Dynamic Relationships of Collagen Extracellular Matrices on Cardiac Differentiation of Human Mesenchymal Stem Cells

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1. Introduction

Myocardial infarction (MI) results in necrosis, inflammation and scar formation in the myocardium. Such pathological insults place increasing mechanical demands on surviving cardiomyocytes (Boudoulas & Hatzopoulos, 2009). As cardiomyocytes have limited regenerative potential, loss of functional healthy tissue and subsequent left ventricular (LV) remodelling, eventually leads to pathological hypertrophic cardiomyopathy. Hypertrophy of the LV has been documented as a chronic response to MI and invariably progresses to heart failure (Hannigan et al., 2007). Chronic heart failure is a major health problem with patients experiencing a debilitating quality of life.

Cardiac remodelling after MI is characterised by progressive and pathological interstitial fibrosis. During acute phase of cardiac repair, degradation of myocardial extracellular matrix (ECM) coupled with an influx of inflammatory cells and cytokines permits deposition of granulation tissue in the infarct region. At the site of tissue injury, granulation tissue composes of macrophages, myofibroblasts and neovascularisation. Activated myofibroblasts synthesise collagen and other ECM proteins to form dense scar tissue in the infarct in response to inflammatory mediators such as angiotensin II (Ang II) and transforming growth factor-β1 (TGF-β1). Macrophages drive the production of TGF-β1, an essential growth factor for fibroblast production, collagen synthesis and inhibition of collagen degradation (O'Kane & Ferguson, 1997; Sun & Weber, 2000). At the site of MI, increased expression of adhesion molecules (inter-cellular adhesion molecule-1, ICAM-1) and chemoattractant cytokines (monocyte chemotactic protein-1, MCP-1) facilitate migration of inflammatory cells (e.g. macrophages) enabling scavenging of necrotic tissues (Lu et al., 2004). This couples with elevated expression of matrix metalloproteinase-1 (MMP-1) results in remodelling of myocardial ECM by degradation of existing collagen I and III in the injured myocardium (Lu et al., 2004). Furthermore, MMP-9 has been implicated in tissue remodelling by cleaving collagen V at the amino-terminus (Niyibizi et al., 1994). Consequently, this process compromises structural integrity of the ventricles, resulting in myocyte slippage, wall thinning and rupture

(Cleutjens et al., 1995b). Derangements in cardiomyocyte-ECM interactions cause the loss of cellular tensegrity and initiates anoikis in neighbouring healthy tissue (Michel, 2003). It is now well recognised that structural changes in the myocardial ECM can alter collagen-integrin-cytoskeletal-myofibril relations, thus affecting overall geometry and function of the heart (Spinale, 2007).

In non-cartilaginous tissues like the heart, collagen I, III and V are the predominant subtypes of the ECM (Breuls et al., 2009; Linehan et al., 2001). Collagen I is primarily a structural element of the myocardial ECM while collagen V represents a minor, but important component sequestered within collagen I fibres. However, collagen V levels increase in inflammation and scar tissue. The relative resistance of collagen V to mammalian collagenases makes it transiently available during tissue remodelling. The temporal availability of collagen V during active extracellular remodelling implies that it may play an important role in ECM remodelling and tissue stiffness (Breuls et al., 2009; Ruggiero et al., 1994). In fact, collagen V plays a deterministic role in collagenous fibril structure, matrix organisation and stiffness (Fichard et al., 1995).

Binding of ECM to integrins provides a linkage between the ECM and cellular cytoskeleton. Integrins are heterodimeric receptors composed of non-covalently bound α and β subunits. (Brancaccio et al., 2006). Dynamic integrin-ECM interactions result in bidirectional signalling and determines cell morphology, gene expression, migration, proliferation, differentiation and death. Perkins et al. (2010) showed that integrin-mediated adhesion is mandatory for maintenance of the sarcomeric architecture. They proposed that disintegration of the Z-line and progressive muscle degeneration can occur once the adhesion complex comprising of integrins, talin or integrin linked kinase (ILK) is not replenished. In the myocardium, integrins can function as mechanotransducers that transmit mechanical ECM cues to the myocyte, resulting in changes to myocyte biology and function (Ross & Borg, 2001). Integrins $\alpha_2\beta_1$, $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$ are collagen binding heterodimers and adhesion to collagen V has been reported to be primarily mediated by integrin $\alpha_2\beta_1$ and $\alpha_1\beta_1$ (Ruggiero et al., 1994). Integrins $\alpha_2\beta_1$ and $\alpha_1\beta_1$ may play a significant role in remodelling of the heart where there is increased collagen synthesis and collagen V expression, although we have previously shown $\alpha_v\beta_3$, but not $\alpha_2\beta_1$, in a collagen V associated cardiac differentiation of human mesenchymal stem cells (hMSCs) (Tan et al., 2010).

Increased ejection fraction (EF) and fractional shortening (FS) parameters, coupled with a reduction in the amount of fibrotic scar tissue have been highlighted following cellular therapy (Chacko et al., 2009). Our previous study showed that cardiomyocyte-like cells (CLCs) that were differentiated from MSCs, improved systolic performance without compromising end-diastolic pressure of the infarcted myocardium when compared to MSCs. CLCs may facilitate hemodynamic recovery by preserving tissue elasticity in the collagen V-expressing peri-infarct borders. This unique cell/matrix relationship may be more conducive to a functionally adaptive remodelling response in maintaining contractile efficiency of post-infarcted myocardium (Tan et al., 2010).

Experimental data show that MSC transplantation inhibits LV remodelling and improves heart function in animals with MI (Xu et al., 2005). Despite the ability of angiogenic mechanisms to reduce infarct mass, only partial restoration of ventricular contraction occurs as myocytes are not regenerated (Gaudette & Cohen, 2006). In addition, cardiac differentiation and retention of surviving transplanted MSCs in-vivo is limited (Feygin et al.,

2007). Influence of ECM proteins and integrin interactions on MSC differentiation have been widely investigated for chrondrogenic and osteogenic differentiation (Djouad et al., 2007; Gronthos et al., 2001). Conversely, studies investigating ECM role in cardiac differentiation of MSCs is limited. Unravelling of integrin roles in cardiac differentiation of MSCs would aid in understanding of mechanisms leading to retention and integration of stem cells in myocardium.

We have previously reported in-vitro differentiation of human MSCs towards CLCs and shown that collagen V promoted adhesion and cardiac gene expression in CLCs (Shim et al., 2004; Tan et al., 2010). In the present study, we further examine the role of individual integrins in cardiac differentiation of CLCs.

2. Materials and methods

2.1 Isolation and culture of bone marrow derived MSCs

Bone marrow was isolated from the sternum of patients undergoing open-heart surgery. They were collected in 17 IU/ml heparin using a 23-gauge needle. Bone marrow aspirates were topped up to 15 ml with Dulbecco's modified Eagle's medium-low glucose (DMEM-LG, GIBCO) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin-streptomycin (Gibco, Invitrogen). To deplete bone marrow asiprates of mature blood lineages, 15 ml of bone marrow blood mixture was overlaid onto 15 ml of Histopaque®-1077 (Sigma-Aldrich) and centrifuged for 1500 rpm (Kubota Centrifuge) for 30 minutes at 4°C. The enriched cell fraction was collected from the interphase, washed once with 5 ml of media and centrifuged at 1200 rpm (Kubota Centrifuge) for 10 minutes. Resuspended cells were then transferred into tissue culture flasks with basal normal growth medium (NGM) comprising DMEM-LG supplemented with 10% FBS for 9 - 11 days to yield plastic adherent MSCs. Subconfluent cells were harvested using 1X Trypsin-EDTA solution for endothelial cell culture (Sigma-Aldrich), 14 – 21 days after initial plating and maintained as MSCs in basal NMG or differentiated towards CLCs in a myogenic differentiation medium (MDM) as previously described (Shim et al., 2004).

Type V collagen (Sigma-Aldrich) and Type I collagen (BD™) were coated on 6-well plates or tissue culture flasks at 10μg/cm^2 for 3 hours at room temperature. Plates and flasks were washed twice with phosphate buffered saline (PBS) and kept at 4°C until required.

2.2 Fluorescence microscopy

Frozen tissue sections of the explanted ventricular rat hearts were fixed in 4% paraformaldehyde (PFA), permeabilised with 0.1% Triton X-100, and further blocked in 5% bovine serum albumin (BSA). This was followed by overnight incubation at 4°C with primary antibodies, including collagen I (Southern Biotech), collagen III (Affinity Bioregent) collagen V (Biotrend) and anti-α-sarcomeric actinin (Sigma-Aldrich) diluted in 1% BSA. Sections were incubated with Alexa Fluor® 488/555/660 - conjugated secondary antibodies (Molecular Probes) in 0.1% BSA at room temperature for 3 hours before staining the nuclei with DAPI. Immunofluorescence microscopy was performed with Zeiss Axiovert 200 M fluorescence microscope, using the Metamorph software (version 6.2, Molecular Devices) or Leica MZ 16 FA Fluorescence Steromicroscope, using the Leica Application Suite software (Version 3.3.0, Leica).

2.3 Flow cytometry

Sternum-derived bone marrow MSCs were differentiated into CLCs and characterised by flow cytometry after 14 days in a MDM. CLCs cultured on uncoated, collagen I or V coated tissue culture flasks were stained with antibodies directed towards integrin subunits α_1 (Abcam), α_2 (Santa Cruz), α_v (Fitzgerald), β_1 (Chemicon) and β_3 (Cell Signaling). Cells were treated with Fix & Perm® Cell Permeabilisation Kit (Invitrogen) and subsequently blocked in PBS containing 5% BSA, 1% FBS and 5 mM ethylenediaminetetracetic acid (EDTA) for 30 minutes at 4oC on a roller. CLCs were then incubated with directly conjugated antibodies for 30 minutes at 4oC. Indirectly conjugated antibodies were incubated for 2 hours at 4oC and subsequently stained with their respective Alexa Fluor® 555 conjugated secondary antibodies (Invitrogen) for 2 hours at 4oC. Isotype controls were stained in parallel with the test samples. Samples were washed in PBS containing 2% BSA, 2% FBS and 5 mM EDTA after each antibody staining and fixation step. All samples were fixed in PBS containing 4% PFA/PBS, washed and resuspended in PBS containing 2% FBS and 0.09% sodium azide (NaZ). Data analysis was performed using FACSDiva software (version 6.1.2, BD™), FlowJo software (version 6.4, Tree Star, Inc.). Histogram overlays were performed and the change in median fluorescence intensity and overton subtraction percentages were computed.

2.4 Integrin neutralisation assays

Integrin neutralisation assays were performed on CLCs using neutralising antibodies against the integrin α_1 (Millipore) subunit and $\alpha_v\beta_3$ (Millipore) heterodimer, at 1µg/ml and 10 µg/ml respectively. CLCs treated with 1µg/ml or 10 µg/ml isotype IgG (Abcam/Dako) antibodies and untreated CLCs served as controls. After trypsin digestion, CLCs were incubated with neutralising and isotype control antibodies for 2 hours at 4oC. 50,000 untreated and treated CLCs were seeded on collagen V pre-coated 6-well plates. Plated CLCs were harvested after 72 hours of culture at 37oC, 5% CO_2. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and treated with RNAse free DNase solution (Qiagen). DNAse treated RNA samples were stored at -80oC until required.

2.5 Real-time reverse transcriptase polymerase chain reaction for quantitation of cardiac gene expression

First strand cDNA was synthesised from total RNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen) and equal concentrations of cDNA were loaded into tubes containing QuantiFast SYBR Green PCR mastermix (Qiagen). Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed on the Rotor-Gene Q thermocycler (Qiagen) using standard cycling parameters and relative gene expression of the following cardiac transcripts was quantitated using the $\Delta\Delta C_T$ method. These transcripts include β actin (BA), cardiac α-actin (CAA), skeletal muscle α-actin (SKAA), troponin T (Trop T), troponin C (Trop C), Nkx2.5 and GATA4 (Sigma-Aldrich). Target gene expression values were normalised relative to the untreated CLCs. BA served as a housekeeping gene for the real time RT-PCR experiments. No template controls were concurrently processed with test samples to rule out the presence of contaminated reagents and nucleic acids.

Gene	Acession Number	Primer Sequence	Product Size (bp)
CAA	NM_005159	5'-CTTCTAAGATGCCTTCTCTCTCCA-3' 5'-TAT TAG AAG CAC AAA CAA ATT GCA-3'	147
SKAA	NM_001100.3	5'-CGAGACCACCTACAACAGCA-3' 5'-GCGGTGATCTCTTTCTGCAT-3'	132
Trop C	NM_003280.2	5'-CTACAAGGCTGCGGTAGAGC-3' 5'-CAGCACGAAGATGTCGAAGG-3'	76
Trop T	NM_000364.2	5'-ATCCCCGATGGAGAGAGAGT-3' 5'-ACGAGCTCCTCCTCCTCTTT-3'	128
Nkx2.5	NM_004387	5'-GATTCCGCAGAGCAACTCG-3' 5'-GGAGCTGTTGAGGTGGGATCG-3'	105
GATA4	NM_002052.3	5'-TCCAAACCAGAAAACGGAAG-3' 5'-AAGGCTCTCACTGCCTGAAG-3'	77
BA	NM_001101	5'-TCCCTGGAGAAGAGCTACGA-3' 5'-AGCACTGTGTTGGCGTACAG-3'	194

Table 1. Primer sequences for real time reverse transcriptase polymerase chain reaction (RT-PCR). Transcripts obtained from RT-PCR assays were all less than 200 bp. CAA, cardiac α-actin; SKAA, skeletal muscle α-actin; Trop T, troponin T; Trop C, troponin C; BA, β actin.

2.6 Cell labelling

CLCs were labelled with 1 mmol/L Vybrant CellTracker chloromethyldialkylcarbocyanie (CM-Dil; Molecular Probes) overnight at 37°C and rinsed 3 times before trypsin disgestion and transplantation. MSCs were labelled with 10mmol/L Vybrant carboxy fluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes). Cells were resuspended in a final concentration of $1 \times 10^6/0.1$ ml to $5 \times 10^6/0.2$ ml.

2.7 Rat myocardial infarction model

MI was created in n=20 female Wistar rats per group. Each rat weighed approximately 350 - 400g in body weight. The animals were subjected to left thoracotomy and the left anterior descending artery (LAD) was exposed and ligated. After which rats were allowed a week for recovery before given treatment of either injection with labelled cells or placebo to the area of infarction. Cyclosporin A was administered at a dose of 5mg/kg body weight at 3 days before and daily following treatment for 6 weeks until end point.

2.8 Echocardiography

Baseline echocardiography was performed on each rat before MI and 6 weeks after treatment. Echocardiography images were acquired using Vivid 7 ultrasound machine (General Electric VingMed) equipped with i13L linear probe operated at 14MHz. Rats were anaesthetised using 1% - 2% isofluorane with 1L/hr oxygen and then fixed in the supine position on a heated platform. Rats were then shaved at the chest and abdominal areas before electrocardiography (ECG) electrodes were placed onto the left and right leg as well as the left upper extremity. All analysis was performed offline with EchoPAC workstation (General Electric Healthcare).

2.9 Statistical analysis

One-way analysis of variance (ANOVA) was used to determine statistical significance between different treatment groups. Tukey Honestly Significant Difference (HSD) post-hoc analyses were used to determine statistical significance between treatment groups using SPSS 13 software (SPSS Inc.). $p < 0.05$ was considered statistically significant. All data are presented as mean ± standard deviation (SD).

3. Results

3.1 Integrin expression and cardiac differentiation

Flow cytometric analysis showed that α_v and β_1 were the predominant subunits of integrins in CLCs, independent of substrate surface (Table 2). In comparison to collagen V matrix, CLCs cultured on collagen I showed a higher expression of integrin α_1 (59.4 ± 13.7% vs. 78.0 ± 0.9%) and β_3 (44.7 ± 10.6% vs. 56.0 ± 21.8%) subunits. Furthermore, with the exception of α_1 subunit, α_2, α_v, β_1 and β_3 integrins in CLCs cultured on either collagen matrices showed a reduction of expression in comparison to CLCs cultured on polystyrene tissue culture surface.

Integrin Subunit	CLC		
	Uncoated	Collagen I	Collagen V
α_1	58.5 ± 1.8	78.0 ± 0.9	59.4 ± 13.7
α_2	59.4 ± 6.1	39.9 ± 11.5	44.3 ± 12.8
α_v	91.6 ± 0.5	83.8 ± 5.7	81.8 ± 4.6
β_1	93.4 ± 0.8	78.5 ± 8.6	79.1 ± 8.6
β_3	57.6 ± 2.4	56.0 ± 21.8	44.7 ± 10.6

Table 2. Flow cytometric analysis showed that integrin α_v and β_1 were the predominant subunits in CLCs. CLCs cultured on collagen I showed increased levels of α_1 and β_3. Data were derived from 3 independent experiments and the overton percentage positive results are expressed as mean ± SD. MSCs, Mesenchymal stem cells; CLCs, Cardiomyocyte-like cells.

3.2 CLCs enhance cardiac gene expression via integrin α_1 and $\alpha_v\beta_3$ on collagen V matrices

We previously reported that collagen V matrix enhanced cardiac gene expression when compared to CLCs seeded on collagen I matrix. Collagen V selectively upregulated expression of cardiac transcription factors (GATA4, Nkx2.5), calcium handling transporter (RyR2) and sarcomeric myofilament proteins (Trop T, Trop C, SKAA) in CLCs (Tan et al., 2010). Neutralisation of $\alpha_v\beta_3$ integrin or α_1 subunit in this study did not affect CAA and SKAA gene expression in CLCs that were cultured on collagen V matrix. Furthermore, no significant changes in Nkx2.5 or GATA4 expression was observed in α_1 subunit neutralised CLCs. However, Nkx2.5 down regulation was observed in CLCs neutralised with $\alpha_v\beta_3$, although similar down regulation was also evident in the isotype control experiment. Gene expression of Trop C reduced significantly after $\alpha_v\beta_3$ integrin neutralisation. In contrary, α_1

subunit neutralisation upregulated Trop C expression. Furthermore, there was a concomitant upregulation of Trop T following α_1 integrin neutralisation.

Integrin Neutralisation Antibody	CAA		SKAA		Trop C		Nkx2.5		Trop T		GATA4	
	Isotype	Test	Isotype	Test	Isotype	Test	Isotype	Test	Isotype	Test	Isotype	Test
$\alpha_v\beta_3$	0.87 ± 0.00	1.00 ± 0.10	1.32 ± 0.13	1.15 ± 0.00	0.94 ± 0.10	0.12 ± 0.02	0.23 ± 0.21	0.22 ± 0.16	N.D.	N.D.	N.D.	N.D.
α_1	0.52 ± 0.05	0.91 ± 0.02	0.99 ± 0.12	1.02 ± 0.10	1.17 ± 0.11	1.45 ± 0.40	1.02 ± 0.22	0.90 ± 0.22	1.04 ± 0.05	1.63 ± 0.16	0.87 ± 0.00	0.63 ± 0.45

Table 3. CLCs cultured on collagen V were treated with integrin α_1 (1 μg/ml) neutralising antibodies. Untreated and isotype IgG (1 μg/ml) treated CLCs served as controls for this experiment. Optimal concentrations of test and control antibodies were predetermined in a series of titration experiments. CLCs cultured on collagen V were treated with integrin $\alpha_v\beta_3$ (10 μg/ml) neutralising antibodies. Untreated and isotype IgG (10 μg/ml) treated CLCs served as controls for this experiment. Results are expressed as mean ± SD. CAA, cardiac α-actin; SKAA, skeletal muscle α-actin; Trop T, troponin T; Trop C, troponin C, N.D., not done; MSCs, Mesenchymal stem cells; CLCs, Cardiomyocyte-like cells.

3.3 CLCs integrate into collagen V-rich cardiac syncytium

Consistent with our previous report (Tan et al., 2010), collagen I as the main constituent of cardiac ECM in intact rat myocardium, was found to co-localise with collagen III matrix in the epicardium and perimysial space between major muscle bundles dispersed throughout the myocardium (Fig. 1A). On the other hand, collagen V was predominantly observed in the endomysial space surrounding healthy cardiomyocytes and in the perivascular structures within the myocardium. Following MI by ligating the LAD artery, significant wall thinning was observed in the anterior wall of the LV (Fig.1B). Accumulation of collagen matrices was evident in the infarcted and non-infarcted zones 7 weeks post infarction. Spatial remodelling and redistribution of collagen matrices were observed whereby perimysial collagen I diminished significantly and upregulation of collagen I and III were observed in the pericardium and epicardium of infarcted as well as non-infarcted zones. Furthermore, fibrosis consisted mainly of collagen I matrix was prominently found in the endocardium of infarcted zone while it co-localised with collagen III matrix in the pericardium/epicardium of the non-infarcted and infarcted zones (Fig. 1Bii & 1Biii). In contrast, collagen V fibrils were sparsely detected in the epicardium of infarct, but were prominently found in the peri-vascular structures within the infarct (Fig. 1Biii). In contrast to redistribution of collagen I matrix, collagen V remained in the endomysial matrix of individual muscle fibres in the non-infarcted borders (Fig.1Bii) and also surrounding isolated, but viable cardiac fibres in the infarct.

Myocardial transplanted CLCs were closely associated with collagen V matrix in the endomysial space in the peri-infarct border of the myocardium (Fig. 2A). In contrast, similarly transplanted MSCs were only found in collagen I-rich infarct despite the presence of isolated, collagen V-expressing, myofibres at the infarct borders (Fig. 2B). Furthermore, CLCs were often intimately engrafted among α-actinin stained native cardiomyocytes that

were surrounded by collagen V, but not collagen I, matrices (Fig. 2C and 2D). On the contrary, transplanted MSCs were sequestered in the infarct that was dominated with collagen I matrix and isolated from viable and α-actinin stained myocardium that expressed collagen V matrix (Fig. 2E).
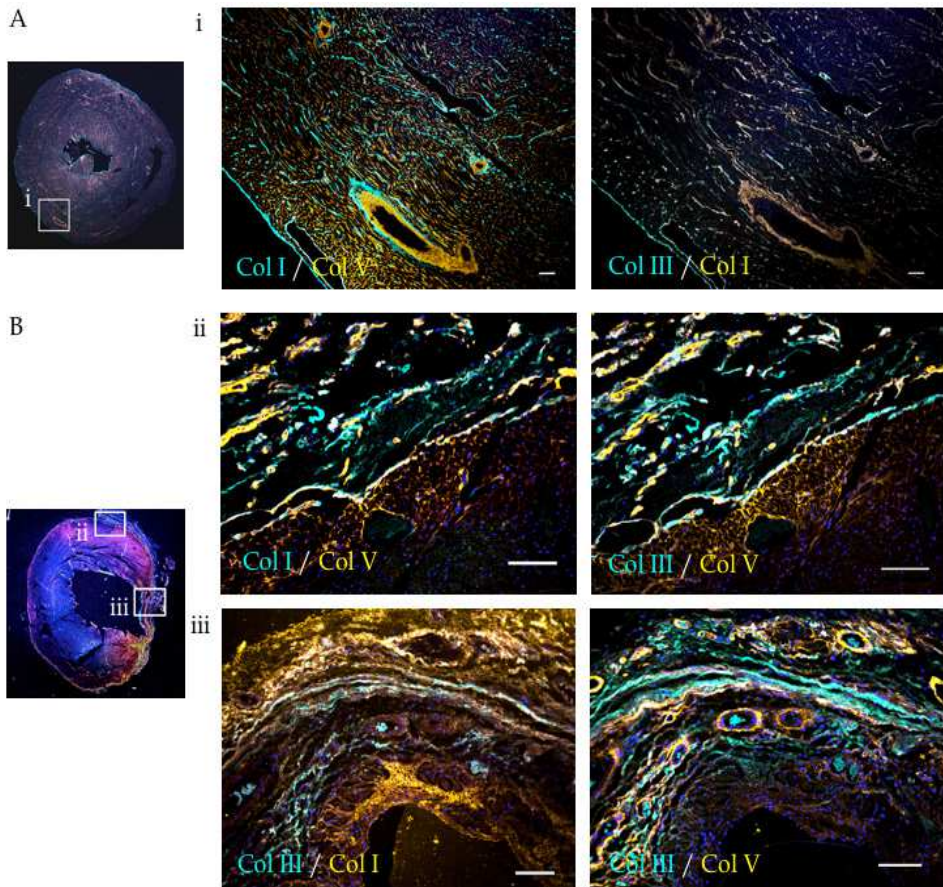

Fig. 1. (A) Collagen distribution in an intact myocardium. (Ai) Higher magnification of the boxed area showing collagen I and III distribution in the perimysium while collagen V was expressed in the endomysial space. (B) Collagen distribution in an infarcted myocardium. (Bii) Higher magnification of the myocardium, epicardium and pericardium at the boxed area. Collagen V was predominantly expressed at the peri-infarct border surrounding viable myocytes and vasculature structures in the infarct region. Collagen I and III were positively stained in the infarcted epicardium and pericardium. (Biii) Higher magnification demonstrating severe thinning of the LV anterior wall. Co-localisation of collagen I and III extended from the pericardium into the infarcted myocardium whereas collagen I was primarily localised in the endocardium. Collagen V was expressed in the vessels and sparsely in the infarct. Scale bar: 200µm.
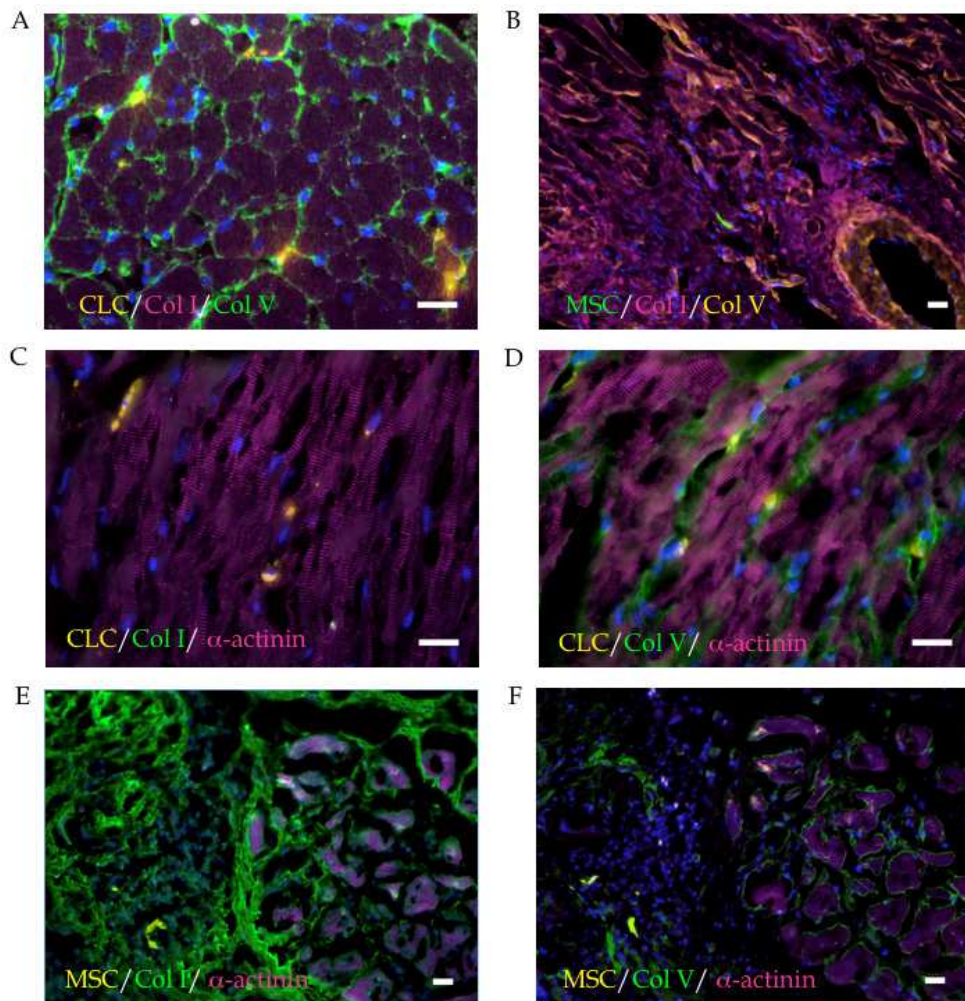
Fig. 2. (A) Transplanted CLCs preferentially home to the collagen V-rich myocardial ECM. (B) Transplanted MSCs localised in the collagen I enriched infarct zone away from the collagen V peri-infarct region. (C) Engraftment of CLCs in the α-actinin stained myocardium (D) showing an affinity towards collagen V matrix in the absence of collagen I staining. (E) MSCs were embedded in the collagen I-rich infarct zone and were isolated from α-actinin expressing cardiomyocytes. (F) Collagen V was sparsely distributed in the infarcted region, but mainly surrounded viable myocytes at the peri-infarct border. Scale bar: 20μm. MSCs: Mesenchymal stem cells; CLCs: Cardiomyocyte-like cells; Col I: Collagen I; Col V: Collagen V.

3.4 CLC therapy at high doses improve cardiac hemodynamics

Consistent with their muscular engraftment, LV echocardiography confirmed a better cardiac performance of transplanted CLCs, 6 weeks post cell transplant (Table 4).

Transplanted CLCs (2.2 ± 0.3 mm, p<0.05), but not MSCs (2.1 ± 0.3 mm), improved LV anterior wall thickness as compared to control infarcted animal (1.8 ± 0.4 mm). Nevertheless, other cardiac parameters indicated that CLCs and MSCs contributed comparably to functional improvements by reducing chamber dilatation and moderating negative LV remodelling.

M-Mode	SF (n=15)	MSC (n=19)	CLC (n=17)	Statistical Significance
LVIDed (mm)	7.7 ± 0.9	7.3 ± 0.6	7.1 ± 0.7	NS
LVIDes (mm)	5.0 ± 1.1	4.2 ± 0.6[+]	4.1 ± 0.9[*]	[+]p<0.05 vs. SF [*]p<0.01 vs. SF
IVSed (mm)	1.3 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	NS
IVSes (mm)	1.8 ± 0.4	2.1 ± 0.3	2.2 ± 0.3[*]	[*]p<0.05 vs. SF
AWT (%)	49.8 ± 16.3	53.6 ± 14.7[+]	54.0 ± 15.6[*]	[+]p<0.05 vs. SF [*]p<0.05 vs. SF
FS (%)	35.0 ± 5.6	42.4 ± 5.3[+]	43.1 ± 6.6[*]	[+]p<0.05 vs. SF [*]p<0.005 vs. SF
EF (%)	60.4 ± 7.9	68.7 ± 6.3[+]	69.8 ± 9.5[*]	[+]p<0.05 vs. SF [*]p<0.005 vs. SF

Table 4. Ultrasound echocardiography assessment of post cellular therapy treated rats. 2D ultrasound echocardiography assessments showed significant improvements in cell transplanted animals. SF: Serum free control; CLC: Cardiomyocyte-like-cells; MSCs: Mesenchymal stem cells; LVIDed: Left ventricular internal dimension at end diastolic; LVID: Left ventricular internal dimension at end systolic; IVSed: Interventricular septum at end diastolic, IVSes: Interventricular septum at end systolic; AWT: Anterior wall thickening; FS: Fractional shortening; EF: Ejection fraction.

4. Discussion

Integrins and ECM are important modulators of stem cell behaviours. To date, cardiac cell therapy supported only modest benefits, likely due to low engraftment of transplanted cells in the infarcted myocardium. Exploration of specific integrin/ECM interaction may improve engraftment and survival of transplanted cells and ultimately, mechanical function of the heart. Our current study examines integrin/ECM interactions on cardiac gene expression of CLCs and distribution of transplanted CLCs in infarcted myocardium.

The distribution and quantity of type I and III collagens in the heart play an important role in maintaining cardiac function. Alterations of collagen population and distribution in the myocardium affect size and shape of the heart chambers as well as myocardial diastolic and systolic function (Cleutjens et al., 1995a; Janicki & Brower, 2002). However, it is unclear if such alterations could affect stem cell migration and differentiation in the myocardium.

We have previously demonstrated that CLCs showed preferential adhesion to collagen V over collagen I matrix by interacting with subsets of integrins (Shim et al., 2004; Tan et al., 2010). van Laake et al. (2010) reported that pre and post transplanted human embryonic

cardiomyocytes (hESC-CM) express integrins matching ECM types they encountered in their environment. Therefore, the integrin modulating role of collagen V may aid in the observed retention of the myocardial transplanted CLCs. Furthermore, intimate engraftment of the transplanted CLCs with collagen V-expressing, α-actinin positive, native cardiomyocytes supports an unique role of collagen V in the myocardium. Moreover, differential expression of α_1 and β_3 integrin between collagen I and V cultured CLCs coupled with the preferential homing demonstrated between transplanted MSCs and CLCs suggested a key role of collagen V matrix, not only in cellular retention, but cardiac differentiation of the transplanted stem cells. This is consistent with modulation of cardiac gene expression of CLCs demonstrated in relation to α_1 and $\alpha_v\beta_3$ neutralisation in vitro, although such relationship was not examined in vivo. Nevertheless, the comparable cardiac outcomes achieved in spite of selective homing of the transplanted cells, indicate that different reparative mechanisms may be initiated by MSCs and CLCs. Despite a positive trend of systolic improvement by CLCs, further mechanistic studies are warranted to discern their specific contribution to systolic and diastolic components of cardiac performance.

Integrin α_1 is known to transduce ECM signals to the cytoskeleton that activate downstream mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase 1 (ERK1) signalling pathways that phosphorylate and activate GATA4 (Akazawa & Komuro, 2003). However, GATA4 expression was unaffected by integrin α_1 neutralisation despite the upregulated Trop C and Trop T belonging to downstream genes known to be activated by GATA4 (Liang et al., 2001; Tidyman et al., 2003). Similarly, neutralisation of $\alpha_v\beta_3$ integrin attenuated Trop C expression despite GATA4 was previously shown to be unaffected by neutralisation of $\alpha_v\beta_3$ (Tan et al., 2010). It is unclear if the modulation of myofilamental gene expression demonstrated was secondary to other nuclear transcription factors. However, integrins are known to mechanotransduce signals to activate Raf-MEK-ERK-1/2 cascade that has been shown to elicit cardiomyocyte growth, increased fetal-gene expression and cytoskeletal reorganisation in neonatal cardiomyocytes (Lorenz et al., 2009). Nevertheless, it is unclear if reduced expression of integrin demonstrated on either collagen surface as compared to CLCs cultured on uncoated polystrene surface was associated with enhanced proliferation of CLCs as previously reported (Tan et al 2010). However, contrary to our previous data, SKAA was not down regulated by integrin $\alpha_v\beta_3$ neutralisation in the current study. This could be due to donor variations. Indeed, donor variation in integrin expression has been documented from different bone marrow isolates and passage numbers, resulting in different growth and proliferation potential (ter Brugge et al., 2002).

Despite beneficial effects of collagen V on cardiac gene expression and stem cell distribution, it should be noted that collagen distribution in the infarcted rat hearts may be different from humans during MI. Furthermore, a 3D structure like the heart may transmit different environmental cues to integrins as compared to 2D environments provided in tissue culture experiments. It remains to be determined whether inhibitory antibodies may transactivate other integrin receptors during epitope occupancy. In addition, the promiscuity of integrins renders it technically challenging to identify whether a single integrin or interplay of synergistic interactions between a few integrins is required for regulation of cardiac gene expression. Future studies employing siRNA techniques that selectively silence α_1 or $\alpha_v\beta_3$ integrin may provide additional information regarding the regulation of cardiac gene expression of CLCs on collagen V matrix ex vivo or in the transplanted milieu of infarcted myocardium.

5. Conclusion

In conclusion, our study indicates that α_1 and $\alpha_v\beta_3$ integrins drive cardiac gene expression of CLCs. Integrin families and ECM are important regulators of cardiac differentiation and myocardial distribution of adult MSCs and CLCs. Specific modulation of interaction between subclasses of collagen and integrin subunits in the post-infarct myocardial ECM could potentially offer a unique opportunity in cardiac regenerative medicine.

6. Acknowledgements

7. References

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