Published in final edited form as:

Biomaterials. 2016 August ; 97: 51–61. doi:10.1016/j.biomaterials.2016.04.031.

Inosculation and Perfusion of Pre-Vascularized Tissue Patches Containing Aligned Human Microvessels after Myocardial Infarction

Sonja B. Riemenschneider, BS¹, Donald J. Mattia, BS², Jacqueline S. Wendel, PhD², Jeremy A. Schaefer, BS², Lei Ye, MD, PhD^{3,4}, Pilar A. Guzman, DVM, MS⁵, and Robert T. Tranquillo, PhD^{1,2,*}

¹Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, MN, USA

²Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, USA

³Department of Medicine, University of Minnesota, Minneapolis, MN, USA

⁴National Heart Research Institute Singapore, National Heart Centre Singapore, Singapore

⁵Integrative Biology and Physiology Phenotyping Core, University of Minnesota, Minneapolis, MN, USA

Abstract

A major goal of tissue engineering is the creation of pre-vascularized tissues that have a high density of organized microvessels that can be rapidly perfused following implantation. This is especially critical for highly metabolic tissues like myocardium, where a thick myocardial engineered tissue would require rapid perfusion within the first several days to survive transplantation. In the present work, tissue patches containing human microvessels that were either randomly oriented or aligned were placed acutely on rat hearts post-infarction and for each case it was determined whether rapid inosculation could occur and perfusion of the patch could be maintained for 6 days in an infarct environment. Patches containing self-assembled microvessels were formed by co-entrapment of human blood outgrowth endothelial cells and human pericytes in fibrin gel. Cell-induced gel contraction was mechanically-constrained resulting in samples with high densities of microvessels that were either randomly oriented (with 420 ± 140 lumens/mm²) or uniaxially aligned (with 940 ± 240 lumens/mm²) at the time of implantation. These patches were sutured onto the epicardial surface of the hearts of athymic rats following permanent ligation of the left anterior descending artery. In both aligned and randomly oriented microvessel patches, inosculation occurred and perfusion of the transplanted human microvessels was maintained,

*Corresponding Author: Robert T. Tranquillo, 7-114 Nils Hasselmo Hall, 312 Church St SE, Minneapolis, MN 55455, Phone: 612-625-6868, Fax: 612-626-6583, ; Email: tranquillo@umn.edu

Conflicts of Interest

None declared.

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proving the *in vivo* vascularization potential of these engineered tissues. No difference was found in the number of human microvessels that were perfused in the randomly oriented (111 ± 75 perfused lumens/mm²) and aligned (173 ± 97 perfused lumens/mm²) patches. Our results demonstrate that tissue patches containing a high density of either aligned or randomly oriented human pre-formed microvessels achieve rapid perfusion in the myocardial infarct environment - a necessary first-step toward the creation of a thick, perfusable heart patch.

Keywords

microvascular; perfusion; myocardial infarction; tissue engineering; inosculation; pre-vascularized

Introduction

The creation of engineered tissues containing microvascular networks that can be rapidly perfused within a few days following implantation remains a major goal of tissue engineering.^{1,2} Applications that involve highly metabolic tissues, such as myocardium and liver, are especially dependent on the presence of a rapidly perfusable microvascular network to prevent the formation of a necrotic core beyond the diffusion limit in the implanted tissue.³⁻⁵ Recruitment of vasculature via angiogenesis is a common method for vascularizing tissue engineered constructs *in vivo*, but this vascularization strategy is too slow to permit the survival of thick, highly metabolic tissues.² Implantation of tissues with pre-formed microvascular networks could enable rapid perfusion of thick engineered tissues by inosculation of the implanted microvessels with adjacent host blood vessels.

Many examples of creating “engineered microvessels” exist,⁶⁻¹¹ and many groups are striving to create functional microvessel patches in macroscopic, implantable materials.^{6,12-18} The most impressive of these studies demonstrate that implantation of pre-cultured human microvessels in immune-compromised mice can result in inosculation with the host vasculature and perfusion of the human microvessels.^{13,14,17} However, these studies were carried out in the healthy subcutaneous environment, and no study has implanted pre-cultured human microvessels in the especially challenging myocardial infarct environment. With the ultimate goal of treating myocardial infarctions and heart disease with a perfusable, beating cardiac patch containing both microvessels and cardiomyocytes, the only fair way to assess the vascularization potential of a precursor patch containing only microvessels would be to implant it at the site of a myocardial infarction. This way, the insights gained from such a study could be leveraged in the future development of a patch that also contained cardiomyocytes.

Many studies in rodents have administered tissue-engineered heart patches onto myocardial infarcts containing various cell types,¹⁹⁻²¹ but implantation of human microvessels has not yet been reported. The Okano Group has shown that thin microvascular cardiac patches made from neonatal rat cardiomyocytes and rat endothelial cells can be perfused by the host and result in improved cardiac function after 4 weeks.²² In our lab’s prior studies we have implanted heart patches made from either neonatal rat heart isolates or human induced pluripotent stem cell derived cardiomyocytes co-entrapped with human pericytes. These

heart patches were made in fibrin gel and cultured for 1–2 weeks to align the cells and fibrin. In both studies, the patches had no microvessels at the time of implantation, yet we observed perfused host-derived capillaries invading the patch as early as 1 week.^{19,20} These results suggest the possibility that a similar patch that contained pre-formed human microvessels could rapidly inosculate with the invading host vessels and be perfused within 1 week.

The microvessel density and organization also needs to be addressed. In tissues that require efficient perfusion, the capillaries are generally highly organized in microvascular beds, with many capillaries spanning across the same arteriole and venule. In myocardium, the capillaries are not only very dense, but they are also aligned.^{23–25} Therefore, emulating these two features in the engineered microvessels of a heart patch is highly desirable. Previous studies of pre-vascularized engineered tissues do not control microvessel alignment, nor do they achieve lumen densities within the same order of magnitude as native adult myocardium (2,000 lumens/mm²)^{23,24}. To date, reports of pre-implant cross-sectional lumen density range from fewer than 200 lumens/mm² up to 650 lumens/mm², the latter of which was reported by our lab.^{6,26–29}

A necessity for a heart patch that would aim to restore mechanical function would be rapid connection to blood flow to maintain viability of the large number and high density of transplanted cardiomyocytes in a thick tissue-like patch. Ideally, this would be achieved by implanting a cardiomyocyte patch containing a pre-formed, perfusable microvascular network that could rapidly anastomose with the host and maintain perfusion. As a step toward that goal, the present study sought to determine the rapid vascularization potential of a remodeled fibrin patch containing either an aligned or randomly oriented microvascular network, but no cardiomyocytes, implanted onto a myocardial infarction for 6 days.

In this study we investigated the rapid *in vivo* vascularization potential of tissue patches pre-vascularized with either aligned or non-aligned human microvessels and implanted over myocardial infarcts. Patches containing human microvessels, were made by entrapping human blood outgrowth endothelial cells (BOECs)³⁰ and human pericytes (PCs) in fibrin gel, and allowing self-assembly of a microvascular network of tubules. Patches with aligned human BOEC/PC microvessels (“aligned microvessel patches”) were anchored at both ends by porous plastic spacers, and aligned via cell-induced gel compaction, as previously described.¹² Briefly, as the samples compact laterally and remain constrained in the longitudinal direction by the spacers, the fibrils, cells, and the formed microvessels become aligned in the longitudinal direction. To investigate the effects of a patch lacking microvessels, patches made in a similar manner with only aligned PCs (“aligned PC patches”) were also investigated. Patches with non-aligned microvessels (“isotropic microvessel patches”) were made by maintaining gel adhesion to the bottom surface of the culture plate and preventing lateral compaction and longitudinal alignment.

The patches were sutured onto the infarcted region of the heart of nude rats immediately following LAD ligation, as we previously described for heart patches containing cardiomyocytes,^{19,20} and were implanted for 6 days. Perfusion of the patches was evaluated with species-specific endothelial-binding fluorescent labels injected into the left ventricle to circulate throughout the bloodstream prior to sacrifice. Immunohistochemistry on

histological sections from explanted hearts was used to quantify the total number of human vessels in the patches. Aligned microvessel patches were predicted to have a greater number of perfused vessels after 6 days and isotropic microvessel patches were predicted to have some perfused vessels, but less than the aligned microvessel patches. Aligned PC patches were expected to recruit some host-derived microvessels by 6 days, but the total number of perfused microvessels (human + rat) was expected to be much lower than in the pre-vascularized patches.

While we did not expect these patches to have an effect on cardiac function or infarct size, as they were lacking cardiomyocytes, ejection fraction and fractional shortening were measured before implantation and at sacrifice to ensure any changes were recorded. The infarct was characterized by measuring the percent of the left ventricular wall occupied by scar as well as the left ventricular wall thickness.

Methods

Culture of human blood outgrowth endothelial cells and human pericytes

Human BOECs were isolated from adult peripheral blood by the lab of Dr. Robert Hebbel at the University of Minnesota – Twin Cities³⁰. Briefly, BOECs were screened for VE-cadherin, flk-1, vWF, CD36, and CD14 (negative). Passage 5 BOECs were thawed and plated on 0.05 mg/ml collagen I – coated flasks in BOEC medium (EGM-2 bulletkit medium (Lonza) supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco)). Medium was changed every other day and BOECs were passaged after 4 days, then plated and cultured for 4 more days prior to harvest.

Human brain vascular PCs (ScienCell, fetal, characterized by immunofluorescence with antibody specific to α -smooth muscle actin) were transduced to express GFP and obtained from the lab of Dr. George Davis at the University of Missouri. Passage 6 PCs were thawed and plated on 1 mg/ml gelatin-coated flasks in PC medium (13% FBS, 1% penicillin/streptomycin (Gibco), 10 ng/ml gentamicin (Gibco) in low-glucose DMEM (Lonza)). Medium was changed every 2–3 days and PCs were harvested after 10 days.

Creation of aligned microvessel patches

Rectangular molds (18.4 mm x 5 mm) were created by melting ridges into the bottom of a 6 well tissue culture plate. Porous polyethylene spacers (5 mm x 5 mm) were placed on top of a dollop of sterile vacuum grease at both ends of the rectangular mold leaving a central rectangular well (8.4 mm x 5 mm). Droplets of fibrin gel solution containing BOECs and PCs were pipetted onto the edge of the two spacers and dragged toward the center to fill the central rectangular well (Figure 1A). This ensures that the gel is integrated with the porous spacers and is anchored in place. The gel solution was made up of 2.55 mg/ml fibrinogen (Sigma), 200 ng/ml of stem cell factor (SCF), interleukin-3 (IL-3), and stromal derived factor 1 α (SDF-1 α) (R&D Systems), 2×10^6 BOECs per mL and 0.4×10^6 PCs per mL, 1.25 U/ml thrombin (Sigma) and Medium 199 basal medium (Gibco) (M199). The total volume of each gel was 112 μ L. Samples were incubated at 37°C, 5% CO₂ for 20 min before adding BOEC medium. Medium was replaced after 1, 3, 5, and 7 days. In these samples, a

microvascular network of tubules self-assembles and PCs are recruited to the abluminal side of the vessels after 5 days of *in vitro* culture.⁶ We will hereafter refer to these structures as microvessels and microvascular networks.

After 5 days of culture, samples were detached from the bottom surface of the tissue culture plastic by sliding the spacers along the bottom back and forth. To re-anchor the spacers, samples suspended between the two spacers were carefully transferred to a new well by picking up the spacers and placing them on fresh droplets of sterile vacuum grease. Spacers were re-anchored the same distance apart to maintain a constant sample length. The samples were then free to compact laterally via cell-induced compaction (Figure 1B). It has been shown that lateral compaction causes alignment of the microvessels and fibrin fibrils in the longitudinal direction.¹² Aligned microvessel patches were harvested after 8 or 9 days for implantation.

Creation of aligned PC patches

Aligned PC patches were created identically to the aligned microvessel patches with the exception that the BOEC cell suspension volume was replaced by M199 (Gibco). PC density was identical to BOEC/PC patches to maintain similar levels of paracrine factors released by the PCs in the patch.

Creation of isotropic microvessel patches

Isotropic microvessel patches were included in the study to assess the importance of vessel alignment in achieving perfusion of pre-formed vessels *in vivo*. Isotropic patches were designed to maintain similar size and identical cell loading as aligned microvessel tissue patches. Maintaining the same length (8.4 mm) ensured that the patches spanned the infarct region. Isotropic patches were implanted as a single patch, while aligned patches were implanted as three parallel strips, thus the isotropic patches were made to contain 3 times the cell number as the aligned patches. The minimum width that would hold the required volume without spilling the gel-forming solution was 7 mm. In this way the cell content was identical between the isotropic and aligned microvessel patch groups, while the patch area was marginally different (50.4 mm² for three aligned microvessel patches vs 58.8 mm² for a single isotropic microvessel patch).

Rectangular wells (8.4 mm x 7 mm) were created by melting ridges into the bottom of a 6 well tissue culture plate (Figure 1C). Fibrin gel-forming solution identical to the aligned microvessel patch formulation (but triple the volume - 336 µl) was pipetted carefully to fill the rectangular well without spilling outside the ridges. Samples were left to gel in the cell culture hood untouched for 15 min due to their precariously high liquid height, then transferred to the incubator at 37°C, 5% CO₂ for 15 min to complete the gelation process. Once gelation was complete, BOEC medium was added to cover the samples. Medium was replaced after 1, 3, 5, and 7 days. Samples remained adhered to the bottom culture surface throughout the culture period to prevent compaction-induced alignment. The resulting isotropic microvessel patches were harvested after 8 or 9 days for implantation.

Patch Characterization

Patches not implanted (at least 6 per group) were harvested after 8 or 9 days of *in vitro* culture and fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) for 10 min at room temperature, then rinsed in phosphate buffered saline (PBS) (Corning). Samples were cut in half longitudinally and one half was reserved for histology and the other was saved for whole-tissue immunofluorescence staining.

Whole-tissue samples were blocked in 5% Normal Donkey Serum (Jackson ImmunoResearch) for 2 hours, then incubated in 1:40 antibody to human CD31 (hCD31) (Dako) in blocking serum for one hour. Three 5 min PBS rinses were performed, then the samples were incubated in 1:200 donkey anti-mouse secondary antibody conjugated with Alexa Fluor® 594 (Jackson ImmunoResearch) in PBS for 1 hour. After secondary antibody incubation, samples were incubated in 1:10,000 Hoescht 33342 (Invitrogen) in PBS for 10 min, followed by two 5 min rinses in PBS. All steps were performed at room temperature on an orbital shaker. Whole-stained patches were visualized with confocal microscopy (Zeiss LSM 510) and three random fields of view from were captured for each sample to assess microvessel alignment, microvascular network length, network connectivity, and PC recruitment and alignment.

For each image, the angle and length of each microvessel segment was measured in Fiji and exported to Excel. Total network length was calculated by taking the sum of all vessel segment lengths for each image and dividing by the area in the field of view. Microvessel alignment was verified by calculating an anisotropy index. The x and y components of each vessel segment were calculated according to the angle and segment length, with x being the longitudinal direction. The anisotropy index was calculated by dividing the sum of the x components by the sum of the y components for each image. Alignment of the PC patches was quantified using a similar method, except rather than measuring microvessel segments, individual PCs were measured by drawing line segments along the major axis of each cell. An anisotropy index of 1 indicated random orientations of cells or microvessels and values increasing from 1 indicated vessels and PCs that were more strongly aligned in the longitudinal direction.

Samples reserved for histology were placed in infiltration solution 1 (30% w/V sucrose in PBS) at 4°C overnight and then transferred to infiltration solution 2 (50% infiltration solution 1, 50% embedding medium (Tissue-Tek OCT)) at room temperature for 4 hours. Samples were then frozen in embedding medium and cross sections were cut by cryosectioning 9 µm thick cross-sections for immunohistochemical staining, as is common-practice for measuring lumen density in aligned tissues. Sections were stained for hCD31 similarly to the whole-tissue samples except incubation steps were not performed on an orbital shaker. hCD31 stained cross-sections were viewed with confocal fluorescence microscopy and three random fields of view were captured for each sample. Lumen density was assessed by manual counting and lumen diameters were measured manually in ImageJ based on hCD31 staining. PC recruitment was assessed by counting the number of PCs in contact with hCD31+ cells and dividing by the total number of PCs in each image.

Patch width was determined from photographs taken prior to harvesting the samples and thickness was measured from histological sections. The fibrin concentration at harvest was estimated by simplifying the patch shape to be two side-by-side trapezoids with constant thickness and calculating the fibrin concentration based on the volume reduction from the initial gel formulation. This estimate assumes no degradation of fibrin occurred over the 8–9 days of *in vitro* culture.

Experimental Design

Four groups were evaluated in this study: 1. MI + aligned microvessel patch (n=6), 2. MI + isotropic microvessel patch (n=6), 3. MI + aligned PC patch (n=5), and 4. MI only (n=6). Patches were applied on the left ventricle below the ligation suture immediately after ligation in patch groups, while MI only received no other treatment. The aligned microvessel patch contained a dense network of aligned microvessels to serve as the predicted optimal treatment group. The isotropic microvessel patch functioned to evaluate the importance of microvessel alignment in a pre-vascularized heart patch. The aligned PC patch functioned as a non-vascularized control patch. Since cell-mediated gel contraction is essential for remodeling the fibrin gels during *in vitro* culture, an acellular control patch was not tested because a cell-free fibrin patch could not be made with physical properties similar to cell-containing gels.

Implantation of patches into an acute nude rat infarct model

Procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee (Protocol ID:1501-32275A) and Research Animal Resources at the University of Minnesota and conform to NIH guidelines for care and use of laboratory animals.

Twenty-three female Foxn1^{nu} nude rats (Harlan Sprague-Dawley) were used in this study, aged 6–8 weeks old and weighing 150–200 g. Isoflurane was administered for anesthesia, and rats were intubated prior to surgery. Depth of anesthesia was monitored throughout the surgery by pinching the toe and tail of the animals. The chest wall and pericardium was opened to expose the heart, and the LAD was permanently ligated to achieve an MI. Once an MI was established, either an aligned microvessel patch, an isotropic microvessel patch, or an aligned PC patch was applied for the three treatment groups. Aligned patches were cut free from the porous polyethylene spacers and three patches were sutured parallel to each other over the epicardial surface of the left ventricle below the ligation suture, approximately parallel to the alignment of the surface myocardium and covering the width of the infarct (Figure 1D), similar to our previous studies.^{19,20} Isotropic patches were detached by sliding a spatula between the tissue and the bottom culture surface to free the tissue. A single isotropic patch was placed over the epicardial surface in the same location as the aligned patches. Once the patch was sutured in place, the chest was closed and the animal was allowed to recover. Ketoprofen (2.5mg/kg, Pfizer) and Enrofloxacin (15mg/kg, Bayer) were administered to the rats daily for 3 days.

Cardiac Functional Measurements and Perfusion Assessment

Both prior to the initial surgery and 6 days post-implantation, echocardiography (Vevo2100) was performed on the animals to assess left ventricular ejection fraction and fractional shortening. During each session, at least two measurements were recorded for both short and long axis views. After echocardiography was performed on day 6, isoflurane was administered for anesthesia, and the chest was re-opened. Immediately prior to sacrifice, rhodamine-conjugated ulex europaeus agglutinin – I (UEA-I) (Vector Laboratories) and fluorescein conjugated griffonia simplicifolia lectin I, isolectin B4 (IB4) (Vector Laboratories), endothelial labels for human and rodent, respectively, were injected directly into the chamber of the left ventricle at a dose of 1.2 µg/g body weight. Blood was allowed to circulate for 1–2 min, then the animal was euthanized via a potassium chloride intracardiac injection and the heart was removed for characterization.

Histological Assessment

Upon explant, hearts were rinsed in PBS for 5–15 min, then cut in two pieces by making a transverse cut below the ligation suture. The two pieces were transferred to 4% PFA for overnight fixation at 4°C on an orbital shaker. After fixing, the hearts were rinsed 3 times for 15 min in PBS on an orbital shaker. Then another parallel cut was made half-way between the apex and the first cut, resulting in three total transverse sections from each heart. Sections were placed in infiltration solution 1 for 24 hours, then infiltration solution 2 for 48 hours at 4°C on an orbital shaker. Heart pieces were then frozen in embedding medium and cryosectioned in 9 µm sections for immunohistochemistry and histological analysis.

Infarct Assessment

Masson's trichrome stain was applied to transverse heart cryosections to evaluate infarct size and left ventricular (LV) wall thickness for three different regions of the heart. The three regions were approximately: below the ligation suture, half-way between the ligation suture and the apex, and near the apex. Infarct size was calculated by averaging the percentage of the LV free wall area occupied by scar. LV wall thickness was calculated by averaging three measurements of the thickness of the LV wall in infarcted regions of three sections from different regions of the heart. When all three trichrome-stained heart sections showed no evidence of infarction, the animal was excluded from the study.

Perfusion Analysis

Heart cryosections were stained for hCD31 to visualize human vessels in the explanted patches. Images of the patch region were captured with confocal fluorescence microscopy using sequential excitation to avoid crossover from multiple wavelength excitation. Nuclei (labeled with Hoescht 33342, 405 nm excitation), PCs (expressing GFP, 488 nm excitation), perfused rat vessels (labeled *in vivo* with IB4, 488 nm excitation), perfused human vessels (labeled *in vivo* with UEA-I, 561 nm excitation), and all human vessels, perfused and non-perfused (labeled with hCD31, 633 nm excitation), were visualized. The number of UEA-I positive vessels and hCD31 positive vessels were counted manually from 40x images captured from at least 10 random locations within the patch. GFP PCs and IB4+ rat vessels (both viewed in the green channel) were differentiated by morphology. When looking at

cross sections, a PC that was wrapped fully around a human vessel sometimes looked like a lumen, so green lumen-like structures were considered recruited PCs if the interior of the green lumen contained hCD31 positive staining and all other green lumens were considered rat vessels. Green structures that did not contain a lumen were considered PCs. Lumen diameters were also measured from these images, as described previously. Sections were also stained with hematoxylin and eosin (H&E) and separately, an antibody for rat red blood cells (RBC) (Rockland) to confirm perfusion of human vessels in the patches.

Statistical Analysis

Data are represented as a mean $\pm$ standard deviation. Students t-tests were performed in Excel. Multiple groups were compared with 1-way analysis of variance (ANOVA) with Games-Howell post-hoc tests in Minitab. P-values < 0.05 were considered significant.

Results

In Vitro Characterization of Patches

Aligned patches compacted vertically throughout the culture period and compacted laterally once they were detached from the bottom culture surface after 5 days of culture. Isotropic patches were left constrained by the bottom culture surface throughout the culture period, thus they only underwent vertical compaction (Figure 1C). The final patch dimensions and their estimated fibrin concentrations are summarized in Table 1.

Aligned microvessel patches contained a dense network of longitudinally aligned microvessels with PCs recruited to the abluminal side of the microvessels (Figure 2). Isotropic microvessel patches formed a network of microvessels with recruited PCs, but showed no alignment. Aligned PC patches contained elongated PCs aligned in the longitudinal direction.

Image analysis of CD31 stained non-implanted tissue patches (Figure 2) revealed that the aligned microvessel patches contained 940 ± 240 lumens/mm². In comparison, isotropic microvessel patches had a reduced lumen density of 420 ± 140 lumens/mm². Similarly, network length for the aligned microvessel patches was higher at 33 ± 6 mm/mm² versus 16 ± 4 mm/mm² for the isotropic patches. The percent of PCs recruited to the abluminal side of the vessels was not different, at $77 \pm 10\%$ versus $75 \pm 9\%$. The anisotropy index, a measure of the alignment of the microvessels or PCs (random = 1, aligned > 1), was different for each group. The anisotropy index for aligned microvessel, isotropic microvessel, and aligned PC patches was 3.1 ± 0.8 , 1.0 ± 0.2 , and 5.0 ± 2.0 , respectively.

Patch Engraftment and Alignment

All patches remained located on the epicardial surface of the left ventricle after the 6 day implantation period, covering all or a portion of the infarct (Figure 3D). No evidence of a necrotic core was found in any of the patch groups, which all showed uniform cell density throughout the patch thickness (Figure 3A–C). Generally, human vessels near the edges of the patch appeared to have a higher prevalence of perfusion, from visual inspection, but perfusion also occurred in the center region. The thickness of the explanted patches were

410 ± 120 µm for aligned microvessel, 400 ± 140 µm for isotropic microvessel, and 360 ± 90 µm for aligned PC. Trichrome staining showed a mix of fibrin (red) and collagen (blue), and a cell-dense, collagenous interface between the patch and the myocardium (Figure 3E). Subsequent immunohistochemical analysis revealed many rat vessels spanning this interface between the patch and myocardium. Aligned patches maintained their alignment after 6 days implantation, while isotropic patches remained isotropic (Figure 3F–H).

Microvessel Characterization and Perfusion Assessment

Total human vessels remaining in the patch after 6 days implantation were counted from hCD31 stained sections (Figure 4). Aligned microvessel patches contained 435 ± 98 human vessel lumens/mm² and isotropic microvessel patches contained 374 ± 98 human vessel lumens/mm². Due to the oblique sectioning angle with respect to the alignment of the patch microvessels (a necessity for obtaining transverse heart sections), the apparent lumen density of the aligned microvessel patches was reduced compared to *in vitro* sectioning and characterization (Supplementary Figure 1). Also, explanted patches could not be distinguished by eye from the surrounding tissues, thus the precise position of the patch at explant was uncertain, and oblique sectioning would have been unavoidable in any case.

The perfused human and rat endothelial labels, UEA-I and IB4, were used to quantify perfused human and rat vessels in the patch. Aligned microvessel patches contained 173 ± 97 perfused human vessel lumens/mm², meaning 40 ± 23% of the human vessels were perfused. Isotropic microvessel patches contained 111 ± 75 perfused human vessel lumens/mm², meaning 30 ± 18% of the human vessels were perfused. No differences in total vessel density, perfused vessel density, or perfused fraction were found between aligned and isotropic microvessel patches. Aligned PC patches were perfused by invading rat vessels (92 ± 80 rat vessels/mm²), as were the other two patch groups to a similar extent.

Inosculature of the human microvessels with rat vessels was visualized in several histological sections as lumens stained for UEA-I that connected with lumens stained for IB4, primarily near the patch-heart interface (Figure 4B). Sections stained for rat RBCs (Figure 4C) as well as H&E (Figure 4D) confirmed perfusion by visualizing RBCs in lumens, providing further evidence of patch microvessel perfusion and inosculature with the host.

Lumen diameters for aligned and isotropic microvessel patches were found to be in the 5–10 µm range of normal human capillaries^{23–25} both before and after implantation with a small increase in mean diameter from 6 µm to 8 µm observed for aligned patches (Figure 5).

Infarct Histology Assessment

Infarct size, defined as the percentage of the LV free wall area occupied by scar (averaged from three different regions of the heart), was evaluated for each patch group as well as the MI only control (Supplementary Figure 2D). Infarct sizes for the three treatment groups were, on average, smaller than the MI only control, but none of the comparisons were statistically different. Similarly, the LV wall thickness for the treatment groups were not statistically different from the MI only control, except for the isotropic microvessel group,

which showed less thinning of the LV wall compared to the MI only control (Supplementary Figure 2E).

Cardiac Function Measurements

Ejection fraction and fractional shortening measurements from baseline and day 6 echocardiography showed a decrease in ejection fraction and fractional shortening at 6 days compared to baseline measurements prior to MI (Supplementary Figure 2B,C). While the average ejection fraction and fractional shortening values were approximately 10% higher for the three treatment groups, no statistical differences were observed.

Discussion

This is the first report of pre-formed human microvessels being implanted and perfused in a myocardial infarct model. This is a challenging environment for cell survival, but despite the challenging infarct environment, both the aligned and isotropic microvessel patches demonstrated a high degree of perfusion of human microvessels after 6 days of implantation. The results from these precursor microvessel patches highlight the potential for future pre-vascularized cardiomyocyte patches to be rapidly perfused after implantation onto a myocardial infarct.

To the best of our knowledge,^{6,14,31} the aligned microvessel patches used in this study contained the highest reported density of microvessel lumens achieved for engineered tissues *in vitro* (940 ± 240 lumens/mm²). Perfused lumen densities at explant were comparable to high reports for pre-formed engineered microvessels^{13,32,33}, yet they are the highest among aligned pre-formed microvessels. With a lumen density well above that of human skeletal muscle³⁴ and half the density of human adult cardiac muscle, the most capillary-dense tissue in the body, these pre-vascularized tissue patches could be translated to a wide variety of clinical applications with the addition of therapeutic cells. Our ability to create either aligned or isotropic microvascular networks and tune the alignment of these microvessel patches (shown in previous studies¹²) makes them especially versatile.

Explant patches maintained a high density of human vessels with physiological lumen diameters. The high degree of PC recruitment to the vessels *in vitro* likely helped maintain them for 6 days *in vivo*, as PCs enhance microvessel stability.³⁵ The vessel alignment in the aligned patches was also preserved *in vivo*, indicating these patches presented a robust template to maintain alignment upon remodeling. It should be noted that lumen densities for explanted aligned microvessel patches are lower than the pre-implant measurements, while the isotropic microvessel patches had no change in lumen density pre- and post-implantation. It is likely that most, if not all, of the reduction seen in the aligned patches is an artifact of sectioning at an oblique angle, rather than orthogonal to the alignment direction, as was done for *in vitro* characterization and as is common-practice in the field. This would cause fewer vessels with wider-appearing lumens to be presented per unit area for the aligned patches, but would cause no change for the isotropic patches due to the random orientation of the microvessels (Supplementary Figure 1). In addition to the reduction due to oblique sectioning, it is possible that some lumens collapsed or vessels regressed during the implantation period.

The majority of vessels present in the patches after 6 days implantation were human, with a small fraction of them being from the host (Figure 4E). None of the patches had formed a necrotic core after 6 days of implantation, including the aligned PC patches, which were also invaded by vessels from the host. It is not entirely surprising that the aligned PC patches lacked a necrotic core, as the patches used in this study were relatively thin and they did not contain a highly metabolic cell type such as cardiomyocytes, which would likely have had greater oxygen and nutrient demands. Human vessels, evidenced by hCD31 staining, were found to be uniform in density for both the aligned and isotropic patches. Perfusion of the human vessels, on the other hand, was more pronounced near the patch-host interface. This suggests that there were disconnected vessel networks present in the patches, and only those that inosculated with the host vessels were perfused. Greater perfusion efficiency could potentially be achieved with longer implantation time or by pre-conditioning these patches with flow⁶ to promote greater network connectivity.

While no perfusion benefit was observed for aligned microvessel patches, it is possible that a longer implant duration would reveal differences in remodeling and perfusion efficiency. It is also possible that with the high density of microvessels in both aligned and isotropic patches, the improved efficiency that might come from an aligned microvessel network was unnecessary for meeting the metabolic demands of the patch and therefore, was not observed. If this was the case, the benefits of alignment could be elucidated with the addition of a highly metabolic cell type, such as cardiomyocytes.

The implantation of a microvascular patch for 6 days had no effect on the ejection fraction, fractional shortening, or infarct size, confirming our expectation that a microvessel patch lacking cardiomyocytes would not be efficacious itself. Several studies have shown that the transplantation of cardiomyocyte patches can improve cardiac function after a myocardial infarction.^{20,22,36} Rather than serving a therapeutic role, the microvessel patches in the present study could be combined with a cardiomyocyte patch to provide rapid blood flow to the implanted heart patch. This could allow for the implantation of larger patches and higher numbers of cardiomyocytes.

The perfusion of these engineered tissue patches containing aligned human microvessels is promising for the future of tissue engineering, especially in the creation of highly metabolic tissue constructs. The next step would be to integrate these aligned patches with a third cell type, such as cardiomyocytes, to create pre-vascularized heart patches. Even more, these patches would greatly benefit from the development of a hierarchical vascular network containing microvessels, such as those created in this study, connected to larger diameter arteriole and venule-like engineered vessels. The addition of larger diameter vessels integrated within the microvessel patches would allow for direct microsurgical attachment to host vasculature and therefore, immediate perfusion.

Conclusion

These pre-vascularized aligned tissue patches, which contained the highest reported density of engineered microvessels at implantation, inosculated with host vessels and were perfused within 6 days of implantation on the epicardial surface post-infarction. In this study we

report the highest perfused lumen density of aligned pre-vascularized microvessels, with no difference in the percentage of human microvessels that were perfused for the aligned versus isotropic patches at this early time point. The rapid inosculation and perfusion of these aligned, pre-vascularized tissue patches containing capillary-size microvessels is a major step forward toward the goal of creating a thick, beating heart patch that mimics native anatomical structure and can sustain high metabolic demand.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported by the National Institutes of Health [R01 HL108670 to R.T. and 5T32GM008347-24 to S.R.] and the University of Minnesota UROP [to D.M.].

The authors would like to thank Patrick Zhang and Ling Gao for surgical assistance, Naomi Ferguson for cell culture assistance, Susan Saunders for cryosectioning and histology, Sandy Johnson for technical assistance, and Jake Siebert for assistance with image quantification.

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Figure 1.

Patch Alignment and Implantation. A. Aligned patch before compaction and alignment and B. after compaction and alignment. C. An isotropic patch that was restricted from compacting laterally. D. Implantation of aligned patches onto the infarcted region of the left ventricle. Arrow = LAD ligation suture, arrowheads = patch, A = apex. Scale bar = 5 mm.

Figure 2.

Pre-Implant Patch Characterization. Top-down view of an A. aligned microvessel patch (n=14), B. isotropic microvessel patch (n=6), and C. aligned PC patch (n=12) via confocal microscopy. D. Cryosection orthogonal to the longitudinal direction for an aligned microvessel patch, E. isotropic microvessel patch, F. aligned PC patch. Red-hCD31, green-GFP transduced PCs, blue-Hoescht-labelled nuclei, scale bar = 100 μm . G. Lumen density of patches measured from histological cross-sections, H. PC Recruitment, or fraction of PCs in contact with vessels, I. Network length, or the sum of the vessel lengths per mm^2 , J. Anisotropy index, a measure of the degree of alignment with a value of 1 being isotropic (dashed line). Data represented as the mean $\pm$ standard deviation. * indicates a difference from isotropic microvessel patch, \$ indicates a difference from aligned PC patch, $p < 0.05$, student's t-test (G-I), 1-way ANOVA + Games-Howell post hoc test (J).

Figure 3.

Patch Cellularity and Organization. Patches engrafted with the host and maintained a high density of human microvessels and PCs throughout the patch thickness for all treatment groups. A. An aligned microvessel patch (n=6), B. an isotropic microvessel patch (n=6), and C. an aligned PC patch (n=5) all showing uniform cell density throughout the patches. A. shows increased perfusion of human microvessels near the edge of the patch. Red-UEA-1 human perfusion label, magenta-hCD31, green-GFP-PCs or IB4 rat perfusion label, blue-Hoescht, scale bar = 100 μ m. D. A heart section stained for cardiac troponin T (red), imaged and stitched together. GFP-PCs mark the patch location. LV = left ventricle, RV = right ventricle, scale bar = 500 μ m. E. Trichrome stain showing an isotropic microvessel patch and the cell-dense collagenous interface between the patch and the myocardium. F. Patch alignment 6 days post-implant for aligned microvessel patch, G. isotropic microvessel patch, and H. aligned PC patch. Double arrows indicate alignment direction, scale bar = 100 μ m.

Figure 4.

Patch Perfusion. A. Confocal micrograph displaying an area of an isotropic microvessel patch that had both perfused and non-perfused vessels. A few perfused human vessels (hCD31-magenta and UEA-1-red) are marked by white arrowheads and an example of a non-perfused vessel (magenta only) is marked by a thin white arrow. The bold white arrows mark rat vessel lumens (IB4-green) among the transplanted PCs (GFP-green). B. An example of inosculature of a perfused human vessel (magenta and red) with a rat vessel (green). Arrow heads point at the human vessel and bold arrows point at the inosculating rat vessel. C. Rat RBC stain (yellow) showing many RBCs inside perfused human microvessels (UEA-I-red) D. H&E stain showing an aligned microvessel patch with many lumens containing RBCs. E. Vessel density of perfused rat, perfused human, and total human vessels in the patch for aligned microvessel patch (n=6), isotropic microvessel patch (n=6), and

aligned PC patch (n=5). F. Fraction of human vessels that were perfused in the patch. Scale bar = 50 μ m. Data represented as mean $\pm$ standard deviation. 1-way ANOVA (E) and student's t-test (F) revealed no differences among the groups ($p > 0.05$).

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Figure 5.

Lumen Diameters. A. Summary of lumen diameters for Aligned and Isotropic microvessel patches pre-implant (n=14 aligned, n=6 isotropic) and 6 days post-implant (n=6 aligned, n=6 isotropic). B. Histogram of lumen diameters for aligned microvessel patches and C. isotropic microvessel patches to show the variation in lumen diameter pre- and post-implant. * indicates a difference from pre-implant lumen diameter by student's t-test, $p < 0.05$.

Patch dimensions before implant, and their estimated fibrin concentration. * indicates a constrained direction. Data represented as mean $\pm$ standard deviation. Student's t-test comparing aligned microvessel and aligned PC patches revealed no difference in width (aligned microvessel patch n = 31, aligned PC patch n = 40), 1-way ANOVA among all patch groups revealed no differences in thickness (p > 0.05) (aligned microvessel patch n = 18, isotropic microvessel patch n = 9, aligned PC patch n = 14).

Table 1

Patch	Length (mm)	Width (mm)	Thickness (mm)	Est. [Fibrin] (mg/ml)
Aligned Microvessel	8.4 *	1.6 $\pm$ 0.4	0.31 $\pm$ 0.08	33
Aligned PC	8.4 *	2.2 $\pm$ 0.3	0.38 $\pm$ 0.12	24
Isotropic Microvessel	8.4 *	7 *	0.39 $\pm$ 0.12	36

* These dimensions were constrained