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Fate of modular cardiac tissue constructs in a syngeneic rat model

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Abstract

Modular cardiac tissues developed both vascular and cardiac structures *in vivo*, provided the host response was attenuated by omitting xenoproteins from the modules. Collagen gel modules (with Matrigel™) containing cardiomyocytes (CM) alone or CM with surface seeded endothelial cells (EC; CM/EC modules) were injected into the peri-infarct zone of the heart in syngeneic Lewis rats. After 3 weeks, donor EC developed into blood vessel-like structures that also contained erythrocytes. However, no donor CM were found within the implant sites, presumably because host cells including macrophages and T-cells infiltrated extensively into the injection sites. To lessen the host response, Matrigel™ was omitted from matrix and modules were rinsed with serum-free medium prior to implantation. Host cell infiltration was attenuated, resulting in a higher degree of vascularization with CM/EC modules, than with CM modules without EC. Most importantly, donor CM matured into striated muscle-like structures in Matrigel™-free implants.

1. Introduction

Myocardial infarction (MI) is caused by the interruption of blood supply to the heart and can lead to massive cell death. The extracellular matrix and cell debris that are left behind undergo extensive remodeling mediated by inflammatory cells and myofibroblasts. The inflammatory region eventually stabilizes into collagen-rich, acellular and non-contractile scar tissue. The scar tissue reduces the overall contractile force of the heart, and its inelastic nature increases overall cardiac load. Therefore there is a need to develop therapeutic strategies that aim to replace scar tissue with functional tissue and ultimately restore cardiac function.

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Current research focuses on two strategies to achieve this goal. Cells, either differentiated myocytes (Farahmand, *et al.*, 2008, Patila, *et al.*, 2009, Sherman, *et al.*, 2009) or undifferentiated progenitor cells (embryonic (Laflamme, *et al.*, 2005, Nussbaum, *et al.*, 2007) or mesenchymal (Amado, *et al.*, 2005, Mathiasen, *et al.*, 2009, Yoon, *et al.*, 2010) stromal cells) may be injected into or around the infarcted region in an attempt to repopulate the sites with functional cells. Alternatively scar tissue is replaced with engineered cardiac muscle consisting of myocytes (CM or skeletal myoblast (Giraud, *et al.*, 2008, Itabashi, *et al.*, 2005, Sekine, *et al.*, 2008, Zimmermann, *et al.*, 2002, Zimmermann, *et al.*, 2006)) and other supporting cell types, including endothelial cells (EC) and fibroblasts (Lesman, *et al.*, 2010, Stevens, *et al.*, 2009). Unfortunately, these strategies suffer from low cell survival caused by a combination of poor cell engraftment and a lack of functional vasculature at the implant/injection sites.

Using modular tissue engineering, EC seeded collagen modules formed functional blood vessels in a rat omental pouch (Chamberlain, *et al.*, 2011). It is proposed that a similar method with collagen embedded cardiomyocytes and surface seeded EC would result in a cardiac construct with uniformly distributed EC that may accelerate graft vascularization *in vivo*. Modular tissue engineering assembles large constructs from small individual components (modules) resulting in uniform, scaleable and vascularized constructs (McGuigan and Sefton, 2006). However, *in vivo* remodeling can alter the structure and function of the resulting construct and that was the focus of interest here. While *in vitro* characterization results have been reported elsewhere (Leung and Sefton, 2010), here, modular cardiac constructs were injected in syngeneic rats in order to assess the *in vivo* fate of the constructs. Modules embedded with CM and surface coated with EC were injected into the peri-infarct zone of an acute MI in a Lewis rat (Figure 1A). At this location, the modules were expected to survive, being adjacent to oxygenated tissue, while remaining close to the infarct zone to be exposed to damaged tissue. The focus was on comparing modules with EC to those without EC, with a view to seeing benefits in increased vasculature and corresponding changes in CM survival and morphology.

2. Materials and methods

2.1 Isolation of rat primary CM and EC

Primary CM and EC were isolated from green fluorescent protein (GFP) transgenic neonatal Lewis rats (Lewis-Tg [EGFP] F455; Rat Resources and Research Center, RRRC, University of Missouri, Columbia, MO). The aorta and atrium were trimmed away and the remaining tissues were cut into small pieces. The tissues were incubated in bovine trypsin (0.6mg/mL in Hanks buffered salt solution, HBSS, Mississauga, ON) overnight at 4°C, followed by serial digestion in collagenase type II (1 mg/mL in HBSS, Worthington Biochemical, Lakewood, NJ) at 37°C. To enrich the CM content, the cell suspension from freshly isolated rat hearts was pre-plated on TCPS for 1 hour at 37°C so that non-myocytes adhered to the plates. After pre-plating, the CM enriched cell suspension was retrieved and loaded onto a Cytospin® slide and stained for troponin I and prolyl-4-hydroxylase (see below) to identify CM and fibroblasts, respectively: the mixture consisted of 50% cardiomyocytes and 40% fibroblasts. The remaining 10% was presumed to consist of other cardiac cell types,

including EC. Harvested cells were maintained in native CM medium consisting of high glucose (25 mM) DMEM medium (Gibco, Grand Island, NY) supplemented with 10% bovine serum (BS, Sigma, St Louis, MO), 1% HEPES buffer (10 mM, Gibco, Grand Island, NY), and 1% Pen/Strep (50 unit/mL Penicillin and 50 µg/mL Streptomycin, Gibco, Grand Island, NY).

Primary rat cardiac EC were isolated from the population of attached cells left behind after pre-plating. At the end of pre-plating, the CM-rich cell suspension was removed and the attached cells were maintained with native EC-medium (MCDM-131 with 10% fetal bovine serum). After 2 days, attached cells were trypsinized and the EC were labeled with mouse anti-rat CD31 FITC conjugated antibody followed by anti-FITC magnetic beads (Miltenyi Biotec, Auburn, CA), and labeled cells were separated using a magnetic cell sorting column. Isolated EC were maintained with native EC medium in fibronectin coated flasks. Over 90% of the cells were EC, as indicated by their uptake of Dil-Ac-LDL (10µg/mL, 4 hours at 37°C, Molecular Probe, Eugene, OR). In some *in vitro* experiments primary rat aortic endothelial cells (RAEC) were used as described elsewhere ⁽¹⁸⁾.

2.2 Modules fabrication and culture

CM-rich cell mixture was embedded in a type I bovine collagen matrix supplemented with 25% Matrigel™ (v/v, BD Bioscience, Bedford, MA), such that the concentration of collagen and Matrigel™ was 3 mg/mL, cast into modules, and seeded with cardiac EC using methods as described previously (McGuigan, *et al.*, 2006). Briefly, type I bovine collagen supplemented with Matrigel™ was mixed with the CM-rich cell isolate from transgenic-GFP, neonatal Lewis rat hearts, at a concentration of 2×10^7 cell/mL. Matrigel™-free modules were made with type I bovine collagen only (3 mg/mL). Modules were cast in polyethylene tubes with inside diameters of 540 µm and cut to 1mm in length after gelation. Based on the volume and cell concentration, we calculated that each module should contain approximately 4500 cells. The mean post-contraction diameter was approximately 220 µm. For EC coating, each milliliter of settled modules was mixed with 2×10^6 primary rat cardiac EC and incubated for 1 hour.

2.3 Peri-infarct module injection model

Two series of modules were studied in this model. In one series, small diameter collagen/Matrigel™ modules were tested. In the other series, the Matrigel™ was omitted to reduce the xenogeneic protein burden. In both series, GFP⁺CM-only modules were maintained in native CM-medium until implantation at day 7. Co-culture modules were made by seeding cardiac GFP⁺EC (passage 3-5) onto CM embedded modules at day 3 post-fabrication and kept in co-culture medium from day 3 to 7 in culture before injection. The collagen only modules were rinsed and incubated for 1 hour in defined serum-free medium as described by Kessler-Icekson *et al.* (Kessler-Icekson, *et al.*, 1984) prior to injection. This medium consisted of 1:1 mixture of Ham's F12 and DMEM supplemented with 10 mM HEPES at pH 7.3, additional 1mg/mL glucose, 0.1 µM hydrocortisone, 25 µg/mL insulin, 25 µg/mL transferrin, 1 mg/mL fetuin from bovine calf serum (Sigma, Oakville, ON).

The myocardial infarction rat model was created by ligating the left anterior descending coronary artery (LAD) of wild type Lewis rats (12-20 weeks old) for 7 days prior to the module injection. Small diameter modules (with or without EC) were injected into the peri-infarct zone using a 28G needle (3 sites per animal, equal distance apart). Approximately 50 modules were injected per site for a total of 150 modules per animal (Figure 1B-C). Animals were sacrificed at 21 days post injection and the hearts were fixed in 4% paraformaldehyde for histological analysis. Three animals were used for each experimental condition.

2.4 Immunostaining

Modules cultured *in vitro* for 7 days were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton-X100 solution. Samples were then triple stained for troponin I, F-actin and cell nucleus. Embedded CM were identified using rabbit anti-rat troponin I (Chemicon, Temecula, CA) antibody followed by Alexa Fluor™ 488 goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR). Alexa Fluor™ 568 phalloidin (Molecular Probes, Eugene OR) and Hoechst (Sigma, Oakville, ON) were used to stain for actin cytoskeleton and cell nucleus, respectively. Samples were visualized using a Zeiss LSM510 confocal microscope. Morphologies of surface seeded EC were visualized using goat anti-rat VE-cadherin primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by rabbit anti-goat IgG AlexaFluor™ 488 secondary antibody (Molecular Probes, Eugene, OR).

Explanted tissues were fixed in 4% paraformaldehyde for 48 hours at 4°C, followed by incubation in 30% sucrose solution for 24 hours. Tissue samples were rinsed in 70% ethanol before being embedded in paraffin. Tissue sections were stained with H&E and Masson's trichrome for tissue morphology assessment. Macrophages and T-cells were stained with monoclonal mouse anti-rat CD68 (Serotec, Raleigh, NC) and mouse anti-rat T cell receptor (BD Pharmingen, San Jose, CA) antibodies respectively. Implanted cells were tracked by staining tissue sections with polyclonal rabbit anti-GFP antibody (Abcam, Cambridge, MA), or double staining with GFP and CD31 (or myosin heavy chain, MHC). For MHC staining, tissue sections were sequentially stained with polyclonal rabbit anti-GFP antibody (Abcam, Cambridge, MA) visualized with 3,3'-diaminobenzidine (DAB), followed by monoclonal mouse anti-rat MHC antibody (α - and β - isoforms, Abcam, Cambridge, MA) visualized with Vector Red. Donor derived blood vessels were detected by sequentially staining tissue sections with polyclonal goat anti-rat CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) visualized with DAB, followed by polyclonal rabbit anti-GFP antibody (Abcam, Cambridge, MA) visualized with Vector Red.

The number of blood vessel-like structures for each sample was determined by averaging the number of structures in three hot spots in a single section (de Jong, *et al.*, 2000). As is used in the tumour literature, the hot spot method enabled the systematic quantification of blood vessel density, even though the vessel distribution was heterogeneous. Hot spots were identified by scanning each section under low magnification for blood-vessel rich areas. The number of CD31 positive blood vessel like structures in each hot spot was counted under higher magnification. Vessel densities were calculated by dividing the averaged vessel count of each sample by the area of view (0.459 mm²).

2.5 Statistical analysis

Statistical analysis including calculations of standard error of means and mean blood vessel density comparisons (t-test or one-way ANOVA) were performed using Prism (version 5.0, Graphpad Software, La Jolla, CA).

3. Results

3.1 *In vitro* morphological characterization of modules

Unlike the previously used and described (Leung and Sefton, 2010), “large” 500µm modules, here smaller diameter modules (average diameter approx. 220 µm) were prepared using collagen/Matrigel™ and collagen only. There were no notable differences in structure between the previously described modules and these smaller modules nor was there any notable difference, morphologically between the two matrices (Figure 2A-D). After 7 days in culture in CM medium, cells on the perimeter appeared elongated in collagen-only modules as well as in collagen/Matrigel™ module. Moreover, troponin I positive cells near the module core in both cases appeared rounded compared to cells on the perimeter.

We also investigated the effect of passage through a fine needle on adherent junctions between ECs. To do this we seeded RAEC over small diameter, CM embedded, collagen/Matrigel™ modules. At 7 days post seeding, modules were loaded into a syringe and then passed through a 28G disposable needle as used for *in vivo* injection. Immediately after injection the modules were fixed and stained for VE-cadherin (Figure 2E-F). Confocal microscopy showed no significant loss of EC in modules which passed through the needle when compared with modules that did not pass through the needle. However, the VE-cadherin staining indicated that the EC junctions were disturbed by the process of being forced through a needle.

3.2 Peri-implant injection

After 3 weeks, injected modules appeared as white dots near the infarct site, which took on a pale color compared to healthy myocardium due to the lack of blood flow (Figure 1C). Injected collagen/Matrigel™ modules were extensively remodeled by host cells, yet in some cases they could be identified by histological staining (Figure 3). Moreover, there was a significant amount of collagen deposited at the injection sites by host tissue. Histological sections also indicated thinning of the heart wall secondary to the infarct in the injection model (Figure 3A and B).

GFP⁺ cells were found throughout the injection sites (Figure 3E and F), but the use of GFP⁺EC did not lead to a substantial change in GFP⁺ cell density in collagen/Matrigel™ modules, implying little to no effect of donor EC on overall CM survival. Cells that were GFP⁺/CD31⁺ were found in blood vessel-like structures within the injection sites (Figure 4A and B). In some cases, blood vessel-like structures were lined entirely with GFP⁺/CD31⁺ (i.e., donor EC, Figure 4D) or partially with GFP⁺/CD31⁺ cells (i.e host EC, Figure 4F), together with donor EC. Furthermore, red blood cells were found inside the lumen of these vessels, which suggest that these vessels may have connected with the host vasculature. Similar structures were also found in CM-only injections. However, these vessels were lined

with only GFP⁻/CD31⁺, suggesting that the EC were host-derived in this group (Figure 4C and E).

To determine if donor CM survived and matured into muscle-like structures, we stained for both isoforms of myosin heavy chain in these collagen/MatrigelTM modules. MHC⁺ structures, which were also GFP⁺ (i.e., donor cells), were found in the CM-only injection group (Figure 5A and C). In the co-culture injection group (Figure 5B and D), MHC⁺ structures did not co-express GFP.

3.3 Host response and the removal of MatrigelTM

The presence of MatrigelTM in the modules elicited a strong macrophage response (Figure 6A and B) as well as T-cell infiltration (Figure 6E and F). These responses were attenuated by removing MatrigelTM from the matrix and rinsing the modules in serum-free medium prior to implantation. After 3 weeks, there was substantially less macrophage and T-cell infiltration into the injection sites in the absence of MatrigelTM (Figure 6C, D, G and H).

The removal of MatrigelTM also resulted in an abundance of muscle-like structures throughout the implants (Figure 7). Unlike implants with MatrigelTM, bundles containing GFP⁺/MHC⁺ cells were found in both CM-only and CM/EC injection groups in MatrigelTM-free implants. Blood vessel like structures lined with GFP⁺/CD31⁺ cells were found throughout CM/EC co-culture peri-infarct module injections (Figure 8D). In contrast, similar structures in CM-only implants were lined with CD31⁺ cells that were host derived (i.e., GFP negative) (Figure 8B).

3.4 Vessel densities

When quantified, implants containing CM-only modules and CM/EC modules (containing MatrigelTM) had no difference in total vessel density (Figure 9A). Nonetheless, we found an increased number of CD31⁺ vessels lined with GFP⁺ (i.e., donor) cells in the CM/EC implants compared to CM-only (Figure 9B). On the other hand, removing MatrigelTM resulted in a significantly higher vessel density count for CM/EC injections compared to the same with MatrigelTM and even to CM-only MatrigelTM-free implants (Figure 9A). Interestingly, when compared with collagen/MatrigelTM modules, the removal of MatrigelTM did not alter the percentage of vessels that contained no donor EC versus vessels that were partially or solely comprised of GFP⁺ EC (Figure 9B) in implants containing donor EC.

4. Discussion

Previously we showed that modules consisting of MatrigelTM supplemented collagen were able to maintain the viability and function of embedded CM and surface coated EC and that the modules could be used to form macroporous sheets *in vitro* (Leung and Sefton, 2010). Here the goal was to determine the fates of EC coated CM modules *in vivo*. Modules were deployed as individual “microtissues” near damaged cardiac tissue.

Modules were injected into the peri-infarct region of an acute infarct in the left ventricle, created by ligating the left anterior descending coronary artery (LAD). This location was chosen due to its proximity to the infarct region so that implanted cells could contribute to

tissue regeneration. In contrast to the ischemic infarct region, the peri-infarct zone may provide a more hospitable environment for implanted cells by providing better perfusion than the infarct zone.

Histological analysis after 3 weeks showed the expected heart wall thinning and decellularization within the infarct zone. Injected modules were found in the peri-infarct zone with most of the module matrix (collagen with or without Matrigel™) still intact. This is in contrast to our observations in modules implanted as a patch sandwiched between collagen foams, where the modules were mostly disintegrated and became indistinguishable from the collagen foam (unpublished data). However, a larger than expected number of macrophages and T-cells infiltrated into the injection site (with collagen/Matrigel™ modules), suggesting that the adaptive host immune response had been triggered against implanted cells or matrix. The immune contribution of Matrigel™ is often reduced by using athymic hosts and masked by the use of immunosuppressant in allogeneic models (Yildirim, *et al.*, 2007, Zhang, *et al.*, 2006, Zimmermann, *et al.*, 2006). In syngeneic models, the presence of Matrigel™ have lead to massive immune response and poor cells survival in the absence of immunosuppressant (Giraud, *et al.*, 2008). Zimmermann *et al.* (Zimmermann, *et al.*, 2004) has noted a similar degree of immune reaction toward implanted cardiac construct in their syngeneic model, and administered immunosuppressant in order to avoid construct destruction.

In this study we chose to inject CM and CM/EC modules in a manner similar to that of cell therapies based on intramyocardial cell injection. The potential advantages of injecting endothelial cell covered modules rather than a cell suspension were expected to be better cell retention, cell survival and integration into a vascularized tissue construct. The modules were physically lodged within the injection site and no modules escaped through the needle puncture hole after injection.

4.1 Cardiomyocyte survival and maturation

After 3 weeks, implanted cell derived (GFP⁺) muscle bundles were found throughout collagen-only modules. In contrast, these same structures were mostly present on the perimeter of collagen+Matrigel™ modules, and few were found near the core of the modules. Only in one *in vivo* collagen+Matrigel™ sample did we find GFP⁺ muscle bundles penetrating through the core. Interestingly, GFP⁺ cells were evenly distributed in both types of modules, suggesting that CM near the core of collagen+Matrigel™ modules may be viable but simply did not mature to the same degree as the ones in collagen-only modules. The improvement in CM maturation in collagen-only modules may be related to the higher vessel density compared to collagen-Matrigel™ modules. It is also likely that in both types of modules, CM near the perimeter of modules experienced different chemical and mechanical signals compared to the ones near the core, and these differences resulted in different degrees of maturation and organization (Jacot, *et al.*, 2008).

Unlike adult cardiac tissue, neonatal myocardium in rodent possesses significant regenerative capacity. Porrello *et al* has shown that 1-day old neonatal mice can regenerate after partial resection (Porrello, *et al.*, 2011), suggesting that rat neonatal CM may have similar proliferative capacity. More importantly, genetic fate mapping showed that this

newly regenerated cardiac tissue arose from preexisting CM and not from cardiac progenitor cells. Therefore, even though the modular constructs may contain some cardiac progenitor cells, we do not expect them to contribute significantly to the growth and remodeling of muscle bundles in the module. A recent study by Sato et al. showed that rat neonatal CM injected, with medium, into the adult rat heart ventricle matured, after 2 weeks, into a mixture of elongated CM with good sarcomeric structures and hypercontracted round CM. At later times (4-6 weeks) the proportion of elongated cells increased and the contractility of the cells also improved (Sato, *et al.*, 2010). CM explanted from our model match the description of 2-4 week explants from Sato et al., suggesting CM in modules may not yet be fully developed by 3 weeks.

Another factor that contributes to the lower number of mature CM in the middle of the module may be poor perfusion and low oxygen tension. Although the small size of the module is intended to support cell viability through passive diffusion without perfusion (Corstorphine and Sefton, 2010), it is conceivable that for specific cell types, such as CM, the diameter used in this study may be too large to sustain their functional needs. Therefore, further optimization of module geometry, size, as well as matrix composition may be useful to facilitate CM survival and maturation.

4.2 Vascularization and host response

The ultimate goal of cardiac tissue engineering is to restore lost function to the heart. In our context, this goal depends on the survival and function of implanted cells as well as their interactions with the host tissue. At the most basic level, the new tissue must contain contractile CM and the necessary vasculature to support them.

Previous studies have demonstrated that allogeneic EC coated on collagen modules were capable of forming at least partially functional blood vessels in an immunosuppressed animal within 3 weeks (Chamberlain, *et al.*, 2011, Kelm, *et al.*, 2006, Lesman, *et al.*, 2010, Stevens, *et al.*, 2009). Here similar vessel-like structures were seen near and among injected modules (with or without Matrigel™). It is likely that these vessels anastomosed with the host vasculature since erythrocytes were found within their lumens, although this by itself is not indicative of function. In our model, we found vessels lined with GFP⁺/CD31⁺ cell when EC were added to the CM embedded modules, which suggests that these vessels were derived from the donor GFP⁺ EC. Vessels consisting of host derived EC alone or a mixture of host and donor EC were also found within the vicinity of implants. These observations suggest that the donor EC not only formed vascular structures, but may also be involved in inducing host EC migration and perhaps even playing a role in stabilizing new blood vessel formation at the implant/injection site (Sekine, *et al.*, 2008). The critical factors responsible for new blood vessel formation remain to be determined in future studies.

Seeding EC onto the cardiomyocyte containing modules increased vessel density, only in the absence of Matrigel™. It is presumed that the host response against foreign proteins masked the difference in vessel density between CM-only and CM/EC injections, with Matrigel™ based modules. Removal of Matrigel™ and the pre-implantation wash of modules in serum-free medium substantially reduced the amount of macrophage and T-cell infiltration. The removal of Matrigel™ may also alter the viability of cells and subsequent secondary cellular

responses, such as the release of IL-1 α during necrosis (Cohen, *et al.*, 2010), which may aggravate immune responses. Regardless of the mechanism, the reduction of immune response helped improve CM survival. Although these Interestingly, in the presence of Matrigel™, implants with or without EC coating had similar blood vessel density. This could be due to the presence of diffusible factors in implants containing Matrigel™ which may have mobilized host EC to initiate angiogenesis. Although we saw an increase in vessel density in Matrigel™-free CM/EC implants compared to CM/EC implants with Matrigel™, the percentage of chimeric vessels, which consisted of donor and host EC, remained the same despite the reduction in macrophage and T-cell infiltration.

Several studies (Kelm, *et al.*, 2006, Lesman, *et al.*, 2010, Stevens, *et al.*, 2009) have highlighted the fact that a functional vasculature is needed to support the metabolic needs of CM, which is essential for their development and organization into differentiated muscles. Using MHC as the primary marker of tissue development, few implanted CM survived in the presence of Matrigel™, presumably due to the above noted inflammatory response, attributed to xenogeneic protein. Others have shown that the presence of xenogeneic protein in Matrigel™ and bovine serum used during culture provokes a strong host immune response (Giraud, *et al.*, 2008, Yildirim, *et al.*, 2007), which can significantly reduce CM survival (Zimmermann, *et al.*, 2006). Without Matrigel™, there was a corresponding increase in CM survival and the presence of GFP⁺/MHC⁺ striated muscle structure in both CM-only and CM/EC peri-infarct injections. Surprisingly, the increase in vessel density due to the presence of above noted donor EC did not translate to a significant increase in CM survival after 3 weeks, relative to the case without donor EC. Perhaps the benefit of EC will be more pronounced at later time points, when functional blood vessels may become fully mature.

The presence of primitive vessels at the implant site may pave the way for tissue regeneration, but that may not be sufficient to support long-term cardiac tissue growth and functional remodeling. Therefore, strategies that promote vessel maturation may be needed to improve CM survival. One way to achieve this is by reducing the host response towards the implant, as we have demonstrated here. Another way may be to alter the tissue remodeling landscape by adding additional cells such as mesenchymal stromal cells (MSC). Recent studies from our group have showed that embedded bone marrow derived MSC in EC coated modules accelerated vessel maturation by appearing to differentiate into pericyte-like supporting cells and migrating to surround newly formed blood vessels (Chamberlain, *et al.*, 2011). This is consistent with the emerging realization that MSC may have been pericytes prior to isolation (Corselli, *et al.*, 2010, Crisan, *et al.*, 2008). More importantly, embedded MSC appeared to foster a pro-tissue repair microenvironment, perhaps by altering macrophage polarization. It is reasonable to speculate that co-injection of bmMSC embedded modules with CM embedded modules may improve EC survival, vessel maturation and improve overall cardiac tissue regeneration.

5. Conclusion

In this study we have demonstrated that module based cardiac tissues survived up to 3 weeks when implanted in a syngeneic animal model. When injected into the peri-infarct region,

donor EC formed vessel-like structures that appeared to have anastomosed with host vasculature. These vessels were lined with a mixture of donor and host derived EC, while in CM-only implants vessels only contained host derived EC. The presence of Matrigel™ and bovine serum triggered significant host responses, which may be responsible for the loss of implanted CM. The removal of foreign protein substantially reduced macrophage and T-cell infiltration and significantly increased overall vessel density with CM/EC modules. It also led to an increase in striated muscle bundles containing donor CM. These results serve as the basis for future optimization studies toward the realization of a modular cardiac construct as a method to replace damaged cardiac tissue.

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Figure 1.

(A) Schematic diagram of peri-infarct module injection site. Individual small diameter modules were injected into the peri-infarct zone. (B) Schematic diagram of left anterior descending coronary artery (LAD) ligation and module injection site. The infarct was induced by LAD ligation 7 days prior to module injection. Modules were injected into the peri-infarct zone (3 sites, 50 modules/site) in a wild type Lewis rat using a 28G needle. (C) Modules (white arrows) were visible around the infarct site (white dotted circle) when injected after 3 weeks.

Figure 2.

Immunofluorescence staining of CM embedded modules after 7 days in static culture containing 10% BS (green - troponin I, red - F-actin, blue - cell nucleus). Cells on the perimeter appeared elongated in both collagen-only (A and C) and collagen/Matrigel™ (B and D) modules. In both groups cell density decreased toward the core of modules (scale bar = 100 μm). (E, F) VE-cadherin staining (green - VE-cadherin, blue - cell nucleus) of CM/EC modules before (E) and after (F) passage through a 28G needle. Matrigel™ supplemented CM modules were coated with RAEC and static cultured for 7 days. There was no significant cell loss associated with injection, however EC junctions (white arrows) appeared more disorganized (scale bar = 100 μm).

Figure 3.

Masson's trichrome staining of peri-infarct injected collagen/Matrigel™ modules at 3 weeks (A-D). Injected CM-only and co-culture modules remained visible in the peri-infarct zone after 3 weeks (A and B, scale bar = 1mm; C and D, white dotted lines highlight the perimeters of modules, scale bar = 100 μ m). Black boxes in A and B correspond to the area of view in C and D, respectively. Immunohistochemical staining revealed the presence of GFP⁺ donor cells scattered throughout the implant sites (E and F). The expression of GFP suggested that donor cells remained viable. However, the presence of EC in CM/EC implants did not translate into a substantially higher GFP⁺ cell density (scale bar = 500 μ m).

Figure 4.

Blood vessel-like structures were found in collagen/Matrigel™ implants containing CM-only (A) and CM/EC (B) modules, (black arrows, scale bar = 100 μ m). The presence of red blood cells suggested that vessels connected to the host vasculature. Blood vessels were evenly distributed throughout the implants. Higher magnification images of blood vessels showing the presence of GFP+/CD31+ donor derived EC (pink and brown) or GFP-/CD31+ host derived EC. The former were seen, wholly (D) or partially (F), with co-culture implants, while in CM-only implants vessels did not stain for GFP (C and E). Panels E and F show different fields than panels C and D; both sets at the same magnification, scale bar = 20 μ m.

Figure 5.

Double staining for GFP (brown) and MHC (pink) showed donor derived CM developed into striated muscle-like structures in CM-only injections (A and C). In contrast, muscle bundles in CM/EC injections consisted of GFP⁻/MHC⁺ cells only (B and D), suggesting these structures were derived from host cells. Scale bar for A and B = 100 μm, C and D = 20 μm. Collagen/MatrigelTM modules.

Figure 6.

Immunohistochemical staining of CD68 and T cell receptor showed a large degree of macrophage (A and B) and T cell (E and F) infiltration in implantation with Matrigel™. Matrigel™-free CM-only and co-culture peri-infarct module injections lead to a significant reduction in macrophage (C and D) and T cell (G and H) infiltration after 3 weeks, as shown by the reduction of CD68⁺ and TCR⁺ cells. Scale bar = 100 μ m.

Figure 7.

Donor CM in MatrigelTM-free CM-only (A and B) and CM/EC (C and D) peri-infarct injections formed striated muscle-like structures after 3 weeks. Under high magnification striated muscle bundles were found. These structures contained both host derived CM (GFP⁻/MHC⁺, pink) as well as donor derived CM (GFP⁺/MHC⁺, pink and brown). A and B, scale bar = 100 μ m; C and D, scale bar = 20 μ m.

Figure 8.

Blood vessel-like structures were found in Matrigel™-free CM-only (A and C) and CM/EC (B and D) peri-infarct injections after 3 weeks. Under higher magnification, blood vessels partially comprised of GFP⁺/CD31⁺ donor derived EC (pink and brown) were found in co-culture injection implants (D). In CM-only injections (C), vessels were lined with GFP⁻/CD31⁺ cells (brown), suggesting the vessels arose from host derived EC. The presence of red blood cells in the lumens suggested the vessels connected to host vasculature (A and B, scale bar = 100 μm; C and D, scale bar = 20 μm).

Figure 9.

(A) Vessel density counts showed no significant difference in vessel densities between CM-only constructs and their CM/EC counterparts with MatrigelTM. In MatrigelTM-free injection implants, however, the presence of EC significantly increased vessel density compared to CM-only implants and co-culture injection implants with MatrigelTM (*, $p < 0.05$, $n = 3$). (B) In all cases, donor EC cells participated in vessel formation, as shown by the increased proportion of blood vessels that were either partially or completely lined with GFP⁺EC.