

Force Generation of Different Human Cardiac Valve Interstitial Cells: Relevance to Individual Valve Function and Tissue Engineering

Sally Smith^{1,2}, Patricia M. Taylor¹, Adrian H. Chester¹, Sean P. Allen¹, Sally A. Dreger¹, Mark Eastwood², Magdi H. Yacoub¹

¹Heart Science Centre, National Heart and Lung Institute, Imperial College at Harefield Hospital, Harefield, Middlesex,

²Centre for Tissue Engineering Research, School of Biosciences, University of Westminster, London, UK

Background and aim of the study: Cardiac valves perform highly sophisticated functions that depend upon the specific characteristics of the component interstitial cells (ICs). The ability of valve ICs to contribute to these functions may be related to the generation of different types of tension within the valve structure. The study aim was to characterize cellular morphology and the forces generated by valve ICs and to compare this with morphology and forces generated by other cell types.

Methods: Cultured human valve ICs, pericardial fibroblasts and vascular smooth muscle cells were seeded in 3-D collagen gels and placed in a device that accurately measures the forces generated. Cell morphology was determined in seeded gels fixed in glutaraldehyde, stained with toluidine blue and visualized using a high-definition stereo light microscope.

Results: Valve ICs generated an average peak force of

30.9 ± 10.4 dynes over a 24-h period which, unlike other cell types tested, increased as cell density decreased ($R = 0.67$, $p < 0.0001$). The temporal pattern of force generation in mitral valve cells was significantly faster than in aortic or tricuspid cells ($p < 0.05$). Microscopic examination revealed the formation of cellular processes establishing a cell/cell and cell/matrix network. When externally induced changes in matrix tension occurred, the valve ICs - unlike the other cell types - did not respond to restore the previous level of tension.

Conclusion: Human cardiac valve ICs produce a specific pattern of force generation that may be related to the individual function of each heart valve. The specialized function of these cells may serve as a guide for the choice of candidate cells for tissue engineering heart valves.

The Journal of Heart Valve Disease 2007;16:440-446

Human cardiac valve interstitial cells (ICs) are a heterogeneous population of cells, which have been described as fibroblasts or myofibroblasts and have some of the characteristics of smooth muscle cells (SMCs) (1-3). Cultured valve ICs express genes encoding structural components of the cardiac and skeletal contractile apparatus and the skeletal muscle-specific regulatory factor myogenin (4). Previously, it has been demonstrated that cultured valve ICs display a phenotype similar to that found in the native valve, particularly when seeded in collagen scaffolds (5). These cells also exhibit a number of specific functions that include an ability to produce matrix remodeling enzymes, the secretion of extracellular proteins, and the ability to contract in response to a range of vasoactive agents (6-

8). These specialized cells have a crucial role to play in cardiac valve function. Although, initially, heart valve leaflets were thought to open and close passively, it is now known that valve movement precedes the onset of aortic blood flow (9,10). In addition, recent findings suggest that the cellular components of the valve may play a role in its dynamic and sophisticated functions, and may also modulate basal valve tone (11). The requirement of viable tissue for a fully functioning valve is illustrated by the superior long-term results for valve replacements achieved with the Ross procedure in which a living valve is used (12).

The mechanisms through which cells can directly influence the distribution of tension within the valve cusp, and thereby affect the function of the valve, have not been addressed. Interestingly, it has recently been reported that valve ICs respond to local tissue stress by altering cellular stiffness and collagen synthesis (13). In the present study, a culture force monitor (CFM) was used to investigate the contractile force generated by valve ICs (14). This device enables quantification of the

Address for correspondence:

Dr. Patricia Taylor, Heart Science Centre, Harefield Hospital, Harefield, Middlesex, UB9 6JH, UK
e-mail: patricia.taylor@imperial.ac.uk

development of contractile forces generated by cells within a three-dimensional (3-D) matrix by producing a continuous, computer-based force-generation profile. In addition, a tensioning-culture force monitor (t-CFM) was used to apply precise mechanical loads to cells in 3-D collagen gels, via a computer-controlled stepping motor; this enabled the physical response of cells to changes in their surrounding matrix to be represented graphically (15). Previous studies have shown that fibroblasts from different origins produce different force-generation profiles, which may be related to their different functions *in vivo*, and that morphological changes in cells within the collagen gels at different time points influenced the profile of the development of tension in the matrix induced by the cells (14,16-19).

The study aim was to characterize cellular morphology and the forces generated by valve ICs in 3-D collagen gels, and to compare this with morphology and forces generated by other cell types.

Materials and methods

Cell culture

Human valve ICs were cultured from four aortic valves, two mitral valves, one tricuspid valve and pericardial fibroblasts from two samples of pericardium. The valves (two aortic, one mitral) and pericardium were obtained from four explanted hearts (from two male and two female patients; age range: 2 to 46 years) at the time of cardiac transplantation, or were removed during valve replacement surgery (two aortic valves, one mitral, one tricuspid) in four patients (one male, three females; age range: 62 to 66 years;). Only non-diseased specimens or non-calcified areas of cusp were used. The study was approved by the Royal Brompton and Harefield NHS Trust Ethics Committee, and patient consent was obtained.

Cells were isolated by collagenase digestion of the tissue as described previously (3). In addition, vascular SMCs were obtained from samples of saphenous vein and cultured using an explant method (20). Cells were grown to confluence in Dulbecco's Modified Eagle's medium (DMEM; Gibco Chemicals, Paisley, UK), supplemented with 20% fetal calf serum (First Link, West Midlands, UK), penicillin/streptomycin (150 U/ml) and 2 mM L-glutamine (Sigma). Cells were passaged using 0.25% trypsin in 1 mM EDTA (Gibco Chemicals) and used from passages 4 to 9.

Seeding of collagen gels

The collagen gel was prepared by mixing 4 ml of a 1 mg/ml solution of native acid-soluble type I rat-tail collagen (First Link) with 0.5 ml of 10× strength Minimum Essential Medium (MEM; Gibco Chemicals) and neutralized by the drop-wise addition of 1 M

NaOH. Cells were resuspended in 0.5 ml of supplemented DMEM and the gel solution added to give a total volume of 5 ml with a final cell density ranging from 0.2 to 1×10^6 cells/ml. The gel/cell suspension was poured into a silicone elastomer hydrophobic culture chamber (75×25×15 mm) containing two attachment bars made from hydrophilic perforated plastic (Darice, USA). The gel was allowed to set in a humidified incubator at 37°C, whereupon it became attached to the bars along its long axis. The gel was then gently released from the sides of the mold and the culture well filled with complete DMEM. The bars with the gel attached floated to the surface of the culture chamber, producing an almost friction-free bearing surface. The complete culture system was placed in the CFM in a humidified 5% CO₂ incubator at 37°C. Force measurements were recorded immediately and continued for 24 h.

Culture force monitor

A schematic diagram of the CFM is shown in Figure 1. Calibration and data capture from the CFM have been described previously (16). In brief, data capture from the CFM was at the rate of one reading per second, and computer software averaged 600 readings to produce one graphical data point every 10 min.

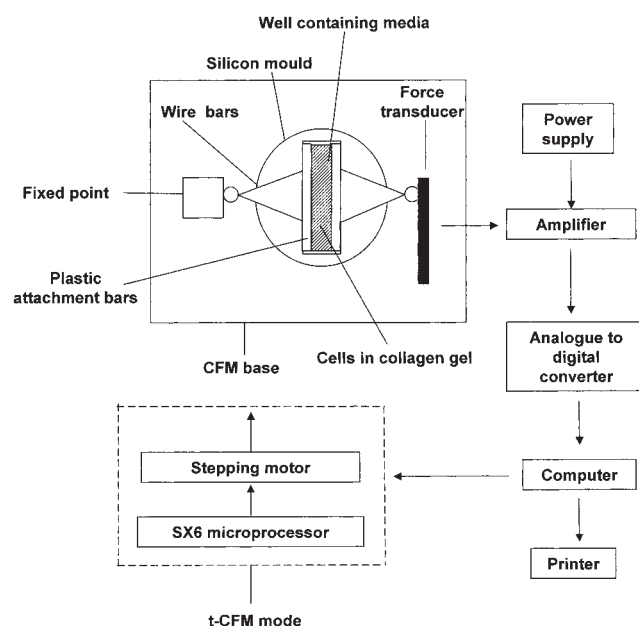


Figure 1: Schematic diagram of the culture force monitor (CFM/t-CFM). Cell-generated contraction of the collagen gel produces an electrical signal via the force transducer which is amplified, converted from analogue to digital and recorded on a computer for later analysis. When used in t-CFM mode, a separate computer program allows different loading regimes to be applied to the culture via a stepping motor that facilitates movement of the CFM base to increase or decrease the tension in the collagen gel.

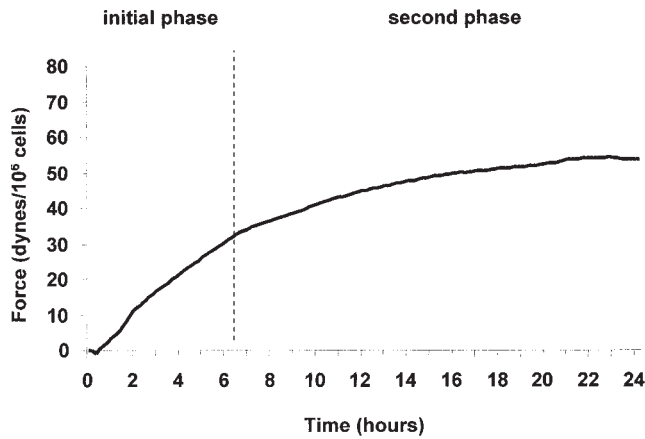


Figure 2: Representative contractile curve generated by 1×10^6 valve interstitial cells (ICs)/ml collagen gel. In this example the rate of force generation during the initial part of the experiment was 4.97 dynes/h, producing 61% of the peak force generated. Following this the rate of force generation decreased to 1.18 dynes/h.

Results were normalized to show the amount of force generated per million cells.

The tensioning-culture force monitor (t-CFM)

The t-CFM utilizes the same force transducer, data collection system and experimental set-up as the CFM, but tension can be applied to the cultures via a microprocessor-controlled microstepping motor (Micro-mech, Braintree, UK) that moves the CFM base, thereby increasing the tension within the collagen gel as it is stretched (15).

Cell morphology

To examine cell morphology over time, CFM experiments were stopped at 1, 2, 8 and 24 h. The gels were fixed immediately in 1% glutaraldehyde for 24 h at 4°C and then washed three times with phosphate-buffered saline solution. For stereomicroscopic examination, the gels were stained with 1% toluidine blue (destained with distilled water) and examined with an Edge high-definition stereo light microscope (Edge Scientific Instrument Corporation, Los Angeles, USA) (21).

Statistical analysis

The profile of the development of force in each gel was assessed by calculating the time required to achieve 50% of the peak force generated by each cell isolate. Data were expressed as the mean \pm SD, and differences were analyzed.

Results

Force generated by valve ICs

Valve ICs contracted the collagen gel and generated force over a 24-h period. The peak force generated

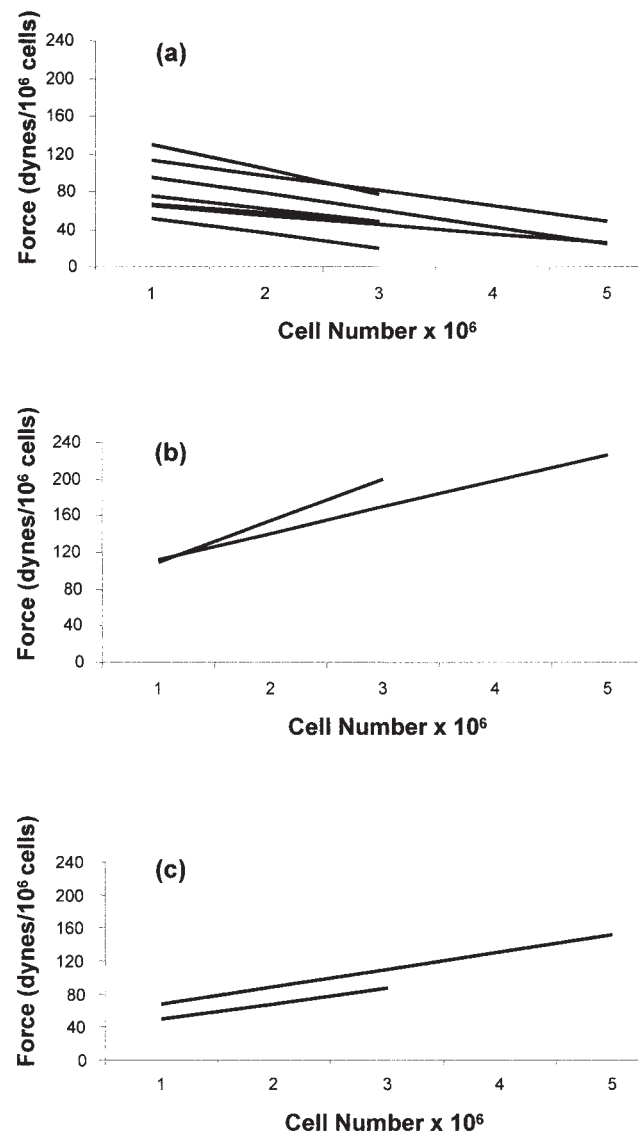


Figure 3: a) Force generated by valve ICs isolated from four aortic valves, two mitral valves and one tricuspid valve.

For each isolate an inverse relationship between the amount of force generated and cell density can be seen; the peak force/10⁶ cells generated decreased as cell density increased. (b) Force generated by cells from two saphenous vein isolates and (c) force generated by cells from two pericardium isolates: the peak of force/10⁶ cells generated was increased as the cell density of the gel increased.

ranged from 21 to 44 dynes per 10⁶ cells, with a mean value of 30.9 ± 10.4 dynes per 10⁶ cells that was relatively constant within each isolate ($n = 7$), although there were some notable differences between isolates. Every isolate studied showed a contractile curve generated by cardiac valve ICs, characterized by a rapid and near-linear increase in force, when most force was generated, followed by a slower, steady increase in force to the end of the experiment (Fig. 2). An analysis of the

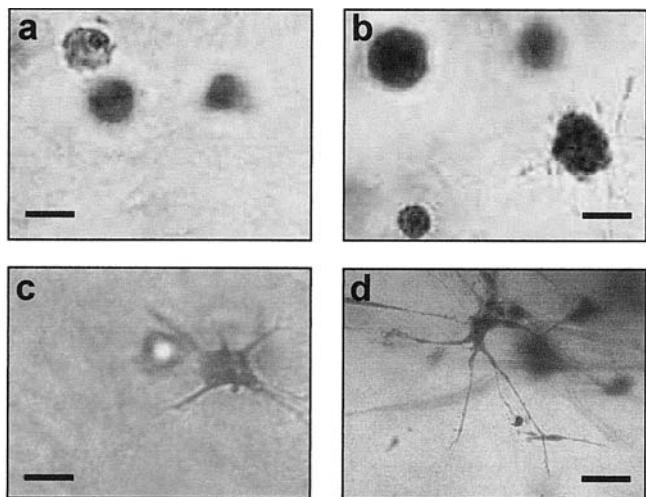


Figure 4: Morphological examination of aortic valve ICs in collagen gels stained with toluidine blue: a) 1 h; b) 2 h; c) 8 h; and d) 24 h. Scale bar = 200 μ m.

force generated by the seven mitral, seven tricuspid and 11 aortic valve cell isolates showed that the average time taken for aortic valve cells and tricuspid valve cells to produce 50% of the peak force was similar at 5.2 ± 1.4 and 5.6 ± 2.2 h, respectively. In contrast, mitral valve cells generated 50% of the peak force in 3.1 ± 1.4 h, which was significantly faster than the other valves ($p < 0.05$). The time taken to reach 50% of the peak force was also relatively constant within each isolate.

Effect of cell density on force generated

In order to assess the effect of cell density on the total force generated by the cells, the number of cells in each gel was varied from 0.2 to 1×10^6 /ml. The amount of force generated per 10^6 cells was seen to increase as the density of cells decreased (Fig. 3a; $R^2 = 0.67$, $p = 0.001$). This inverse relationship between force generated and cell density was not seen for saphenous vein SMCs (Fig. 3b), or pericardial fibroblasts (Fig. 3c), where the amount of force generated per 10^6 cells increased with increasing cell number.

Morphological examination of cells

Stereomicroscopic examination of the gels containing aortic valve ICs at 1 to 8 h (Fig. 4a-c) revealed the spreading and elongation of cell bodies and the formation of cellular processes. Examination of the gels at 8 and 24 h revealed extensive cellular process formation during this time (Figs. 4c and d, and Fig. 5a).

It can be seen from Figures 5b and c that gels seeded with saphenous vein SMCs and pericardial fibroblasts displayed smaller elongated bodies. In addition, the number and length of the processes was not as extensive as those seen in the gels seeded with aortic valve ICs.

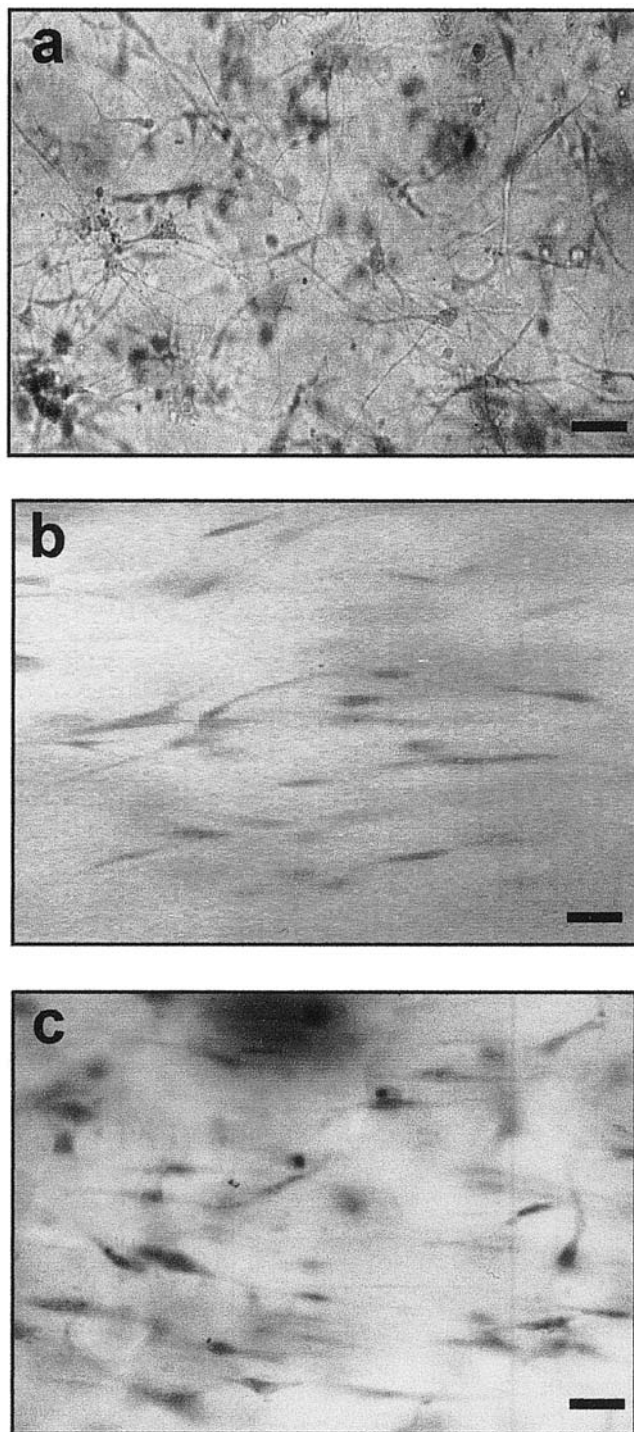


Figure 5: a) Morphological examination of aortic valve ICs in collagen gels after 24 h in the CFM reveals a network of cells connected via cellular processes. b) Saphenous vein smooth muscle cells and (c) pericardial cells do not display a connecting cellular network. Scale bar = 400 μ m.

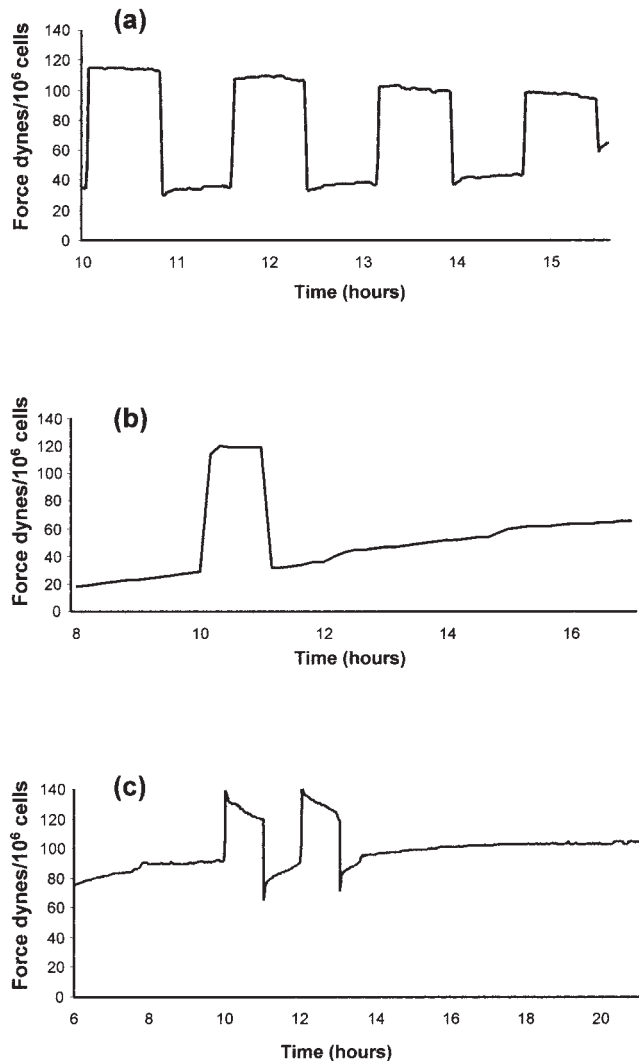


Figure 6: The response of cells to changes in matrix tension. a) Heart valve ICs and pericardial fibroblasts show little or no response to changes in matrix tension. b) Saphenous vein smooth muscle cells clearly respond to the increase in matrix tension attempting to return the matrix tension to the pre-loaded level, thereby displaying 'tensional homeostasis' (c).

Effect of increasing the tension within collagen gels

Once tension had stabilized within the gel, increased force was applied to the matrix via the t-CFM. When increased tension was maintained, there was very little or no response by heart valve ICs to the increase in matrix tension (Fig. 6a). Similarly, pericardial cells did not respond to an increase in matrix tension (Fig. 6b); however, saphenous vein SMCs appeared to decrease the tension in the matrix towards the pre-loaded level (Fig. 6c). The latter situation is referred to as displaying 'tensional homeostasis'.

Discussion

The results of the present study revealed unique properties of valve ICs to form networks of cells within a collagen gel, and spontaneously to interact with their matrix and generate contractile forces. Importantly, the characteristics of this response differ between ICs from each valve type, as well as from other cell types. The data demonstrate that an inverse relationship exists between cell number and force generation, which was observed only in valve ICs and not in the other cell types examined. In addition, these cells have a specific pattern of force generation, both under static conditions and in response to induced tension. Analysis of the cells in the gels illustrated their potential for morphological adaptation as the tension develops.

Cardiac valve ICs are a heterogeneous population of cells, many of which express the contractile protein smooth muscle alpha-actin as well as other specific contractile proteins and muscle transcription factors (1,3,4,22). A number of factors may influence the amount of force generated by cells. It is known that factors such as the nature of the collagen gel affect the force and rate of contraction, as does the sensitivity of the force transducer and the way the gels are attached to the recording equipment (23,24). By using identical equipment and the same collagen gel as in the present study, Eastwood et al. (14) reported that dermal fibroblasts produced an average of 45.9 ± 17.5 dynes per 10^6 cells, a value similar to that found for valve ICs in the present study. The same authors also reported large variations in the total force generated by different isolates of dermal fibroblasts, although within each isolate the peak force generated was reasonably constant.

In the present study the peak force generated by valve ICs from the same isolate was also found to be fairly constant, although large differences were seen between cells from different isolates. Heart valves contain a mixed population of cells, which have been described as fibroblasts or myofibroblasts, some of which share characteristics with SMCs (2,25). In the present experiments, the decision was made to allow those cells seeded in the gel potentially to reflect the cellular heterogeneity of cusp tissue, as the plasticity of the valve ICs made it impossible to obtain pure populations of individual cell phenotypes. Whilst there was a difference in magnitude of the tension generated by different isolates, the pattern of force generation remained constant across isolates. This suggests that factors related to the source of different isolates, such as the degree of calcification present in the valve or the age and gender of the donor, may dictate the magnitude of the effect seen with each individual isolate.

The present results showed that cardiac valve ICs generate an initial near-linear increase in force, fol-

lowed by a continued slow and steady increase in force. This is unlike the profile of other cell types, which reach a plateau in their force generation (14). Interestingly, mitral valve cells generated 50% of the total force significantly faster than either aortic or tricuspid valve cells. As the mitral valve leaflets are subjected to a unique rising stress profile (26), the present findings suggest that the functional properties of cells from each type of heart valve reflect and are suited to the particular mechanical function performed by each valve.

The results of previous studies have shown that the force generated by dermal fibroblasts in collagen gels increased linearly as a function of cell density (27). In contrast, in the present study, although this was found to be true for pericardial cells and saphenous vein SMCs, valve ICs displayed an inverse relationship between cell density and the force generated. This effect may be due to the extensive cellular processes that develop between the valve ICs and between valve ICs and the matrix, allowing efficient contraction of the gel by low numbers of cells. The presence of an extensive network of cellular processes has been previously reported in both human and animal valve leaflets, as well as in animal cell cultures (2,28,29).

Morphological examination of valve ICs within the collagen gels indicated that the force generated during the initial phase was associated with cell spreading, elongation and process extension. However, the maintenance and slower gradual increase in force appeared to be due to continued and extensive cellular process formation, thus creating a network of cells within the collagen gel. Given that heart valve ICs reside in a highly dynamic mechanical environment, the intensive direct cell-to-cell and cell-to-matrix communication may reflect a mechanism that contributes to the maintenance of tension within the valve cusp during systole.

When subjected to increased levels of tension, the valve ICs - unlike saphenous vein SMCs - failed to adjust their tension back to the pre-load levels. This lack of ability to display tensional homeostasis, which is seen in other fibroblastic cells, may shield the cells from the repeated cyclical changes in tension seen during the cardiac cycle. Interestingly, pericardial fibroblasts, which are also subjected to the cyclic changes in force generated by the heart (30), also failed to show tensional homeostasis. Tensional homeostasis is cell-mediated, and as such may be related to a regulatory mechanism whereby the cells respond to mechanical cues from their environment (15). Subjecting the cells to an increase in tension may result in an alteration in cell morphology and/or phenotype, although this was not investigated in the present study. It has been shown previously that cusp tissue can generate tension in response to a wide range of biologically active molecules, via specific receptor interactions (6). The regu-

lation of valve tone may therefore be directed by external stimuli as well as a sensing mechanism by the cells within the cusp.

The contribution of valve ICs to the complex function of each heart valve depends on their ability to form a network capable of generating specific types of force commensurate with valve function. This property may be crucial in maintaining valve tissue homeostasis, and is thus an important consideration when choosing a cell source for future tissue-engineered constructs. Shinoka et al. (31) reported that tissue-engineered constructs populated with dermal fibroblasts failed to maintain and remodel into a viable valve leaflet. The possibility exists that only cells which share functional properties, such as the ability to generate tension as valve ICs, will be appropriate for the tissue engineering of heart valves. It will be important for the future success of any tissue-engineered construct that the cells used respond to their new in vivo mechanical environment in the same way as the native cells.

Study limitations

Among limitations, the present study was designed to assess the response of ICs cultured from human valves and to compare this response to that of other cell types. The acquisition of 'normal' human valve tissue from which to isolate the cells remains a challenge, however, and consequently cells were isolated either from non-diseased valves from transplant recipients or from non-calcified areas of leaflets from valve replacement patients. In addition, large numbers of cells were required to populate the gels, which restricted any ability to validate fully the experimental reproducibility. A further limitation was the issue of whether cultured cells behave in similar manner to cells in situ. Here, however, comparison is made between the responses of valve cells and of cells cultured from other tissues.

The results obtained, although subject to these limitations, were considered of interest and may have important implications not only for valve function but also for tissue engineering.

Acknowledgements

The authors acknowledge The Magdi Yacoub Institute for the funding of these studies.

References

1. Brand NJ, Roy A, Hoare G, Chester A, Yacoub MH. Cultured interstitial cells from human heart valves express both specific skeletal muscle and non-muscle markers. *Int J Biochem Cell Biol* 2006;38:30-42
2. Messier RH, Jr., Bass BL, Aly HM, et al. Dual structural and functional phenotypes of the porcine aortic valve interstitial population: Characteristics of the leaflet myofibroblast. *J Surg Res* 1994;57:1-21

3. Taylor PM, Allen SP, Yacoub MH. Phenotypic and functional characterization of interstitial cells from human heart valves, pericardium and skin. *J Heart Valve Dis* 2000;9:150-158
4. Roy A, Brand NJ, Yacoub MH. Molecular characterization of interstitial cells isolated from human heart valves. *J Heart Valve Dis* 2000;9:459-464
5. Taylor PM, Allen SP, Dreger SA, Yacoub MH. Human cardiac valve interstitial cells in collagen sponge: A biological three-dimensional matrix for tissue engineering. *J Heart Valve Dis* 2002;11:298-306
6. Chester AH, Misfeld M, Yacoub MH. Receptor-mediated contraction of aortic valve leaflets. *J Heart Valve Dis* 2000;9:250-254
7. Dreger SA, Taylor PM, Allen SP, Yacoub MH. Profile and localization of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in human heart valves. *J Heart Valve Dis* 2002;11:875-880
8. Latif N, Sarathchandra P, Taylor PM, Antoniow J, Yacoub MH. Localization and pattern of expression of extracellular matrix components in human heart valves. *J Heart Valve Dis* 2005;14:218-227
9. Higashidate M, Tamiya K, Beppu T, Imai Y. Regulation of the aortic valve opening. In vivo dynamic measurement of aortic valve orifice area. *J Thorac Cardiovasc Surg* 1995;110:496-503
10. Thubrikar M, Boshier LP, Nolan SP. The mechanism of opening of the aortic valve. *J Thorac Cardiovasc Surg* 1979;77:863-870
11. Kershaw JD, Misfeld M, Sievers HH, Yacoub MH, Chester AH. Specific regional and directional contractile responses of aortic cusp tissue. *J Heart Valve Dis* 2004;13:798-803
12. Hon JK, Melina G, Wray J, Yacoub MH. Insights from 36 years' follow up of a patient with the Ross operation. *J Heart Valve Dis* 2003;12:561-565
13. Merryman WD, Youn I, Lukoff HD, et al. Correlation between heart valve interstitial cell stiffness and transvalvular pressure: Implications for collagen biosynthesis. *Am J Physiol Heart Circ Physiol* 2006;290:H224-H231
14. Eastwood M, McGrouther DA, Brown RA. A culture force monitor for measurement of contraction forces generated in human dermal fibroblast cultures: Evidence for cell-matrix mechanical signalling. *Biochim Biophys Acta* 1994;1201:186-192
15. Brown RA, Prajapati R, McGrouther DA, Yannas IV, Eastwood M. Tensional homeostasis in dermal fibroblasts: Mechanical responses to mechanical loading in three-dimensional substrates. *J Cell Physiol* 1998;175:323-332
16. Brown RA, Talas G, Porter RA, McGrouther DA, Eastwood M. Balanced mechanical forces and microtubule contribution to fibroblast contraction. *J Cell Physiol* 1996;169:439-447
17. Eastwood M, Porter R, Khan U, McGrouther G, Brown R. Quantitative analysis of collagen gel contractile forces generated by dermal fibroblasts and the relationship to cell morphology. *J Cell Physiol* 1996;166:33-42
18. Porter RA, Brown RA, Eastwood M, Occlleston NL, Khaw PT. Ultrastructural changes during contraction of collagen lattices by ocular fibroblasts. *Wound Repair Regen* 1998;6:157-166
19. Talas G, Adams TS, Eastwood M, Rubio G, Brown RA. Phenytoin reduces the contraction of recessive dystrophic epidermolysis bullosa fibroblast populated collagen gels. *Int J Biochem Cell Biol* 1997;29:261-270
20. Bundy R, Marczin N, Chester AH, Yacoub M. Differential regulation of DNA synthesis by nitric oxide and hydroxyurea in vascular smooth muscle cells. *Am J Physiol* 1999;277(5 Pt.2):H1799-H1807
21. Greenberg G, Boyde A. Novel method for stereo imaging in light microscopy at high magnifications. *Neuroimage* 1993;1:121-128
22. Zacks S, Rosenthal A, Granton B, Havenith M, Opas M, Gotlieb AI. Characterization of Cobblestone mitral valve interstitial cells. *Arch Pathol Lab Med* 1991;115:774-779
23. Ehrlich HP, Rajaratnam JB. Cell locomotion forces versus cell contraction forces for collagen lattice contraction: An in vitro model of wound contraction. *Tissue Cell* 1990;22:407-417
24. Harris AK, Stopak D, Wild P. Fibroblast traction as a mechanism for collagen morphogenesis. *Nature* 1981;290:249-251
25. Taylor PM, Batten P, Brand NJ, Thomas PS, Yacoub MH. The cardiac valve interstitial cell. *Int J Biochem Cell Biol* 2003;35:113-118
26. Kunzelman KS, Cochran RP, Chuong C, Ring WS, Verrier ED, Eberhart RD. Finite element analysis of the mitral valve. *J Heart Valve Dis* 1993;2:326-340
27. Delvoeye P, Wiliquet P, Leveque JL, Nusgens BV, Lapiere CM. Measurement of mechanical forces generated by skin fibroblasts embedded in a three-dimensional collagen gel. *J Invest Dermatol* 1991;97:898-902
28. Bairati A, DeBiasi S. Presence of a smooth muscle system in aortic valve leaflets. *Anat Embryol (Berlin)* 1981;161:329-340
29. Filip DA, Radu A, Simionescu M. Interstitial cells of the heart valves possess characteristics similar to smooth muscle cells. *Circ Res* 1986;59:310-320
30. Goto Y, LeWinter MM. Nonuniform regional deformation of the pericardium during the cardiac cycle in dogs. *Circ Res* 1990;67:1107-1114
31. Shinoka T, Shum-Tim D, Ma PX, et al. Tissue-engineered heart valve leaflets: Does cell origin affect outcome? *Circulation* 1997;96(9 Suppl.):II-7