

Phenotypic Characterization of Isolated Valvular Interstitial Cell Subpopulations

Tracy L. Blevins¹, Joshua L. Carroll¹, Alina M. Raza², K. Jane Grande-Allen¹

¹Department of Bioengineering, Rice University, Houston, ²Department of Biomedical Engineering, University of Houston, Houston, TX, USA

Background and aim of the study: Valvular interstitial cells (VICs) demonstrate a heterogeneous range of phenotypes such as variable expression of smooth muscle α -actin (SM α A). Myofibroblast-like VICs, expressing high levels of SM α A, are thought to be involved in myxomatous degeneration of mitral valves. The inability to isolate specific cell types has restricted potential investigations of valvular disease mechanisms. Thus, investigations were conducted into methods of isolating different cell subpopulations from primary VICs as a preparatory step for cell type-specific evaluations of heart valve disease.

Methods: VICs were isolated from porcine valves, cultured to 80% confluency, and subdivided using differential detachment or adhesion. The subdivided cells were further cultured and analyzed phenotypically by immunocytochemistry and flow cytometry to characterize SM α A expression. Roundness and growth rates were also analyzed.

The nascent field of heart valve biology has the potential to revolutionize the treatment of valve disease worldwide. Interest in this field has been invigorated by recent progress in developing tissue-engineered valves, and in determining the biochemistry and structure of the valvular extracellular matrix (ECM). Many difficult questions remain, however, concerning the biology and dysfunction of the valvular interstitial cells (VICs). These cells are located throughout the valve interior and are believed to coordinate the biological and potentially the mechanical function of the valve. A key characteristic of VICs is that they exhibit a regionally diverse phenotype of fibroblastic, myofibroblastic, and smooth muscle cell characteris-

Results: VICs that were relatively sensitive to trypsinization expressed low and heterogeneous levels of SM α A (15-35%), whereas more-adherent VICs expressed higher and homogeneous levels (>98%) suggestive of a myofibroblast-like phenotype. The more-adherent cells also had lower growth potential and were less round than less-adhesive VICs. Separated cell subtypes were found to maintain their phenotype through several cell passages.

Conclusion: VICs are a mixed population of cells, many of which express high levels of SM α A. Differential detachment and adhesion can effectively separate cell subpopulations from primary cultures of VICs. The ability to study valve cell subpopulations has substantial implications for future analyses of valvular biology, disease, and tissue engineering.

The Journal of Heart Valve Disease 2006;15:815-822

tics (1-5). It remains unclear whether this mixed phenotype is caused by a single population of cells that express many features simultaneously (1,5) and/or can switch between multiple phenotypes (6), or is actually a population of several types of cell (7). Myofibroblasts, which exhibit characteristics of both fibroblasts and smooth muscle cells, are particularly abundant in myxomatous valves (4) as well as in fibrotic diseases of the kidney (8,9) and other organs. The cause of this overabundance of myofibroblasts, and their role in the myxomatous degeneration of the valve, is unknown.

Although VICs as a group are generally attributed with the production of valvular ECM, it seems likely that a specific subpopulation of the heterogeneous cells - potentially the myofibroblasts or their specific VIC precursors - may be responsible for the development of the diseased valve phenotype. Distinct VIC populations have been difficult to explore functionally, however, because the mixed cells have proven difficult to separate in culture. Because valve diseases are characterized not only by shifts in the heterogeneous cell

Presented as a poster at the Third Biennial Meeting of the Society for Heart Valve Disease, 17th-20th June 2005, Vancouver Convention and Exhibition Centre, Vancouver, Canada

Address for correspondence:
K. Jane Grande-Allen PhD, Department of Bioengineering, MS 142,
Rice University, P.O. Box 1892, Houston, TX 77251-1892, USA
e-mail: grande@rice.edu

phenotypes but also by alterations in the ECM (4,10), it was hypothesized that the cellular production of ECM might serve as the basis for a cell-separation technique. In particular, cells that have a pericellular matrix rich in hyaluronan and versican - as found in myxomatous mitral valve disease (10) - have been shown to detach readily from tissue culture plastic (11,12). It has also been observed that valve cells in general trypsinize very heterogeneously, with some cells detaching rapidly while others remain attached for more than 10 minutes. Thus, the study aim was to separate a mixed population of VICs into two subpopulations based on their rates of detachment and/or adhesion. The resulting subpopulations were then analyzed phenotypically, using both microscopy and flow cytometry.

Materials and methods

Isolation of primary VICs

VICs from porcine aortic valves were used in these studies because the porcine aortic valve has been widely used as a model for human valves (13); moreover, these cells have been well characterized in previous reports (1,2,5).

Porcine hearts were obtained from an abattoir and returned to the laboratory. Within 24 h, the valve leaflets were dissected from the heart and rinsed thoroughly in sterile phosphate-buffered saline (PBS). To loosen the endothelial cells, the leaflets were placed in 15 ml collagenase II solution (Worthington Biochemical Corp., Lakewood, NJ, USA; 2 mg/ml in serum-free Dulbecco's modified Eagle's medium (DMEM); Mediatech, Herndon, VA) and agitated on an incubated rotary shaker (Barnstead MaxQ 4000, Dubuque, IA, USA) for 20 min (150 rpm) at 37°C. The leaflets were then removed from the collagenase II and all outer surfaces swabbed with a sterile cotton swab to remove endothelial cells. The leaflets were finely minced with a sterile scalpel, placed into 15 ml collagenase III solution (1 mg/ml collagenase, 0.1 mg/ml DNase, 0.1% hyaluronidase in serum-free DMEM; all enzymes from Worthington), and incubated in a 150-rpm shaker for 4 h at 37°C. Following treatment, the mixture was poured through a 70- μ m cell strainer and the cells pelleted by centrifugation for 5 min at 1500 \times g. The resulting cells were counted and seeded into T-75 flasks at a density of 10,000 cells/cm². The cells were incubated at 37°C with 95% humidity and 5% CO₂ in DMEM:F12 with low glucose, HEPES buffer, 10% bovine growth serum (BGS; Hyclone, Logan, UT, USA; all other culture solutions from Mediatech), and 1% antibiotic-antimycotic solution. The cells were cultured in the incubator for approximately three days, at which point they were almost 80% confluent. Cells were then imaged at 10-20 \times using a Leica DFC320 CCD camera

connected to a Leica DMIL inverted microscope (Wetzlar, Germany).

To confirm that VIC cultures contained minimal valvular endothelial cells, porcine VICs and 5- μ m slices of paraffin-embedded porcine mitral valves (before and after collagenase II treatment) were incubated with antibodies against CD 31 (MAB 1393; Chemicon International, Temecula, CA, USA) and von Willebrand factor (generously donated by Dr. Joel Moake, Rice University) for 1 h at 25°C. Immunohistochemistry was completed using a biotin-SP-conjugated anti-mouse IgG secondary antibody (1 h, 25°C), followed by a 30-min incubation of the ABC Elite reagent (Vector Labs, Burlingame, CA, USA) and a final 10-min incubation with 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA).

Sorting of cell subpopulations

The first method proposed to separate VIC subpopulations was differential detachment via trypsinization. When the VIC cultures reached 80% confluency, the media was aspirated from the flasks and the cells washed once with sterile PBS. Trypsin (2 ml, diluted 1:10 with PBS) was added to the flask and rocked back-and-forth once over the cells to maximize enzymatic detachment. After 1 min, the trypsin and detached cells were removed from the flask and placed into a 15-ml conical tube containing 8 ml media with 10% BGS. Another 2 ml diluted trypsin was added to the same T-75 flask, and the preceding process was repeated 14 times. Following the 14th detachment using diluted trypsin, 2 ml full-strength trypsin was added to the T-75 flask to detach the strongest-adhering cells. After 2 min, the trypsin and cells were removed and placed in a 15-ml conical tube with 8 ml media containing 10% BGS. This full-strength trypsinization was repeated once. The cells within the resulting 16 conical tubes with the cell mixtures were pelleted, resuspended in 0.1 ml 10% BGS in DMEM, and counted.

The fastest 25% ('FAST') and slowest 25% ('SLOW') of cells to detach were then resuspended in 10 ml medium/10% BGS, separately seeded into T-75 flasks, and grown in a tissue culture incubator. Upon reaching 80% confluency, the aforementioned method was repeated to isolate the second generation of FAST cells (FAST2) from the first 25% of cells to detach from FAST. The SLOW2 subpopulation was likewise isolated from the last 25% of cells to detach from SLOW. A third round of trypsin-based separation was used to create FAST3 and SLOW3. The numbers of cells detached per minute were tracked in all of these separations in order to characterize the phenotypic detachment rate of the cell subpopulations.

Later, a second method of separation was tested based on the cells' differential adhesion to tissue cul-

ture plastic. Cultured porcine aortic VICs (P0) were grown to 80% confluency, trypsinized using full-strength trypsin, pelleted, resuspended in 10 ml media/10% serum, then placed in a T-75 flask and incubated for 30 min. After incubation, the media and unattached cells were replaced with fresh media and 10% serum. The unattached cells and medium were placed into a new T-75 flask for an additional 30 min. This process was repeated until cells were grouped by adhesion times of 30 min (most adherent), 1 h, 4 h and 1 day (least adherent). All four flasks were then grown to 80% confluence before further analysis.

Microscopy and image analysis

The phenotypic shapes of the FAST2 and SLOW2 cells were compared using phase-contrast imaging. Following trypsinization, these VIC subcultures were seeded into separate six-well plates at a density of 10,000 cells/cm², and allowed to adhere for 72 h. The cells were then imaged at 10× and 20× magnification in five different areas of four randomly selected wells for each of FAST2 and SLOW2. All cells in each image were digitally traced using Image-Pro Express (Media Cybernetics, Silver Spring, MD, USA), which calculated the area and perimeter of each cell. The roundness of each cell was quantified using the formula:

$$\text{roundness} = (4\pi) * \left(\frac{\text{area}}{\text{perimeter}^2} \right) * (100\%) \quad (14).$$

Histograms for roundness were created for both cell lines. Roundness means were compared using a two-tailed Student's *t*-test, with significance accepted at *p* < 0.05.

The phenotypic abundances of smooth muscle α -actin (SM α A) in the primary VICs and the cell subpopulations from trypsinization and adhesion analysis were analyzed using immunocytochemistry. Cells were seeded onto sterile cover slips and grown for two days prior to fixation, staining, and imaging. The cell-seeded coverslips were rinsed in PBS at 37°C and then covered by cold acetone at -20°C for 1 min. Following acetone fixation, the cells were rinsed twice with cold PBS (4°C), and then blocked with goat serum buffer (2% in PBS/0.02% Triton X-100) for 30 min at 37°C. After blocking, the cells were incubated in 50 μ l mouse anti-human SM α A (0.5 μ g/100 μ l in goat serum buffer; Dako Cytomation, Glostrup, Denmark) at room temperature for 1 h. Each cell sample was then washed in PBS (3×5 min) and incubated in 50 μ l fluorescein-conjugated goat anti-mouse IgG (0.5 μ g/100 μ l in goat serum buffer; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 60 min at 37°C. Finally, the cells were rinsed with PBS (3×5 min), wet-mounted with Vectashield containing DAPI (Vector,

Burlingham, CA, USA), and placed cell-side down on a microscopic slide. Cells were imaged using a Coolsnap HQ camera (Photometrics, Tucson, AZ, USA) attached to a fluorescence microscope (Zeiss, Göttingen, Germany). Images and image overlays were processed using Metamorph software (v.6.1r5, Limerick, Ireland).

Growth study

The FAST3 and SLOW3 VIC subpopulations (generated as described previously in 'Sorting of cell populations') were seeded at a density of 10,000 cells/cm² in six-well tissue culture plates and cultured in triplicate for six days. Each day, the VICs in one well of each plate were trypsinized and counted using a hemocytometer. The FAST3 and SLOW3 cell numbers at each time point were compared using a two-tailed paired Student's *t*-test, and significance was accepted at *p* < 0.05.

Flow cytometry

The phenotypic SM α A staining in the VIC subpopulations (from both detachment- and adhesion-based sorting) were again analyzed by flow cytometry. Cell subpopulations at 80% confluence were trypsinized using full-strength trypsin. Each cell sample was washed with PBS and fixed with 100 μ l fixation buffer (1% paraformaldehyde in PBS, pH 7.5), 20 min, 25°C. Following incubation, flow cytometry buffer ('FC buffer', 1% bovine serum albumin and 0.1% Na Azide in PBS; Fisher Chemical, Fairlawn, NJ, USA) was added to each sample and centrifuged (5 min, 1500×g). The pelleted cells were then permeabilized in 0.1% Triton X-100 100 μ l FC buffer for 5 min at 25°C. Next, each cell subpopulation was washed, resuspended in

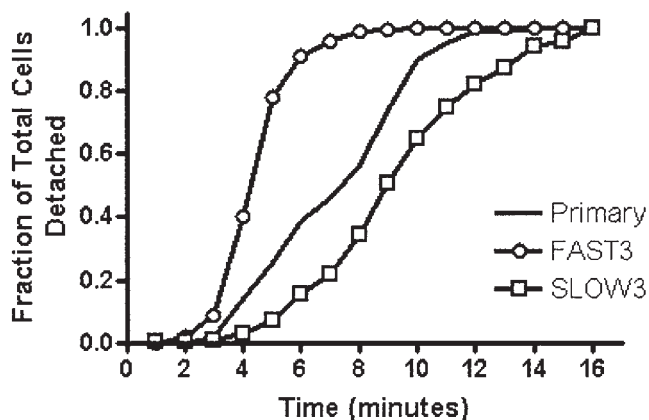


Figure 1: Cumulative percentage of cell detachment per minute from primary valvular interstitial cell (VIC) cultures and separated VIC subpopulations. The detachment rates of the FAST3 and SLOW3 subpopulations indicate the persistence of the different phenotypes throughout passaging.

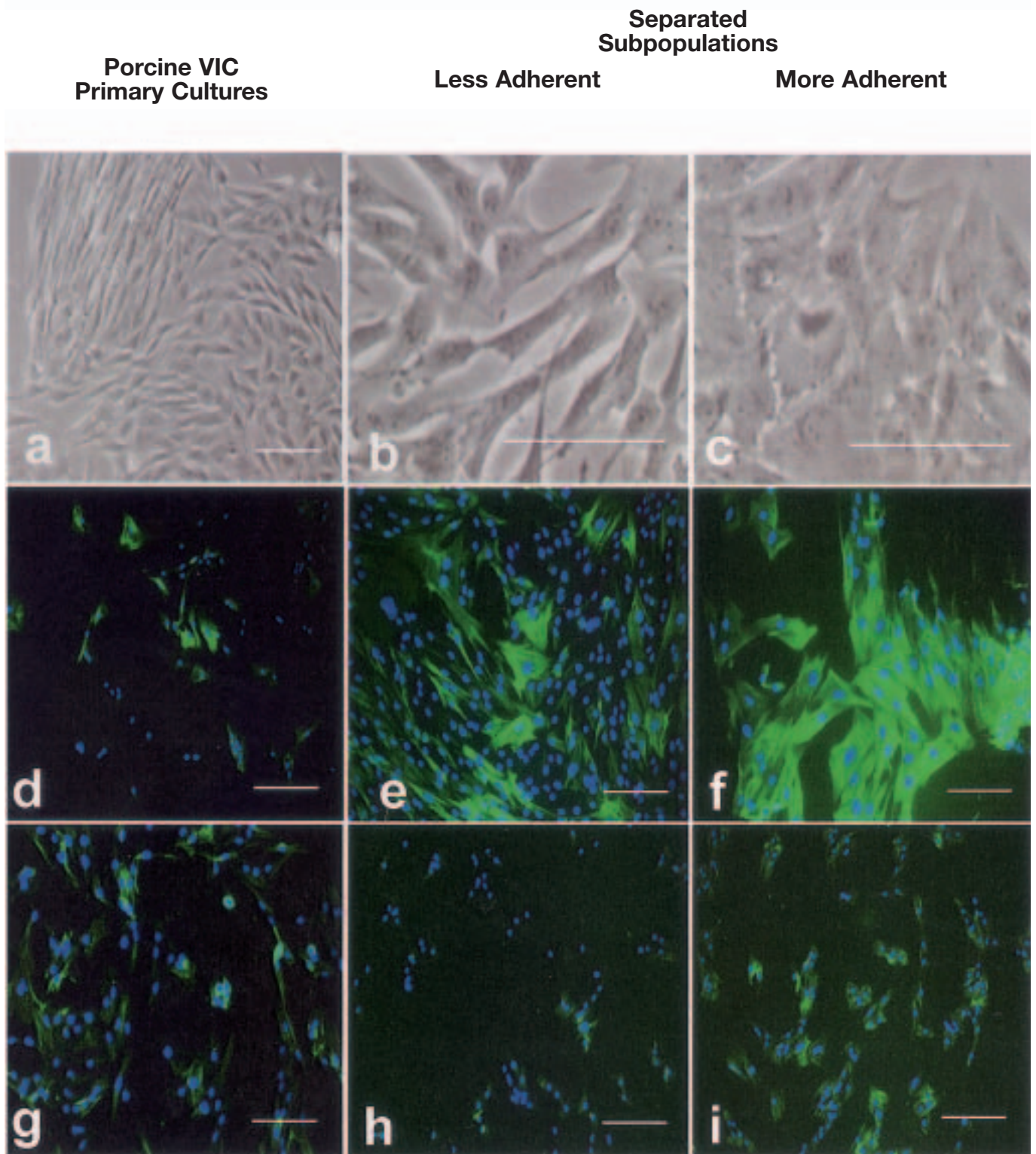


Figure 2: Porcine valvular interstitial cell (VIC) primary cultures expressing inherent heterogeneity (panels a, d, and g), less adherent subpopulations that are more round and less flattened (panels b, e, and h) and more adherent subpopulations that are flatter by comparison and stain strongly for smooth muscle α -actin (panels c, f, and i). Panels (a-c) were imaged in grayscale phase contrast; all other images were of cells stained with DAPI and a fluorescein isothiocyanate (FITC) green-tagged antibody against smooth muscle α -actin. Panels (a-f) represent porcine VICs before and after separation using differential detachment; panels (g-i) represent porcine VICs before and after separation using differential adherence. Scale bars = 100 μ m.

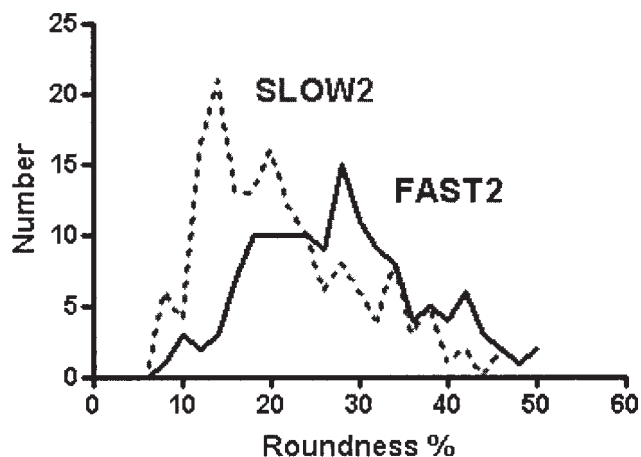


Figure 3: Roundness frequency polygons of FAST2 and SLOW2 valvular interstitial cell (VIC) subpopulations. The SLOW VICs have a lower proportion of area to circumference-squared, indicating a greater proportion of focal adhesions and filipodia.

100 μ l FC buffer containing mouse anti-human SM α A (1:200), for 30 min on ice. Each cell sample was then washed and resuspended in 100 μ l FC buffer containing fluorescein-conjugated goat anti-mouse secondary antibody (1:100) for 30 min on ice. Following incubation, the eight samples were again rinsed and resuspended in 2 ml fresh FC buffer. Each sample was then analyzed by flow cytometry (FACScan and CellQuest Pro software; BD Biosciences, Franklin Lakes, NJ, USA) to obtain histograms and statistics (medians) of the fluorescence-staining intensities.

Results

Subpopulation development

Cell cultures and heart valve tissue slices (post-collagenase II treatment) stained negatively for CD31 and von Willebrand factor, indicating that this isolation procedure isolates VICs with little contamination from valvular endothelial cells (data not shown).

Differential detachment using diluted trypsin resulted in a gradual detachment of cells over time. In general, the first 25% of cells (FAST) detached within 3-5 min, while the final 25% of cells (SLOW) required at least 8 min to detach. After repeating the detachment-based separation on these cell subpopulations for three passages, their detachment rates were compared with that of the original VIC primary culture (Fig. 1). Compared to the detachment rate of the original primary culture, the FAST3 cells detached earlier and at a faster rate, whereas the SLOW3 cells detached later but at approximately the same rate. At 5 min, almost 80% of the FAST3 cells were detached compared to less than 10% of the SLOW3 cells.

The differential adhesion procedure clearly demon-

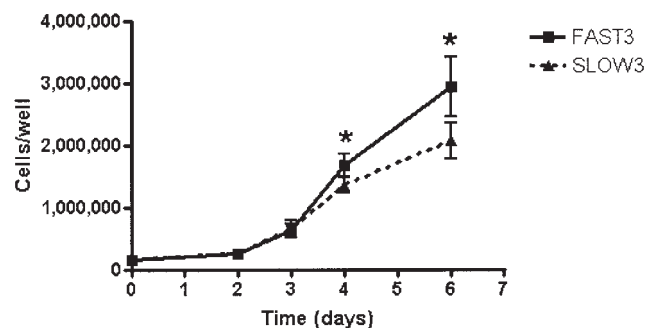


Figure 4: The FAST3 valvular interstitial cell subpopulation demonstrated more growth than the SLOW3 cells at days 4 and 6 (*, $p < 0.05$). Error bars represent standard deviations.

strated that the VIC heterogeneity extended to their adhesion to tissue culture plastic. Approximately 25% of the VICs adhered within the first hour, 25% more between 1 h and 4 h, relatively few (<10%) between 4 h and one day, while the remainder took longer than one day to adhere.

Cell morphology, roundness, and growth

Unseparated VICs displayed a mix of elongated fibroblast-like and cobblestone morphologies (Fig. 2a). The cell subpopulations separated using differential detachment demonstrated more distinctive morphologies; the SLOW2 VICs had more ruffled edges (multiple filipodia) and appeared flatter and more adherent to the tissue culture flasks than did the FAST2 VICs (Fig. 2b and c). The SLOW2 cells were also less quantitatively round ($21.5 \pm 8.9\%$, $n = 135$) than the FAST2 cells ($27.5 \pm 9.5\%$, $n = 157$; $p < 0.0001$; Fig. 3). In addition, the FAST3 cells grew significantly faster than the SLOW3 cells, with 24% higher cell density at four days ($p = 0.020$) and 42% higher density at six days ($p = 0.038$; Fig. 4).

SM α A staining

The VIC subpopulations that were less adhesive and more sensitive to trypsinization demonstrated heterogeneous immunocytochemical staining for SM α A, with approximately 35% of cells within the field of view staining moderately to intensely (only 15% were stained intensely; Fig. 2e and h). Both groups of more-adhesive cells, in contrast, stained uniformly (>98%) and strongly for this phenotypic marker (Fig. 2f and i). Similarly, flow cytometry on the VIC subpopulations showed that more-adherent cells resulting from both methods expressed SM α A more strongly, with a nar-

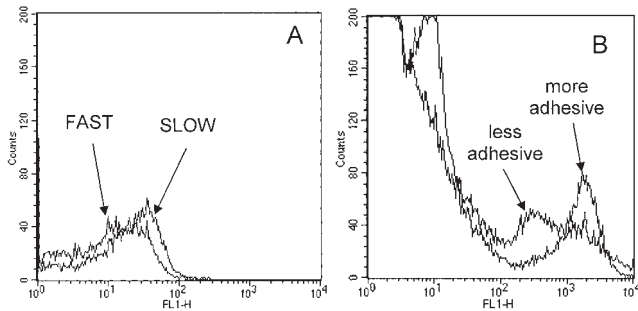


Figure 5: Flow cytometric quantification of smooth muscle α -actin abundance in porcine aortic valvular interstitial cells (VICs) separated by differential detachment (A) and adherence (B) to tissue culture plates. FL1-H indicates fluorescence intensity (log scale). The more-adherent cells separated by either technique demonstrate stronger and more uniform staining than less-adherent cells. In panel (B), the tall peaks at the left-hand side are cellular debris remnants from the primary culture of VICs from heart valves that bound non-specifically to the FITC-labeled secondary antibody.

rower histogram peak, than both less-adherent cell subpopulations (Fig. 5). In general, the median fluorescence intensity of staining for SM α A of the more-adherent VICs was 1.5- to 2-fold greater than that of the less-adherent VICs.

Discussion

In these studies, a series of *in-vitro* techniques was developed to separate and semi-purify cell subpopulations from primary cultures of porcine VICs, based on differential detachment from, or adhesion to, tissue culture plates. These subculturing techniques resulted in the isolation of two different cell subpopulations. One type was flattened with multiple filipodia, tightly adherent to tissue culture plates, relatively slow growing, and expressed highly uniform levels of SM α A (>98%), suggestive of myofibroblasts. The other subpopulation was less adherent, faster growing, and had a lower but still heterogeneous expression of SM α A (35% overall with strong staining in only 15%). These values straddle - but are both distinctly different from - the reported amount of SM α A staining in heterogeneous VIC cultures (57%) (6). Based on these results, it was concluded that differential adhesion characteristics could be used to separate cell subtypes in porcine aortic VIC cultures.

Although a variety of methods for cell separation have been used in investigations relevant to heart valves, there have been no previous successful attempts to isolate VIC subpopulations. Fluorescence-activated cell sorting was successful in separating a mixed group of vascular cells (smooth muscle cells and

endothelial cells) for use in the tissue engineering of heart valves, but no actual valve cells were used in that study (15). Similarly, specially treated beads that bind to endothelial cells have been shown to isolate valvular endothelial cells from VICs in whole-valve collagenase digests (6).

The decision of the present authors to investigate differential adhesion for the separation of cell subpopulations was motivated by observations from passaging the VICs. Differential adhesion and detachment methods have previously been used to eliminate unwanted cell types (such as in the isolation of stem cells (16,17)) or to investigate subpopulations of aortic smooth muscle cells demonstrating different thicknesses of pericellular matrix (12). In the present authors' laboratory, it was frequently noted that many cells detached quickly after the application of full-strength trypsin, while others required aggressive agitation of the flask in order to detach from the tissue culture plates. The finding that differential adhesion and differential detachment resulted in approximately the same subpopulations, however, suggested that the basis for separation was likely dictated by adhesion receptors rather than an anti-adhesive pericellular matrix. In the differential adhesion technique, the trypsinized cells should not have had enough time to synthesize a substantial pericellular matrix. Integrin populations such as the $\alpha_9\beta_3$ or $\alpha_v\beta_3$ integrins previously reported in bovine and primate VICs (18), may differ between these two cell groups.

The more adhesive of the two cell subpopulations appeared to be myofibroblasts, a class of special, highly contractile fibroblasts. Myofibroblasts produce and secrete components of the ECM during development (19), in response to injury and disease (20), and in response to various growth factors, cytokines and, most notably, to mechanical stimulation (20,21). Myofibroblasts stain densely for SM α A filaments, which provide these cells with an exquisite sensitivity to mechanical perturbations through a protein complex known as the fibronexus that connects the actin filaments to the cell surface and thence to the ECM (22,23). In fibrotic diseases such as cirrhosis and scleroderma, the activation of myofibroblasts by inflammatory cytokines results in excess collagen synthesis. In contrast, myxomatous mitral valves also demonstrate an abundance of activated myofibroblasts (4), but are more characterized by excesses of the glycosaminoglycan hyaluronan, the proteoglycan versican (10), and matrix metalloproteinases (4) than by collagenous fibrosis. Although the activated myofibroblasts tend to be co-localized with the metalloproteinases, it is unknown whether the myofibroblasts or another cell subpopulation produces the excess gelatinous matrix. In supporting the idea that the cells involved in myxo-

matous degeneration are myofibroblasts (4), it has been observed that undivided cell populations cultured from myxomatous mitral valves express abundant SM α A (though still heterogeneously) and trypsinize very easily when passaging, but will not adhere to tissue culture flasks for several hours or days. This observation could be associated with the elevated abundance of hyaluronan and versican within myxomatous valves; both compounds have anti-adhesive properties and contribute to a thick, slippery pericellular matrix (11,12).

These studies have also been extended to other VIC lines isolated from myxomatous human valves and slaughter-aged bovine and porcine mitral valves, with mixed results. Differential adherence resulted in an enrichment of SM α A populations in the highly adherent VIC subpopulations in diseased human VICs, but not in bovine or porcine mitral VICs. Differential trypsinization has not yet been performed on human VICs, and was unsuccessful at creating an SM α A-enriched subpopulation with bovine mitral VICs, but was repeatedly successful at separating porcine mitral VICs into subpopulations.

The ability to separate VIC subpopulations has significant implications for future investigations into valvular disease mechanisms, biology, and tissue engineering. For example, a cell subtype-specific analysis of myxomatous mitral valve disease could pinpoint the intracellular signal transduction pathway responsible for the degenerative remodeling. Cell segregation would also enable many biological studies to be conducted, such as how heterogeneous cell populations communicate with each other and with the valvular endothelial cells. If one cell subpopulation were to secrete substantial amounts of a particular ECM component, it might be advantageous to seed tissue-engineered heart valve scaffolds with that specific cell line. Further analysis of the differential adhesive characteristics of these VIC subpopulations, and especially their adhesion receptors and adhesion to modified surfaces, should also provide a rich area for future study.

Study limitations

There were several possible limitations to the present study. First, the process of culturing cells can itself cause an increase in SM α A; hence, to limit this effect the sequential isolations of VICs were commenced at low passages only (<P3), passaging them three times during subculture for a final passage number of P6 for FAST3 and SLOW3. VIC cultures may also vary substantially in SM α A expression. In addition, the process of enzyme-based detachment might also affect the cells' phenotype artificially; for this reason, an adherence-based separation was also tested. Much like the trypsin-separated cells, the porcine aortic VICs that

had never been in culture (and never exposed to trypsin) were found to display heterogeneous binding to tissue culture plates, while the most-adherent cells expressed higher levels of SM α A than the less-adherent cells. Furthermore, most phenotyping performed on these separated VIC subcultures used gross morphology and growth patterns, whereas only SM α A was used as a molecular marker. A much more comprehensive biochemical and morphological investigation of these cells would be required to understand their basic role in valve biology and disease. An additional complication is that no data are yet available describing the relative numbers of these subpopulations in native valves.

In conclusion, it has been demonstrated that differential detachment and adhesion to tissue culture plastic can be used to separate different cell subpopulations from primary cultures of porcine aortic VICs. The more-adherent and slowly growing cell subpopulation isolated by either technique demonstrated a uniformly high expression of SM α A, a phenotypic marker for myofibroblasts. This novel means of separating valvular cell subtypes should have a profound impact on future studies of valvular biology, remodeling, disease, and tissue engineering.

Acknowledgements

The authors express their sincere appreciation to Nancy Turner BS, Joel Moake MD and Darryl Pilling PhD for instruction in flow cytometry; to Damian Dalle Nogare BS for assistance with fluorescence microscopy; and to David Engler PhD for helpful discussions. These studies were funded by the Whitaker Foundation and a Brown Foundation Undergraduate Research Award to J.L.C.

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