Published in final edited form as:

Biomaterials. 2013 December ; 34(36): . doi:10.1016/j.biomaterials.2013.08.017.

The Effect of Bioengineered Acellular Collagen Patch on Cardiac Remodeling and Ventricular Function post Myocardial Infarction

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Abstract

Regeneration of the damaged myocardium is one of the most challenging fronts in the field of tissue engineering due to the limited capacity of adult heart tissue to heal and to the mechanical and structural constraints of the cardiac tissue. In this study we demonstrate that an engineered acellular scaffold comprising type I collagen, endowed with specific biomechanical properties, improves cardiac function when used as a cardiac patch following myocardial infarction. Patches were grafted onto the infarcted myocardium in adult murine hearts immediately after ligation of left anterior descending artery and the physiological outcomes were monitored by echocardiography, and by hemodynamic and histological analyses four weeks post infarction. In comparison to infarcted hearts with no treatment, hearts bearing patches preserved contractility and significantly protected the cardiac tissue from injury at the anatomical and functional levels. This improvement was accompanied by attenuated left ventricular remodeling, diminished fibrosis, and formation of a network of interconnected blood vessels within the infarct. Histological and immunostaining confirmed integration of the patch with native cardiac cells including fibroblasts, smooth muscle cells, epicardial cells, and immature cardiomyocytes. In summary, an acellular biomaterial with specific biomechanical properties promotes the endogenous capacity of the infarcted myocardium to attenuate remodeling and improve heart function following myocardial infarction.

Keywords

cardiac tissue engineering; scaffold; collagen; heart; cardiomyocyte; angiogenesis

1. Introduction

Due to the impaired self-renewal capacity of adult cardiomyocytes and the limited number of cardiac progenitors, the human heart exhibits insufficient ability to restore heart function

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and structure following injury [1–4]. In recent years, clinical trials to treat severe heart injuries, primarily focused on cell transplantation, have shown modest success due to a number of limitations; these include low cardiac engraftment efficiency, immune system reaction, poor penetration, deficient viability and retaining of the cells at the site of injury, exorbitant costs, and the lack of control of the fate of the grafted cells [5–9]. Therefore, there is a critical need for the development of alternative cardiac regenerative approaches.

The field of cardiovascular tissue engineering is recently receiving growing attention as a new therapeutic modality to treat heart injuries [1,10,11]. This multidisciplinary field applies the principles of engineering, cell biology, and clinical therapies, towards generating tissue replacement (i.e. scaffold or cardiac patch) that maintains, restores, or improves the function of damaged or diseased cardiac tissue [11,12]. Engineered tissue scaffolds are aimed to provide: I) a mechanical support for tissue replacement/regeneration; II) an environment that favors cell migration and integration, proliferation, and differentiation for the desired therapeutic effect; and III) a permeable construct that allows efficient nutrient and waste transfer. The chemical composition, biomechanical properties (e.g., stiffness), and 3D architecture of the scaffolds have been shown to play key roles in determining cell-microenvironment interactions and the fate of stem cells [13–15].

Whereas the outcomes of the studies utilizing various systems of cardiac patches have been modest but promising [16–19], the clinical application of these techniques has been limited. The main obstacles include poor control of the patch's structure and properties, insufficient vascularization, infection, immunogenicity, and lack of appropriate biomechanical cues to promote cardiogenesis [11,20–22]. Moreover, incorporating exogenous cells into the patch can cause additional complications including diffusional limitations of oxygen/nutrients transport, the necessity of an extensive, functional vascular network, poor integration and differentiation of the cells, and immune system responses [23–25].

Considering the challenges and restrictions on the *in vivo* application of cellular patches, *acellular* biomaterial matrices have been recently evaluated as mechanical-structural supports for lesions, as well as for their potential to provide a favorable physiological milieu for new tissue formation [26–30]. Among various biomaterials, *in vitro* reconstituted three-dimensional (3D) collagen type I scaffolds are increasingly being used as protein substrates in diverse biomedical applications, particularly, in tissue repair, via providing a biomimetic environment for cell growth [15,31–33]. Their main attributes include favorable fibrous structure, mechanical properties, biocompatibility, and biodegradability [20,31,32].

The goal of this study is to engineer a myocardial-friendly environment through the use of biologically compatible, type I collagen gel with optimized biomechanical properties in order to organize and condition the cardiac cells already present in the damaged heart tissue. Plastic compression technique was used to rapidly generate dense tissue scaffolds with optimal elastic modulus to support myocyte contractility [34–37]. We assessed the epicardial delivery of the engineered collagen scaffold to treat acute transmural myocardial infarction (MI) in the mouse.

2. Materials and methods

2.1. Collagen gel preparation – plastic compression

Highly hydrated collagen gels were produced by adding 0.5 and 0.6 ml of 10X and 1X DMEM (Sigma, MO, US), respectively, to 0.9 ml of sterile rat tail-derived type I collagen solution in acetic acid (3.84 mg/ml, Millipore, MA, US) and neutralizing with 5 M NaOH. The collagen solution (0.9 ml) was then cast into 24-well plates (15.6 mm in diameter) and placed in a tissue culture incubator for 30 minutes at 37°C for polymerization. Compressed

gels – used as cardiac patch in this study – were produced as previously described [34–36]. Briefly, as cast highly-hydrated collagen gels were transferred to a porous support comprising (bottom to top) absorbent paper blot layers, a steel mesh, two polymer meshes, and two fabric meshes (Figure 1A). Subsequently, a static compressive stress of either 340, 1000, or 1400 N/m² was applied to the hydrated scaffolds for 2–5 minutes in order to remove water and produce a dense biomaterial with improved biological and mechanical properties.

2.2. Atomic force microscopy – mechanical characterization of the patch

Elastic moduli of the compressed collagen gels were assessed by atomic force microscopy (AFM) in nanoindentation mode, using a force trigger that resulted in a minimal local strain of less than 10% (indentation of ~100 nm) in order to minimize the effect of substrate-related artifacts. For this purpose, a custom-designed flat AFM tip was fabricated using focused ion beam milling and utilized to probe the gel stiffness by scanning areas of 90 μm $\times$ 90 μm (Figure 2). A minimum number of ($n>$) 3 gels were tested per each condition. For each gel specimen, a minimum of 3 areas were probed. The surface topography and phase contrast images of the compressed collagens were also assessed by AFM, in semi-contact mode with rounded cantilevers ($R_{\text{tip}} = 40$ nm) for prolonged scanning and minimal surface damage.

2.3. Mouse model of myocardial infarction – epicardial application of the patch

All procedures involving animal use, housing, and surgeries were approved by the Stanford Institutional Animal Care and Use Committee (IACUC). Animal care and interventions were provided in accordance with the Laboratory Animal Welfare Act. Male 10–13 weeks old C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and studied in four different groups: I) control mice underwent sham ligation with no infarct/patch, II) MI-only group which had infarct with no treatment (Figure 1B–D), III) healthy myocardium treated with the patch, and IV) infarcted myocardium grafted with patch ($n>5$, Figure 1B, E–F). For this purpose, mice were anesthetized utilizing an isoflurane inhalational chamber. This was followed by endotracheal intubation using a 22-gauge angiocatheter (Becton, Dickinson Inc., Sandy, Utah) that was connected to a small animal volume-control ventilator (Harvard Apparatus, Holliston, MA). A left thoracotomy was performed via the fourth intercostal space and the lungs retracted to expose the heart. Once the pericardium was opened, a 7-0 suture was placed to ligate the left anterior descending artery (LAD) ~2–3 mm below the edge of the left atrium (group IV, Figure 1B). Criterion for occlusion success was an immediate color change in the left ventricular (LV) wall (red to pale). For experimental groups treated with patch (groups III and IV), prepared collagen patch was sutured (at two points, Figure 1B) onto the surface of the ischemic myocardium. Animals were kept on a heating pad until they recovered.

2.4. Echocardiography

In vivo heart function of sham control, MI-only, and MI treated with patch groups was evaluated by performing echocardiography two and four weeks after ischemic injury. Two-dimensional (2D) analysis was performed using a GE Vivid 7 ultrasound platform (GE Health Care, Milwaukee, WI) equipped with a 13 MHz transducer. The mice were anesthetized with isoflurane (100 mg/kg, inhalation), shaved, and placed on a heated platform in the left lateral decubitus position to facilitate echocardiography. 2D clips and M-mode images were obtained using a short axis view from the mid-LV at the tips of the papillary muscles. Fractional shortening (FS, %) and ejection fraction (EF, %, via extrapolation of 2D data) were calculated from LV dimensions in the 2D short axis view. LV internal diameter (LVID) and posterior wall thickness (LVPW) were measured at end diastole and systole. A minimum number (n) of 5 mice per study group was used for the

echo evaluations. Measurements were performed by two independent observers blinded to study group.

2.5. Histology and immunohistochemistry analysis

Histological analysis was conducted following standard protocols for paraffin embedding. Mounted heart samples were stained for Hematoxylin and eosin (HE, to visualize nuclei in blue, and cytoplasm and connective tissue in pink), Masson's trichrome (TC, to label muscle fibers in red, collagen in blue, cytoplasm in pink, and nuclei in dark brown), and von Willebrand factor (vWF to visualize endothelial cells in the vessel walls in dark brown). For immunohistochemistry, embedded samples were sectioned at a thickness of 7 μm . Antibodies used are listed in Table 1. A minimum of 5 sections per stain were used for histology and 4 for immunohistochemistry studies. The inclusion criterion for the patch engraftment was that the patch covered > 70% of the infarct (controlled by histology).

2.6. Statistical analysis

The number of samples (n) used in each experiment is noted in the text. Dependent variables are expressed as means $\pm$ SEM. The differences in the means were tested using ANOVA and Student T-test to check for statistical significance ($P < 0.05$).

3. Results

3.1. Plastic compression of collagen gels

In order to assess stiffness of collagen gel scaffolds in this study, highly hydrated (uncompressed) and plastic compressed collagen gels (Figure 1A) were probed by AFM in a nanoindentation mode ($n > 3$). While uncompressed gels exhibited relatively low moduli ($E \sim 0\text{--}300$ Pa, blue curve in Figure 2D), application of partial plastic compression levels of 340 and 1,000 N/m^2 produced dense matrices with greater elasticity ranging from 0 to 2,000 and 0 to 4,000 Pa, respectively (red and violet curves in Figure 2D).

In order to generate a single-layer patch structure, an elevated level of compressive stress (~ 1400 N/m^2 for 5 minutes), greater than those required to generate two-layer structure (partial compression, described above) was applied. Hydrated scaffolds that underwent this 'full' compression exhibited a uniform ultrastructure [36], together with a further increase in the elastic modulus (~ 3000 to 10,000 Pa, green curve in Figure 2D). These 'fully-compressed' collagen matrices were used as cardiac patches in the *in vivo* assays.

3.2. Application of the patch *in vivo*

Integration of the collagen patch with myocardial tissue was first assessed by applying the engineered scaffold onto the surface of the healthy myocardium. Histology and immunohistochemistry of heart sections obtained four weeks post grafting demonstrated secure attachment of the scaffold to the host tissue, together with massive cellularization of the patch by the migratory native cells (Figure 3A–E). Moreover, a significant number of new blood vessels were formed in the patch and adjacent to the border zone between native myocardium and patch (Figure 3F–G).

Subsequently, the engineered patch was grafted onto infarcted LV myocardium immediately post myocardial infarction (Figure 1B, E–F). As shown by non-invasive echocardiography at week 4 after injury, hearts treated with patch exhibited significantly improved ejection fraction (EF) and fractional shortening (FS) (Figure 4A). Partial preservation of contractility was associated with inhibition of cardiac remodeling, as detected by a reduction in LV internal diameter at end-diastole and systole (LVIDd and LVIDs, respectively), and

increase in LV posterior wall dimension at end- diastole and systole (LVPWd and LVPWs, respectively) (Figure 4A).

Four weeks post injury the animals were sacrificed and hearts were analyzed. As demonstrated in Figure 4B, a functional network of newly formed blood vessels was observed in the patch in the hearts grafted with engineered patch. Histology (HE, TC, and vWF staining) of heart sections from MI-only hearts showed extensive dilation of the LV chamber, wall thinning, disrupted vasculature, and widespread fibrosis in MI-only hearts, when compared with sham controls (Figures 4C–J). In contrast, patch-treated hearts exhibited limited fibrosis, diminished dilation of the LV chamber, and several evidences of neo-angiogenesis within the patch and at the border zone (yellow arrows, Figures 4K–Q).

In order to further characterize the migratory cells residing within the patch and at the border zone, immunostaining for multiple cardiac markers (Table 1) was conducted. Interestingly, infarcted heart treated with patch contained a significant number of functional vessels stained for the smooth muscle marker (α -smooth muscle actin, SMA) within the patch, as well as in the native myocardium near the border zone (Figure 5A–C), suggesting enhanced angiogenesis. To investigate the presence of cardiomyocytes in the MI+Patch animals, histological sections through the patch and border zone were analyzed for cardiac specific α -actinin marker. A small population of α -actinin⁺ myocytes was found within the patch, mostly in the border with the host tissue. These cells however exhibited disturbed, unorganized sarcomeric pattern (Figure 5D–F). Staining for DDR2 and PDGFR also revealed that significant fractions of the residing cells within the patch were cardiac fibroblasts and epicardial derived progenitor cells, respectively (Figure 5G–L). Staining for cells bearing a T lymphocyte marker (CD3) or macrophage marker (F4/80) demonstrated no significant immune response/inflammation as a result of collagen patch implantation in the mice (Figure 5M–R).

4. Discussion

The adult mammalian heart exhibits a limited ability to repair itself following injuries [1,4]. Tissue-engineered cardiac substitutes, constituted of a 3D scaffold with or without cells, can be utilized as a therapeutic device to protect or activate endogenous and/or exogenous regenerative capacity of the heart [1,11,30]. In this study we explored whether an engineered acellular collagenous patch can be used to attenuate LV remodeling and stimulate cardiac healing after injury.

It is well known that cardiac cells require certain conditions for their survival in the heart's microenvironment, including adequate mass transfer properties (i.e. diffusivity and permeability of the extracellular matrix) in order to traffic nutrients, oxygen, metabolic waste, and signaling/paracrine molecules (e.g. see [15,33]). Another not fully investigated, yet crucial requirement for cardiac cells is the need for anchorage to a substrate providing specific biomechanical cues [10,37–40]. For example, it has been reported that the contractile work and development of immature cardiomyocytes can be significantly improved by tuning the substrate elasticity [37]. Moreover, matrix stiffness has been shown to not only regulate the maturation of differentiated cardiomyocytes but also affects the induction and proliferation of myocytes from undifferentiated progenitors [38,41].

Plastic compression technique has been recently developed to rapidly produce dense biomimetic scaffolds with precise biomechanical properties (e.g. pore size, permeability, and elastic modulus), while it has no adverse effect on the viability and metabolism of resident cells [15,34,42,43]. Highly hydrated collagen gels exhibit poor structural integrity, insufficient for tissue replacement [34]. Therefore, in this study, multiple levels of plastic compression were applied to cast collagen gels in order to produce compressed scaffolds

with improved elastic moduli (E) as candidates for myocardial tissue replacement (Figure 2). The increases in E for *partially* and *fully* compressed gels were ~6–14 folds and ~35 folds, respectively.

As we previously reported [15,35,36], application of *partial* compression levels to hydrated collagen scaffolds led to the formation of a two-layer gel microstructure, with a significant increase in collagen fibrillar density, accompanied by an increase in the macro compressive modulus, measured by a thermal mechanical analyzer. It is noteworthy that the microstiffness measurements conducted in the current study using AFM were in close agreement with the bulk stiffness values measured previously [36]. The fully compressed collagen scaffolds exhibited elastic moduli (2–10 kPa) that was comparable to the optimal range of substrate elasticity, reported by Engler et al. [37], in order to achieve maximum myocyte contractility and development of immature cardiomyocytes ($E \sim 1\text{--}14$ kPa). Interestingly, this range of elasticity matches that of embryonic epicardium, whereas adult epicardium and fibrotic cardiac tissues exhibit significantly greater stiffness values that are far from this range [37]. Further consolidation of collagen gels beyond this level (via an increase in compressive stress or time of compression) has shown to have infinitesimal effect on the scaffold properties [35,36]. Therefore, we utilized the latter state of compression to produce dense collagen matrices with optimal properties to serve as cardiac patch.

In analogy to the healthy hearts grafted with patch, attachment of the engineered patches on infarcted LV of mice demonstrated integration between the tissues, as well as extensive cell migration into the patch (Figures 3 and 4). Moreover, echocardiography at weeks 2 (data not shown) and 4 after MI showed significant improvement in cardiac function (EF and FS), neo-angiogenesis, diminished fibrosis, and inhibition of LV dilation and wall thinning (LVID and LVPW, respectively) (Figure 4). These results indicate that biomimetic collagen patches exert beneficial *in vivo* effects on cardiac remodeling and function after injury.

Immunostaining analysis of heart sections, obtained 4 weeks post infarct, demonstrated that a significant number of native cardiac cells including smooth muscle cells (forming new vessels), cardiomyocytes, cardiac fibroblasts, and epicardial derived progenitor cells migrated into the patch. These results, together, suggest a number of processes as potential mechanisms responsible for the improvement of cardiac function in animals treated with an engineered patch: a) mechanical support of the grafted scaffold to the infarcted tissue; hence, reduced dilation and fibrosis, and an increase in the thickness of LV wall; b) enhanced angiogenesis at the infarct zone, in the patch, and at the border zone. This in turn can result in improved oxygen/nutrient delivery and therefore, a decrease in the amount of ischemic tissue; c) presence of new cardiac muscle cells on the two sides of the border zone, in the patch and in the damaged myocardium, can improve the contractility of the neo-tissue. In addition to the beneficial mechanical effects, these engineered patches can be utilized as a local, sustained-delivery device to preserve cardiac function and induce regeneration, thus constituting a platform for treating cardiac disease.

5. Conclusion

In summary, we developed an engineered cardiac patch out of compressed Type I collagen that improves recovery of the heart after acute myocardial infarction through several processes, independently from exogenous cells and other factors. The main mechanisms responsible for this effect include the mechanical support of the scaffold, facilitation of cell migration and angiogenesis, and partial preservation of the heart muscle cells within the lesion and the area beneath the patch. Further investigations on therapeutic factors and/or

cells that can be seeded within the engineered patch can usher in a new clinical therapy for cardiac repair following severe heart injuries.

Acknowledgments

This work was supported by NIH grants HL65484 and HL086879 (to PRL). KW was a SBMRI California Institute for Regenerative Medicine postdoctoral fellow (TG2-01162). VS is an Oak Foundation postdoctoral fellow at Stanford. MJB was supported by K08 AI079268 and MJB and AW by the Stanford BioX Interdisciplinary Initiatives Program and NSF NSEC Grant No. PHY-0830228. DB was supported by NIG grant HL061535.

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Figure 1.

Schematic representation of inducing myocardial infarction (MI) via permanent ligation of LAD artery (A) which was either treated with patch (B, C) or left untreated (D, E). Panel C depicts a cross-sectional view of the patch located on the infarct, describing various processes involved in protective role of engineered patch. The inset on the right shows electron microscopy of the dense collagen scaffold fibrillar structure (reconstructed from [44]). Panel E shows HE staining of the MI heart with no treatment.

Figure 2.

Evaluation of mechanical properties of engineered patch. Elastic modulus of compressed collagen gels by atomic force microscopy utilizing a custom-designed flat tip (A, B), manufactured using focused ion beam. Stiffness measurements were performed by scanning areas of $90\ \mu\text{m} \times 90\ \mu\text{m}$ (C). D: Distribution of measured moduli of patches, plotted for non-compressed, as cast hydrogel (blue), partially compressed matrices (red and violet), and fully compressed scaffold used as patch in this study (green). Grey region highlights the range of substrate stiffness that supports maximal contractile work from immature myocytes [37]. The inset in panel D shows the phase map of the fully compressed scaffold used as cardiac patch in this study, obtained by AFM. The yellow arrows in this image highlight the characteristic banded pattern of periodic striations found in collagen fibers.

Figure 3.

Application of the engineered patch onto the healthy myocardium. A: Stereomicroscopy image of the whole heart section (trichrome staining). B–D: HE, trichrome, and von Willebrand factor staining images of healthy heart grafted with the patch, respectively. Panels E–G show the effect of the patch in greater magnification. Yellow lines and arrows demonstrate patch-tissue boundary and newly formed vessels, respectively. These results confirm patch integration with the host tissue along with massive cell migration and new vessels formation.

Figure 4.

A: Cardiac function assessed by echocardiography. Echo analysis four weeks post-MI (normalized by dividing to pre-surgery baseline values) demonstrated a remarkable attenuation in LV dilation (LVID) and an increase in LV posterior wall thickness (LVPW) in the patch-treated hearts (blue, $n>4$) compared with those of sham control and MI-only animals (green and red, respectively). In addition, ventricular ejection fraction (EF) and fractional shortening (FS) were improved significantly in the treated hearts. B: image of the whole heart treated with patch after MI. Yellow arrow highlights formation of functional blood vessels (with blood stream) within the patch. C–Q: Stereomicroscopy image of the whole heart, together with HE, trichrome, and von Willebrand factor (vWF) staining of sham controls (C–F), MI-only (G–J), and MI treated with patch (K–Q), respectively. These images demonstrate extensive fibrosis, LV dilation and wall thinning in MI-only group, when compared to sham. On the contrary, treating MI with the patch showed an effective integration of the tissues, associated with widespread cellularization and angiogenesis within the patch. The yellow lines in the panels indicate approximate location of the interphase between the patch and myocardium. The yellow arrows point to the new vessels formed inside the patch and at the border area. Panels O–Q show greater magnifications of panels L–N, respectively.

Figure 5.

Immunohistochemical analysis of hearts 4 weeks post treatment in sham controls (left) and infarcted hearts treated with patch (two right columns). A–C: Immunostaining with α -smooth actin (red) in the demonstrating formation of new vessels inside the patch and at the border zone. D–F: Immunostaining of the cardiomyocyte marker α -actinin (red) in the hearts showing a small number of positive cells inside the patch and a few more at the border zone and infarcted tissue under the patch. Immunofluorescence staining for DDR2 (cardiac fibroblasts) and PDGFR α (epicardial cells) in the heart sections (G–I and J–L, respectively). Moreover, heart sections were stained for various inflammatory signals including CD3 for T cells and F4/80 for macrophages (M–O and P–R, respectively) demonstrating an insignificant immune response. In all of the images green is the auto fluorescence and blue is the nuclear staining using DAPI.

Table 1

List cell types and corresponding antibodies used for the immunohistochemical analysis

Cell type	Marker	Cell type	Marker
Cardiomyocytes	-actinin (sigma, A7811)	Epicardial derived progenitor cells	PDGFR (Abcam, ab61219)
Cardiac fibroblasts	DDR2 (Santa Cruz Biotech, sc-7555)	Smooth muscle cells	-SMA (Sigma, A5228)
T-Cells	CD3 (Abcam, ab6326)	Macrophages	F4/80 (Abcam, ab100790)