200 µL; 5 µg/mL; BT902, Biomedical Technologies) reagent was added to one cell chamber and incubated for 4 h in 5% CO_2 at 37 °C. Slides were washed three times with PBS and were examined by fluorescence and bright field microscopy.

Image capture and processing

Cell morphology was observed by light microscopy during the culture period and assessed for cell morphology and confluence. Representative images were captured using a camera connected Zeiss Axiover 40 microscope. Immunofluorescence and DiI-Ac-LDL fluorescent channel images were captured using a Leica Firecam. For optimal visualisation of the fluorescence signals, the antibody and DAPI staining images were converted into greyscale with Image J software. Original antibody and DAPI channel colour images were merged using Adobe Photoshop CS6 software (Adobe System). For DiI-Ac-LDL labelling, bright field images were taken from the same sampled area demonstrating cell distribution and were converted into greyscale images with Image J software.

Table 2
Details of the antibodies used for canine mitral valve endothelial cell (VEC) and valve interstitial cell (VIC) immunocytochemistry characterisation.

	Species raised	Catalogue number and supplier	Dilution
Primary antibody			
Anti-CD31	Rabbit polyclonal	Ab28364, Abcam	1:200
Anti-SMemb	Rabbit polyclonal	Ab24761, Abcam	1:400
Anti-vimentin	Mouse monoclonal	V6389, Sigma	1:1600
Anti-α-SMA	Mouse monoclonal	A2547, Sigma	1:400
Secondary antibody			
Anti-rabbit IgG (H + L) Alexa Fluor568	Goat	A11011, Invitrogen	1:500
Anti-mouse IgG (H + L) Alexa Fluor488	Goat	A10667, Invitrogen	1:500