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Fibronectin-Based Isolation of Valve Interstitial Cell Subpopulations: Relevance to Valve Disease*

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Abstract

Myxomatous mitral valves (MVs) contain elevated proportions of unique cell populations such as myofibroblasts. Without a reliable technique to isolate such cell populations, however, it has been difficult to study the role of these cells. The goal of this study was to use fibronectin (FN) to isolate distinct cell subpopulations from normal porcine MVs.

Cells from porcine posterior MV leaflets were separated based on time-dependent adhesion to either tissue culture plastic (TCP) flasks or FN-coated flasks. The resultant “FAST” and “SLOW” adhering subpopulations from each technique were phenotyped using flow cytometry and immunocytochemistry to detect expression of myofibroblast markers, enzymes for collagen synthesis, and MAP kinases. Compared to FN SLOW, FN FAST showed significantly higher expression of prolyl 4-hydroxylase, heat shock protein-47 (HSP47), smooth muscle alpha-actin (SM α A), non-muscle myosin (Smem), extracellular-related signaling kinase (ERK) 1, ERK2, and phosphorylated-ERK. In contrast, TCP FAST showed higher expression of only HSP47, SM α A, and Smem compared to TCP SLOW.

In conclusion, differential adhesion to FN successfully separated a myofibroblast-like subpopulation from the posterior leaflet of the MV. This subpopulation may be useful in studying myxomatous MV disease, although additional studies remain to verify that this myofibroblast-like population resembles that observed in myxomatous MV disease.

Keywords

fibronectin; mitral valve; adhesion; valvular interstitial cells; flow cytometry

INTRODUCTION

While myofibroblasts are known to be involved in disease processes in multiple tissues including the kidney,^{1,2} lung,³ pancreas,⁴ and heart valves,⁵ it has been difficult to isolate these cell subpopulations from valves to allow their systematic study. In order to develop novel medical treatments for these diseases, it will be important to understand the

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phenotypic regulation of the cells involved and how these cells contribute to disease progression. The myofibroblast-like valve cell phenotype in particular is common to a number of valve diseases and thought to be influential in the development and progression of diseases such as myxomatous degeneration.^{5,6} Numerous publications have described the valvular interstitial cells as a heterogeneous population with some fibroblast-like cells and others that appear more myofibroblast-like.⁷⁻⁹ The myofibroblast-like valve cell, when characterized in situ or induced via treatment with transforming growth factor- β , is marked by greater production of matrix, expression of matrix metalloproteases, expression of muscle related markers, and contractility.^{5,6,10} Furthermore, many of the valve diseases that are characterized by altered proportions of distinct cell phenotypes also contain altered composition of valvular extracellular matrix.^{5,11} It was therefore hypothesized that matrix components could be used to isolate specific valvular interstitial cell (VIC) subpopulations, which are critical for further studies regarding the etiology and treatment of valve diseases. It was the general goal of this study to develop a matrix-based technique for the isolation of this myofibroblast-like cell phenotype.

Previous studies from our laboratory explored the utility of two related methods to isolate the myofibroblast subpopulation from aortic valve cells:¹² differential detachment (trypsinization) from tissue culture plastic (TCP) and differential adhesion to TCP. Although it was observed that these two methods yielded similar cell subpopulations,¹² it was speculated that the detachment method could cause the loss of the very proteins that make this adhesive population unique. It was further speculated that differences in adhesiveness could be the basis for phenotypic differences among VICs;¹² therefore, this study was developed to investigate the nature of differentially adherent cells. In addition, our previous study used only one phenotypic marker, smooth muscle alpha-actin (SM α A), whereas this study involved a much more comprehensive assessment of cell phenotypes. Furthermore, the present study utilized VICs harvested from the posterior leaflet of the mitral valve, since these cells will be more relevant to future studies of the mechanisms of myxomatous mitral valve disease (which primarily affects the posterior leaflet).

Most importantly, this study investigated the phenotypes of cell subpopulations resulting from differential adhesion to fibronectin, a protein that is relevant to both normal and diseased cell-matrix interactions and is present in both normal and myxomatous valves.^{10,13,14} Fibronectin was evaluated for this matrix-based technique to isolate myofibroblasts for several reasons. Myofibroblasts are known to exert force on their adhesive substrates through fibronectin-linked, enlarged focal adhesions (also known as the fibronexus).^{15,16} It was thus hypothesized that the myofibroblast subpopulation within a mixture of primary cultured VICs would adhere to fibronectin more strongly than other VIC phenotypes. Fibronectin is also more abundant in injured and regenerating tissues.¹⁷⁻²² In renal fibroblasts, for example, differentiation to the myofibroblast phenotype results in extracellular fibronectin accumulation.²³ In heart valves, it has been shown that activated VICs remodel the orientation of fibronectin fibers in the matrix.⁶ Furthermore, fibronectin has been associated with valve cell “injury” in that VICs that migrate into a region devoid of cells (using a scrape wounding in vitro model) then secrete fibronectin.²⁴ For these reasons, fibronectin may be an appropriate matrix component to serve as the basis for isolating myofibroblastic VICs associated with valve injury.

Therefore, the purpose of this investigation was to compare the utility of fibronectin-coated vs. uncoated tissue culture plastic for differential adhesion-based isolation of the myofibroblast-like subpopulation of VICs. Following cell separation on these two substrates, flow cytometry and immunocytochemistry were used to assess a number of phenotypic characteristics relevant to myofibroblasts.

MATERIALS AND METHODS

Isolation of valvular interstitial cells

This investigation utilized cultured VICs, which have been previously reported to demonstrate phenotypes largely representative of valve cells *in vivo*⁸ and to maintain aspects of this phenotype over multiple passages.^{25–27} These VICs were cultured from porcine valves, a widely used, anatomically comparable model for human heart valves.^{10,28–31} Porcine hearts from healthy 6-month-old pigs were purchased from Fisher Ham and Meats, Spring Cypress, Tex., USA; valves from several hearts were pooled together (between 3–10 hearts per cell harvest) were harvested for primary culture according to standard methods.^{32–34} Valve tissues were pooled to limit potential effects of inherent variability between animals. The isolation of primary cells from pooled valve tissues was performed on at least six different occasions. The posterior leaflets of the mitral valves were dissected from the hearts, trimmed of attached chordae, placed in 2 mg/mL collagenase II (Worthington Biochemical Corp., Worthington, VA) in serum-free Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) containing 1% antibiotic/antimycotic/antifungal solution (Mediatech), and incubated in a shaker for 20 min (37°C, 140 rpm).³⁴ Afterwards, the loosened endothelial cells were removed by wiping the leaflet surfaces with sterile cotton swabs. The tissues were then finely minced and dissociated in a solution of collagenase II and hyaluronidase (1 mg/ml and 0.1 mg/mL, respectively in serum-free medium) in an incubated shaker for 4 hours. The resulting VICs were counted, seeded in tissue culture flasks at a density of 10,000 cells/cm², and maintained at 37°C, 95% humidity, and 5% CO₂. Cells were grown in medium containing 10% bovine growth serum (BGS, HyClone, Logan, UT) and 1% antibiotic/antimycotic/antifungal solution and passaged after reaching 90% confluence. Experiments were performed on cells between their first and third passages.

Isolation of subpopulations

VICs were separated into subpopulations based on adhesion time to either uncoated or fibronectin-coated tissue culture plastic (TCP). Primary cultures of VICs were removed from culture plates using trypsin, pelleted, and resuspended in 10 ml of media. These VICs were then seeded into an uncoated TCP T-75 flask (Fig. 1, flask #1) and incubated. After 30 minutes approximately 20% of the cells had adhered to the flask. The media and unattached cells were then removed from flask #1 and placed into a new T-75 flask (flask #2) while fresh media was added to flask #1. After 2 more hours, the unattached cells and media from flask #2 were removed and seeded into a new T-75 flask (flask #3). The subpopulation that adhered within 30 min (in flask #1) was called TCP FAST, while the population that had not adhered after 2.5 hours (grown in in flask #3) was called TCP SLOW. While the TCP SLOW population may have been exposed to an increased number of dead cells, previous

studies of viability and phenotype of valve cells after various durations of ischemia suggest that it would be unlikely for the presence of non-viable cells to alter the phenotype and viability of the surviving cells³⁵ The same procedure was repeated with VICs on fibronectin (FN)-coated TCP flasks (Becton Dickinson, Franklin, NJ) but the time intervals were shortened because VICs adhered to FN-coated flasks faster than to normal TCP flasks. Therefore, in the case of FN-coated flasks the subpopulation that adhered within the first 15 minutes was called FN FAST and the subpopulation that had not adhered after 1.5 hours was called FN SLOW. To demonstrate repeatability, this adhesion-based separation process was performed on six separate groups of primary cell cultures. Cells attached to the #2 flasks were considered to have an intermediate rate of adhesion and a mixed phenotype (neither distinctly FAST nor SLOW), and were discarded.

Adhesion of subpopulations to TCP

Each of the separated VIC subpopulations (FN FAST, FN SLOW, TCP FAST, TCP SLOW) were seeded within quadruplicate wells of a TCP 6-well plate at a density of 4.0×10^4 cells/cm². Control cells, which were primary cells (harvested as described above from the pooled valve tissues) that had not been subjected to the separation process, were seeded in identical conditions. After 30 min, 90 min, 150 min, and 24 hours, the medium containing the unadhered cells were removed and the adherent cells were trypsinized and counted using trypan blue staining and a hemocytometer.

Flow cytometry

The various adhesive VIC subpopulations (FN FAST, FN SLOW, TCP FAST, TCP SLOW) were evaluated using flow cytometry to assess their expression of several phenotypic characteristics. These markers included prolyl 4-hydroxylase (P4H, Chemicon, Temecula, CA) and heat shock protein-47 (HSP47, Abcam, Cambridge, MA), both markers of active collagen synthesis; smooth muscle alpha-actin (SMαA, Dakocytomation, Denmark), non-muscle myosin heavy chain (Smem, GeneTex, San Antonio, TX and Covance, Berkeley, CA), which are markers of VIC activation and the myofibroblast phenotype; smooth muscle myosin (SMM, Sigma), which differentiates myofibroblasts from smooth muscle cells; and extracellular signal-regulated kinase (ERK)-1, ERK2, and p-MAP kinase1/2 (pERK, Chemicon). ERK1, ERK2, and pERK are all signaling proteins in the mitogen activated protein (MAP) kinase pathway that are involved in fibroblast activation to myofibroblast in a number of tissues,^{23,36–38} and have been shown to be responsive to tension such as that the cell experiences during adhesion.^{39,40} These proteins are also related to the production of matrix metalloproteases,⁴¹ which are abundant in myxomatous mitral valves.

In preparation for flow cytometry, after 2–4 days, the separated VIC subpopulations were trypsinized, counted, washed twice with 10 ml of PBS, and placed in fixative (1% paraformaldehyde in PBS) at 2.00×10^6 cells/ml. After 1 hour, the fixed cells were incubated in permeability buffer (0.1% Triton-X 100 in PBS; 1 ml per 1×10^6 cells) for 10 minutes. Separate tubes containing 1×10^6 cells were prepared for each antibody and for a negative isotype control (to be treated with IgG and secondary antibody only). An additional tube of cells, which would remain untreated, was prepared for a “blank” control and used for gating purposes. The cells were mixed with 1 mL of flow cytometry (FC) buffer (PBS

containing 0.5% BSA, 0.05% sodium azide), then pelleted by centrifuging for 1 minute at 13,200 rpm. The cells were then incubated in 100 μ L of primary antibody (or IgG for controls) for 1 hour on ice, then mixed with 1 mL of FC buffer and pelleted by centrifuging for 1 minute at 13,200 rpm. Next, the cells were mixed with 100 μ L of FITC-conjugated secondary antibody and stored in the dark. After 1 hour, 500 μ L of FC buffer was added, samples were spun for 1 minute at 13,200 rpm, and the supernatant was decanted. The pellet was resuspended in 250 μ L of FC buffer and transferred to 5 ml tubes.

A FACScan flow cytometer with Cell Quest Pro software (Becton Dickinson) was used for data acquisition and analysis. Data was gated based on size (forward scatter), complexity (side scatter), and non-specific fluorescence following calibration with the isotype control samples and unstained samples. The fluorescence-based gate was set such that less than 1% of the non-specific fluorescence fell within the gate. Data reported includes only that located within the above gates. Each sample was analyzed at least twice to confirm the mean fluorescence values, which has been shown to be proportional to the number of proteins on the cells.⁴² Flow cytometry measurements were performed three times on VIC subpopulations from 3 different adhesion-based separations. Data was reported as an average of all 3 measurements.

Immunocytochemistry

Cells from the 4 different adhesive subpopulations were seeded at a density of 5,000 cells/cm² on glass microscope slides and maintained in medium/10% serum/1% antibiotic solution in an incubator. After 2 days, the cells were rinsed with PBS and fixed in 100% acetone at room temperature. The slides were incubated in a solution containing 5% goat serum in PBS for 1 hour to block non-specific binding. The slides were then incubated in primary antibodies that were diluted in PBS containing 1% bovine serum albumin and 0.1% TritonX-100 (Roche Diagnostics, Mannheim, Germany). Antibodies used were the same as those in the flow cytometry assessment as well as vimentin (Dako). All primary antibodies were diluted 1:100 except for vimentin (1:200). After washing three times in PBS, the slides were incubated in appropriate secondary antibodies for 1 hour (goat anti-mouse and anti-rabbit IgG FITC, Jackson ImmunoResearch, West Grove, PA, 1:100 dilution). Negative controls were prepared without primary antibodies but with secondary antibodies. The slides were washed three times with PBS, drained, mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA), coverslipped, and sealed. This staining procedure and the imaging described below was repeated three times on three different batches of subpopulations.

The stained slides used for quantification of fluorescence were imaged using the 10 $\times$ and 20 $\times$ objectives of an Axioplan fluorescent microscope (Zeiss, Thornwood, NY) and Metamorph software (Molecular Devices, Sunnyvale, CA). Three 20 $\times$ images were taken from non-overlapping, randomly chosen regions of each slide. The total number of cells in each image was manually and automatically counted using the DAPI nucleic acid marker. The results of manual and automatic counting were comparable (data not shown), therefore, the automatic count was applied to all samples. All images for the same marker were obtained using identical acquisition parameters in one imaging session. Cells staining

positive were evaluated to obtain the fluorescence intensity per pixel (from 0 to 4025, where 4025 was the highest fluorescence.) The background intensity, due to non-specific binding, was eliminated by establishing a threshold of 700–800. Once the threshold had been applied, the integrated intensity of an area was divided by the number of nuclei in that same area to give the average intensity per cell.

Preliminary analysis of human myxomatous valves and VICs

In a preliminary translation of these methods to human valve studies, these FN and TCP-based subpopulation isolation techniques were also performed on VICs isolated from 4 human myxomatous mitral valves. Myxomatous mitral valve specimens were obtained from 4 patients after surgery to correct severe mitral regurgitation due to a prolapsing valve. These four patients (3 females aged 50, 61, and 69 years old and one 59 year-old male) underwent quadrilateral resection of the posterior leaflet and implantation of an annuloplasty ring. These tissues were obtained from the Cooperative Human Tissue Network and their use was authorized by the Institutional Review Board. Following the adhesive separation process on cells from passages 4–5, the 4 human VIC subpopulations were subjected to flow cytometry analysis for SmaA, Smem, pERK, and ERK2.

We also performed immunohistochemistry to confirm the presence of fibronectin in human normal and myxomatous mitral valve leaflets. Posterior leaflets from four myxomatous mitral valves (mean patient age 65.0 ± 10.5) removed during valve repair surgery and three normal valves (no history of cardiac disease, mean subject age 64.7 ± 14.5) removed at autopsy were formalin-fixed, paraffin-embedded, and sectioned to a thickness of 5 μm . Slides were stained using an antibody to fibronectin (Chemicon, Temecula, CA) after citrate-based antigen retrieval, visualized using Vectastain Elite ABC and diaminobenzidine kits (Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI). The use of these valve tissues for research was approved by the Institutional Review Board.

Statistical analysis

Statistical analysis was performed using SigmaStat software (SPSS, Chicago, IL). When the data was normally distributed, an ANOVA test was used. When the data set was not normally distributed, a rank transform was performed before the ANOVA. In both cases the level of significance was set at 0.05. The Holm-Sidak all-pairwise multi-comparison method was used for post-hoc testing. Statistical comparisons of immunocytochemical data, as well as the myxomatous cell flow cytometry data because of the limited nature of that data set, were performed using a student's paired t-test and the same level of significance (0.05).

RESULTS

Confirmation of FAST and SLOW subpopulations adhesion rate

After the TCP and FN separation techniques were performed, the resulting subpopulations' adhesion rates to TCP were evaluated to confirm that distinctly adherent FAST and SLOW subpopulations had been generated (Fig. 2A, B). For both the FN and TCP separation techniques, the FAST cells adhered more rapidly than did the SLOW cells, and both were

different from the control (unseparated cells). Furthermore, the final percentage of cells adhered in the FAST subpopulations was higher than the SLOW subpopulation for both surfaces. Lastly, the final percentage of adhered cells for FN FAST was higher than TCP FAST and similarly the final percentage of adhered cells for FN SLOW was higher than TCP SLOW.

Flow cytometry – porcine VICs

Compared with the cells that adhered slowly to fibronectin (FN SLOW), the cells that adhered quickly to fibronectin (FN FAST) showed significantly higher expression of the markers of collagen synthesis P4H ($p<0.001$) and HSP47 ($p=0.043$), markers of the myofibroblast phenotype SM α A ($p<0.001$) and Smem ($p=0.002$), and proteins in the MAP kinase pathway ERK1 ($p=0.005$), ERK2 ($p=0.011$), and pERK ($p<0.001$) (Fig. 3A). In contrast, following differential adhesion to tissue culture plastic, only ERK1 was significantly different between the fast and SLOW subpopulations ($p<0.001$), and in fact was lower in the TCP FAST group (Fig. 3B). There were also trends of increased ERK2 and pERK expression in the TCP FAST cells compared to the TCP SLOW cells ($p=0.074$ and $p=0.064$ respectively). SMM expression was negligible in all groups, confirming that these isolated cells were not smooth muscle cells.^{5,43,44} These same expression patterns were found in all replicate flow cytometry measurements, regardless of whether the cells had been cultured for 2 or 4 days following the subpopulation separation procedure.

When comparing the FN FAST group to the TCP FAST, both of which were the ~20% most adhesive VICs isolated from the posterior leaflet, the FN FAST cells showed higher expression of two markers, ERK1 ($p=0.0002$) and Smem ($p=0.0001$). In contrast, when comparing the two least adherent VIC subpopulations, those separated using fibronectin had lower expression of ERK1, pERK, and SM α A than the TCP SLOW ($p=0.002$).

Flow cytometry and immunohistochemistry – human myxomatous valves

In the small group of human VIC subpopulations, FN FAST tended to show greater expression of the markers SM α A, NMM, pERK, and ERK2 compared to FN SLOW (Fig. 4A, $p=0.064$ by paired t-test). However, no difference was detected between TCP FAST and TCP SLOW ($p=0.452$). Immunohistochemical staining of a representative set of myxomatous and normal posterior mitral valve leaflets demonstrated positive staining for FN in all samples (Fig. 4B).

Immunocytochemistry

Immunocytochemical results were similar to the flow cytometry results. In immunocytochemistry FN FAST showed higher expression of ERK2 ($p<0.001$), pERK ($p<0.001$), HSP47 ($p<0.006$), SM α A ($p<0.01$), and Smem ($p<0.001$) compared to FN SLOW (Fig. 5A), while the TCP subpopulations were not as markedly different with TCP FAST only having higher expression of HSP47 ($p=0.021$), SM α A ($p<0.001$), and Smem ($p<0.001$) in TCP FAST compared to TCP SLOW (Figs. 5B and 6). Although the immunocytochemical data from different cell labeling preparations and different cell subpopulation separations could not be averaged because of possible variations in staining

and imaging parameters, the statistically significant differences between subpopulations were consistent across experiments.

DISCUSSION

Differential adhesion to fibronectin was successful in isolating VICs with an activated, myofibroblast-like phenotype as evidenced by greater expression of myofibroblast-related markers by the FN FAST subpopulation compared to FN SLOW. Although the phenotyping presented in this paper was performed on freshly isolated subpopulations, our previous studies have shown that subpopulations retain their adhesive and phenotypic properties over multiple passages.¹² Therefore, this method may be an effective means of isolating the myofibroblast-like valve cell population for further study. Overall, these results confirmed and extended the results of our previous subculturing study.¹² These cells that adhered the most rapidly to fibronectin demonstrated stronger expression of Smem as well as other proteins associated with the myofibroblast phenotype including markers of collagen synthesis^{10,45} and the MAP kinase pathway.^{46–48} While the mechanism behind the differential adhesive properties these VIC subpopulations is still under investigation, myofibroblasts expressing SMαA have been shown to exert stronger forces than their SMαA-negative counterparts,^{49,50} a feature that is attributed to more abundant cell-matrix contacts,⁵¹ isoform-specific terminal peptide adhesions,⁵² and specialized, enlarged focal adhesions incorporating unique proteins such as tensin.¹⁵

The findings that the VICs that were more adhesive to fibronectin showed the greatest expression of the myofibroblast markers, compared to tissue culture plastic, suggests that the fibronectin aids in promoting the myofibroblast phenotype. Fibronectin was utilized in this study because it is present throughout mitral valve leaflets (Fig. 4) and chordae¹⁴ and because it is involved in myofibroblast adhesion.^{15,16} Others have shown that adhesion to fibronectin causes fibroblasts to differentiate towards the myofibroblast phenotype²¹ and that VICs cultured on fibronectin-coated plates had significantly higher SMαA expression compared to VICs cultured on TCP.⁵³ Therefore, it is possible that the fibronectin coating both enables the separation of cell subpopulations and manipulates the cell phenotype of the cells that adhere. Given that the FN SLOW cells did not become myofibroblast-like, we speculate that the ability of fibronectin to potentiate activation of the myofibroblast phenotype is specific to certain cell types. The slow to adhere cells may represent a more true fibroblastic phenotype.

Our demonstration that the abundance of unphosphorylated and phosphorylated ERK was more abundant in the FN FAST cells is promising given that the activation of valve cells to myofibroblasts is thought to occur via the MAP kinase pathway. Correspondingly, it was recently shown that the use of heparin to prevent the activation of the FGF2 receptor complex, which inhibits MAP kinase, resulted in an increase in the myofibroblast phenotype of VICs.⁵⁴ The link between MAP kinase pathway activation and SMαA has been shown in VICs^{46–48} as well as cardiac fibroblasts,³⁶ and the role of the MAP kinase pathway in fibroblast activation to myofibroblasts has been shown in cells derived from a number of tissues including vascular adventitia,³⁸ liver,³⁷ and kidney²³ among others.

Both immunocytochemistry and flow cytometry showed that differential adhesion to fibronectin (vs. TCP) resulted in a more definitive isolation of the myofibroblast phenotype. However, there were some differences between the results of these two assessment methods. The difference in SM α A expression between TCP FAST and TCP SLOW was detected by immunocytochemistry, but not by flow cytometry. On the other hand, the greater expression of ERK1, ERK2, and pERK by the TCP FAST cells (vs. TCP SLOW) was only detected by flow cytometry, as was the greater ERK1 expression in FN FAST compared to FN SLOW. Some differences are to be expected between the results of these two methods given that in each technique the cells are in different adhesive states. In immunocytochemistry the cells are actively attached to a surface, while in flow cytometry the cells have been removed from their surface. The process of the cells removal and the cells' adhesive state may affect the states of signaling. It should also be noted that in immunocytochemistry, the tested cells had been cultured on glass for several days, rather than TCP or FN-coated flasks as in the case of the cells tested by flow cytometry. This difference in cell substrate could have also contributed between differences in results between flow cytometry and immunocytochemistry, as substrate stiffness has been shown to alter fibroblast⁵⁵ and valve cell phenotype,²⁷ and given that glass is significantly stiffer than TCP.⁵⁶ Interestingly, in the present study the TCP FAST cells did not show significantly higher expression of SM α A by flow cytometry as was found in the previous study. This discrepancy may be because mean fluorescence after gating was used to assess marker expression in flow cytometry rather than the median fluorescence as was done in the last study. For both immunocytochemistry and flow cytometry, it would be beneficial to use a multi-labeling/imaging technique to determine which markers are expressed together on a given cell.

Fibronectin often serves as a substrate in a number of cell-matrix adhesions including focal adhesions and fibrillar adhesions. These two types of adhesions are quite different structurally. Focal adhesions are large rod-shaped complexes and contain a number of cytoplasmic plaque proteins.^{57,58} In contrast, fibrillar adhesions are smaller consisting mainly of $\alpha_5\beta_1$ integrins and tensin, lacking the cytoplasmic plaque proteins found in focal adhesions.^{57,58} Based on these structural differences it is likely that these two types of adhesions have significant differences in function. Fayet et al. have recently shown that VICs growing in monolayers form fibrillar adhesions at the edges of a scrape wound "injury."²⁴ Future studies into this FN-based separation technique could involve using antibodies unique to fibrillar adhesions to determine if this differential adhesion method is separating the cell population principally based on focal adhesions or fibrillar adhesions, or both. Characterizing the specific method of isolation in relation to these two types of adhesions could have important implications for normal and diseased VIC-matrix interactions. Indeed, given that fibrillar adhesions form in response to VIC injury,²⁴ investigating proteins specific to fibrillar adhesions, such as tensin, may be useful in characterizing this myofibroblastic, injury-related VIC population. Clearly, much research remains to be done in this area.

While this paper has provided a means for separating the myofibroblast-like VIC phenotype from the mitral posterior leaflet, and preliminary results suggest that the method is also effective for human myxomatous valve cells, it will be important to substantiate the utility of this method in the study of myxomatous mitral valve disease with a larger sample number of

diseased valves. While myxomatous valves contain greater numbers of myofibroblasts,⁵ myxomatous valve cells may secrete excess GAGs and PGs into their pericellular matrix, which may make them less adhesive, but this remains to be shown. Elucidating the basis, mechanism, and optimal substrate for differential adherence of these cells will be essential in isolating the myofibroblast-like cells from a pool of myxomatous mitral valve cells. It is possible that further study will show that the myofibroblast-like population from myxomatous valves shares expression of many of the same markers as the myofibroblast-like cells isolated in this study.

In conclusion, this study demonstrated the successful separation of VIC subpopulations using differential adhesion. Differential adhesion to fibronectin produced a larger difference between subpopulations and moreover the FN FAST subpopulation demonstrated the phenotype associated with the VIC myofibroblast. This study demonstrated that an extracellular matrix component, fibronectin, which is abundant in tissues undergoing remodeling, can be used to isolate a unique cell population that is also commonly found in remodeling and diseased valves. Developing and validating such techniques for isolating specific cell subpopulations will be crucial in enabling the study of these subpopulations in diseased heart valves.

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Fig. 1. Schematic illustrating the process of isolating subpopulations from plastic flasks. The same method was used for subpopulations isolated from fibronectin-coated flasks, except with different time increments.

Fig. 2.

A) Adhesion to TCP over time using subpopulations previously separated using TCP. B) Adhesion to TCP over time using subpopulations previously separated using FN-coated flasks. Our laboratory has previously reported that such VIC subpopulations retain their adhesive properties over multiple passages.¹²

A. FC Marker Fluorescence for FN Differential Adhesion Subpopulations

B. FC Marker Fluorescence for TCP Differential Adhesion Subpopulations

Fig. 3.

A) Flow cytometry marker fluorescence of TCP differential adhesion subpopulations. Error bars indicate standard error of the mean, as the graph includes data from 3 independent experiments. $*=p<0.05$. B) Flow cytometry marker fluorescence of FN differential adhesion subpopulations. Error bars indicate standard error of the mean, as the graph includes data from 3 independent experiments. $*=p<0.05$, $\wedge=p<0.075$.

A. Differential Marker Expression in Human Myxomatous Valve Cell Subpopulations

B.

Fig. 4.

A) Ratio of FN FAST to FN SLOW flow cytometry marker mean fluorescence for human myxomatous VIC subpopulations. Error bars indicate standard error of the mean. B) Immunohistochemical staining illustrates the presence of fibronectin in both normal (left, 79 year-old male) and myxomatous heart valves (right, 70 year-old male). Scale bar represents 200 μ m.

Fig. 5.

A) Immunocytochemistry marker integrated optical density per cell of TCP differential adhesion subpopulations. Error bars indicate standard deviation, as the graph includes data from one representative experiment. Data between experiments showed the same differences between cell subpopulations, but varied in integrated optical density. *=p<0.05. B) Immunocytochemistry marker integrated optical density per cell of FN differential adhesion subpopulations. Error bars indicate standard deviation, as the graph includes data from one

representative experiment. Data between experiments showed the same differences between cell subpopulations, but varied in integrated optical density. $\ast=p<0.05$.

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Fig. 6.

Representative images of immunocytochemistry for given markers on TCP differential adhesion subpopulations. Images were captured using 10× objective. Scale bar represents 100 μ m and applies to all images.