IMPLANTATION AND CHARACTERIZATION OF
TISSUE ENGINEERED MICROVASCULAR GRAFTS

by

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DEDICATION

This is for my family.
You’ve afforded my every opportunity to succeed.
I had no choice.

To my beautiful wife Sarah:
Every man should be so lucky as to feel your love, support and understanding.
My greatest achievement is our family.

“All who are treated by this remedy recover in a short time, except those whom it does not help, who all die. Therefore, it is obvious that it fails only in incurable cases.”
Galen, modified by Holmes
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ABSTRACT

The socioeconomic constraints generated by patients with cardiovascular disease necessitates the development of novel treatment strategies for the pathologies associated with disease progression. One promising field of active research and development is Cardiovascular Tissue Engineering. It is believed this discipline will ultimately provide alternative strategies for the development of vascular bypass conduit, bioprosthetic valves, functional microvascular networks, and solid organ replacement tissue. The primary goal of this project was to test the hypothesis that, following implantation, tissue engineered microvascular grafts are capable of inosculation with host coronary vasculature and attenuating the loss of ventricular function following acute myocardial infarction. To test this hypothesis, microvascular grafts were constructed of adipose-derived microvascular fragments suspended in a 3-dimensional matrix. These tissue engineered grafts were transplanted and evaluated in a number of in vivo research scenarios. Research protocols were designed to critically evaluate the potential of microvascular network grafting in multiple tissue sites, and in differing pathophysiologic conditions. Microvascular grafts were initially implanted and studied in a subcutaneous position in recipient animals. Following implantation, the microvessel network within the grafted construct established spontaneous anastomotic connections with the host. Inosculation of the grafted microvessles and host circulation occurred rapidly following surgical placement, with evidence of significant vascular remodeling within the graft. The experimental grafts were also evaluated in the cardiac position following acute
cardiac injury. Perfusion was realized through the grafted microvascular tissue. The resulting microvasculature was complete with well-formed arterioles, venules, and capillaries. It was established that development of left ventricular dysfunction following experimental coronary artery occlusion was abated in animals treated with epicardial placement of microvascular grafts. Interestingly, while there was overwhelming evidence of microvascular remodeling in both the subcutaneous and cardiac position, there was a noted tissue-specific adaptation that occurred. Grafts in the cardiac position had a higher vascular density than those in the subcutaneous position, and developed a vessel-type distribution that was approximate to that observed in native epicardium. The results described in this dissertation project support the utility of tissue engineered microvascular grafts for the treatment of pathophysiologic tissue within the cardiovascular system proper, as well as in peripheral systems.
1. Introduction

Cardiovascular Disease -

The prevalence of Cardiovascular Disease (CVD) continues to rise in the Western world at an alarming rate despite continued allocation of funds at the levels of treatment, research and prevention. Based on 1998 statistics released by the American Heart Association there are an estimated 60,800,000 Americans with one or more forms of CVD. This includes 50,000,000 people with high blood pressure, 12,400,000 people with coronary heart disease, and 4,500,000 people with stroke related illnesses. Additionally, in recent years there has been an explosion of obesity and Type II Diabetes – the associated complications of these diseases are hypertension, hyperlipidemia and atherosclerotic vascular pathologies. In fact, among the ten most common causes of death, Cardiovascular Disease has a higher mortality rate than the next 5 leading causes combined. In the year 2000 CVD claimed roughly 40% of lives in the US, or 1 in every 2.5 deaths. While the overall mortality due to Cardiovascular Disease has shown a decline over the past 20 years, the number of hospital discharges due to CVD has continued to rise (Figure 1.1). As a result of the increasing age of the American population this is a phenomenon that demonstrates devastating potential with respect to the socioeconomic structure of the American health care system and the American public in general.

Within the cardiovascular system proper, the most significant manifestation of CVD is coronary artery disease (CAD) and peripheral vascular disease (PVD). The gravity of the problem is well illustrated by the fact that in 2000 there were over 700,000 deaths as a
result of CAD alone. This mortality rate is slightly more than 1 in every 5 deaths in the US for the calendar year of 2000. There are over 12 million Americans that have been diagnosed with CAD, and roughly 1.1 million will experience a new or recurrent coronary attack each year.

It has long been a myth that Cardiovascular Disease is a “man’s disease”. This myth is not only being discounted by recent studies, but is being shown to be incredibly erroneous. CVD in the American female population is incredibly prevalent, and continues to rise at a rate much greater than that of their male counterparts. The overall mortality rate from CVD has been greater for females than for males in every year since 1984, and the disparity between the two sexes has continued to worsen in every year since that time. In 1984 there were approximately 460,000 deaths from CVD in both men and women. In the year 2000 there were approximately 400,000 male deaths and 505,000 female deaths as a result of CVD (Figure 1.2). The death rate for females as a result of CVD is greater than the next seven leading causes of death combined. To further illustrate this point it should be noted that breast cancer was responsible for 1 in every 29 deaths and CVD responsible for 1 in every 2.4 deaths in women in 2000.

Furthermore, while this is certainly a significant health care issue for both men and women in the United States, the incidence and progression of CVD is a health care concern of paramount proportions worldwide. According to statistics published by the World Health Organization and the American Heart Association in 2003, 16.6 million people around the globe die of cardiovascular diseases each year. In 2001 CVD contributed to nearly one third of all deaths. By the year 2010 the WHO estimates that
Hospital Discharges for Cardiovascular Diseases

Figure 1.1 Data taken from the American Heart Association® Heart Disease and stroke Statistics Update illustrating the rise in hospital discharges associated with cardiovascular disease since 1970. There has been a steady rise in the number of hospital discharges since 1970 and it is predicted that as the mean age of the western population rises, so will the incidence of CVD.
CVD will be the leading cause of death in all developing countries and that by 2020 there will be 25 million deaths worldwide attributed to CVD.

The indirect and direct costs in the treatment of CVD are currently a major economic limitation and liability for developed nations and the impact of the necessary resource allocation will continue to worsen as the incidence of CVD continues to rise. The National Heart Lung and Blood Institute estimates that the total direct and indirect cost of the treatment of CVD in 2003 alone was $351.8 billion. The socioeconomic ramifications of CVD are especially devastating in developing countries where those individuals who are at the peak of their mid-life productivity are those that are stricken with the disease. In developing countries, those individuals of lower socioeconomic classes have a higher prevalence of risk factors such as tobacco use, hypertension, poor nutrition and, therefore, have a higher incidence of disease and mortality.

While CVD is a broad term for many pathologic states including atherosclerosis, coronary artery disease, stroke, peripheral vascular disease and congestive heart failure, the underlying etiology common to all of these pathologic conditions is vascular dysfunction. The primary and often focal cause of the vascular injury that leads to CVD is chronic endothelial cell injury. This endothelial cell injury can be caused by a number of different things including persistent low-grade chronic inflammation, hypertension, viral infection, and the release of reactive oxygen species. The underlying vascular dysfunction can be manifested at the large vessel level, as in the setting of abdominal aortic aneurysms, or at the microvascular level as is seen in diabetic retinopathy, or global myocardial ischemia and hibernating myocardium. Additionally, because CVD is
truly a systemic problem that is rarely, if ever, localized to a single organ system or tissue site, the clinical symptoms associated with CVD are usually progressive and cumulative. As a result of the large scale of vascular involvement in advanced CVD, this is the primary target for preventative and therapeutic intervention and treatment of CVD.

Indeed, while the symptoms associated with advanced CVD are manifested throughout many organ systems including the renal, and respiratory systems, disease of the cardiovascular system is primary cause of these failures. The progression of CVD from the cardiovascular system itself to additional organ systems exquisitely illustrates the integrated role of the vascular system in all organ systems of the body. For example, pathologic involvement of the renal system in CVD, which can result in chronically compromised renal function and, ultimately, renal failure, is secondary to focal vascular pathologies at the level of the renal vasculature and/or the whole animal level as a result of systemic hypertension. The vascular involvement in these patients ranges from focal lesions of the renal arteries, to diffuse renal infarction and necrosis as a result of microvascular obstruction and/or systemic hypertension. Furthermore, the consequences of the pathophysiologic condition of the renal system further exacerbate the state of CVD at the whole-animal level through activation of the renin-angiotensin-aldosterone system.

**Cardiovascular Disease – Coronary Artery Disease**

The overwhelming majority of deaths due to CVD are as a result of Coronary Heart Disease (CHD) also known as Coronary Artery Disease (CAD). In fact 54% of deaths related to CVD in 2000 were due to CHD. As is the case with hospital discharges due to
Leading Causes of Death for All Males and Females in the United States: 2000

Figure 1.2 Data from the American Heart Association® indicating the leading cause of death in the United States for the year 2000.
CVD in general, the number of hospital discharges due to CHD has continued to rise since 1970 for both males and females.

The major cardiovascular disease states that involve the heart are coronary artery disease, hypertensive hypertrophy, congestive heart failure, bacterial endocarditis, rheumatic heart disease, idiopathic cardiomyopathy, ischemic cardiomyopathy, valvular dysfunction, and congenital heart defects (Figure 1.3). Again, of all the heart related manifestations of CVD, CAD is responsible for a large majority of the morbidity and mortality associated with. In the year 2000 there were over 510,000 deaths as a result of CAD and 47,500 of Congestive Heart Failure.

**Coronary Artery Disease - Atherosclerosis**

The underlying cause of most cases of CAD in the adult population is atherosclerosis. Atherosclerosis is a chronic disease state of elastic arteries that is accurately defined as a progressive narrowing of the arteries that will often lead to complete arterial occlusion and cessation of distal blood flow. There are many different risk factors for the development of clinical atherosclerosis, however the atherogenic process is thought to be primarily the same in most cases. Atherogenic lesions occur in certain tissue sites more readily than others and it is in these areas that the majority of clinical symptoms occur in the general patient population. The vascular sites most often affected by atherosclerosis are the coronary arteries, abdominal aorta, popliteal artery, thoracic aorta, and the circle of Willis. It is currently believed that the initiation of plaque formation is due to chronic endothelial cell injury. The onset and sustention of endothelial injury can be due to a
Percentage Breakdown of Deaths from Cardiovascular Diseases

Figure 1.3 A graphical representation of the contribution of specific manifestations of Cardiovascular Disease to the overall death rate attributed to CVD. The overwhelming majority of CVD-related deaths occur as a result of Coronary Heart Disease (54%).
number of things including chronic low-grade inflammation, repeated physical trauma, viral or bacterial infection, toxins from cigarette use, hypertension, hyperlipidemia, and irregular flow patterns. As a result of repeated endothelial cell injury, there is a resulting endothelial dysfunction that leads to increased permeability, leukocyte adhesion, monocyte adhesion (Duplaa 1996), and diapedesis (Ross 1986), (Ross, 1993). These areas become laden with macrophages and T-Cells, which engulf low density lipoprotein (LDL) and very low density lipoproteins (VLDL), thus becoming foam cells.

The initial development of foam cells is noted pathologically as the deposition or presence of fatty streaks. These fatty streaks are though to be precursors to focal areas of atheroma formation. The continuing development of foam cells within these fatty streaks leads to the local release of potent mitogens from the foam cells themselves. The release of localized mitogens results in the proliferation of medial vascular smooth muscle cells (SMC), deposition of extracellular matrix and other extracellular lipids. The proliferating SMCs also ingest a vast amount of lipids, which leads to a worsening of the problem through the further development of foam cells and subsequent release of potent mitogens which will cause increasing levels of SMC proliferation.

The developing atheroma poses a number of significant potential problems including reduction in luminal size, reduced distal blood flow, increased vessel wall stress, vessel occlusion, weakening of the vessel wall, aneurysm formation and plaque rupture. The development of arterial occlusion can occur as an acute event following plaque rupture, emboli formation and occlusion distal to the focal lesion, or as a result of thrombus formation and occlusion at the site of the lesion itself. In both cases, immediate cessation
of blood flow causes initial ischemia and hypoxia to distal tissue, and, unless blood flow is restored, this will lead to infarction and tissue necrosis.

**Coronary Artery Disease – Myocardial Infarction**

The maintenance of coronary blood flow is critical to the survival and function of cardiac myocytes. While atherosclerotic lesions can affect many different vascular and tissue sites, the setting of coronary atherosclerosis is particularly devastating to the myocardium. As the pathologic condition of coronary arteries affected by atherosclerosis worsens the likelihood of adverse cardiac events increases. Occlusion of the coronary arteries can occur in this setting as a result of a number of different developments including primary occlusion due to thrombus formation, plaque rupture and distal occlusion of the coronary vasculature, and occlusion due to circulating emboli (Figure 1.4).

Unrelieved occlusion of a major epicardial artery can lead to transmural infarction of the myocardium, usually within the ventricular wall (Reimer, 1977). Furthermore, if the distal tissue is not revascularized the initial infarct site will continue to grow for a period of time in a “wavefront” fashion as a result of normal wound healing and inflammation. In the setting of acute myocardial infarction, a cascade of events is started that will ultimately lead to apoptotic cell death and tissue necrosis. Because cardiomyocytes lack any appreciable regenerative capacity, the loss of cardiomyocytes following myocardial infarction is permanent. While there is emerging evidence of a limited subpopulation of cardiac myocyte capable of regeneration in the adult human heart (Nadal-Ginard, 2003),
Figure 1.4 Figure illustrating the progression of plaque deposition in the coronary arteries, and the reduced lumen size as plaque deposition increases. This reduced lumen size leads to a reduction in coronary blood flow which causes myocardial ischemia. The area of ischemia distal to an occluded coronary artery is illustrated at right.
it is clear that the adult heart lacks the ability for adequate repair following such an injurious assault as AMI. It has yet to be demonstrated in any mammalian species, let alone human, that there is any evidence for substantial repair within the myocardium that would restore ventricular function, de novo. Indeed, while there may be survival of the initial events following myocardial infarction, many patients die following infarct expansion or as a result of left ventricular systolic failure as functional units of the myocardium are lost.

When a coronary artery is occluded and there is not timely restoration of blood flow, there is a permanent process of myocardial ischemia and infarction that occurs. This process of myocardial infarction, although due to an acute event, is not an acute development. Myocardial necrosis usually begins within 20-30 minutes after coronary artery occlusion. The most pronounced area of perfusion deficit and infarct formation is usually the subendocardial region. Because this area experiences low perfusion under normal circumstances and it is exposed to extremely high wall pressure and compressive forces, it is an area that is predisposed to ischemia and infarction. The ultimate size of the infarct is dependent on the location in the vascular tree that the occlusion occurred, the condition of the surrounding vasculature, and the time period in which thrombolysis or mechanical opening of the occlusion occurs (Waller, 1988).

In the initial period immediately following coronary occlusion there is an episode of acute inflammation characterized by the infiltration of polymorphonuclear cells. Over the next two to three days this acute response is replaced by a more chronic inflammation and repair response that is characterized by the presence of macrophages, lymphocytes
Inflammation and Wound Healing

Figure 1.5 Time course of inflammation and wound healing in the myocardium following coronary artery occlusion. The acute phase is characterized by infiltrate of PMNs while the macrophage and fibroblast involvement predominate in the chronic stages.
and fibroblasts (Figure 1.5). As the population of macrophages continues to engulf the dead cardiac myocytes, this muscular tissue is replaced by fibrous scar tissue that is produced by resident and recruited fibroblasts. (Narula, 1996) Additionally, at or around seven to 14 days there is a significant vascular response that is seen within the granulation tissue that is forming. This tissue experiencing a robust angiogenic response will undergo a subsequent angioregression and leave behind an acellular, avascular myocardial scar (Olivetti, 1997), (Searle, 1982), (Cannon, 1977), (Parodi, 1993), (Cheng, 1995). The resulting size of the infarct is of critical importance to patient survival. It has been shown that infarcts $\geq 40\%$ of the left ventricle are predictors of cardiogenic shock and death (Page, 1971).

**Myocardial Ischemia and Angiogenesis**

The clinical condition of myocardial ischemia is as significant as that of acute myocardial infarction. The primary manifestation of myocardial ischemia in the patient is angina pectoris, or chest pain. There exists a large patient population with angina following moderate exertion as well as those patients that suffer from angina brought on by the mildest of physical demands. For the large majority of these patients, pain is only relieved by pharmacologic intervention, and there are a select few for whom this option is also inadequate.

As is the case with myocardial infarction, the primary cause of myocardial ischemia is vascular dysfunction as a result of atherosclerosis. Because the heart is an organ that has a very low ischemic tolerance, the development of prolonged episodes of regional or
global ischemia has significant pathologic consequences. The persistence of ischemia within the myocardium will ultimately lead to areas of infarction. It is important to note that the development of infarction can occur as a result of persistent ischemia and does not require occlusion of a major coronary artery (Piek JJ, 1988), (Falk, 1985), (Andersen 1989).

In response to prolonged bouts of ischemia, the mammalian heart undergoes a number of adaptive responses aimed at revascularizing the ischemic tissue and restoring perfusion levels. The most widely studied adaptive process to date is that of angiogenesis. Angiogenesis is the process by which new capillaries sprout and differentiate from pre-existing post-capillary microvascular networks. The angiogenic process can proceed through a number of different pathways including dilation and enlargement of post-capillary venules, elongation and increased branching of these venules, and physical interruption of existing vessels (intussusception), which produces new branching segments. In the setting of angiogenesis in response to ischemia, the new vasculature is comprised of primarily capillaries, which is thought to be an adaptive response aimed at increasing nutrient exchange in the ischemic/hypoxic tissue.

The initiation of the angiogenic response is caused by tissue hypoxia and leads to increased production of a family of transcription factors known as HIFs or hypoxia inducible factors. The HIFs are HIF-1β, HIF-1α, and HIF-2α, and mediate the response to hypoxia by binding to specific DNA sequences, which regulate the transcription of genes that govern the cellular response to hypoxia, several of which are regulators of
Angiogenesis (Sellke, 1995). The specific angiogenic growth factors that have garnered the most scientific and clinical attention are VEGF, FGF and the Angiopoietins. The creation of new blood vessels requires the presence of angiogenic growth factors, however this is not sufficient to drive the angiogenic production of new vascular conduit. There must be basement membrane degradation, extracellular matrix remodeling and capillary lumen formation. These processes are dependent on the presence of a myriad of proteases and protease inhibitors. Critical to the angiogenic development of blood vessels is the activity of the matrix metalloproteinases (MMPs) and the plasminogen activators u-PA and t-PA, which convert plasminogen to plasmin. The presence of plasmin will lead to the activity of MMPs which are responsible for the breakdown of proteins in the extracellular matrix such as fibronectin, laminin, and the core of the proteoglycan glycocalyx (Dvorak, 1986), (Haas, 1999), (Mignatti and Rifkin, 1996). The further steps in the angiogenic process require the presence of the aforementioned specific factors, VEGF, bFGF, nitric oxide, endothelin, platelet derived growth factor, and Angiopoietin-1. The release of this compliment of growth factors, at the appropriate levels, and in the appropriate temporal sequence leads to endothelial migration, proliferation, new vessel formation, maturation, and the production of function vascular segments (Buschmann and Schaper, 2000), (Griffioen and Molema, 2000), (Henry, 1999), (Tomanek and Schatteman, 2000). It is critical to the physiologic angiogenic response that the proper growth factors be expressed in a coordinate manner. In the setting of coronary ischemia, NO plays a crucial role in regulating proper VEGF function (Matsunaga, 2000), which may in turn be
regulated by the presence of endothelin-1 (Goligorsky et al., 1999). The recruitment of additional cell types that are needed for vascular development has been hypothesized to be dependent on the release of PDGF. The steps of vessel maturation and stabilization are thought to rely on the expression of TGF-β and the glycoprotein Angiopoetin-1 (Folkman, 1996).

**Hibernating Myocardium**

The pathogenesis of myocardial ischemia is not fully encompassed by a discussion of myocardial infarction and angiogenesis. The condition of chronically hypoperfused myocardium can also produce a condition known as hibernating myocardium (HM). The traditional assumption in the treatment of patients with ventricular dysfunction was that heart failure arose from either reversible ischemia or irreversible forms of vascular insufficiency. However, since the mid-1980s the condition of hibernating myocardium has been recognized as a distinct state of diminished resting ventricular function in the setting of coronary artery atheromatous narrowing (Rahimtoola, 1985). Recent clinical data suggest that it occurs in at least one third of patients with chronic coronary insufficiency. The development of hibernating myocardium is thought to be protective in nature – the condition of chronic hypoperfusion of the myocardium leading to downregulation of myocardial contractility leading to a loss in ventricular function. The current clinical hypothesis also holds central the concept that therapeutic intervention and restoration of perfusion will partially or completely restore the loss in myocardial contractility.
There have been a number of clinical observations recorded that have lead to the discovery of the pathophysiologic state of hibernating myocardium. The restoration of normal wall motion following coronary revascularization has been reported as early as 1970s (Chatterjee, 1973), (Chatterjee, 1977). Additionally, Stinson and Billingham published reports of left ventricular wall abnormalities in hearts that were absent of left ventricular scarring, suggesting that the abnormal wall motion was not due to myocardial infarction (Stinson and Billingham, 1977).

Of patients with atherosclerotic disease of the heart, it is predicted that as many as 40% have clinically significant development of HM (Lewis et al., 1991), (Schelbert and Buxton, 1988). In the condition of HM the contractile cells of the myocardium appear to downregulate much of their contractile machinery, yet remain viable. At the microscopic level there are a number of structural changes that occur including depletion of contractile elements and changes in the extracellular connective tissue matrix. The changes in contractile elements are seen as a loss of myofilaments, loss of sarcoplasmic reticulum, aggregation of glycogen, glycogen plaques, reduction in T-tubular invaginations and sarcomeres, and increases in endoplasmic reticulum, and number of mitochondria (Heusch, 1998), (Schwarz, 1996), (Ausma, 1998), (Hennesey, 1998), (Ausma, 1995), (Willems, 1996). There is a disorganization of cytoskeletal proteins such as desmin, tubulin, and vinculin. Extracellular matrix modifications that are characteristic of HM are primarily a function of increased interstitial fibrosis. The matrix is filled with increased levels of collagen type I, type III, type VI, laminin and fibronectin, but not, interestingly, collagen type IV (Matsuzaki, 1983).
While the cumulative effects of these changes to the myocardium can result in the regional loss of ventricular function, it is essential to note that the definition of HM provides that this function can be restored through revascularization of the coronary circulation. Thus HM represents a form of adaptation and not necessarily a situation of irreversible degeneration of the myocardium. For the patient with diffuse disease of the coronary vasculature and subsequent severe loss of ventricular function there is promise for the restoration of that function through treatment modalities aimed at increasing resting perfusion levels to areas of hibernating myocardium.

**Microvasculature in Cardiac Pathophysiology**

The myocytes of the myocardium extract 70% of the oxygen available in the blood during resting conditions. This level of oxygen extraction is comparable to the brain, and provides insight into the metabolic demands of this muscle bed. Consequently, in order for increases in metabolic demand to be met, there must be increased blood flow to the tissue, as there is no reserve in the level of oxygen extraction in order to supply the myocardium with more oxygen and energy. Accordingly, there is an extraordinarily high level of vascularization of the myocardium at the level of the microcirculation. In fact, the vasculature makes up 37% of the volume of the myocardium, with the majority of this vascular tissue being microvasculature (Weber et al., 1987). Thus, dysfunction at the level of the microvasculature will have significant repercussions for the myocardium and, consequently, cardiac performance and function.
Microvasculature in Ischemic Heart Disease and Heart Failure

Because of the extreme level of heterogeneity found in the microvasculature of the heart, there are specific areas of the heart that are more susceptible to ischemia than others. The heterogeneous response that is shown to injury is not well understood but should be well accepted. These variations in regional perfusion can be demonstrated using a variety of imaging techniques including scintigraphy, myocardial contrast echocardiography, and positron emission tomography (Schofer et al., 1985), (Widimsky, et al., 1988), (Schelbert, 1994), (Schelbert, 1994), (Schmermund et al., 1997).

Under normal conditions, the myocardium responds to ischemic injury by increasing vascular density. The increase in vascular density is due to a higher number of both capillaries and arterioles per volume tissue via angiogenesis and/or arteriogenesis as well as remodeling of existing vasculature (Jantunen, 1989), (Zielinski, 1991), (Zielinski, 1994). While there has not been large amount of research devoted to the phenomenon of microvascular dysfunction in cardiac pathology, recent studies are starting to elucidate the role of microvascular deficiencies in patients with LV dysfunction. Using myocardial contrast echocardiography it has been shown that patients with defective myocardial segments are more vulnerable to ischemia and left ventricular wall motion abnormalities (Shimoni et al., 2002). Furthermore, because of the large degree of heterogeneity in the microvascular system, there can be localized areas of ventricular dysfunction in the
Figure 1.6 Schematic representation of Coronary Artery Bypass Grafting to the Right Coronary, Left Anterior Descending, and Left Circumflex Arteries.
setting of disease states that may affect the whole heart such as ischemia reperfusion, microvascular spasm, and myocarditis. Interestingly, while these results would suggest that ischemia leads to LV dysfunction the reverse may also be true.

While there has been much energy focused on revascularization strategies for the major epicardial arteries and distal tissue, a patent epicardial artery does not equate with tissue perfusion at the microvascular level. The concept of no-reflow, where blood flow through the microvasculature is stopped, even in the presence of a patent feeder artery, was first noticed in the brain (Majno et al., 1967). However, this phenomenon has been noted extensively in the heart. Research animals and Patients that have undergone coronary artery bypass grafting or percutaneous transluminal coronary angioplasty have been observed to have limited perfusion to certain areas of tissue perfused by the re-opened coronary artery (Kloner et al., 1974), (Wilson et al., 1989).

It has been proposed that the presence of a microvascular bed that is resistant to flow is a selective adaptation in that it reduces flow to areas of necrotic tissue that are prone to vessel wall rupture and aneurysm formation. However, this reduction in flow to areas of necrosis also reduces flow to areas of viable myocardium surrounding the necrotic core. This re-distribution of blood flow predisposes the areas surrounding necrosis to further infarction, leading to further loss of ventricular function (Gavin et al., 1998).

In the situation of heart failure, where large portions of the ventricle have been lost to infarction, the microcirculation is responsible for meeting greater demands of the viable myocardial tissue. Microvascular dysfunction in the spared myocardial tissue would lead to further myocyte loss, which would severely compromise the function of this tissue as
well. This is illustrated by studies that have shown that in areas of myocardium adjacent to myocardial infarction, the presence of patent microvasculature confers functional significance. There was greater functional impairment in areas at risk in those patients that lacked a patent microvasculature (Gerber et al., 2000). Furthermore, it has been shown in animal models that restoration of blood flow results in improved function for non-infarcted areas of the heart, supporting the concept that infarct expansion can be attenuated at the level of the microcirculation (Michael et al., 1999), (Richard et al., 1995). Clearly, the microvasculature is critical to the maintenance of normal cardiac function, and to the progression of cardiac pathophysiology.

**Therapeutic Approaches to Myocardial Revascularization**

The history of therapeutic intervention to correct myocardial ischemia dates back to the early 1930s. Beck was the first surgeon to attempt to revascularize the heart through surgical intervention. Beck used sandpaper, emory boards or special metal burs to abrade the surface of the epicardium. In doing so, he caused a large local inflammatory response that would generate a large amount of granulation tissue accompanied by a neovascular and angiogenic response. In addition, to provide an extracorporeal circulation to the heart, a portion of the pectoralis muscle was dissected free and attached to the heart. This procedure bore resemblance to the future attempts at cardiomyoplasty as well as the Vineberg procedure (Beck, 1932), (Beck, 1935).
Coronary Artery Bypass Grafts

Attempts at surgical revascularization of the myocardium were greatly progressed in the early 1960s by Vineberg (Vineberg, 1964). The Vineberg procedure involved the isolation, dissection and transsection of the left internal mammary artery. The freed LIMA was then anastomosed into a tunnel that had been made into the myocardium. While this was not a true Coronary Artery Bypass Graft as is done today, the concept of providing a new supply of arterial blood to the myocardium was the same. The Vineberg procedure was the first grafting procedure to show evidence of myocardial revascularization and did show success in a number of patients. There is angiographic evidence of Vineberg grafts remaining patent for as long as 27 years (Marx, 2001).

The procedure now known as Coronary Artery Bypass Graft (CABG) was first performed in 1967 by Favaloro and was later refined by DeBakey and Favaloro (Favaloro, 1968). Today there are over 600,000 CABG procedures performed annually, and the majority of those procedures use either saphenous vein grafts, internal mammary artery grafts, or synthetic grafts (Taggart, 1999), (Tomizawa, 1995), (Canver, 1995). Following revascularization of the myocardial tissue distal to the bypassed lesion, the increased perfusion leads to improved wall motion and ventricular function. This is thought to be due to the rescue of hibernating myocardium (Rinaldi et al., 2000).
**Percutaneous Transluminal Coronary Angioplasty**

Efforts to minimize the stress, morbidity, and mortality associated with surgical intervention, as well as the financial burden incurred by hospital and patient, have lead to the development of minimally invasive techniques to treat atherosclerotic coronary lesions and myocardial ischemia. Percutaneous Transluminal Coronary Angioplasty (PTCA) was first performed by Andreas Gruntzig in 1977 and is now performed nearly 600,000 times annually in the US (Gruntzig, 1978). The procedure involves the insertion of a catheter into the arterial circulation and advancement of that catheter into the coronary circulation. The catheter is then placed in the lumen of the stenosed coronary artery and a balloon around the catheter is inflated. The opening of the balloon allows for a physical widening of the coronary artery and improved distal flow. Additionally, this approach allows for minimally invasive intervention of coronary artery grafts that have undergone a stenosis injury themselves (Kaul, 1999). This method of percutaneous intervention avoids what is known as a “re-do” procedure that carries with it markedly increased levels of morbidity and mortality (Kaul, 1999).
Figure 1.7 Schematic representation of Percutaneous Transluminal Coronary Angioplasty. A catheter is inserted into the stenosed vessel and a balloon is then inflated around the catheter in order to re-open the vessel lumen.
Novel Therapeutic Interventions

Despite continued advances in CABG, PCTA, coronary stenting, and medical therapy through the use of ACE inhibitors, β-blockers, and statins, an ever-growing number of patients exists that cannot be managed by these treatment modalities. Furthermore, while many patients are initially helped by the aforementioned therapeutic interventions, a significant number of patients undergo continued disease progression leading to a state where they are longer amenable to these treatment options (Atwood et al., 1990), (Bourassa et al., 1992). Fueled by increasing numbers of CVD patients, the human genome project, advances in tissue engineering, and stem cell research, there has been a surge in the development and research of new therapeutic interventions to treat cardiovascular disease in recent years – specifically ischemic heart disease and heart failure. These treatments have included the transplantation of adult stem cells into infarcted and ischemic myocardium, the introduction of angiogenic growth factors through gene therapy, and the placement of tissue engineered constructs and polymers that secrete angiogenic factors. In the laboratory many of these approaches have shown promise, but the clinical benefits of these treatment modalities have not been realized on a large scale.

Therapeutic Angiogenesis

One of the most widely studied alternative therapies for the treatment of both CAD and PVD is therapeutic angiogenesis. The primary goal of therapeutic angiogenesis is essentially the pharmacologic augmentation of collateral circulation formation through
the use of angiogenic growth factors and/or the genes encoding these growth factors. The two growth factors that have been studied most extensively, both in the laboratory and in the clinic, are VEGF and FGF. These growth factors have each been administered in protein form as well as the genes encoding these proteins, and have been given both through intravenous and direct intracoronary injection routes.

The administration of VEGF and FGF, all isoforms, has given mixed results, at best. In pre-clinical animal studies there has been positive, if inconsistent, results demonstrated for both methods of therapeutic angiogenesis. However, in the clinical setting, the results that have been shown following therapeutic angiogenesis treatments have either been transient in nature or not statistically different from placebo groups enrolled in the same studies. In fact initial studies with aFGF were not only inconclusive but in one study demonstrated the possibility of vascular occlusion secondary to smooth muscle cell proliferation following aFGF administration (Ware and Simons, 1997), (Uchida et al., 1995), (Banai et al., 1991).

Clinical studies using these growth factors have also been incomplete and often produced contradictory findings. The first randomized clinical study of any angiogenic growth factor was the Vascular Endothelial Growth Factor in Ischemia for Vascular Angiogenesis (VIVA) trial (Henry et al., 1999). The VIVA study demonstrated a lack of clear improvement following VEGF treatment versus placebo groups. There was no difference between treatment and placebo groups in exercise treadmill time, rest and exercise perfusion imaging or angina class and quality of life measurements at 2 and 4 months after treatment.
It has become increasingly apparent that patient selection is one of the most critical steps in the future development of therapeutic angiogenesis as a viable treatment option. There is a large cohort of patients for which therapeutic angiogenesis is not a prudent therapeutic approach, and an equally large group of patients that may see some benefit from such procedures. There are patient characteristics that appear to be better suited for therapeutic angiogenesis and they include the presence of a single occlusion that supplies viable myocardium, and multi-vessel diffuse disease with evidence of inducible ischemia. These patients will have undergone a level of natural collateralization and angiogenesis within the myocardium, and while they may be deficient in these processes this appears to be a critical element to the success of these approaches.

With these selection criteria in mind, it should be noted that these criteria exclude those patients with resting angina, and those patients that suffer from acute events. The exclusion of these patients is due primarily to the time course of vascularization required during the administration of angiogenic growth factors or the genes encoding them. There is approximately a 14 to 21 day period required for the development of new vessels in this setting and this does not allow for timely revascularization for the patient suffering from unstable angina, and more critically, the patient experiencing acute coronary episodes. Furthermore, there are a host of cardiac medications that are know to be anti-angiogenic in nature and as a result would be contraindicative. Aspirin, isosorbide dinitrate, spironolactone, furosemide, captopril and lovastatin are all medications that may be critical for the cardiac patient, but that would inhibit angiogenesis following growth factor treatment. As a result, while these patients may be in need of medical
intervention at the level of the cardiac microvasculature, without removal of these pharacologic agents, therapeutic angiogenesis would not be a viable option.

**VEGF**

VEGF was discovered in the early 1980s as a factor that made the vasculature leaky and was initially given the name Vascular Permeability Factor (VPF). (Senger, 1983), (Dvorak, 1995) Years later it was discovered that VPF was able to stimulate endothelial cell migration and replication and that VPF was a potent angiogenic factor in vivo and it subsequently took the name Vascular Endothelial Growth Factor (Connolly, 1989). VEGF has been found in five isoforms and those isoforms are VEGF 121, 145, 165, 189, and 206, with the numbers referring to the amino acids in each variant. VEGF₁₆₅ is the predominant form of VEGF produced and as a result has become the most widely used and studied form of VEGF. In situ hybridization and mouse knockout studies have shown that VEGF is ubiquitously expressed during development and that it plays a major role in angiogenesis. Loss of a single copy of the VEGF gene results in embryonic lethality. Further developmental and in vitro studies have revealed that VEGF associates primarily with two receptor tyrosine kinases, flt-1, and flk-1/KDR. The expression of these receptors is primarily on the Endothelial Cell surface and therefore the primary effects this cytokine are at the level of Endothelial Cell.

Studies that have involved the use of solely VEGF as an angiogenic strategy have demonstrated that the resulting vasculature is comprised of primarily a capillary component and that these vessels lack the pericytes and mural cells normally associated
with larger caliber vessels. As a result of this highly capillary rich environment, there can be some improvement in basal perfusion levels, however, stress perfusion, either exercise or pharmacologic, does not improve after treatment. This is believed to be due to a lack of arterioles present in the newly developing collateral vasculature (Mack, 1998), (Hendel, 2000). Furthermore, over administration of VEGF can result in systemic hypotension and edema formation.

**FGF**

Fibroblast Growth Factor (FGF) is another prevalent protein found in the setting of angiogenesis. Currently the FGF family consists of 15 members, and are all heparin-binding growth factors. There are two very common forms of FGF, basic FGF and acidic FGF. The more commonly studied form of FGF is bFGF, and it is found to exist in four isoforms characterized by different molecular weights. The exact role and mechanism of the FGFs in vivo, has been difficult to understand, and is still has not been completely elucidated. Knockout studies have provided some insight as well as some additional questions to the mechanisms behind the FGFs. Homozygous FGF knockout mice appear to be normal, and are found to have decreased vascular tone and low blood pressure (Zhou et al., 1998). Additional knockout studies have shown that the FGF2 mice have delayed wound healing and some show evidence of neuronal defects. Despite these phenotypic changes, the most notable discovery from these knockout studies is that the animals survived and were viable. This suggests that FGF, while obviously present in
such angiogenic circumstances as wound healing and tumor development, is not critical to either neovascularization or angiogenesis.

Reports from studies where FGF was administered to ischemic myocardial tissue show that there is an increase in the presence of arterioles and not in capillaries, which further suggests distinct roles for FGF and VEGF in angiogenesis (Watanabe, 1998). However, while there have been reports of clinical success using bFGF at the time of CABG, there have been additional studies that indicate there is no consistent pattern of improvement in either global stress perfusion or inducible ischemia.

Cell Transplantation – Cellular Cardiomyoplasty

Currently, the only viable option for patients with end stage heart failure is organ transplantation. However, due primarily to an organ shortage, the number of heart transplant procedures each year is greatly outnumbered by the number of patients that die as a result of heart failure and associated complications. Improvements have also been made in Ventricular Assist Devices as a destination therapy, however these devices are large and cumbersome and have an inherent rate of device malfunction and failure (Rose et al., 2001). Additionally, new surgical techniques and procedures are being developed for the treatment of the end stage heart failure patient. These procedures such as ventricular reconstruction (Dor et al., 1998), and endocardial patch plasty require the implantation of a material to be used as a replacement for akinetic, dilated ventricular
tissue. While the necrotic, infarcted, akinetic ventricle is removed and/or reinforced during these procedures, there is no new ventricular tissue that is generated as a result of these procedures. Accordingly, because there is no new myocardial tissue, there are no new ventricular myocytes, and while the process of ventricular decompensation may be attenuated there are no functional units added to the contractile apparatus of the heart. The central assumption in the treatment and understanding of heart failure is that failure develops when a critical number of cardiomyocytes has been permanently lost as a result of ischemia and infarction. Therefore, while these procedures may relieve the burden that the native heart is experiencing, for those patients that are truly in the latter stages of heart failure, without the addition of contractile units there can be no permanent recovery of ventricular function.

One potential method of adding functional contractile units to the adult heart is through cell transplantation or cellular cardiomyoplasty (CCM). While cell transplantation has been tried outside the cardiovascular system in efforts to treat other diseases such as Parkinson's Disease and Alzheimers, as well as to treat Diabetes (which is closely associated with CVD) the primary cardiovascular system application is in the heart. The process of cellular cardiomyoplasty aims to use an adult stem cell type that can be transplanted to the myocardium in an area of ventricular dysfunction, with the hypothesis that the transplanted cells will become functional cardiomyocytes. Cellular Cardiomyoplasty has been tried with a number of potential mesenchymal cell types including, hematopoietic stem cells, endothelial cells, fibroblasts, and skeletal muscle
stem cells, or satellite cells (Taylor, 2001), (Atkins, 1999), (Sim, 2003), (Menasche, 2003), (Menasche, 2003), Menasche, 2003), (Hagege, 2000), (Scorsin, 2000).

The potential success of CCM is reliant on two central beliefs which are 1) heart failure develops as a result of the loss of a critical number of myocytes and, 2) function of dead myocardium can be rescued through the repopulation of these areas with cells that are, or can become, contractile and participate in ventricular contraction. There have been results from both laboratory and clinical studies that demonstrate the potential for benefits with CCM. Animal models of both myocardial infarction and global heart failure have shown functional improvement following treatment with potential stem cell therapy. Clinically, the patients that have undergone CCM, regardless of cell type used, have done coordinate with another intervention, such as angioplasty or CABG. Therefore, it becomes difficult to separate the effects of the cellular transplantation from the primary coronary intervention. Sole therapy with CCM in a double blinded study will provide the insight needed to determine the effects of the cellular transplantation.

However, CCM has some crucial potential side effects as well as some significant obstacles to overcome. The first concern is cell retention at the site of injection. The issue of cell retention is a concern for both the success and failure of the therapy. Animal studies indicate that the level of ventricular benefit is proportional to the number of cells transplanted. Taylor’s group has shown that the ventricular function recovery following CCM was greater in animals that received a higher amount of cells than those with lower amounts of cells. However, the level of cellular retention at the site of injection has been reported to be as low as 10% in some cases, and this raises the critical issue of cellular
release into the circulation of the animal or patient undergoing this procedure. If only 10% of the cells are remaining immediately following injection this means that upwards of 90% of the cells are lost either to the circulation or the thoracic cavity, depending on the mode of injection (i.e., epicardial versus percutaneous or endocardial). This poses a serious potential side effect due to the high likelihood of the lost cells aggregating in the peripheral circulation and become emboli that could cause serious injury. For example, if the patient undergoes an endoventricular route of cell injection the lost cells could potentially be lost to the brain where they could embolize one of the cerebral arteries resulting in stroke.

Furthermore, assuming 10% of the initial cell volume remains at the site of injection, the remaining cells almost certainly do not all survive. It has been well documented that cells remaining at the site of injection undergo a predictable level of cell death as a result of physical forces, inflammation, apoptosis, and hypoxia. Perhaps the most important of these factors is hypoxia due to a lack of a functional vascular supply to serve the transplanted cells. This is illustrated by research results that indicate that the survival of cardiomyocytes grafted into highly vascularized granulation tissue is twofold higher than that for cells grafted into acutely necrotic myocardium. Accordingly, there have been attempts to augment cell survival through the co-administration of angiogenic growth factor, or the co-delivery of cells that overexpress certain angiogenic factors (Zhang et al., 2001), (Sakakibara et al., 2002), (Suzuki et al., 2001), (Suzuki et al., 2000). It is also critical to consider the timing of cell administration to optimize cell survival. If the cell transplantation procedure is performed too quickly following infarction, the massive
inflammatory response will lead to significant cell death of the transplanted cells (Li et al., 2001). However, it is also clear that because the perfusion level of the tissue positively affects cell survival, waiting too long following infarct will cause a precipitous drop in cell survival. Thus, it has been hypothesized, that improving the perfusion to necrotic tissue undergoing CCM will lead to increases in cell survival and more drastic improvements in ventricular function.

Finally, it has also been reported that CCM leads to the development of potentially fatal arrhythmias. While there is limited evidence of connexin expression in the transplanted cells, the large majority of studies have found that while the transplanted cells may begin to express some cardiac-specific contractile proteins, they remain electrically isolated from the surrounding native myocardium. Because the transplanted cells do not express the appropriate connexins and make connections with the surrounding cardiomyocytes, the injected cells can act to slow down cardiac conduction and thus lead to re-entrant arrhythmias that can cause sudden cardiac death (Whittaker et al., 2003), (Zhang et al., 2002).

**Tissue Engineering**

There is a current push towards the development of not only additional, efficacious medical and pharmacologic therapies to treat CVD, but also in the development of treatment modalities that would augment or replace diseased and damaged tissues. Because of the limited supply of autologous tissue needed for CVD surgical intervention and the increases in CVD patient population, treatment options that would allow for the
use of allogeneic tissue, as well as biologically inert scaffolds for tissue structure, are in increasing demand. This clinical need has given rise to the relatively nascent field of Tissue Engineering. The term Tissue Engineering was first used at an NSF workshop in 1987 and was defined as “an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function” (Langer, 1993).

In recent years there has been significant progress towards the development of successful tissue engineered cardiovascular implants. There are, however, three major limitations that must be optimized in order for tissue engineering, as a field, to be ultimately successful. These three fundamental objectives are scaffold selection, cell sourcing, and tissue/construct vascularization.
Figure 1.8 Image of a Tissue Engineered Microvascular Construct after 14 days implantation on the epicardial surface of a mouse heart that has undergone coronary ligation and subsequent myocardial infarct formation.
Tissue Engineering and Scaffold Selection

The identification of the ideal scaffold for tissue engineered intervention is of paramount importance to the long term function of tissue engineered devices. However, while the objective is clearly defined, the solution is complicated by the optimization of many differing variables, dependent on the specific tissue site being addressed.

Proper scaffold selection in the cardiovascular system includes permission of nutrient and solute exchange, migration and proliferation of cells, proper tensile strength and handling characteristics, and appropriate biodegradation characteristics. Each of these variables, however, will change dependent on the tissue being engineered and the implantation site of the construct. For example, while it may be advantageous for a tissue engineered heart valve to be mechanically strong, and somewhat rigid to withstand the dynamic forces of the heart and the aorta during the cardiac cycle, tissue engineered coronary vascular grafts must be compliant enough to allow for surgical manipulation and movement of the organ and the patient following implantation. Additionally, while such devices as valves must be comprised of substances that are not biodegradable, or degrade at an extremely slow rate, it may be advantageous for certain tissue substitutes to utilize completely biodegradable scaffolds.

Strategies for the development of tissue engineering have focused not only on how the scaffold interacts with the host tissue, but also with the cells that comprise the tissue engineered device. It has been well documented that the composition of selected scaffolds can greatly affect the behavior and phenotype of the cells that are placed on and within the scaffold materials (Chandy, 2003), (Mann, 2001), (Hutmacher, 2001).
Ultimately, the most critical element in the selection of a proper tissue engineering scaffold is the criteria that there is good biocompatibility with the host tissue – that there is good tissue-tissue engineered integration. This integration would include the seamless connection of the implanted construct and the host tissue at the level of cell-cell contact, neural integration, electrical coupling, and macrovascular and microvascular anastomoses.

**Tissue Engineering – Optimal Cell Sourcing**

While there is intensive investigation ongoing in the area of optimal cell-type identification for cellular transplantation, there is also a major focus in optimizing cell sourcing for tissue engineering. As is the case with scaffold selection, cell type selection is a process that is constrained by many variables that are dependent on the desired tissue implantation site. Additionally, fully functional tissue engineered constructs must be comprised of multiple, distinct cell types. Constructs that are generated using a single cell type are doomed to failure as a result of over-simplification of the biologic processes of human tissues.

Native myocardium is comprised of a number of distinct cell types, and a truly functional myocardial substitute must include those cells as well. This includes not only cardiomyocytes, but also, neural cells, fibroblasts, endothelial cells, vascular smooth muscle cells, and vascular pericytes. In order to recreate these cell populations in vitro, there must be a careful evaluation of optimal cell sourcing. Current trials in cell transplantation have used mesenchymal stem cells, endothelial progenitor cells, skeletal
Figure 1.9 An assortment of biomaterial currently used in the treatment of patients. Pictured devices include prosthetic vascular grafts, artificial joints, oxygenator, artificial heart, artificial heart valve, and skin substitute.
muscle satellite cells, hematopoietic stem cells, and smooth muscle cells. There have been moderately successful attempts in the laboratory to generate tissue engineered myocardial constructs using ventricular myocytes, atrial myocytes, and fetal myocytes. However, these approaches have used primarily a single cell type and the transplanted grafts have been sufficiently thin to allow for diffusion of nutrients to allow for cell survival within the grafted construct. Furthermore, it is important to understand the cellular adaptations that occur in vitro during construct formation. The phenotypic changes that occur to cells in culture can be a result of a number of things including the surface on which they are cultured, additional cell types present in the culture system, shear stress, mechanical stretch, and strain experienced by the cells in the culture environment.

Accordingly, the success of tissue engineering, especially for large, solid organs, will depend greatly on the identification and the availability of the correct cell types.

Tissue Engineering – Vascularization of Constructs

In order for tissue engineering interventions to be successful, there must be complete integration of the grafted tissue with the host tissue. This integration includes not only physical connection of the grafted material and grafted cells through surgical approximation but cell-cell contact between graft and host, and vascular continuity from the host through the grafted tissue. Furthermore, the development of large constructs will require rapid vascularization of the grafted tissue to allow for the survival of the grafted cells.
Figure 1.10 Figure illustrating the continuum of tissue engineered approaches to the treatment of disease. These devices and treatments range from the engineered inert metals to the utilization of engineered stem cells. The most complex tissues will require the combination of elements from both ends of the continuum.
Currently, the limitations of vascularizing tissue engineered constructs have restricted the development of tissue engineered constructs to those that are limited in three-dimensional size. In this way, the cells of the grafted device are capable of surviving through the diffusion of nutrients and metabolic waste products. Thus, the current clinically available tissue engineered products are limited to such things as heart valves, bladder substitutes, skin equivalents, and vascular conduit. These products are sufficiently thin to allow for diffusion to meet metabolic demands or for the development of a vasculature after implantation. While the neovascularization of tissue engineered products can work for thin products that do not have high metabolic demands, in the case of solid organs such as the kidney, liver and heart, with high metabolic demands, and relatively high tissue density, there is a need for a more rapid establishment of perfusion throughout the graft.

Attempts at vascularizing tissue engineered constructs have been made through the administration of angiogenic growth factors, use of polymers that release angiogenic factors, seeding of cells that secrete angiogenic growth factors, and the utilization of single cellular elements that are thought to become blood vessels within the graft. These approaches have attempted to drive vascularization through both the driving of the host to vascularize the graft (angiogenesis) and the development of a new vascular network within the graft (neovascularization).

While many of these strategies have been encouraging, they have been incomplete in their results. This approach to vascularizing constructs is inherently flawed in at least two important areas. First, the development of a vasculature through either angiogenesis
Figure 1.11 Ink perfusion of a tissue engineered graft on the epicardial surface. Ink was perfused into the coronary circulation via retrograde perfusion through the aorta and antegrade perfusion through the coronary artery vasculature. The filling of the vessels within the graft indicates vascular continuity with the underlying cardiac vasculature.
or neovascularization generates a significant latency period in tissue perfusion as the vasculature develops. The development of blood-carrying vessels can take up to as long as 7 days, and this is a time frame that will assure cellular necrosis in areas of the construct that are outside the limits of diffusion, or roughly more than 200μm from a vascular supply (Folkman, 1973). The second problem with driving vascularization of tissue engineered devices through angiogenesis or neovascularization is that the vascular network that develops will consist of primarily of capillaries and will be devoid of arterioles and venules. Administration of single growth factors for therapeutic angiogenesis, especially VEGF, has generated increased vascular density primarily through the development of increased numbers of capillaries. The lack of arterioles and venules in these vascular networks presents a situation where the grafted tissue cannot adjust blood flow to meet physiologic changes in metabolic demand. For example, in the setting of tissue engineered myocardial tissue, while capillaries are critical to the delivery of oxygen to the myocytes, if there is an increase in systemic oxygen demand, the heart must meet these changes, and the cardiomyocytes will experience an increased oxygen demand, as well. Without feeder vessels to the tissue, the transplanted tissue will be unable to increase oxygen delivery and thus will be unable to sustain increases in contractility. Rapid and complete vascularization of tissue engineered devices and constructs is essential to the successful augmentation or replacement of diseased tissues and organs.
Significance and Research Plan

Currently, Cardiovascular Disease remains the leading cause of death in the United States and the rest of the western world. The incidence of Cardiovascular Disease has continued to rise steadily since 1970 and as the mean age of the American population continues to rise, it is expected that the occurrence of CVD will also rise. Of all the forms of CVD, the most significant, with respect to morbidity and mortality, is Coronary Heart Disease. Coronary Heart Disease is responsible for approximately 54% of the deaths related to CVD, and by itself is responsible for roughly the same number of deaths in the United States as cancer, the second leading cause of death.

In recent years, there have been significant research developments and clinical improvements in the treatment of Coronary Heart Disease, at the levels of medical management, minimally invasive intervention, and surgical intervention. However, a significant number of patients become refractory to medical management and frontline surgical intervention. Over time, as the body is continually exposed to drugs, pharmacologic treatments fail. Diseased arteries that have been re-opened following coronary angioplasty or stenting demonstrated a significant restenosis rate, requiring multiple interventions. The use of coronary artery bypass grafting has proven to be critically important in the management of Coronary Heart Disease patient. However, the poor availability of autologous bypass conduit and presence of diffuse disease leads to fatal disease progression in many patients.
The primary target for therapeutic intervention in patients suffering from Coronary Heart Disease and/or Ischemic Heart Disease, is the maintenance or restoration of major epicardial coronary artery patency. The revascularization of ischemic myocardium through balloon angioplasty, coronary stenting or coronary artery bypass grafting, is performed at the level of the major coronary arteries, and, currently, cannot feasibly be done at the microvessel level. However, a patent epicardial artery is not an adequate marker of myocardial perfusion distal to the corrected lesion. True tissue perfusion in the myocardium can only be realized at the level of the microvasculature. For those individuals who have failed medical management and surgical intervention due to diffuse microvascular dysfunction there is currently no viable treatment option.

Cardiovascular Tissue Engineering promises to provide alternative strategies for the development of bypass conduit, bioprosthetic valves, functional microvascular networks, and myocardial replacement tissue.

The primary goal of this project was to test the hypothesis that Following implantation, Tissue Engineered Microvascular Grafts are capable of inosculation with underlying host coronary vasculature and attenuating the loss of ventricular function following acute myocardial infarction.

Specific Aim 1: Using intact microvessel fragments, develop a prevascularized tissue engineered construct in vitro that, when implanted, becomes a patent, functional microvascular network. Our laboratory has previously isolated microvessel fragments from adipose tissue and cultured these fragments in a 3-dimensional collagen
matrix. The microvessel fragments have been isolated from rat, mouse, and human fat
and, thus, all of these animal models can be evaluated. The isolation of intact
microvascular fragments provides a unique model system where all cell types associated
with an in vivo microcirculation are present. The presence of not only endothelial cells,
but vascular smooth muscle cells, vascular pericytes, and fibroblasts is critical to the
development of a mature microcirculation. Furthermore, this cellular composition should
accelerate the process of vascular remodeling and maturation when compared to model
systems that utilize a single cell type.

Hypothesis 1: Following implantation in the subcutaneous or epicardial position,
Tissue Engineered Microvascular Grafts will inosculate with the host vasculature,
and the vascular elements within the graft will become a stable functional
vasculature. The implanted microvascular grafts will inosculate with the underlying
and/or surrounding host vasculature and become a function microvascular segment within
the host vascular system. The grafted constructs contain microvascular fragments that
will become patent, blood-carrying vascular elements following transplantation into a
host animal. The capability of the constructs to anastomose with the host vasculature will
be determined and evaluated in the subcutaneous position.

Specific Aim 2: Develop a microvascular bypass graft, utilizing a previously
developed and characterized Tissue Engineered Microvascular Graft. By utilizing
the previously characterized Tissue Engineered Microvascular Graft, application of this
microvascular transplantation strategy will allow for the development of a microvascular bypass graft. Currently, clinical coronary artery bypass grafting can only be done at the large vessel level. It is not technically feasible to anastomose bypass grafts at the microvessel level. As a result, patients with diffuse small vessel disease, or patients with well-defined areas of microvascular dysfunction to not have an attractive treatment option. The development of a microvascular bypass graft would provide not only a treatment option for these individuals, but also a platform for the development of large, complex tissue engineered constructs that require the rapid establishment of a vascular connection with the host microvessel circulation.

Hypothesis 2: Tissue Engineered Microvascular Grafts, when implanted in an epicardial position, will serve as a microvascular bypass graft in a murine model of myocardial ischemia and infarction. The grafted microvascular constructs will inosculate with the underlying host coronary vasculature as they did with the peripheral vasculature in the subcutaneous position. These constructs will be implanted onto areas of myocardium that have been made ischemic following coronary artery ligation and acute myocardial infarction. The grafts will be implanted in such a way so as to allow for overlap with visibly non-ischemic tissue to provide for a possible microvascular bypass to the ischemic tissue.

Specific Aim 3: Use a Tissue Engineered Microvascular Graft to attenuate the loss of left ventricular function following acute coronary artery occlusion. The implanted
microvascular graft will form a contiguous vascular connection with the underlying coronary arterial circulation of the host heart. This will allow for the development of a microvascular coronary artery bypass graft. Previous work in our laboratory has shown that increased microvascular density in areas of infarcted myocardium has resulted in improved left ventricular function in mice following acute myocardial infarction. It has been hypothesized that this relation is due to the increased perfusion as a result of increased microvascular density.

**Hypothesis 3: Implantation of a Tissue Engineered Microvascular Graft will reduce the extent of left ventricular dysfunction associated with acute ligation of the Left Anterior Descending coronary artery.** Pressure-Volume relationships will be used to determine the left ventricular function of animals that have undergone acute coronary artery ligation. Animals that have undergone coronary ligation and Tissue Engineered Microvascular Graft placement will be compared to animals that have undergone coronary artery ligation alone as well as animals that have undergone coronary artery ligation and placement of avascular collagen gels in a manner identical to the placement of Tissue Engineered Microvessel Grafts. The presence of a microvascular coronary artery bypass will confer a functional benefit to those animals versus control animals.
2. Rapid Perfusion and Vessel Remodeling in a Microvascular Construct Following Implantation

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**Introduction**

Tissue vascularization, the formation of a new or expanded microvascular bed, is an important component of tissue remodeling, repair and regeneration (Carmeliet et al, 1997). Complete vascularization is the successful integration of angiogenesis, collateralization and vascular remodeling leading to a functional vascular bed capable of meeting tissue metabolic demands (Buschmann et al, 1999), (Carmeliet, 2000). Numerous molecules are known to initiate angiogenesis and affect neovessel formation (Iruela-Arispe et al), (1997; Sato, 2000), an important aspect of tissue vascularization. However, little is known about how angiogenesis is integrated into the vascularization process, including how neovessels interface with other vessels within a vascular network and, a microvessel matures into a specific network segment.

We have previously shown that isolated microvessel fragments containing endothelium and perivascular cells, retain angiogenic potential and are capable of forming a simple capillary-like bed (microvascular constructs) when cultured in a 3-dimensional collagen I gel (Hoying et al, 1996). In the microvascular construct, the vessel fragments undergo stereotypical angiogenesis forming neovessels that maintain patent lumen and perivascular cell associations. Furthermore, the vessel fragments within this culture
system are responsive to pro-angiogenic conditions (Hoying, Boswell, and Williams, 1996), (Carter et al, 1996), (Carter et al, 2000).

Here we report the development and characterization of an experimental model of tissue vascularization based on the implantation of this microvascular construct. Pre-cultured or freshly formed microvascular constructs implanted subcutaneously rapidly connect with the host vasculature and expand into a vascular bed containing heterogeneous vessel elements. With this model, we reproduce the important aspects of vascularization; angiogenesis, inosculcation, and network remodeling. Furthermore, we demonstrate that the model accommodates human-derived vessel fragments, enabling the construction of human-mouse vascular chimeras.
Materials and Methods

All animal procedures were performed according to University of Arizona IACUC approved protocols.

Microvessel Isolation and Culture

Rat fat microvessel fragments (RFMF) were isolated from epididymal fat pads of retired-breeder Sprague Dawley rats using a modification of previously described methods (Hoying, Boswell, and Williams, 1996). Under aseptic conditions, harvested fat pads were washed in 0.1% BSA-PBS, finely minced with scissors and digested in 2 mg/ml collagenase + 2 mg/ml BSA (essentially fatty acid free) in PBS for 8 min at 37°C with vigorous shaking. Tissue debris and large vessel pieces were removed by filtering the suspension through a sterile 500 μm pore-size nylon screen. RFMFs were captured by filtration of the remaining suspension on a 30 μm pore size nylon screen and recovered by vigorous flushing of the screen surface with 0.1% BSA-PBS. The type and lot number of collagenase used was pre-determined to optimize fragment yield while maintaining microvessel structure. RFMFs were suspended (12,000 - 15,000 RFMFs/ml) in ice cold 3 mg/ml rat tail type I collagen (BD BioSciences, Bedford, MA) prepared with DMEM (1x final) and pH-neutralized with 1M NaOH. RFMF/collagen suspensions were plated into individual wells (0.25 ml/well) of a 48 well plate and placed in a 37°C incubator for 20 min. to polymerize the collagen. For culturing, an equal volume of 10% FBS in DMEM is added to each RFMF gel, which was re-fed once vessel sprouting occurred (usually around day 4). For immediate implantation, newly formed RFMF gels were removed from the wells with a sterile spatula and transferred directly to the implant site (see
Human fat microvessel fragments (HFMF) were isolated and cultured from human abdominoplasty tissues using the same procedure.

**Implantation**

Prevascularized constructs and acellular collagen gel controls were implanted in the subcutaneous position on the flanks of SCID mice. Each mouse received two implants: a microvascular construct on one side and an avascular control collagen gel on the other side. For implantation, animals were anesthetized with an intraperitoneal injection of 2.5% Avertin. Dorsal hindlimb and lower back areas were shaved, depilated and cleaned. Using blunt dissection through a small skin incision, a subcutaneous pocket was formed between the skin and the underlying muscle anterior to the pelvis. Each pocket received a construct or a control gel. The incision was then closed with 6-0 suture and the animal allowed to recovered.

**Explant and Imaging**

At the time of explant, the animals were anesthetized as outlined above. The skin above the implant was carefully dissected away so as to expose the implant and underlying muscle tissue. The implant was then photographed and subsequently imaged by orthogonal polarization spectroscopy (Cytoscan®; Cytometrics Inc., Philadelphia, PA) using a 10 times magnification probe for the visualization of blood within vessels down to a depth of 700μm.
Histology and Histochemistry

Implants were removed with the underlying muscle attached, fixed in 2% paraformaldehyde/PBS and processed into paraffin. General histology was determined on deparaffinized, 5-6 μm thick sections stained with hematoxylin and eosin. Vascular elements were identified using a rodent-specific lectin, GS-1 (*Griffonia simplicifolia I*) or the human-specific endothelial cell marker UEA-1 lectin (*Ulex Europaeus Agglutinin I*). Perivascular cells were identified with a monoclonal anti-α smooth muscle actin antibody (clone 1A4; Sigma Immunochemicals) using a horse radish peroxidase (HRP) reporter system (Sigma Immunochemicals). For the lectin and immunostaining, sections were counter-stained with 1% methyl green. Vessel density was determined by counting discreet, GS-1 positive structures in the implanted construct from at least 5 different fields (of defined area) per section from two different implants. Individual counts were divided by the area of each field and averaged for each time point.

Ink perfusion

Mice containing implants were anesthetized with Avertin and placed supine on a dissecting stage. The chest was opened and a catheter (PE 60 tubing) placed into the left ventricle. The mouse was perfused with PBS containing 10U/ml heparin and 10 μM sodium nitroprusside until the perfusate was clear of blood. India ink (Speedball #3398; Hunt Manufacturing Co., Statesville, NC), dialyzed against PBS and filtered through #1 Whatman paper, was perfused into the mouse at a maintained pressure of 90-100 mm Hg until all tissues in the mouse appeared dark (usually this required 2-3 ml of ink solution).
After ink perfusion, the implant was excised and fixed in 4% paraformaldehyde in PBS for 45 min. at 4°C. The fixed implants were rinsed in cold PBS, sliced longitudinally and placed in 100% glycerol for 20 min. to clarify the constructs. The two halves, cut-side up, were sandwiched between a microscope slide and coverslip for viewing with a standard light microscope.

**En bloc immunohistochemistry**

Cultured constructs or explants were rinsed in PBS and fixed for 1 hr in 2% paraformaldehyde in PBS or 4% paraformaldehyde in PBS, respectively. Constructs were washed in cold PBS three times for 15 min. each and placed in blocking buffer (5% nonfat dry milk and 1.5% BSA in TBST buffer) overnight at 4°C. Constructs were incubated overnight at 4°C with a primary antibody directed against rat MHC (clone # OX-18) and conjugated to biotin, diluted 1:50 in blocking buffer. Following three, 1 hour washes at RT with blocking buffer, constructs were incubated with strepavidin conjugated to Oregon Green (1:400 dilution; Molecular Probes, Portland, OR) in blocking buffer for 2 hr at RT. Finally, constructs were washed twice with blocking buffer for 30 min each and twice with PBS for 30 min each, all at RT. Stained constructs were sandwiched between a microscope slide and coverslip for viewing with a standard epifluorescence microscope.

**In Situ Hybridization**

A probe that detects a repeat element on the Y chromosome in rat (Essers, de Stoppelaar, and Hoebee, 1995) was synthesized from rat-tail genomic DNA by PCR (F: ggt tct aga
ctg taa aac cca gac R: act taa aac taa gct tat tgg cca), size-verified and labeled with biotin using the Photoprobe labeling kit (Vector Laboratories, Burlingame, CA). For hybridization, 8 μm sections were deparaffinized, rehydrated, treated with 0.2 M HCl for 15 min., incubated in 0.1% TritonX-100 for 2 min., treated with 10μg/ml of proteinase K in PBS for 2 min and finally rinsed with 2 mg/ml glycine in PBS (1 min.). Prepared sections were post-fixed with 4% paraformaldehyde in PBS, rinsed once in glycine/PBS and once in 0.2X SSC. To release DNA from the chromatin, sections were treated with 0.1M TEA, 0.25M acetic anhydride followed by 2 washes of 5 min. each in 2x SSC. Sections were then dehydrated in ethanol from 50% to 100% prior to pre-hybridization (50% formamide, 20% dextran sulfate in 2x SSC, and salmon sperm DNA (83 μg/ml) for 20 minutes at 55°). The biotin-labeled, Y-chromosome probe was denatured (95°C for 5 min.), added to fresh pre-hybridization buffer and hybridized to sections overnight at 40°C. Final concentration of the probe was 6 ng/μl. Sections were washed twice with 4x SSC for 5 minutes at R.T., 2x SSC for 20 minutes at R.T., 0.2x SSC for 15 minutes at 42°, and finally 0.1x SSC for 15 minutes at 42°. Probe was detected via an HRP-conjugated strepavidin according to manufacturer’s instructions (Photoprobe®;Vector Laboratories, Inc.). For determination of the % Y chromosome positive cells, serial sections (6 micron thick) were stained for the Y chromosome by in situ hybridization or hematoxylin to label all nuclei. The ratios of Y chromosome-positive counts to hematoxylin-positive counts (both derived from serial sections) from at least 5 areas of a section from two different implants were averaged.
Results

Isolated fat microvessel fragments undergo angiogenic sprouting within the first 5 days of culture in collagen I gels (Figs. 1a-c). Sprouting occurs at the ends and from mid regions of individual fragments. Furthermore, the sprouting process is dynamic (Figs. 1b-c). By 11 days in culture, fragments have grown to form a collection of elongated, simple neovessels (Fig. 1d) with an average diameter of 24.8 ± 6.8 microns (n=39). These neovessels contain patent lumen and a relatively low density of alpha-actin positive, perivascular cells (Hoying, Boswell, and Williams, 1996).

Coordinate with angiogenesis, newly formed vessel elements must locate and inosculate with other elements of the vascular network prior to functioning as a blood perfusion circuit. We evaluated the ability of this microvascular construct to interact with an existing vasculature by implanting neovessel cultures into immune compromised (SCID) mice. Constructs were placed under the skin and in direct contact with the dorsal musculature. We analyzed 4 or more implants at each time point (days 1, 3, 5, 7, 10, 14, 21, 28, and 35 post-implantation). All microvascular constructs contained vessels after implantation. Upon gross examination of implants, we observed superficial, blood-filled vessels associated with microvascular constructs (Fig. 2a) but vessels were not observed in collagen gel controls (Fig. 2b). Histologic evaluation of explanted, microvascular constructs revealed the presence of blood in heterogenous vessels of the construct (Fig. 2c-f). We observed the full range of vessel types commonly seen in a mature, functional vascular bed, including small arteries, arterioles, capillaries, venules and veins (Fig. 2e-f).
We perfused ink into the left ventricle of the host mouse to determine when vessels within the construct become contiguous with the mouse vasculature. By day 1, a limited number of microvessels in the implant contained ink indicating continuity with the mouse circulation (Fig. 2.3a). By day 2, the construct vessels are beginning to assemble, continuing to forming interconnections by day 3 (Fig. 2.3b,c). By 4 weeks post-implantation, the vessels have refined into a mature vascular bed (Fig. 2.3d). Orthogonal polarized spectral (OPS) imaging, which selectively detects hemoglobin (Groner et al, 1999), of living implants reveals blood-containing vessel structures throughout the constructs by day 14 (Figs. 2.3e). Only surface blood, due to the dissection was detected with OPS on control, vessel-free collagen gel implants (Figure 2.3f).

Staining of implant sections with the *Griffonia simplicifolia*-1 lectin (Laitinen, 1987) verified the presence of endothelial cell-comprised vessels (Fig. 2.4a,b). Vessel densities (*Gs-1* positive structures) within the microvascular constructs increased over 2 fold from day 5 to day 28 (Fig. 2.4c) suggesting that angiogenesis continued within the implants during this time. Immunostaining of implants for α-SMC actin, to corroborate the mature vessel phenotype observed in the histology, identified positive cells surrounding vessels of different caliber (Fig. 2.4d).

To determine if the vessels within the prevascularized construct were derived from the original, cultured rat microvessels or, alternatively, were replaced by the ingrowth of host mouse vessels, we took advantage of the fact that the rat microvessels were isolated from males and the constructs were placed into female mice. Using a DNA probe specific to a repeat element of the rat Y chromosome (Essers et al, 1995), we analyzed sections from
(Ryan et al, 2000) day 5 and day 28 implants by in situ hybridization. Vessel-like structures and single cells throughout the implant were detected with the Y chromosome-specific probe (Fig. 2.5a). Because no Y-positive cells were observed within the adjacent muscle of the host mouse, it appears that the expansion of the isolated microvessel fragments placed into the construct was limited to the construct boundaries (Fig. 2.5a). In order to determine the percentage of cells within the microvascular constructs that were derived from the original isolate, serial sections from day 5 and day 28 implants were stained either with hematoxylin, to identify all nuclei, or the Y-chromosome probe (Figs. 2.5b-e). Comparing the number of Y-positive cells to the total number of cells indicates that nearly all of the cells (~90%) within the implants were comprised of cells derived from the original rat microvessel isolate (Fig. 2.5f).

The apparent continued angiogenesis and remodeling that occurred within the implant suggested that pre-culturing of the vessel fragments is not necessary to the subsequent formation of a functioning vasculature when implanted. Therefore, we examined the ability of freshly isolated microvessel fragments to establish a vasculature within the implant in the absence of pre-culturing. As before, rat adipose microvessel fragments were isolated and embedded in collagen gels. However, these freshly formed constructs were immediately implanted without prior culturing. As with pre-cultured constructs, the freshly formed microvascular constructs developed many GS-I-positive, vessels of different diameters (Fig. 2.6a,b). These results prompted us to explore the use of human-derived vessel fragments in fabricating microvascular constructs, which would have more clinical relevance. Similar to what we observed with rat microvessels, constructs
established from human sources contained UEA-I-positive vessels after 15 days of implantation, indicating the maintenance of the original human fragments (Fig. 2.6c) with limited mouse (GS-I positive) vessel invasion (Fig. 2.6d).
Discussion

In the present study, we examined the capability of a microvascular bed constructed in vitro to interface with an existing vasculature and subsequently remodel into a functional perfusion circuit. Our goal was to develop an experimental model to enable the detailed study of angiogenesis, vessel inosculation and network remodeling. Microvascular constructs consisted of 3-dimensional cultures of isolated microvessel fragments that formed, via angiogenesis, a complex of new microvessels (Hoying, Boswell, and Williams, 1996). We have previously shown that these neovessels contain patent lumen and retain associated perivascular cells (Hoying, Boswell, and Williams, 1996). We hypothesized that, because the cultured neovessels preserve a capillary-like architecture, they would be capable of rapid integration with an existing vasculature. As hypothesized, the microvessels of the construct quickly inosculated with the host vasculature upon implantation into a subcutaneous pocket, carried blood and remodeled into a heterogeneous vasculature. As shown by ink perfusion of the entire host mouse and histology of construct sections, inosculation begins within the first day of implantation, preceding the remodeling of vessels into arteries, arterioles, capillaries, and venous vessels within the first week.

The relative short time frame for vessels within the construct to inosculate with the host vasculature is similar to that observed in skin grafting in which the construct (the graft in these cases) contains a pre-formed, functional vasculature (the skin microcirculation) comprised of endothelial and perivascular cells (Converse et al, 1975), (Young et al, 1996). This suggests that the microvessels within the microvascular construct, as in the
skin microcirculation, have retained the ability to integrate with another vasculature and thus represent a good model of a remodeling microcirculation in vivo. A typical microvessel consists of endothelial cells arranged into a tube wrapped by one or more layers of perivascular cells (usually pericytes smooth or muscle cells) (Hirschi et al, 1996). The interactions between the endothelial and perivascular cell layers is thought important in maintaining microvascular integrity and stability. For example, perivascular cells can inhibit endothelial cell proliferation (Orlidge et al, 1987), mediate endothelial cell physiology (Dora et al, 1997), and prevent spontaneous hemorrhage (Lindahl et al, 1997). In a recent study involving cultured endothelial cell tubes assembled in an artificial scaffold, endothelial cell tubes within the implant acquired perivascular cells by day 14 post-implantation which coordinated with the time of vessel maturation (the presence of blood cells and loss of VCAM expression) (Schechner et al, 2000). In this study, no perivascular cells were present in the scaffold at the time of implantation and perivascular cell investment was more prevalent at the construct periphery. It was hypothesized that the investing perivascular cells were host derived (Schechner et al, 2000). In the study presented here, perivascular cells were in the original vessel isolate used to form the microvascular constructs. The early and continued presence of perivascular cells with endothelial cells in these constructs may explain why the vascular system was established so quickly upon implantation. Interestingly, there were limited host cells, perivascular or otherwise, within the construct, even after 4 weeks (Fig. 5). This indicates that any additional perivascular cells required during continued growth and maturation of implant vessels were derived primarily from the original microvessel
fragments used to fabricate the construct. Moreover, it suggests that the presence of stable vessel elements, due to the perivascular cells, may preclude or minimize the expansion and/or recruitment of neighboring host vessels into the construct.

In 3-dimensional culture, the fragments, which are derived from a spectrum of microvascular elements (Hoying, Boswell, and Williams, 1996), spontaneously undergo angiogenesis to form an expanded, but homogenous, collection of simple capillary-like vessels. Once implanted and exposed to blood flow, this simple vasculature remodels to contain arteries, arterioles, capillaries, venules and veins. Changes in hemodynamic stimuli to a vessel, namely shear stress and circumferential and axial wall stress, affect vessel diameter and wall thickness as well as possibly controlling arteriole specification (Langille et al, 1986), (Langille et al, 1989), (Skalak et al, 1998), (Pries et al, 2000), (Tuttle et al, 2001), (Jackson et al, 2002), (Sullivan et al, 2002). Thus, it is likely that the stimuli affecting vessel remodeling in the implants are blood flow (shear stress) and pressure (wall stress), consistent with the finding that blood flow within the vessels precedes maturation (Fig. 3). Whether or not blood flow is sufficient to establish a completely mature network architecture capable of providing efficient tissue perfusion meeting a tissue’s specific metabolic and functional needs is not clear. The vessels within the microvascular construct of this study adapt and remodel into a more mature vascular bed (e.g. contains arteries and veins) after implantation, despite the apparent absence of non-vascular tissue cells. However, the relatively tortuous and chaotic appearance of the vascular network in the implants suggests that further network remodeling within the construct is necessary. Based on theoretical simulations, a stable microvascular structure
requires the presence of both hemodynamic stimuli and tissue-derived metabolic signals (Pries et al, 1998), (Pries et al, 2000), (Pries et al, 2001). Thus, inclusion of tissue cells within the constructs may affect further network refinement. Further studies determining the contributions of blood hemodynamics and tissue microenvironment in the vascularization of these constructs will provide valuable information as to the mechanisms of vascular network differentiation.

Cell and engineered tissue implants offer promise in facilitating tissue healing and replacement (Langer et al, 1993). As is the case for most tissues, such implants require an adequate blood supply to maintain viability and function. Therefore, successful implant designs will include approaches for rapid implant vascularization and blood perfusion. With the microvascular constructs developed in this study, we provide an alternative strategy to vascularizing tissue constructs by providing a ready-made vasculature. The rapid, initial perfusion and subsequent remodeling obtained with the microvascular construct would provide early support of stromal cells incorporated into an engineered tissue, earlier than observed with other strategies. Furthermore, it is not necessary to modify the source vascular cells in order to preserve the construct vasculature as is the case with cultured endothelial cells (Nor et al, 1999). In engineering tissue constructs, it should be possible to incorporate the angiogenic fragments directly into a tissue construct or use the precultured microvascular construct as a separate vascular module to which other tissue constructs (native, artificial or biohybrid) could be fused. Finally, the microvascular constructs could potentially serve as a nucleation site for tissue
revascularization, such as in infarcted tissue, or other applications where tissue vascularization or vessel/tissue interactions are important.

In this study, we present a model of vascularization based on the formation of a microvascular construct grown from intact, microvessel elements. The microvascular construct implanted into the SCID mouse serves as a complete model of vascularization and enables evaluation of the molecular, cellular and physiological mechanisms of angiogenesis, vascular specification and other features of an actively remodeling vasculature. An important aspect of the model is the relative absence of host cells in the construct and the persistence of the original vessels when implanted. This aspect of the microvascular construct facilitates experimental design and simplifies interpretation of experimental results. In addition, non-vascular tissues cells can be included with the microvessels of the construct which will permit study of tissue:vascular cell interactions in the absence of confounding tissue cell types from the host (except for circulating blood cells). We also demonstrate the utility of this model in constructing an experimental human microcirculation without the need to modify or engineer the human cells. The resulting human-mouse vascular chimeras will prove useful in further studies exploring the mechanisms and processes of angiogenesis and vascular integration in the human microcirculation as well as explore microvessel:tissue cell interactions such as microvessel differentiation, metastasis and inflammation.
Figure 2.1 Vessel fragments exhibit features of angiogenesis when cultured in 3-D collagen gels. 

a) A freshly isolated arteriole fragment with a capillary branch suspended in the collagen gel (day 0).

b,c) The same vessel fragment shown in at day 4 and day 5 of culturing. Multiple sprouts are present by day 4 which may continue to elongate or regress in one day.

d) A representative field of neovessels (arrows) immunostained with a fluorescent anti-MHC antibody within an intact microvascular construct after 11 days of culturing.
Figure 2.2 Vessels within microvascular implants are heterogeneous in structure and contain red blood cells. (a, b) Gross views of an example of microvascular construct and avascular collagen gel (b) 7 days and 3 days post-implantation, respectively. The long arrow in (a) points to a large vessel entering the construct from the surrounding host tissue. H&E stained sections of characteristic implanted constructs after 14 (c, d, e) or 10 (f) days implantation in a subcutaneous pocket reveal the presence of arteries, arterioles (arrowhead in f), capillaries (arrows), venules (arrowhead in d), veins (V) and RBCs within vessels.
Figure 2.3 Vessels within microvascular constructs inosculate with the host vasculature and are perfusion competent when implanted subcutaneously into SCID mice. a-d represent fields of microvascular constructs harvested day 1(a) day 2(b), day 3(c), or day 28 (d) post implantation following perfusion of the respective host mouse through the left ventricle with ink. e-f Orthogonal polarized spectroscopic images of microvascular implants after 14 days implantation and a day 3, avascular control gel.
Figure 2.4 Microvascular implants contain vascular endothelium and smooth muscle-positive perivascular cells. Example low (a) and high (b) magnification micrographs of tissue sections from day 5 implants stained with the rodent endothelial cell-specific lectin Gs-1. Vessels of various caliber are present. D. Vessel density measurements determined from Gs-1 stained sections of implanted microvascular constructs. Vessel density increases in implanted constructs between day 4 and day 28 post-implantation. c A representative section of a day 21 microvascular implant immunostained for alpha-smooth muscle actin showing numerous positive vessels of various caliber.
Figure 2.5 Smooth muscle coverage of vessel elements changes during vascularization. Confocal image stacks of double stained vessel elements (green = MHC and red=actin) were used to generate vessel and perivascular volumes. Example single image planes of (a) a freshly isolated microvessel fragment (day 0), (b) a day 8 cultured fragment and (c) day 28 post implantation branched vessel element. d Percentage of vessel elements covered by α-actin positive perivascular cells measured at different time points in the model.
Figure 2.6 Nearly all of the cells within the microvascular constructs are derived from the original cultured isolates. a A low magnification micrograph of a day 28 implant section labeled by in situ hybridization to a Y chromosome-specific repeat. The “m” is underlying host tissue while the “I” indicates the graft. The arrow indicates the boundary between the two tissues. Serial sections of a day 28 implant (b,c) labeled by in situ hybridization, or hematoxylin alone (e) to identify graft versus host cells. f The relative percent of graft cells at day 5 and day 28 is reported.
Figure 2.7 Constructs assembled from rat or human-derived freshly isolated microvessel fragments also form a vascular bed upon implantation. A Gs-1 staining of histology sections of rat-derived, day 14 implants show numerous microvessels. Fluorescence immunostaining for all cells (anti-MHC antibody) reveals that vessels within the construct form tree-like structures, and ink perfusion demonstrates vessels are contiguous with the host circulation. C,d Superficial vessel connections between host and construct are visible. Staining of human-derived vessels for vWF and smooth muscle actin indicate vessels are human and mature.
3. Implantation of a Pre-Vascularized Epicardial Graft

Introduction

Cardiovascular tissue engineering promises to be an area of research and development that will be critical to the future health care management of patients with cardiovascular disease. Currently, medical and interventional procedures can limit the disease progression of patients with CVD, but ultimately these patients become refractory to these interventions. The scarce availability of autologous and allogeneic tissues greatly limits the treatment of cardiovascular disease. While interventions such as organ and tissue transplantation are eminently successful, the lack of available tissue leads to an extreme mismatch in the number of donors and potential recipients (Heart and Stroke Statistical Update). First used in 1987, the term “tissue engineering” was defined by the National Science Foundation as “an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function” (Langer and Vacanti, 1993). For the treatment of pathologies associated with CVD, tissue engineering is currently being explored as a means to generate vascular conduit (Williams et al., 1996), (Graham et al., 1980), (Meinhart, et al, 1997), (Weinberg and Bell, 1986), bladder (Baddyak et al., 1998), heart valves (O’Brien et al., 1999), ventricular outflow tracts (Ross, 1998), (O’Brien et al., 1999), and myocardial tissue (McDevitt et al., 2002), (McDevitt et al., 2003).

However, the largest impediment to the development of large, complex three-dimensional structures is the development of a functional vasculature within the construct. Currently,
there are a limited number of commercially available tissue engineered products. A readily noticeable and significant commonality amongst these products is the limited three-dimensional size that each possesses. Tissue engineered skin and cartilage are thin enough to allow for the metabolic demands of the implant to be met through diffusion. TE bladder and heart valves are also sufficiently thin to allow for the development of a vasculature following implantation. However, for a large tissue engineered construct to perform optimally, vascularization of the implant must occur very rapidly to avoid tissue necrosis at the core of the implant (Peters and Mooney, 2000). For the potential bioengineering and construction of solid organs such as liver, kidney and heart, the role of the microvasculature in the development of a successful organ substitute is of paramount importance. In fact, cells are only capable of survival when they are within 100-200\(\mu\)m of a blood supply (Eiselt et al., 1998), (Folkman and Hochberg, 1973). Thus, while it may be technically feasible to engineer tissue constructs that contain vessels larger than 1mm, the tissue distal to these large vessels must be served by an organized microcirculation.

The rapidly progressing field of cardiac tissue engineering provides strategies to develop more complex tissues in vitro, comprised of distinct cell populations with cell-cell interactions and three-dimensionality that more closely approximates conditions which occur in vivo. The utilization of these constructs in vivo will depend on rapid vascular connections between the host tissue and the grafted tissue. There have been a number of previous strategies aimed at vascularizing tissue engineered implants, including the use of growth factors, which relies on the remodeling of the pre-existing host vasculature
(angiogenesis) in response to the added angiogenic factors, as well as the transplantation of cells capable of becoming new vessels (neovascularization or vasculogenesis) (Perets et al., 2003), (Takeshita et al., 1994), (Baumgartner and Isner, 1998), (Lee et al., 2000), (Lazarous, et al., 1996) (Schechner et al., 2000). These strategies however, have significant limitations to the development of more complex tissue engineered constructs that are capable of meeting the variable metabolic demands within a dynamic tissue such as cardiac muscle. The greatest restriction to the establishment of a functional vasculature is the period of vessel genesis required in the previously described models. Utilization of intact vascular elements eliminates the latency period inherent for vascular development. We have previously presented the successful implantation and engraftment of intact microvessels in the subcutaneous position of a mouse. To our knowledge, this is the first in vivo application of a tissue engineering strategy utilizing intact microvessels. Here, we demonstrate the successful implantation of a tissue engineered microvessel graft (MG) that has been implanted on the epicardial surface of an infarcted rat heart. Using adipose tissue, MGs were generated that contained microvessel fragments composed of endothelial cells, smooth muscle cells, and pericytes. Microvessel constructs that had been allowed to proliferate in culture (cMG) as well as MGs that were implanted at the time of vessel isolation (fMG), were used in the study. Both of these constructs, cMG and fMG, demonstrated the ability to inosculate with the host vasculature and carry a blood supply through the construct. These results suggest the use of prevascularized epicardial grafts may provide an alternative therapy to treat diffuse myocardial ischemia as well as support the cellular elements of tissue-engineered myocardium.
**Materials and Methods**

All animal studies were approved by the University of Arizona animal review committee, and animals were housed in American Association of the Accreditation of Laboratory Animal Care approved facilities following the procedures outlined by the National Institutes of Health (NIH) *Guidelines for the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1985).

**Microvessel Isolation and Culture**

Rat fat microvessel fragments (RFMF) were isolated from epididymal fat pads of retired-breeder Fischer 344 rats using a modification of previously published methods (Hoying, 1996). Under sterile preparation, harvested fat pads were washed in 0.1% BSA-PBS, finely minced with scissors and digested in collagenase (2mg/ml) in BSA-PBS (2mg/ml) for 8 min at 37°C with vigorous shaking. Tissue debris and large vessel pieces were removed by filtering the suspension through a sterile, 500μm pore size, nylon screen. Microvessel fragments were captured by filtration of the remaining suspension on a 30μm pore size nylon screen and recovered by vigorous flushing of the screen with 0.1% BSA-PBS. The type and lot number of collagenase used was predetermined to optimize fragment yield while maintaining microvessel structure. RFMFs were suspended (12,000 – 15,000 fragments/ml) in cold rat tail type I collagen (BD BioSciences, Bedford, MA) prepared with DMEM and pH neutralized with NaOH (1M). The tissue engineered microvascular grafts (MG) were plated into individual wells (0.250 ml/well) of a 48 well plate and placed in a 37°C incubator for 10 min. to allow for polymerization of the
collagen gel. Fresh isolate Microvascular Grafts were surgically implanted immediately following gel polymerization and addition of media. Cultured Microvascular Grafts underwent additional tissue culture expansion of the microvessels within the graft. For culturing, 0.250 ml of 10% FBS-DMEM was added to the MG, and each construct was re-fed at 4 days. The total culture time prior to surgical implantation was 7 days.

**Myocardial Infarction and MG Implantation**

On the day of surgery, general anesthesia was induced by placing the animals in an induction chamber infused with gaseous Isoflurane. Once a plane of anesthesia was induced the animals were intubated with a 16G catheter and anesthesia was maintained with inhaled 2.5% Isoflurane. The left lateral chest was then shaved and scrubbed with Nolvasan for sterile procedure. The animal was connected to a small animal ventilator for mechanical ventilation and anesthesia administration during the operative procedure. The heart was exposed through a left lateral thoracotomy. Cryoinjury was then performed on the left ventricular wall in the area defined by the left main and left circumflex arteries. Cryoinjury was performed by cooling a metal probe in liquid nitrogen and applying the chilled probe directly to the epicardial surface for one minute. This was repeated ten times. Immediately following cryoinjury, a cultured Microvascular Graft, a fresh isolate Microvascular Graft, or control collagen gel without microvessels (n=6,6,6), was sewn directly onto the area of observed damage, using two 7-0 prolene stitches. Infarct only controls received no suture or gel placement (n=5). The chest was
closed in layers, the chest evacuated of air, and the animal extubated. The animals were recovered under a heat lamp.

**Explant and Evaluation**

On the day of explant the animals were anesthetized with 2.5% Isoflurane. The abdominal aorta was isolated and cannulated with a 20G catheter. The heart was perfused in a retrograde fashion with a solution of PBS containing CdCl₂ (10%), Sodium Nitroprusside (10μM), and Heparin (10μ/ml). Hearts were rapidly excised and placed in Histochoice® fixative solution (Ameresco). The hearts were then dehydrated and paraffin embedded. Sections of tissue (6μm) were then cut for Hematoxylin and Eosin staining, Masson’s Trichrome staining, and cytochemistry.

Sections to be used for cytochemistry were reacted with the lectin *Griffonia simplicifolia* (*Gs*-1). *Gs*-1 binds carbohydrate domains on endothelial cells, and was visualized by use of a peroxidase recognition system (Dako, Inc. Universal mouse kit).

**Vessel Density and Characterization**

Tissue sections treated with *Gs*-1 were analyzed using a 20X (vessel type) or a 40X water immersion (vessel density) objective and light microscopy. Microvessels were classified using standard histological features. (Weidner, 1991) Capillaries were identified as a single layer of flattened endothelial cells. Arterioles were identified as having and ID ≥ 10μm and by the presence of characteristic layers: endothelium, tunica media, and tunica adventitia. Control infarct animals that did not have implants were only evaluated within
the myocardial scar (n=5). Venules were differentiated from arterioles by their large lumen diameter compared with vessel wall thickness, a thin or absent smooth muscle layer, a less significant tunica adventitia, and an ID ≥ 10μm. Vessel density studies were calculated using Gs-1 reacted sections observed under light microscopy with a 40X water immersion lens. Ten random HPF (54X54 um) were identified within the infarcted myocardium for animals in each group. In each HPF the number of cross sectional or longitudinal vessels that were positive for Gs-1 staining were counted. A one-way ANOVA was used to determine significance between treatment groups.
Results

Following isolation, rat microvessel fragments spontaneously undergo angiogenic sprouting and proliferation within the first four to five days of culture (Figs. 3.1a, b). This angiogenic growth occurs at both the free ends as well along the length of the microvessel fragments. Morphologically, the proliferating cells appear to be endothelial cells, with an elongated, spindle shaped cell body. This sprouting continued for the entirety of the culture period of 7 days. By the end of 7 days, the isolated vascular segments had formed a collection of simple neovessels within the collagen gel (Fig. 3.1c, d).

Once the microvessels had formed an extensive vascular network within the collagen gels, these preformed microvascular grafts were sewn directly to the epicardial surface following cryoinjury and infarct formation. Upon gross observation at the time of explant (14 days), the microvascular grafts appeared to be well integrated with the underlying epicardial surface. There were neovessels visible within the graft at the time of explantation of both cultured microvessel grafts and fresh isolate microvessel grafts (Fig. 3.2a-c). Acellular collagen gels that were used as controls showed no gross evidence of vascularization (Fig. 3.2d). Following 14 days of implantation, the collagen gels appeared completely avascular.

Hematoxylin and Eosin staining of the grafts and underlying cardiac tissue verified the presence of integration with the underlying cardiac tissue. Vascular profiles were noted throughout the cMG and fMG. There were RBCs present within the vascular lumina of the graft indicating blood perfusion of the graft during the implant period. In addition to
capillaries, the presence of arterioles, complete with pericytes and smooth muscle cells, and large venules was confirmed (Fig 3.3a-d). Avascular control gels were found to be completely void of vascular profiles within the collagen gel grafts.

Staining of paraffin embedded sections with the lectin *Griffonia simplicifolia-1* demonstrated the presence of the endothelial component of the graft. Positive staining, and presence of a lumen, was used to determine microvessel density within the graft and underlying host epicardial tissue. The vascular density within the grafts was 1097 +/- 204 vessels/mm² and 761 +/- 120 vessels/mm², for cMG and fMG respectively. The subepicardial vascular density in the tissue directly under the graft was also found to be higher in treated animals than either control animals or sham treated animals, with the epicardial vascular density in tissue underlying cMG 1474 +/- 206 vessels/mm² and tissue underlying fMG 1251 +/- 166 vessels/mm².

Using serial sections, H&E stained sections and alpha smooth muscle actin reacted sections were used to determine the morphologic characteristics of the graft. The distribution of arterioles, venules, and capillaries was found to be 6.4%, 52%, and 42%, respectively, for cMG. For fMG the vessels were found to be 4.4% arterioles, 33.6% venules, and 62.8% capillaries. Native, normal cardiac tissue was found to be vascularized by 2.8% arterial vessels, 49.3% venous vessels, and 47.9% capillaries, while control infarct tissue was found to be 0.5% arterial, 52% venular, and 47% capillaries.
Discussion

In this study we present the use of adipose-derived microvascular fragments to tissue engineer a surgically implantable microvascular graft (MG). The MG consisted of adipose-derived microvascular fragments that were isolated and resuspended in 3-dimensional collagen gels to form an implantable source of microvessel elements. In the current study we evaluated both a model system where the grafts were implanted immediately following microvessel isolation and graft construction (fMG), as well as grafts that were constructed and then allowed to undergo microvascular culture expansion (cMG). The cMG culture expansion process produced an extensive microvascular network within the graft as a result, primarily, of endothelial cell sprouting and proliferation from the intact vascular segments. The hypothesis in the current study was that both fMG and cMG would form vascular connections with the host coronary circulation following surgical placement on the epicardial surface of rat hearts that had undergone cryoinjury. Histologic evaluation and retrograde ink perfusion of the aortic and coronary circulations confirmed inosculation of the graft with the host coronary vasculature.

We have previously characterized this tissue engineered microvascular graft in the subcutaneous position of mice and demonstrated that the grafts form functional vascular connections with the host circulation as early as day 1. Here, when implanted in the epicardial position for 14 days the graft was well integrated with the underlying host cardiac tissue, and the vascular elements within the implanted graft had inosculated with the host epicardial coronary circulation. Histologic evaluation of the grafts revealed the
presence of arterioles, venules and capillaries within the vascular grafts, illustrating the presence of a mature microvascular network comprised of inflow, exchange, and outflow vascular elements.

The cardiovascular tissue engineering approach described in this study uses intact microvessel fragments that have been isolated from fat tissue. During cMG formation the rat microvessel fragments are suspended in a 3-dimensional collagen matrix, where they undergo spontaneous angiogenesis and expand to form a homogenous vascular network that is comprised of simple vessel elements. It is critical to note however, that the entire complement of microvascular cells is present including endothelial cells, smooth muscle cells, and vascular pericytes. This approach to pre-vascularization of the graft provides for increased microvascular density within the graft prior to implantation. Once implanted, the vessels within the cMG inosculate with the host vasculature and the grafted microvessels are exposed to blood flow.

It is likely that this exposure to blood flow leads to heterogeneous changes in the hemodynamic stresses that are experienced by the microvessels within the graft and this drives a vascular remodeling resulting the presence of arterioles venules and capillaries (Langille and O'Donnell, 1986), (Langille, 1989), (Skalak et al., 1998), (Pries et al., 2000), (Tuttle et al., 2001), (Jackson et al., 2002), (Sullivan et al., 2002). The presence of vascular pericytes and vascular smooth muscle cells at the time of MG implantation is also likely to expedite the development of this vascular structure distribution. Contrary to our study, where intact microvessels, complete with ECs, VSMCs and pericytes, are present at the time of inosculation and flow initiation through the graft, previously
published methods of generating complete microvessels in tissue engineered constructs have relied on the primary engraftment of endothelial cells followed by recruitment of additional cell types, presumably from the host circulatory system (Schechner et al., 2001), (Yang et al., 2001).

In many cases the construction and utilization of tissue engineered constructs that are ready for immediate use would be advantageous. In such situations it would be ideal to develop a construct that could be prevascularized and implanted within a single surgical setting. In this study we investigated the potential of a freshly isolated microvessel source (fMG) to establish a vascular connection with the host coronary vasculature. When utilizing fMG that are comprised of adipose derived microvessel fragments that have been suspended in collagen but have not undergone culture expansion, we observed a vascular response that was very similar to that seen with cMG. There were differences between the cMG and fMG implants that indicate the response seen in the former is moderately accelerated. This is evidenced by increased subepicardial vascular density as well as increased vascular density with the graft itself.

The microvascular density within the fMG was lower than that observed in cMG implants. This result can be readily explained by the lower vascular density at the time of implantation in the fMG as well as the proliferative state of the vessels in the cMG. At the time of graft construction the microvessels are suspended at roughly 12,000-15,000 fragments/ml, however in the cMG this number increases as a result of angiogenic expansion during the culture process. Additionally, the subepicardial vascular density was increased in the cMG treated animals. Previous research in our laboratory has
demonstrated that increased epicardial vascular density in myocardial infarction tissue correlates with increased left ventricular function following acute myocardial infarction. This suggests that there may be a functional advantage to treatment with a cMG implant versus treatment with fMG.

These data indicate that there may be an important role in initial vascular density that allows for cMG to undergo an appropriate vascular remodeling process earlier than fMG implants. The lower initial vascular density within the graft may require the graft to continue to undergo an angiogenic response that allows for proper connections with the host vasculature to be established. The higher prevalence of large vessels in the cMG also indicates that vascular remodeling, presumably outward remodeling of smaller vessels, is slower in the fMG. This may be due to more extensive perfusion at an earlier time following surgical grafting, or to the proliferative state of the cMG at the time of MG implantation.

Importantly, we were able to show that while allowing for expansion of the vascular elements in culture may allow for more robust response and accelerated remodeling within the construct, this culture period is not necessary for graft survival and inosculation with the host cardiac tissue. While the process of vascularization appears to be somewhat delayed in the fMG, it does appear to be complete. This is critically important to the application for human patients. Because fMG possesses the innate capability to undergo vascular remodeling and to establish arterial and venous blood systems, this suggests that this model may be applicable to an intra-operative setting. A scenario could then be realized whereby a patient’s own adipose tissue would be used to
isolate microvessel fragments, these fragments then used to add a vascular component to a tissue engineered construct, and the construct implanted – all within a single operative setting.

The development of large, complex tissue engineered constructs is dependent on the rapid establishment of a functional vasculature that is capable of meeting the metabolic demands of the implanted construct (Murphy et al., 2000). The ideal scenario for a large, dense construct would be in vitro generation with a microcirculation that either contains, or is capable of rapidly developing, arterioles venules and capillaries. However, for the critically ill patient needing immediate microvascular grafting, the employment of a technology not necessitating in vitro expansion will be most desirable.

Because of the utility displayed in models of allogeneic grafting, tissue procurement followed by immediate engraftment (fMG), and in vitro culture expansion prior to engraftment (cMG), the experimental MG described in this study offers immense promise for the treatment of myocardial microvascular dysfunction. The utilization of intact microvessels has not been previously described in the epicardial position, and the ability of the model described here to integrate with the host coronary circulation in the setting of acute myocardial injury offers a significant advantage over previously described methods for vascularizing tissue engineered grafts (Schechner et al., 2003). This model of microvessel transplantation may also serve to accelerate our understanding of the role of microvascular adaptation and dysfunction in not only pathologic heart failure, but cardiovascular disease, in general.
Figure 3.1  a-c Photomicrographs of isolated rat fat microvessel fragments in three-dimensional culture. The isolated microvessel fragments demonstrate extensive sprouting of endothelial cells from the parent vessels from day 0, until day 7, forming an extensive microvessel network in the collagen gel. d An MG ready for implantation following 7 days of in vitro microvessel expansion.
Figure 3.2  a, b Representative gross images of implanted cMG following 14 days implantation. Pericardial tissue, seen on the left, was easily freed from the implanted graft. c fMG following 14 days of implantation. d Acellular control gel imaged demonstrating no grossly visible microvessels at explantation.
Figure 3.3  

- **a** H&E staining of cMG demonstrating vascularization throughout the entire graft, 10X.  
- **b** 20X H&E staining showing the presence of arterioles, venules and capillaries within the transplanted cMG. Note the presence of RBCs within the luminal space of the vessels, indicating perfusion of the graft.  
- **c** fMG seen at 20X, H&E staining, also showing the presence of large caliber vessels with RBCs noted in within the walls of the defined vessels.  
- **d** Control gels comprised of collagen gels without microvessel elements remain avascular following 14 days of implantation. There were no vascular profiles noted in any of the control graft implants.
Table 3.1  

*a* Microvascular density was determined for normal cardiac tissue, cryoinjured tissue, and cryoinjured tissue treated with cMG or fMG.  

*b* Vascular density within the microvascular grafts at explantation.  

*c* The microvessel distribution was quantified, and the relative amount of arterioles, venules and capillaries was determined in serial sections of tissue. There was an increase in the number of venules present and a reduced number of capillaries present in the cMG versus the fMG.  

*n* = 6 in all groups.
4. Implantation of a Tissue Engineered Microvascular Graft in a Murine Model of Myocardial Infarction

Introduction

The revascularization of ischemic myocardium is essential to restore and maintain normal cardiomyocyte function. Areas of ischemic myocardium that cannot be revascularized by bypass or stenting are targets for tissue-engineered constructs that would regenerate a functional microcirculation and restore cardiac function. In the setting of acute myocardial infarction and in the development of ischemic cardiomyopathy, revascularization of ischemic myocardium is essential to a number of events which include but are not limited to, minimizing infarct size, the loss of ventricular function, and the potential rescue of hibernating myocardium (Pfeffer and Braunwald, 1990). Following AMI there is an initial area of infarction that will continue to undergo fibrotic remodeling and increase in size (Jennings et al., 1995). As this area of necrosis grows there is a precipitous loss of left ventricular function as a result of the permanent loss of cardiac myocytes. Moreover, there is significant evidence that as the condition myocardial ischemia persists, there develops an area of hibernating myocardium. The condition of hibernating myocardium is caused by critically low perfusion levels to areas of viable myocardial tissue (Rahimtoola, 1985), (Camici et al., 1997). This tissue, while viable, does not contribute to the contractile activity of the cardiac cycle and, therefore, does contribute to the decrease in ventricular function. The contractile function of this dormant tissue could, theoretically, be restored if perfusion to the areas of hibernating myocardium is increased.
The primary treatment modalities for patients suffering from the above conditions are Percutaneous Transluminal Coronary Angioplasty (PTCA) or Coronary Artery Bypass Grafting (CABG). However, there remains a significant patient population that does not receive complete revascularization following these procedures. Approximately 37% of patients undergoing CABG do not achieve complete revascularization, and this is due in large part to diffuse, microvessel involvement of the affected myocardium and consequent “no-reflow” condition (Levin et al., 1982), (Kloner et al., 1974). For these patients a treatment modality aimed at the restoration of a functional microvasculature is of paramount interest.

There have been a number of different strategies employed to limit the size of myocardial infarction, to revascularize ischemic myocardium, and to improve left ventricular function in the setting of myocardial infarction. These strategies have ranged from pharmacologic therapeutics such as ACE inhibitors (Konstam et al., 1992), and β-blockers (Basu et al., 1997), to gene therapy (Henry et al., 2003), (Grines et al., 2003), (Iwakura et al., 2003) cellular cardiomyoplasty (Taylor et al., 1998), (Atkins et al., 1999), and tissue engineering (Kellar et al., 2001), (Matsubayashi et al., 2003), (Fedak et al., 2003). While each of these therapeutic strategies may have potential benefits, there are also limitations to each. Pharmacologic therapeutics and gene therapy, which may limit the growth of infarction and ventricular remodeling, do not restore ventricular function to the patient. Cellular cardiomyoplasty, which is hypothesized to restore function by addition of contractile units to the heart via cell transplantation, is currently limited to the
delivery of single cellular units in a high dose, and these transplanted cells are subject to a very high mortality rate, themselves.

An emerging strategy to treat cardiac patients, that may initially allow for the addition of vascular conduit and, ultimately, the addition of contractile units to the heart, is tissue engineering. In contrast to other therapeutic modalities being explored now, a tissue engineered intervention offers the potential for the development of a device that will ultimately augment or even replace a diseased tissue or organ. We have previously described the use of a pre-formed microvascular graft for use as a tissue engineered microcirculation that can be successfully grafted in both the subcutaneous and epicardial positions. While inosculuation of the microvasculature within the grafted construct and the host vasculature occurred in both of the previously described models, it has not been previously evaluated in a model of acute arterial occlusion and resultant tissue ischemia. The ability to successfully engraft microvessels in a stringent model of tissue ischemia will be an appropriate litmus test for the potential of such a microvascular construct in patient care.

In the present study we have implanted a tissue engineered microvascular graft (MG) comprised of intact adipose-derived microvessel fragments to the epicardial surface of Severe combined immunodeficient (Scid) mice following experimental coronary artery occlusion. Microvessels were isolated from the epididymal fat pads of male rats, and cultured for 7 days prior to surgical engraftment. During the culturing period the microvascular elements undergo extensive spontaneous angiogenic sprouting and produce a microvascular network within the 3-dimensional collagen matrix in which they
are suspended. MG demonstrated inosculation with the underlying host vasculature, as well as vessel remodeling and vascular stability within the graft. The group of animals that received MG treatment had smaller areas of left ventricular infarction at 14 as compared to control animal groups. The attenuated development of left ventricular fibrosis following coronary artery occlusion and acute myocardial infarction indicates a therapeutic potential for the tissue engineering microvasculature to treat pathologic myocardial microvascular dysfunction and negative ventricular remodeling.
Materials and Methods

All animal studies were approved by the University of Arizona animal review committee, and animals were housed in American Association of the Accreditation of Laboratory Animal Care approved facilities following the procedures outlined by the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985).

Microvessel Isolation and Culture

Rat fat microvessel fragments (RFMF) were isolated from epididymal fat pads of retired-breeder Fischer 344 rats using a modification of previously published methods (Hoying, Boswell, and Williams, 1996). Under sterile preparation, harvested fat pads were washed in 0.1% BSA-PBS, finely minced with scissors and digested in collagenase (2mg/ml) in BSA-PBS (2mg/ml) for 8 min at 37°C with vigorous shaking. Tissue debris and large vessel pieces were removed by filtering the suspension through a sterile, 500μm pore size, nylon screen. Microvessel fragments were captured by filtration of the remaining suspension on a 30μm pore size nylon screen and recovered by vigorous flushing of the screen with 0.1% BSA-PBS. The type and lot number of collagenase used was predetermined to optimize fragment yield while maintaining microvessel structure. RFMFs were suspended (10,000 – 12,000 fragments/ml) in cold rat tail type I collagen (BD BioSciences, Bedford, MA) prepared with DMEM and pH neutralized with NaOH (1M). The tissue engineered microvascular grafts (TEMG) were plated into individual wells (0.125 ml/well) of a 96 well plate and placed in a 37°C incubator for 10 min. to
allow for polymerization of the collagen gel. For culturing, 0.125 ml of 10% FBS-DMEM was added to the TEMG, and each construct re-fed at 4 days. The total culture time was 7 days.

**Myocardial Infarction and MG Implantation**

On the day of surgery general anesthesia was induced with 2.5% Avertin (2.5% 2,2,2-tribromoethanol, 2.5 tert-amyl alcohol in PBS: Aldrich) by intraperitoneal injection. The neck and chest were shaved, and excess hair removed using a depilatory solution. The shaved areas were then prepared for sterile surgery using Nolvasan solution. Airway access was obtained through a tracheotomy and a 22-gauge catheter was inserted into the trachea and connected to a small animal ventilator for mechanical ventilation while the chest was open. The heart was exposed through a left lateral thoracotomy. The Left Coronary Artery was ligated with 8-0 nylon suture (Ethicon). Coronary ligation was confirmed via visualization of blanched cardiac tissue distal to the ligation site. Immediately following coronary artery ligation, an MG or control collagen gel without microvessels (n=6,5), was sewn directly onto the area of observed blanching, using a single 8-0 nylon stitch. Ligation only controls received no suture or gel placement (n=5). The chest was closed in layers, the chest evacuated of air, and the trachea was closed. The animal was recovered under a heat lamp.
**Explant and Evaluation**

On the day of explant the animals were anesthetized with 2.5% Avertin, IP. The abdominal aorta was isolated and cannulated with a 24G catheter. The heart was perfused in a retrograde fashion with a solution of PBS containing CdCl$_2$ (10%), Sodium Nitroprusside (10μM), and Heparin (10u/ml). Hearts were rapidly excised and placed in Histochoice® fixative solution (Ameresco). The hearts were then dehydrated and paraffin embedded. Sections of tissue (6μm) were then cut for Hematoxylin and Eosin, Trichrome, and cytochemistry.

Sections to be used for cytochemistry were reacted with the lectin *Griffonia simplicifolia* (*Gs*-1). *Gs*-1 binds carbohydrate domains on endothelial cells, and was visualized by use of a peroxidase recognition system (Dako, Inc. Universal mouse kit).

**Ink Perfusion**

Mice containing implants were anesthetized with Avertin and placed supine on a dissecting stage. The abdominal aorta was exposed and cannulated with a 24G catheter. India ink (Speedball #3398; Hunt Manufacturing Co., Statesville, NC), dialyzed against PBS and filtered through #1 Whatman paper, was perfused into the mouse at a maintained pressure of 90-100 mmHg until all tissues in the mouse appeared dark (2-3 ml ink). After ink perfusion, the heart was excised and dehydrated in graded concentrations of ethanol. After dehydration, the heart was then placed in methyl salicylate for tissue clarification. The clarified TEMG was then imaged for analysis using a dissecting microscope.
Determination of Infarct Size

Ventricular tissue sections (6μm) from the mid-infarct area were used to determine the percent fibrosis of the left ventricle. Tissue sections were stained using Masson’s Trichrome, which stains collagen fibers blue. Digital images of the tissue section were captured and using a morphometric analysis program (Metamorph), the area of infarct was determined by the percent of the left ventricle that stained blue.

Vessel Density and Characterization

Tissue sections treated with Gs-1 were analyzed using a 20X (vessel type) or a 40X water immersion (vessel density) objective and light microscopy. For vessel characterization studies, five random high power fields (HPF) (200X200 μm) were examined within the implant for each animal in both the MG and control implant group (n=6,5). Microvessels were classified using standard histological features (Weidner et al., 1991). Capillaries were identified as a single layer of flattened endothelial cells with an identifiable lumen. Arterioles were identified as having and ID ≥ 10μm and by the presence of characteristic layers: endothelium, tunica media, and tunica adventitia. Control infarct animals that did not have implants were only evaluated within the myocardial scar (n=5). Venules were differentiated from arterioles by their large lumen diameter compared with vessel wall thickness, a thinner or absent smooth muscle layer, a less significant tunica adventitia, and an ID > 10μm. Vessel density studies were done using GS-1 reacted sections observed under light microscopy with a 40X water immersion lens. Ten random HPF
(54X54 um) were identified within the infarcted myocardium for animals in each group. In each HPF the number of cross sectional or longitudinal vessels that were positive for Gs-1 staining were counted. A one-way ANOVA was used to determine significance between treatment groups.
Results

The enzymatic digestion of the removed adipose tissue resulted in the isolation of intact, microvascular fragments. The fragments were comprised of the full compliment of microvascular cell types including endothelial cells, smooth muscle cells, and vascular pericytes. When suspended in a three-dimensional collagen matrix the isolated fragments underwent angiogenic sprouting within the gels. Sprouting of vessel elements occurred primarily though proliferation of endothelial cells, identified morphologically, and possibly some vascular fibroblasts. The endothelial cell sprouting occurred at the ends of the vascular segments as well as along the length of the isolated microvessel. Following 7 days of culture expansion and graft formation, the grafts were surgically sewn to the epicardial surface following coronary ligation (Fig. 4.1).

After 14 days of implantation, the grafts were found to be well integrated with the underlying epicardial surface. Blood vessels were identifiable within the graft with the use of a dissecting microscope. By contrast, avascular collagen gels that were implanted as controls showed no gross signs of vascularization (Fig. 4.2a,b). There were no blood vessels noted in any of the implanted control grafts (Fig. 4.2c,d).

Histologic evaluation, using Hematoxylin and Eosin staining, of the implanted MG demonstrated a robust microvasculature throughout the grafts (Fig. 4.3a-d). The histologic identification of arterioles, venules, and capillaries was noted in all MG. Control grafts consisting of acellular collagen gels were found to be absent of any vascular infiltration. There were a very limited number of infiltrating inflammatory cells found in some control grafts.
The presence of endothelial cells was confirmed by histochemistry using the lectin \textit{Gs-1} (Fig. 4.4 a,b). Microvascular characterization of the vessels within the grafts were done in order to determine vascular density and vessel type distribution. The vascular density in this model within control grafts was found to be 10 +/- 42, while the MG vascular density at 14 days was 802 +/- 244 vessels/mm$^2$, and at 28 days was 1419 +/- 377 vessels/mm$^2$ (Table 4.1). Vessel type distribution was determined using serial sections at 14 and 28 days, and the percent arterioles, venules and capillaries was found to be 3.8%, 37.2%, and 59%, respectively at 14 days and 2.8%, 56%, and 41.2% (Table 4.1).

Vessel density studies were also performed in the area of infarct and the surround peri-infarct tissue (Table 4.2). There was a trend towards increased microvascular density in both the area of myocardial infarction as well as the area of peri-infarct tissue in the animal group treated with MG, however this difference was not statistically significant.

Additionally, double fluorescent imaging was performed to determine the spatial relation of perivascular cells with respect to endothelial cells stained with vWF (Fig. 4.4c). The pan nuclear stain BBI was utilized to visualize all nuclei within the graft, and the same tissue section was also reacted with fluorescently conjugated vWF in order for determination of the spatial relation of the cells in the graft to the vascular structures of the graft (Fig. 4.4d).

When India ink was used to perfuse the coronary vasculature in an antegrade fashion, by retrograde infusion into the thoracic aorta, the vessels within the graft filled further illustrating vascular continuity with the underlying cardiac tissue (Fig. 4.5). The
perfusion of the graft with the ink used occurred in a rapid fashion indicating a contiguous connection with the arterial coronary circulation.

Masson’s Trichrome staining of sections taken at mid-infarct level was used to determine the degree of myocardial infarction in the three treatment groups after 14 days of enrollment. The infarct area was found to be significantly less in the MG treated animals than in the infarct only or the infarct with control graft groups (Fig. 4.6).
Discussion

The development of tissue engineered constructs to treat the maladies associated with ischemic heart disease is an essential step in the future management of cardiovascular disease. In the present study we evaluated the potential for a surgically implanted tissue engineered microvascular graft (MG) to inosculate with underlying coronary vasculature following acute coronary ligation in the mouse. The central hypothesis for this study was that a tissue engineered microvascular graft comprised of adipose-derived microvessels could be surgically attached to the epicardium of mice following coronary ligation, and that this graft would serve as a transplanted unit of vascular conduit. The secondary hypothesis to be tested here was that following inosculation and perfusion through the graft, continued vascular remodeling would produce arterioles venules and capillaries within the graft, and that the presence of this mature microvasculature would result in reduced infarct size.

We have characterized the current tissue engineering model used in this study, previously, in the subcutaneous position and demonstrated that there is rapid perfusion of the microvessel graft following implantation. The initial vascular connection between graft and host is established as early as day 1, and active vascular remodeling continues for as long as three months. Furthermore, using in situ hybridization, we have demonstrated that the transplanted MG is a stable vascular unit with up to 95% of the cells in the graft being derived from the MG at 14 days. Based on the establishment of a rapid vascular connection and the excellent vascular stability of the grafts, we felt that
this microvascular tissue engineering approach was ideally suited for the experimental treatment of acute myocardial infarction following coronary occlusion.

Acute cardiac injury followed by MG engraftment on the epicardial surface was initially studied in a rat model of left ventricular cryoinjury. This model of localized cardiac injury, followed by MG transplantation, offered valuable insight into the capability of the experimental MG to establish a perfusion connection with the underlying host cardiac tissue. Perfusion of the grafted microvessels following placement in a tissue site undergoing pathologic remodeling is a significant step in the development of a microvascular tissue substitute. The relative success of this experimental model must be tempered by the knowledge that the injury created in the cryoinjury study was one that does not remove a significant level of host tissue perfusion. That is, while there is a defined area of cardiac injury, this is not a result of hypoperfusion generating infarcted tissue.

The need to evaluate the MG in a site of critical hypoperfusion caused by arterial ligation has led us to the present study. Here, we have experimentally ligated the left coronary artery of mice to mimic the natural events following acute myocardial infarction, and to determine potential physiologic effects of MG grafting. The results of this study were that the implanted MG was able to inosculate with the underlying epicardial vasculature, establish a functional vascular connection, and to reduce the level of infarct formation at 14 days following coronary ligation. The control graft that was used in this study was a three-dimensional collagen gel that was absent of any cellular elements and was grafted to the epicardium in the same fashion as the MG immediately following coronary
ligation. The acellular control grafts were found to remain avascular for up to 28 days, and had no effect on infarct formation and ventricular remodeling.

The development of treatment options that incorporate tissue engineering is reliant on the rapid establishment of perfusion through the construct. If the engineered constructs are to be of significant size, blood flow must be established early, as diffusion will not meet the metabolic needs of the grafted tissue (Eiselt et al., 1998). While the strategies of utilizing single cell units or the release of angiogenic factors in and around constructs has shown limited efficacy, these approaches have major limitations. First, previous approaches have shown a severe trend towards the production of vascular beds comprised primarily of capillaries with little or no large caliber arterioles or venules. Second, whether the mechanism of vascularization is angiogenesis, or neovascularization, there is a definable latency period required for vascular assembly and for the establishment of flow through the neovessels. This second problem is of major concern when dealing with large constructs comprised of metabolically active cells, especially cardiomyocytes, that would undergo tissue necrosis within the core of the graft if not rapidly perfused following implantation.

Therefore, it would be advantageous to utilize a pre-vascularized construct such as the MG evaluated in this study. The use of a pre-vascularized device would obviate the latency period required for vascular development through either angiogenesis or neovascularization, as is the case with growth factor and cell transplantation strategies in tissue engineering. Important, the distribution of vessels within the MG studied here was found to consist of arterioles, venules and capillaries, which is an important
characteristic of a functional microcirculation. In contrast to many previous attempts to both vascularize tissue engineered constructs and ischemic tissue via therapeutic angiogenesis, which have produced predominantly a capillary rich environment, the implanted MG in this study display the characteristics of a microcirculation capable of meeting varying metabolic demands as would be found in the myocardium. There are histologically identifiable arterioles venules and capillaries within the graft and fluorescent staining using vWF and BBI demonstrated that the majority of cells in the MG were in close association with vWF positive endothelial cells and are, thus, most likely smooth muscle cells and pericytes.

The coronary ligation model system used here is not only an appropriate model for experimental approaches to revascularize myocardium but a stringent one, especially given that re-establishment of blood flow is required for graft success. The placement of the graft onto cardiac tissue that has just been made ischemic as a result of the suture ligation of a major coronary artery provides a very dynamic setting for the recruitment of a vascular supply. By implanting the MG so as to overlay both tissue that was continuously perfused and ischemic tissue created by the coronary occlusion, it is conceivable, if not likely, that the initial graft-host vascular connection occurred in a tissue site that was unaffected by the coronary ligation. Indeed, because arterial blood flow had been severely restricted to the tissue served by the Left Coronary Artery distal to the ligation, it is unlikely that graft perfusion was initiated in this tissue bed. In this proposed setting, the implanted MG would be acting like a microvascular coronary artery
bypass graft and, in fact, be reperfusing distal tissue that was directly affected by the coronary ligation.

Clinically, there are a large number of patients that are affected both by the continued ventricular remodeling that occurs after acute myocardial infarction, as well as by the condition of hibernating myocardium (Vanoverschelde et al., 2001), (Pagano et al., 2001). The growth of scar regions following the initial ischemic event is a process that leads to increased and permanent loss of cardiomyocytes and, as a result, loss in left ventricular function. The restoration of perfusion to tissue that surrounds an infarct, area at risk, may reduce both the negative ventricular remodeling that occurs, as well as the development of hibernating myocardium (Rinaldi et al., 2000), (Adams et al., 1996).

By utilizing the mouse model of coronary ligation we are able to evaluate the degree of myocardial infarction that occurs in animals that have undergone infarction alone as well as those animals that have been treated with MG and control grafts. We report here that animal groups that were treated with MG had reduced infarct sizes versus animal groups of infarct alone or treatment with control grafts. These results support the hypothesis that the MG acted as a microvascular bypass graft and attenuated, to some degree, the negative ventricular remodeling that occurs following acute myocardial infarction. This improved ventricular healing should lead to improvements in measurements of left ventricular function such as ejection fraction and cardiac output.

Regardless of the potential for the described TE model to be applied to the pre-vascularization of large tissue constructs, the most significant finding of this study was the reduced infarct size associated with MG treatment. As of yet, no previous tissue
engineered graft comprised of vascular elements has shown a physiologic benefit to pathophysiologic tissue treated with the experimental construct. These results suggest that the cardiac performance of animals treated with MG would be greater than those not treated and may implicate a role in the attenuation of further development of Ischemic Heart Failure.

The reperfusion of myocardial tissue following acute myocardial infarction is critical to the preservation of that tissue. Furthermore, as a result of the increased incidence of ischemic heart disease in the western world, patients with microvascular dysfunction and diffuse small vessel disease of the heart will require novel therapeutic approaches such as tissue engineering. A tissue engineered strategy that provides a pre-vascularized construct capable of rapid inosculation and perfusion would present an ideal microvascular tissue substitute for those patients. The results of the present study demonstrate the ability of a tissue engineered microvascular graft to revascularize ischemic myocardium following acute myocardial infarction and to attenuate the development of negative ventricular remodeling that occurs following coronary artery occlusion.

In addition to the potential physiologic advantages that may be conferred to the heart as a result of microvessel transplantation, the addition of a pre-vascularized graft may represent additional novel strategies to treat disease. Construction of tissue, ex vivo, that will spontaneously anastamose with the host circulatory system could potentially allow for administration of pharmacologic agents or gene therapies. Combination of the microvessel technology used in this study with genetically engineered cells or controlled
release polymers for drug elution, may greatly aid in the development of revolutionary therapies that are applicable to countless disease states.
Figure 4.1 Light micrographs of a cultured MG at culture day 0 (a), day 5 (b).  
For size comparison, an MG used in the previous rat study is shown, with a 7-0 suture needle imaged for reference.  
(d) MG immediately prior to implantation in a mouse. The markings on the ruler below both grafts indicate millimeters and the mouse graft is pictured with an 8-0 suture needle for size reference.
Figure 4.2 a Barium Sulfate filled coronary vasculature prior to suture ligation and (b) following suture ligation of the left coronary artery. c and d Gross explant images of MG following 14 days implantation. Note the extensive microvasculature and the well-defined vascular arborization. e and f Control gels constructed of collagen gel alone remain avascular.
Figure 4.3 Hematoxylin and Eosin of implanted MG and control grafts. *a* and *b* MG stained with H&E following 14 days implantation reveal the presence of extensive vascularization within the graft. The presence of arterioles, venules and capillaries is noted in the graft. *c* and *d* By contrast, histologic evaluation of control gels does not reveal the presence of any vascular profiles within the control graft proper.
Figure 4.4  

Figure 4.4 a and b Gs-1 reacted tissue sections revealing the presence of endothelial cell-line vascular profiles within the graft. c Fluorescent labeled vWF staining of the MG at 14 days. d This vWF staining is shown with BBI nuclear staining demonstrating the localization of nuclei to the vascular structures in the graft. The majority of cells within the graft are either endothelial cells or cells found associated with the microvascular wall.
Microvessel Density in Graft
Murine Coronary Ligation

Table 4.1 Microvessel Characterization of the vessels within the graft. a The vascular density was found to increase from 14 to 28 days, while control grafts were found to be avascular. b Microvessel characterization was also done at 14 and 28 days and the relative number of large vessels increased from 14D to 28D (n=6).
Table 4.2 Microvascular characterization of the infarct and peri-infarct tissue.  

*a* The microvessel density within the area of left ventricular fibrosis was found to be elevated in the group of MG treated animals versus control groups.  

*b* Evaluation of the peri-infarct tissue revealed the same trend of increased microvessel density in the MG treated animal group.
Figure 4.5 India ink perfusion of the vasculature within the graft. The MG has been dehydrated and clarified in methyl salicylate following retrograde ink perfusion through the aorta and subsequent antegrade perfusion of the coronary vasculature. This figure illustrates vascular continuity with the underlying cardiac vasculature.
Figure 4.6 Trichrome staining of explanted hearts following 14 days of infarct formation. The infarct alone group (a), and infarct plus control graft (b) demonstrated a larger infarct size than the MG-treated infarct group (c). The level of infarct as a percent of the left ventricle is quantified in d.
5. Implantation of Tissue Engineered Microvascular Graft Improves Left Ventricular Function Following Myocardial Infarction

Introduction

The development of novel therapeutic strategies to treat Ischemic Heart Disease (IHD) and Heart Failure (HF) is critical to the future management of Cardiovascular Disease patients. There have, indeed, been improvements in the efficacy, as well as increased numbers, of medical management options for IHD patients, however, the number of patients who prove to be refractory to these therapies continues to rise (Shachar et al., 2003). For those patients who have proven resistant to traditional treatment modalities, continued research and development of new treatments is critical.

The condition of myocardial ischemia and infarction leads to the permanent loss of cardiomyocytes due to the inability of these muscles cells to regenerate in the adult heart. There is a limited, if not contradicted, body of evidence indicating limited myocyte proliferation in the adult heart, but there is no documentation of settings where this regeneration is sufficient to preserve left ventricular function in the setting of acute myocardial infarction (Urbanek et al., 2003), (Nadal-Ginard et al., 2003), (Anversa et al., 2002), (Leri et al., 2002). Thus, while there may be inherent mechanisms in place to recover a certain degree of lost left ventricular function following ischemic insult, it is clear that these reparative mechanisms are insufficient.

The central dogma behind the revascularization treatment strategies for IHD is that primary vascular lesions in the large epicardial coronary arteries lead to areas of
myocardial ischemia and occlusion of these arteries leads to areas of infarction (Patten et al., 1998), (Pfeffer et al., 1985), (Pfeffer and Braunwald, 1990). In order to restore perfusion to the myocardium, these lesions must be re-opened or surgically bypassed. Historic methods of re-establishing blood flow have consisted primarily of Percutaneous Transluminal Coronary Angioplasty (PTCA), Coronary Artery Bypass Grafting (CABG) and coronary stent placement. Each of these interventional methods have been very successful, however, each have significant failure rates as well. For example, the acute restenosis rate for coronary stents is approximately 30% (Rajagopal et al., 2003). Finally, many patients are not only refractory to these primary intervention strategies, but also have microvascular dysfunction that is responsible for global myocardial ischemia and loss of left ventricular function (Strujiker Boudier et al., 2003), (Sagar et al., 2000), (DeWood et al., 1980), (Braunwald, 1990). These patients are not candidates for surgical intervention, and in many cases are deemed “no-option” patients. In order to offer these patients an efficacious treatment option, enormous resources have been allocated to research in the area of novel IHD therapeutics. These approaches have included Transmyocardial Revascularization, Gene Therapy, therapeutic angiogenesis, Cellular Cardiomyoplasty, and most recently Tissue Engineering (Sen et al., 1965), (Mirhoseini et al., 1983), (Horvath et al., 1996), (Oesterle et al., 1998).

The main target of these treatment strategies has been increased myocardial perfusion through increased microvascular density. This increased microvascular density is achieved through either angiogenesis or vasculogenesis. The endogenous process of ischemic angiogenesis and vasculogenesis is hypothesized to be augmented as a result of
the tested experimental treatment. There have been many clinical trials initiated to evaluate the safety and efficacy of these potential treatments. While there have been promising initial results in some cases, these results have been accompanied by notable clinical failures.

The proposed strategies fail in a number of critical areas. The development of new vasculature from de novo vascular components (vasculogenesis) or through modification of existing vascular structures (angiogenesis) requires a latency period of new vessel growth and remodeling. Furthermore, patients who suffer from microvascular dysfunction, necessitating such intervention, may be extremely unlikely to respond to therapies aimed at driving microvascular growth. Finally, because the treatment is aimed at a hypoxic tissue site, the critically low oxygen levels are not amenable to cell survival which is critical to the success of cell transplantation studies such as CCM or cell-mediated therapeutic angiogenesis. Cardiac Tissue Engineering (TE) is a field that aims to regenerate cardiac tissue and augment ventricular function. The success of this field is dependent on the ability to rapidly vascularize tissue engineered constructs. Because the ultimate goal for TE constructs will be the development of grafts too large to allow for diffusion of nutrients and metabolic waste, rapid establishment host-graft vascular connections will be necessary for initial and prolonged graft viability.

In this study we present the successful implantation of a tissue engineered microvascular graft in a murine model of myocardial infarction. Severe combined immunodeficient mice underwent Left Coronary ligation followed by MG implantation. Fourteen days after ligation and MG implantation, left ventricular function was measured using a Millar
Conductance Catheter. Mice who had been treated with MG demonstrated increased hemodynamic function versus control groups of infarct alone, or infarct treated with avascular collagen gels. These results indicate the potential for tissue engineered microvascular grafts to serve not only as a platform for large, three-dimensional tissue engineered constructs, but as a stand-alone treatment for myocardial microvascular dysfunction.
Materials and Methods

All animal studies were approved by the University of Arizona animal review committee, and animals were housed in American Association of the Accreditation of Laboratory Animal Care approved facilities following the procedures outlined by the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985).

Four groups of mice were studied including; normal SCID mice (n=5), infarcted SCID mice (n=5), infarcted SCID mice with MG (n=5), and infarcted mice with avascular collagen gel implanted as a control graft (n=5). At 14 days post-infarction, left ventricular function pressure-volume loops were quantified and tissue samples were collected from all treatment groups. A one-way analysis of variance (ANOVA) was used to determine a significant difference (alpha ≤ 0.05) between the treatment groups. Duncan's multiple comparison test was used to do pairwise comparisons between the treatment groups.

Microvessel Isolation Microvascular Graft Formation

Rat fat microvessel fragments were isolated from epididymal fat pads of retired-breeder Fischer 344 rats using a modification of previously published methods (Hoying, Boswell, and Williams, 1996). Under sterile preparation, harvested fat pads were washed in 0.1% BSA-PBS, finely minced with scissors and digested in collagenase (2mg/ml) in BSA-PBS (2mg/ml) for 8 min at 37°C with vigorous shaking. Tissue debris and large vessel
pieces were removed by filtering the suspension through a sterile, 500μm pore size, nylon screen. Microvessel fragments were captured by filtration of the remaining suspension over a 30μm pore size nylon screen and recovered by vigorous flushing of the screen with 0.1% BSA-PBS. The type and lot number of collagenase used was predetermined to optimize fragment yield while maintaining microvessel structure. Rat Fat Microvessel Fragments were suspended (12,000 – 15,000 fragments/ml) in cold rat tail type I collagen (BD BioSciences, Bedford, MA) prepared with DMEM and pH neutralized with NaOH (1M). The microvascular grafts (MG) were plated into individual wells (0.125 ml/well) of a 96 well plate and placed in a 37°C incubator for 10 min. to allow for polymerization of the collagen gel. For culturing, 0.125 ml of 10% FBS-DMEM was added to the MG, and each construct was re-fed at 4 days. The total culture time for graft formation was 7 days.

**Myocardial Infarction and MG Implantation**

On the day of surgery general anesthesia was induced with 2.5% Avertin (2.5% 2,2,2-tribromoethanol, 2,5 tert-amyl alcohol in PBS: Aldrich) by intraperitoneal injection. The neck and chest were shaved, and excess hair removed using a depilatory solution. The shaved areas were then prepared for sterile surgery using Nolvasan solution. Airway access was obtained through a tracheotomy and a 22-gauge catheter was inserted into the trachea and connected to a small animal ventilator for mechanical ventilation while the chest was open. The heart was exposed through a left lateral thoracotomy. The Left Coronary Artery was ligated with 8-0 nylon suture (Ethicon). Coronary ligation was
confirmed via visualization of blanched cardiac tissue distal to the ligation site. Immediately following coronary artery ligation, a MG or control collagen gel without microvessels (n=5,5), was sewn directly onto the area of observed blanching, using a single 8-0 nylon stitch. Ligation only controls received no suture or gel placement (n=6). The chest was closed in layers, the chest evacuated of air, and the trachea was closed. The animal was recovered under a heat lamp.

**Explant and Evaluation**

On the day of explant the animals were anesthetized with 2.5% Avertin, IP. The abdominal aorta was isolated and cannulated with a 24G catheter. The heart was perfused in a retrograde fashion with a solution of PBS containing CdCl₂ (10%), Sodium Nitroprusside (10μM), and Heparin (10u/ml). Hearts were rapidly excised and placed in Histochoice® fixative solution (Ameresco). The hearts were then dehydrated and paraffin embedded. Sections of tissue (6μm) were then cut for Hematoxylin and Eosin, Trichrome, and cytochemistry. Sections to be used for cytochemistry were reacted with the lectin Griffonia simplicifolia (Gs-1). Gs-1 binds carbohydrate domains on endothelial cells, and was visualized by use of a peroxidase recognition system (Dako, Inc. Universal mouse kit).

**Vessel Density and Characterization**

Tissue sections treated with Gs-1 were analyzed using a 20X (vessel type) or a 40X water immersion (vessel density) objective and light microscopy. For vessel characterization
studies, five random high power fields (HPF) (200X200 µm) were examined within the implant for each animal in both the TEMG and control implant group (n=6,5). Microvessels were classified using standard histological features. (REF from Kellar) Capillaries were identified as a single layer of flattened endothelial cells. Arterioles were identified as having and ID > 10µm and by the presence of characteristic layers: endothelium, tunica media, and tunica adventitia. Control infarct animals that did not have implants were only evaluated within the myocardial scar (n=5). Venules were differentiated from arterioles by their large lumen diameter compared with vessel wall thickness, a thinner or absent smooth muscle layer, a less significant tunica adventitia, and an ID > 10µm. Vessel density studies were done using Gs-1 reacted sections observed under light microscopy with a 40X water immersion lens. Ten random HPF (54X54 um) were identified within the infarcted myocardium for animals in each group. In each HPF the number of cross sectional or longitudinal vessels that were positive for Gs-1 staining were counted. A one-way ANOVA was used to determine significance between treatment groups.

Left Ventricular Function Evaluation

The functional analysis of each animal was performed prior to animal sacrifice, 14 days following infarct surgery and MG implantation. Left ventricular function analysis performed as described in the procedures of Yang et al. and Nemoto et al. (10), (11). Briefly, at 14 days, mice were anesthetized with an intraperitoneal injection of urethane (1,000 mg/kg) and α-chloralose (50mg/kg). The use of the α-chloralose/urethane
anesthesia was selected based upon reports in the literature that describe this anesthesia to have minimal effect on cardiovascular reflexes and that it is well tolerated in mice (12), (13). Respiration was controlled through a tracheostomy cannula connected to a small animal ventilator. The right carotid artery was isolated and cannulated with a 1.4 Fr Millar Conductance Catheter. Thermal cautery was used to ensure minimal blood loss. The conductance catheter was then advanced into the left ventricle and positioned along the cardiac longitudinal axis with the distal electrode in the apex and the proximal electrode below the aortic valve. The external jugular vein was cannulated for volume administration, which was limited to 300 ml of saline:albumin (50:50). Placement of the catheter in the ventricle was confirmed by the profile of the pressure-volume loops generated, and it was easily determined when the catheter was in the aorta and when it had been advanced into the ventricle. A midline abdominal incision was made to expose the inferior vena cava above the liver and below the diaphragm. Real-time pressure and volume data were collected for approximately 5 to 10 seconds (5 to 20 loops) for both baseline and inferior vena cava occlusion time points. Data collection used the Millar Aria-1 system with AD Instruments Powerlab software system. Following data collection subsequent data analysis was performed using Millar PVAN Cardiac Pressure-Volume Analysis software, version 2.9.

Following pressure-volume loop analysis, heart samples were arrested in diastole, perfused with fixative and immediately immersed into Histo-Choice® fixative (Ameresco). Sections (6 µm) of fixed samples were subsequently processed for hematoxylin and eosin (H&E), trichrome, and cytochemical evaluation. Sections for
cytochemistry were reacted with *Griaffonia simplicifolia* lectin (peroxidase-conjugated lectin Gs-1, EY Laboratories; used at a final dilution of 1:100), which binds carbohydrate domains on endothelial cells, and visualized using a peroxidase recognition system (Dako, Inc.; Universal mouse kit) to identify vascular elements.
Results

The isolated microvascular fragments were suspended at an initial density of 15,000 fragments/ml and expanded in culture. The expansion in culture is primarily through angiogenic sprouting of endothelial cells from the primary isolate vessels. Previously published results from experiments in our laboratory with isolated RFMF in collagen gels have demonstrated the vessels present within the gel have are largely homogenous in size with an average diameter of 24.6μm after 7 days of culture. The implanted MG were constructed under identical culture conditions and phase micrographs demonstrated a homogenous vascular distribution like that seen in our previous experiments. A schematic representation of the donor and implantation relationship is shown in Figure 5.1.

At explantation, the grafts were found to be well integrated with the underlying epicardial tissue, and large microvessels were grossly visible within the graft. As early as day 7 large vessels are visible within the graft, and the level of vascularity has increased by day 14 (Fig. 5.2b,c). By contrast, control gels comprised solely of Type I collagen without microvessel elements, were found to be grossly avascular following 14 days of implantation (Fig. 5.2d).

Histologic and cytochemical evaluation of the explanted graft revealed the presence of an avascular control and the residence of a mature microvascular network in the transplanted MG (Fig. 5.3a,b). Using an MG comprised of microvessels isolated from a mouse expressing GFP in association with the Tie-2 receptor, visualization of the grafted microvessels was possible using confocal microscopy. The presence of large caliber
vessels arborizing into smaller vessels was noted in the graft. Additionally, FITC-labeled
dextran was infused into the venous system of the mouse as an intravascular tracer 30
minutes prior to arrest of the heart. The presence of dextran was noted within the walls
of the GFP expressing vessels indicating vascular continuity with the host circulation
(Fig. 5.3c,d).

The abbreviations used for Hemodynamic parameters are outlined in Table 5.1 and the
hemodynamic parameters are reported in Table 5.2 for all groups of mice in the study.
The highlighted parameters in Table 5.2 were found to be statistically different between
the treated group and the two control groups, with \( p < .05 \). The hemodynamic parameters
that were found to be different between the MG treated groups and the control groups
were \( V_{\text{min}} \), \( V_{\text{max}} \), End Systolic Volume, End Diastolic Volume, \( P_{\text{min}} \), Ejection Fraction,
Cardiac Output, and Stroke Work. Graphical representation of each of these parameters
is presented in the bar graphs in Table 5.3.

Representative pressure-volume loops from each of the four groups of mice are shown in
Figure 5.4. Note the upward-right shift of the infarct only animals and the infarct animals
treated with control grafts. The animals treated with MG demonstrated a slight rightward
shift of the pressure volume loop, but this shift, indicative of LV dysfunction and heart
failure, was attenuated in the MG-treated animals.
Discussion

The tissue engineered microvascular graft utilized in this study has been previously characterized by our laboratory following grafting in both the subcutaneous position as well as the epicardium (data in submission for publication). In both tissue sites the implanted MG have demonstrated rapid inosculation with the host circulation. Moreover, the vessels within the graft undergo continual vascular remodeling and establish a vascular architecture comprised of arterioles, venules, and capillaries. For grafts that are implanted with the same initial vascular density, the vascular density within the MG when implanted for 14 days is greater in the epicardial position than in the subcutaneous position suggesting a tissue-specific vascular adaptation.

The current study aimed to evaluate the ability of this previously characterized tissue engineered microvascular graft to confer functional improvement to the LV of mice that had undergone acute coronary ligation. The hypothesis tested was that implantation of a tissue engineered microvascular graft following acute myocardial infarction would attenuate the development of left ventricular dysfunction. By grafting an additional perfusion circuit that would enhance natural collateralization following coronary occlusion, the area of developing infarction can, in turn, be limited and the extent of LV dysfunction modulated.

In order to evaluate LV function in the current study, we utilized a 1.4 Fr Millar Conductance Catheter in order to generate real-time pressure volume loops and gather data associated with the hemodynamic performance of the left ventricle. In order to preserve ventricular and myocardial integrity in the tissue site of interest, the conductance
catheter was introduced into the left ventricular cavity through the carotid artery. This method of catheterization, as opposed to an open chest approach where the catheter is inserted into the left ventricle through the apex, has been previously described by Nemoto et al., and more accurately assesses baseline cardiac function as the thorax is never entered (Nemoto et al., 2003).

Normal Scid mice that had not undergone any surgical procedures were used as controls in this study to establish baseline cardiac function. The hemodynamic parameters that we have reported here are well within the range of values previously published by other groups (Yang et al., 2002), (Liu et al., 2002), (Yu et al., 2002). Additional animal groups evaluated consisted of an infarct only group, infarct treated with avascular collagen gel, and infarct treated with MG. Both the infarct alone and infarct treated with control graft groups demonstrated almost identical hemodynamic parameters.

The animals that underwent coronary ligation alone, or ligation followed by control graft implantation demonstrated characteristics of heart failure development including increased $V_{\text{max}}$, $V_{\text{min}}$, EDP, and Tau, along with reduced EF, CO, and SW. The development of left ventricular dysfunction in the MG treated group of animals was reduced as indicated by measured values that were closer to values of the normal group of animals tested. For example, $V_{\text{max}}$ and $P_{\text{min}}$ in the infarct only group was 21.4 $\mu$L and 8.0 mmHG, respectively. The $V_{\text{max}}$ and $P_{\text{min}}$ values for normal Scid mice was found to be 17.6 $\mu$L and 2.1 mmHG, while $V_{\text{max}}$ and $P_{\text{min}}$ values in the MG treated group were 16.7 $\mu$L and 4.4 mmHG. Additionally in the normal, MG-treated, control graft-treated, and infarct alone groups the ejection fractions were found to be 79%, 64%, 39%, and 36%,.
respectively. Again, MG-treated animals displayed ejection fractions that were statistically greater than the control infarct groups.

The data that has been previously published by our laboratory using tissue engineered constructs suggests that the augmentation of coronary revascularization and collateralization is successful in retarding the development of LV dysfunction following coronary occlusion. Other research groups have had similar success using therapeutic angiogenesis and cellular cardiomyoplasty (Isner et al., 2002), (Kleiman et al., 2003), (Simons et al., 2000) (Taylor et al., 1999), (Li et al., 1999). However, especially in the field of gene therapy and therapeutic angiogenesis, the clinical results of these studies have been inconclusive.

Constructs of appreciable 3-dimensional size have been used in the laboratory as well, with evidence that cellular viability within the core of the graft is compromised due to the lack of perfusion through the graft. Thus, vascularization of large tissue engineered grafts, especially at the microvascular level, is of paramount importance to the ultimate success of the graft (Moldovan et al., 2002). Furthermore, in sites of high metabolic demand, such as cardiac tissue, this establishment of vascular continuity with the host must occur rapidly.

The MG used in this study has demonstrated the potential for both the development of larger 3-dimensional constructs that require a vascular supply as well as the potential for a transplanted microvascular network as a stand alone therapy. Importantly, the MG utilized in this set of experiments does not require the use of any exogenous or added growth factors for vascularization. Inosculation of the grafted microvasculature with the
underlying host cardiac vasculature occurs spontaneously. There is no latency period required for vascularization for the MG as is needed for neovessel formation when employing growth factors, therapeutic angiogenesis, or cell transplantation. Once the graft has been exposed to flow, the heterogenous presence of microvascular calibers can be explained by the presence of flow and shear experienced in the grafted microvessels. It is also critical to note that the MG is comprised of the full complement of microvascular cells (endothelial cells, pericytes, vascular smooth muscle cells, and fibroblasts), which may explain the presence of well defined arterioles. This is also supported by the observation that MGs implanted for 14 days contain a larger proportion of capillaries than MGs implanted for 28 days. The grafts that have been implanted for 28 days have a larger component of arterioles and venules than grafts implanted for 14 days. These results suggest an active vascular remodeling within the MG, which would likely be positively affected by the presence of additional cell types as would be found in a bioengineered graft.

At this time it is not currently understood what is driving the anastomotic connection process to occur. It is likely that the surrounding tissue environment is responsible for the initial impetus to form a vascular connection. There is a critically hypoxic tissue milieu that is created in the cardiac tissue that is served by the arterial blood supply removed through coronary ligation. This hypoxic tissue would release a number of important cytokines including VEGF, and FGF that would affect the vascular remodeling that occurs within the graft. In order for any soluble cytokines to affect the vascular cells within the graft there must be an initial connection established between host and graft
vacular structures, and an initiation of flow through the grafted microvessels, however this signal is likely to be initiated with by host tissue. This is supported by the observation that when using MGs constructed with human or mouse microvessel fragments that do not spontaneously proliferate in culture, inosculcation still occurs with the host. This indicates that a proliferative state the vascular elements within the graft is not necessary for the establishment of perfusion through the graft.

Cardiovascular Tissue Engineering is a field that continues to demonstrate powerful potential to treat the tissue pathologies associated with Cardiovascular Disease. The results of the current study establish that tissue engineered constructs comprised of microvascular elements, when placed in the cardiac position, can attenuate the development of left ventricular dysfunction associated with acute myocardial infarction. Therefore, such microvascular grafts may not only prove eminently useful in the construction of larger 3-dimensional bioengineered cardiac grafts, but may serve as a stand alone treatment option for those patients who suffer from diffuse or localized areas of microvascular dysfunction.
Figure 5.1 Schematic representation of the MG construction and implantation process. Microvessels are isolated from a donor rat (1) and the fragments culture expanded for 7 days (2). At day 7 a Scid mouse is surgically prepared (3) and undergoes coronary artery ligation and acute myocardial infarction (4). Following AMI, the graft is transplanted onto the epicardial surface so as to overlay the ischemic tissue and perfused tissue surrounding the area of ischemic insult.
Figure 5.2  

a MG prior to implantation, imaged with millimeter markings and an 8-0 suture needle for size reference.  
b Photomicrograph of an MG following 7 days implantation in the epicadial position.  
c Gross evaluation of an implanted MG reveals Intense vascularization of the MG at day 14.  
d Control grafts appear avascular at explantation, day 14.
Figure 5.3  
a Hematoxylin and Eosin staining of a control graft illustrating the lack of vascular structures in the control grafts.  
b Demonstrates the presence of arterioles, venules and capillaries within the MG, indicative of a mature microvascular bed.  
c Confocal microscopy of 28 day implant of MG constructed with GFP-expressing vascular fragments.  
d 30 minutes prior to explantation the animal was given an i.v. fluorescent labeled dextran. Note the co-fluorescence of dextran and GFP indicating perfusion through the graft.
<table>
<thead>
<tr>
<th>Hemodynamic Parameter</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (bpm)</td>
<td>HR</td>
</tr>
<tr>
<td>Maximum Volume (uL)</td>
<td>Vmax</td>
</tr>
<tr>
<td>Minimum Volume (uL)</td>
<td>Vmin</td>
</tr>
<tr>
<td>End-systolic Volume (uL)</td>
<td>ESV</td>
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<tr>
<td>End-diastolic Volume (uL)</td>
<td>EDV</td>
</tr>
<tr>
<td>Maximum Pressure (mmHg)</td>
<td>Pmax</td>
</tr>
<tr>
<td>Minimum Pressure (mmHg)</td>
<td>Pmin</td>
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<td>End-systolic Pressure (mmHg)</td>
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<tr>
<td>End-diastolic Pressure</td>
<td>EDV</td>
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<tr>
<td>Stroke Volume (uL)</td>
<td>SV</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>EF</td>
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<tr>
<td>Cardiac Output (uL/min)</td>
<td>CP</td>
</tr>
<tr>
<td>Stroke Work (mmHg*uL)</td>
<td>SW</td>
</tr>
<tr>
<td>Arterial Elastance (Ea)</td>
<td>Ea</td>
</tr>
<tr>
<td>Tau_w (msec)</td>
<td>Tau</td>
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Table 5.1 A list of the hemodynamic parameters reported in this study and the abbreviations that are used to refer to these parameters.
Hemodynamic Parameters

<table>
<thead>
<tr>
<th>Hemodynamic Parameter</th>
<th>Normal</th>
<th>Infarct</th>
<th>Infarct + Control Graft</th>
<th>Infarct + MG</th>
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<tr>
<td>Heart rate (bpm)</td>
<td>442</td>
<td>421</td>
<td>419</td>
<td>402</td>
</tr>
<tr>
<td>Maximum Volume (µL)</td>
<td>17.6</td>
<td>21.4</td>
<td>23.2 *</td>
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<tr>
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<td>15.2 *</td>
<td>5.9</td>
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<tr>
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</tr>
<tr>
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<td>20.0</td>
<td>22.5 *</td>
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</tr>
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<td>82.3</td>
<td>77.8</td>
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<tr>
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<td>8.0</td>
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<td>4.4</td>
</tr>
<tr>
<td>End-systolic Pressure (mmHg)</td>
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<td>78.3</td>
<td>78.7 64.4</td>
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<tr>
<td>End-diastolic Pressure (mmHg)</td>
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<td>13.4</td>
<td>11.6 12.5</td>
<td></td>
</tr>
<tr>
<td>Stroke Volume (µL)</td>
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<td>9.1</td>
<td>8.0 *</td>
<td>10.8</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
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</tr>
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<td>Cardiac Output (µL/min)</td>
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<td>3495.6</td>
<td>3292.5 * 4964.3</td>
<td></td>
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<tr>
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<td>544.9</td>
<td>477.1 657.6</td>
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<tr>
<td>Arterial Elastance (Ea) (mmHg/µL)</td>
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<td>9.3</td>
<td>10.9 7.1</td>
<td></td>
</tr>
<tr>
<td>dP/dt max (mmHg/sec)</td>
<td>8156.1</td>
<td>5659.3</td>
<td>5297.0 5464.7</td>
<td></td>
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<tr>
<td>dP/dt min (mmHg/sec)</td>
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<td>-4509.9</td>
<td>-4423.3 -3982.5</td>
<td></td>
</tr>
<tr>
<td>Tau_w (msec)</td>
<td>8.4</td>
<td>10.2</td>
<td>11.1 9.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 Hemodynamic values for the four groups of mice evaluated in this study. The highlighted rows indicate cardiac performance variables that were found to be statistically different amongst MG-treated animals and the two control groups. n=5 in all groups. * = p<.05.
Table 5.3 Graphical representation of the cardiac function parameters that were improved in the MG-treated animals. MG treatment was compared against Infarct alone and Infarct treated with Control Gel. These values were not compared to normal Scid mice. n=5 in all groups * = p<.05
Figure 5.4 Representative Pressure Volume Loops from each of the four groups of mice evaluated in this study. Note the upward and rightward shift of the Infarct and Infarct + Control groups, indicative of left ventricular dysfunction and failure. While the Infarct + MG animal does undergo a degree of LV dilation, it is not as severe as in the control infarct group.
6. Summary and Conclusion

Summary

The four hypothesis-driven research studies described in this text address the three specific aims outlined in the research plan of the dissertation project. The results of those experiments have identified a number of significant advances in the field of Cardiovascular Tissue Engineering. Those results can be described as establishing the following principles. (1) Isolated microvessel fragments can be utilized to pre-vascularize 3-dimensional tissue engineered constructs that, when implanted in the subcutaneous or epicardial position, will inosculate with the host vasculature. (2) The transplanted microvascular segments within the tissue engineered graft undergo tissue specific vascular remodeling and adaptation within the graft following the establishment of an anastomotic connection with the host vasculature. Further, the vascular elements within the graft act as an intact microvascular unit, neither being replaced with host vasculature, or invading surrounding microvascular beds. (3) A tissue engineered microvascular graft comprised of intact microvessel fragments can be utilized as a microvascular transplant following acute myocardial infarction to attenuate the development of left ventricular dysfunction.
Utilization of Adipose-Derived Microvessel Fragments in Pre-Vascularized Tissue Engineered Grafts

The first specific aim and corollary hypothesis of this project was focused on using adipose-derived microvessel fragments to construct a microvessel graft that would be suitable for implantation into the body. The Rat Fat Microvessel Fragment (RFMF) culture system used in this project was previously described by our laboratory as a method of culturing microvascular segments, as well as evaluating angiogenesis in vitro. The benefit to using this model system, as opposed to cell culture, was multi-fold. First, the 3-dimensional system that was used for RFMF culture generates a culture system that is more similar to in vivo conditions than 2-dimensional cultures that are standardly used for in vitro analysis of cellular behavior (Montesano, 1983), (Canfield, 1990), (Schor, 1983), (Honegger, 1992). The spatial orientation of the microvascular elements in 3-dimensional culture systems has profound effects on the behavior of that culture system and this is well documented in the case of microvascular endothelial cells (Madri, 1988), (Satake, 1998). In large part, observations of cell behavior in 3-dimensional systems is more indicative of cellular behavior in vivo than those made in 2-dimensional models (Bader, 1996). This characteristic of 3-dimensional cultures is important in the development of tissue engineered devices as it allows for a certain level of prediction. If the constructs are to be made in vitro and then surgically implanted into the body, there may be cases where pharmacologic manipulation of the biologic elements in the construct
is desired. In this setting, a 3-dimensional culture system is preferable based on the evidence above.

Secondly, the intact microvessel fragment system allows for the utilization of intact microvessel segments that are comprised of all cells found in the microvasculature. The microvessel fragment system contains not only microvascular endothelial cells but also vascular smooth muscle cells, vascular pericytes, and fibroblasts. The most common attempts at vascularizing tissue engineered constructs have relied on the use of a single cell type, endothelial cells or their precursors, to drive the desired vascular response. However, it is critical to the function of the microvasculature for all cell types to be present, and without the complete complement of cells the microvascular bed loses proper regulatory controls. For example, removal of pericytes from the microvasculature leads to an dysregulated phenotype of endothelial cells, suggesting a regulatory role for pericytes (Lindahl, 1997), (Darland, 2003). Also, smooth muscle cells and endothelial cells have extensive paracrine communication, as in the use of Nitric Oxide, that can lead to quiescent or proliferative states for each cell type. The absence of either cell causes a complete disruption of this interaction. With respect to the field of tissue engineering, this deregulation leads to a vasculature that is immature and perhaps dysfunctional. While the role of micropatterned microvasculatures in tissue engineering may be critical to certain future applications, it is clear that rapidly established functional microcirculatory components will have an integral role in the production of complex solid organ replacements such as artificial renal and cardiac tissue.
The data reported in chapter 2 of this dissertation clearly indicate that microvessel fragments suspended in a 3-dimensional collagen matrix can be utilized as a tissue engineered microvascular graft. When the graft is implanted in the subcutaneous position it is capable of inosculcation with the surrounding host vasculature. Perfusion studies done with India ink, and fluorescent dextran, demonstrate vascular continuity between the host and the grafted vasculature. Additionally, in histologic sections of the graft the presence of RBCs is noted in the vascular structures within the graft. These results support the primary hypothesis of chapter 2, that such a tissue engineered graft can be implanted and following implantation will establish vascular continuity with the host.

An additional important finding from chapter 2 was the presence of arterioles, venules, and capillaries within the graft at explantation. These vascular phenotypes are histologically identifiable as early as day 7, although they may be present at earlier time points.

While the results of chapter 2 demonstrate the potential for use of microvascular grafts in the subcutaneous position, the same type graft was also implanted and evaluated in the epicardial position following cryoinjury or coronary ligation and acute myocardial infarction. In the epicardial position the graft underwent a similar anastomotic process as that observed in the subcutaneous position. The process of graft inosculcation with the host epicardial coronary vasculature occurred following implantation onto freshly cryoinjured myocardium as well as acutely ischemic myocardium following suture ligation of the left coronary artery. The establishment of a graft-host vascular connection in a site of tissue injury is a much more critical evaluation of the potential use for such
approaches to vascularization of tissue engineered products. The realization of graft failure secondary to a lack of a perfusion connection is more likely to occur in a hypoxic, damaged tissue, and the establishment of perfusion in this model is a monumental progression in the field of tissue engineering.

Because the tissue sites of interest in the potential and targeted patient population are tissue areas in a pathophysiologic condition, evaluation of tissue engineered grafts should be done in an appropriate model. In this dissertation two models of injury, both cardiac in nature, were chosen. The utilization of a cryoinjury model of left ventricular damage allowed for the evaluation of the graft in a localized area of injury. In this way, the site of injury could be reproduced in each animal tested and the response to the epicardial injury by the grafted construct evaluated. The experimental results presented in the third chapter of this dissertation clearly outline the use of the MG system in this model of injury and the successful integration and perfusion of the grafted construct with underlying cardiac tissue.

An additional key set of experiments that was reported in chapter 3 was the utilization of freshly isolated microvessel fragments in graft construction as opposed to using fragments that have undergone in vitro culture expansion. The freshly isolated fragments were suspended in a 3-dimensional collagen matrix and implanted immediately following graft formation. The implantation response when using freshly formed MGs was similar to cultured MGs with some notable differences. Using freshly isolated tissue is a paramount step in the development of clinically usable tissue engineered devices, especially in the setting of cardiac disease. Patients that have suffered an acute cardiac
event and are undergoing emergent cardiac surgery are inherently unstable and require immediate intervention. For these patients, the use of tissue engineered constructs that can be generated in a single operative setting, utilizing autologous or freshly prepared allogeneic tissue, are ideal. By using fresh tissue for bioengineering, there can be realization of a situation where a patient undergoes graft construction and implantation within a single surgical setting using autologous tissue. This situation would obviate the need for many additional steps that would take place outside the operating room and over a long period of time after the initial procedure – all conditions that would predispose the graft to failure due to complications such as infection, culture contamination, culture failure, and increased patient morbidity. The successful utilization of freshly procured tissue for the purpose tissue engineering is a significant development towards the goal of clinically utilized tissue equivalents.

Vascular Remodeling and Adaptation in Tissue Engineered Microvascular Grafts

While the establishment of flow through the graft is certainly the primary objective of this tissue engineering project, the vascular response within the graft following initiation of flow will be the ultimate determinant of graft function. In order for the technology employed in the construction of this graft to support the development of larger bioengineered grafts, there must be the presence of a mature, functional microcirculation that is comprised of all vessel types normally found in the microvasculature. The presence of larger caliber vessels including arterioles and venules is indicative not only of
a mature vascular network, but also suggests that there is active vascular remodeling occurring within the graft. At the time of graft implantation, the overwhelming majority of the vessels are of approximately the same caliber. This vascular remodeling most likely occurs following anastomosis with the host vasculature. Once flow is sensed by the vessels within the graft there are a number of mechanical forces that will affect the phenotype of the vessel. While it is not well understood in this model system what determines the differentiation path of a venule or an arteriole, the differentiation of these vessel types is probably a complex interaction of soluble proteins and cytokines, as well as the mechanical forces that are transduced through the host vessel to the grafted vessel. By differentiating vessel type in this fashion, the graft is capable of responding to tissue needs and establishing a vascular network that is most beneficial to the implant site. This is an ideal attribute for a tissue engineered construct, as the construct is usually generated in vitro and is not exposed to the tissue environment prior to implantation. As a result of this isolation of the bioengineered device from the implantation site there can be no unique or individual graft constructed for use in a specific person or tissue site. This specialization is critical because the tissue milieu of the implant site will have a significant impact on the requirement of the implanted bioengineered device. For example, in the cardiac position, if the vascular component of the bioengineered graft is to be utilized to perfuse bioengineered cardiac muscle, the vascular density must be very high, with a fairly low amount of arterioles, but a high, and roughly equivalent, level of capillaries and venules. When compared to a tissue site like liver or kidney, the
microvascular distribution described for cardiac tissue is entirely different than those tissues.

The unique model that has been used in this dissertation has demonstrated the ability to respond to different tissue sites by producing a different vascular phenotype. The data in chapter 3, in which the graft was used in the epicardial position following cryoinjury, suggest that there is a similar anastomotic process occurring in each system, but the vascular distribution within the epicardial graft is significantly different when compared with the MG implanted in the subcutaneous position. In the epicardial position, the vascular density within the graft is much higher than the subcutaneous position. The likely reason for this difference is the underlying host tissue that inosculates with the graft. In the damaged epicardium there are a number of factors that are driving an angiogenic response in the host cardiac tissue and a number of these factors are soluble factors including angiogenic and arteriogenic growth factors. Once perfusion through the implanted MG is established these soluble factors will be delivered to the cells of the graft, thus having the same type of effect on these cells.

Furthermore, it is likely that there is a tissue specific response and interaction between the graft and the host tissue that is not dependent on pathologic physiology. That is to say that the graft may adapt to the surrounding tissue milieu and assume a vascular profile that is suitable to that environment. This would explain the development of a lower vascular density within the MG following grafting in the subcutaneous position. This type of tissue-specific adaptation and healing is an ideal characteristic for a tissue engineered vascular intervention and if this hypothesis is further supported by the
grafting of MGs to additional tissue sites such as kidney, intestine, liver, with unique vascular distributions in each tissue site, it represents a seminal development in the area of vascularizing tissue constructs.

Of particular interest to the theory that vascular remodeling occurs in response to an integration of signals from the host and from within the graft, is the differential vascular response noted when comparing cultured vessel fragments with freshly isolated microvessel fragments used in MG implantation. There was a quantifiable difference in the vascular density as well as the vessel type distribution within the graft in the cultured MG and the fresh MG. The vascular density within the graft following 14 days of implantation was less in the grafts formed with fresh fragments than grafts formed with cultured fragments. Furthermore, there was a shift in the type of vessels present within the graft – fewer capillaries and more arterioles and venules were found in the cultured grafts than in the fresh isolate. These results would indicate that there is active remodeling that occurs within the graft following the establishment of perfusion and that this remodeling is accelerated in the grafts implanted following culture expansion of the vascular fragments. However, while the cultured MG may appear to have an accelerated adaptive response, it is clear that the fresh MG does undergo a similar vascular remodeling response and it is likely that over extended periods of time the fresh MG would establish a vasculature identical to that in the cultured MG.

When evaluating the vascular density in the epicardial tissue directly below the MG in the rat model of cryoinjury used in chapter 3, it is noted that there is an increased microvascular density in the tissue under MG versus that tissue that experienced
cryoinjury alone or control graft implantation. The increased microvascular density in the epicardial tissue indicates the probable interaction between graft and host vasculature, and is ample evidence that presence of the vasculature within the graft has an effect on the tissue underlying the graft. This is an interesting theory because the data presented in chapter 1 in the subcutaneous tissue, as well as limited data using GFP-tagged fragments in the epicardial position, does not show the presence of grafted vessels in the host tissue. Also, there is an extremely low amount of host cells found in the graft even at 28 days, which reveals a situation more consistent with microvascular transplantation. That is to say, the MG acts like a transplanted microvasculature as opposed to providing an avenue for vascular invasion by the host.

This data also supports the concept that the bioengineered MG is a functional microvascular unit that is capable of responding to the metabolic demands of surrounding tissue as well as future cell types that may be integrated within the MG that was studied here. This issue of graft vascularization is the most critical obstacle to be overcome in the development of a truly functional bioengineered cardiac graft. The data presented in chapters 2 and 3 support the belief that isolated microvascular fragments can be utilized to pre-vascularize tissue engineered grafts and to establish a microvasculature within the graft that could augment tissue perfusion as well as support the cells of a bioengineered graft.
Physiologic Benefits to Microvascular Graft Implantation

The second and third specific aims were directed at the cardiac applications and the physiologic benefits from the implantation of MGs following myocardial infarction. The second specific aim of the dissertation project was to develop a microvascular bypass graft with the tissue engineered construct utilized in specific aim one. The hypothesis associated with this second aim is that the implantation of an MG following acute coronary ligation will result in reduced left ventricular remodeling and infarct size. There have been many recent advancements in the treatment of ischemic heart disease at the level of pharmacologic management, left ventricular assist devices, cellular cardiomyoplasty, and tissue engineering. These advances are all critical due to the continued disease progression of an alarming number of ischemic heart disease patients. In laboratory studies, the most common model used to analyze the efficacy of potential therapeutic options is the small animal model of myocardial infarction. Myocardial infarction in the small animal is most often created in the rat or mouse using either the cryoinjury or coronary ligation model of acute myocardial infarction. While there are certain benefits to each model system, each of the models can be used in the critical evaluation of new treatments and each has been employed in this dissertation. The cryoinjury model was used to establish the potential of graft perfusion in the cardiac position. The murine coronary artery ligation model was employed to characterize graft
survival and perfusion in site of tissue ischemia and low blood flow, as well as to establish a functional benefit of MG implantation.

In order to determine the potential physiologic benefit of the epicardial placement of a tissue engineered microvascular graft, the mouse model of coronary ligation causing acute myocardial infarction and resultant left ventricular dysfunction was used. In the evaluation of a microvascular graft this model of cardiac injury is arguably the most stringent model available for laboratory testing considering the extreme nature of the occlusion injury. In order for the MG to have a physiologic benefit to the animal following coronary ligation, the MG must enhance perfusion to a tissue site that has become acutely ischemic. In many ways, increasing perfusion to distal tissue in this manner is similar to coronary artery bypass grafting – in this case, the MG acts as a microvasular coronary artery bypass graft. This concept of microvascular bypass is one of the more novel, exciting and difficult to prove ideas presented in this dissertation. However difficult to prove, the physiologic data presented below allow certain inferences to be made as to the nature of the anastomotic connections established, and support the concept of microvascular bypass grafting.

The data presented in chapter 4 shows that implanting MG following acute myocardial infarction in the mouse leads to a reduction in infarct size at 14 days. The reduced infarct size observed in treated animals supports the concept that perfusion to ischemic tissue is improved following MG implantation. By establishing perfusion into the MG and from the MG to distal tissue sites that are affected by the coronary occlusion (effectively revascularizing ischemic heart tissue as in Coronary Artery Bypass Grafting), the extent
of ventricular remodeling is modified. Following coronary ligation there is an initial area of intense ischemia that results in infarcted myocardium. Over time, this area of infarct will undergo a continued level of fibrosis and remodeling that results in an infarct area that is larger than the initial zone of infarction. In the clinical population, this infarct growth is detrimental to left ventricular function and, in many cases, leads to the condition of heart failure.

This infarct size growth occurs in what is known as a “wavefront” fashion that sweeps across the injured left ventricle. Because this is a progressive condition it presents an ideal place for intervention with a tissue engineered vascular construct that would limit this infarct expansion. Furthermore, because the microcirculation is often damaged and diseased in many patients suffering from this condition, an intervention aimed at limiting infarct growth utilizing a microvascular transplant would be beneficial. Analysis of the results of the experiments performed in chapter 4, primarily reduced infarct size in MG treated animals following coronary occlusion, lead to the hypothesis that implanting such a tissue engineered MG would attenuate the wavefront development of infarct growth and confer functional benefits to the heart undergoing coronary ligation.

While morphometric analysis allows for accurate determination of infarct size, histopathologic evidence of reduced infarct size is not a sufficient determinant of the level of left ventricular dysfunction associated with coronary occlusion. In order to more accurately measure the true potential physiologic benefit of MG implantation, left ventricular function must be directly evaluated.
The final specific aim of this dissertation project, and, arguably, the most ambitious and clinically relevant aim, was to evaluate the potential functional benefit of a tissue engineered microvascular graft implanted immediately following coronary artery ligation. The set of experiments reported in chapter 5 were done to measure left ventricular function of control animals and animals treated with MG implantation following coronary ligation. The studies were performed on Scid mice that had undergone coronary ligation and were either not treated, treated with control grafts, or treated with MG. Left Ventricular function was measured using a conductance catheter system that generated real time pressure volume measurements in order to evaluate left ventricular hemodynamics.

The results of chapter 5 support the final, and critical, hypothesis of this dissertation – that an implanted MG would attenuate the development of left ventricular dysfunction and result in improved left ventricular performance. Based on the previous finding that infarct size is reduced with MG treatment, this result is somewhat expected, but cannot be downplayed as it is essential to quantify the level of benefit conferred by MG transplantation. There have been previous research studies done utilizing the conductance catheter system to evaluate the left ventricular function of mice following coronary artery ligation and subsequent treatment. The conductance catheter system, while technically very challenging to employ in the laboratory, is quickly gaining acceptance as the gold standard of cardiac performance analysis, and has proven an invaluable tool in the description of this dissertation. Research performed in our laboratory previously described the utilization of a tissue engineered 3-dimensional
fibroblast culture to attenuate left ventricular function loss in mice with the same method of analysis being used to determine LV functional enhancement. However, this previous study utilized a technology that contained a culture of single cell elements applied to the heart as a sheet of single cellular elements. These cells release a high concentration of angiogenic growth factors that are hypothesized to affect the vascularization and collateral development of infarcted myocardium. While this approach proved to be successful in laboratory tests, it may not be an ideal way to vascularize a large tissue engineered construct, and may not work well in tissue sites that demonstrate a resistance to physiologic angiogenesis.

The utilization of a prevascularized epicardial graft has not been previously demonstrated to confer functional benefits to a left ventricle having undergone coronary ligation and myocardial infarction. Furthermore, while efforts have been made to stably introduce cells capable of forming vessels in a matrix for tissue engineering, no previous studies have demonstrated physiologic benefit, and none have utilized intact vascular segments. The results outlined in chapter 5 build on the previous body of evidence in this dissertation project that supports the use of tissue engineered vascular constructs as microvascular bypass and transplant. It also is consistent with the current belief that the development of bioengineered cardiac grafts is a feasible approach to augmenting left ventricular function in the failing heart.
Conclusion

The results of this dissertation will greatly aid in the advancement of vascularizing tissue engineered constructs for tissue and organ augmentation. The most critical element of successful tissue engineering is the rapid establishment of a vascular network within the graft, and the development of pre-vascularized grafts that contain all the elements of a functional microcirculation will expedite the progression of complex bioengineered tissue substitutes. The microvascular graft that has been characterized in this dissertation project has also demonstrated the ability to be utilized as a stand alone microvascular transplant which may prove an integral part in the future treatment options for patients suffering from extensive microvascular dysfunction.

It is my sincerest hope, and expectation, that the principles of Tissue Engineering not only in the Cardiovascular System, but solid organ engineering and tissue augmentation, will be greatly influenced by the innovations presented in this dissertation. It is my feeling that the elegantly constructed previous body of work in this field has been profoundly advanced by the results of the experiments presented in this dissertation and, as a result of this work, we are now closer to the realization of artificial tissue and organ replacements.
Appendix A

Portions of this appendix were submitted for peer review and publication in the journal *Circulation*, as of July 2004.

**Reversal of Myocardial Ischemia and Infarction by Transplantation of a Tissue Engineered Microvascular Graft**

**Background** – The formation of a new, functional microcirculation will be essential to regeneration of hibernating cardiac tissue after myocardial infarction, cell based therapies to regenerate new myocardium and in reversing microvascular ischemia.

**Methods and Results** – We describe a microvascular coronary artery bypass graft constructed from isolated, intact microvascular segments stabilized in a three-dimensional matrix. This microCABG was implanted as a patch on the epicardial surface of mouse and rat hearts subjected to left ventricular cryoinjury (rat) or coronary artery ligation (mice). After 14 and 28 days of implantation, left ventricular function was assessed (mice), and grafts evaluated via histology and cytochemistry. Inosculation of microvessels within the graft with host cardiac vasculature occurred in both the cryoinjury and coronary ligation models of injury. Evaluation of the grafts revealed arterioles, venules and capillaries, and erythrocytes within vascular lumina. Control grafts of collagen alone remained avascular. Left ventricular infarct size was reduced, and left ventricular function improved in treated animals.

**Conclusions** – A microvascular graft may provide therapeutic benefit as a primary treatment or serve as a microvascular platform for cardiac repair and regeneration.
Introduction

The utilization of pre-formed, functional microvascular networks for the purpose of tissue engineering may prove an integral step in future therapeutic management of patients with cardiovascular disease. Currently, medical and interventional procedures can limit the disease progression of patients with CVD, but ultimately these patients become refractory to these interventions (Packer, 1992), (Mann, 1999), (Young, 1991), (Eriksson, 1995). While organ and tissue transplantation are eminently successful, the lack of available tissue leads to an extreme mismatch in the number of donors and potential recipients, necessitating alternative approaches to disease management, such as tissue engineering (Hosenpud et al., 1995). Additionally, there is a large patient population suffering from microvascular dysfunction that derives minimal clinical benefit through large vessel (e.g. CABG) surgical revascularization, and would be exceedingly well served by the grafting of functional microvascular units.

Cardiovascular and specifically cardiac tissue engineering provides strategies to develop functional tissues in vitro, comprised of distinct cell populations with cell-cell interactions and three-dimensionality that more closely approximates conditions which occur in vivo. The utilization of these constructs in vivo will depend on a nutrient supply provided by vascular connections between the host tissue and the grafted tissue. There have been a number of previous approaches to support a functional microcirculation in tissue implants, including the use of growth factors to stimulate the formation of a microcirculation from the pre-existing host vasculature (angiogenesis) (Zisch et al., 2003), (Perets et al., 2003), (Yamamoto et al., 2003), (Pandit et al., 1998),
(Fournier and Doillon, 1996). Alternatively, the transplantation of cells capable of becoming new vessels (neovascularization or vasculogenesis) (Schechner et al., 2000), (Peters et al., 2002), (Takeshita et al., 1994), (Baumgartner and Isner, 1998), (Lazarous et al., 1996), (Koike et al., 2004) has been proposed. These strategies have significant limitations to the development of complex tissue engineered constructs that are capable of meeting the variable metabolic demands within a dynamic tissue such as cardiac muscle. The utilization of vascular-inducing growth factors or single cells capable of differentiation into new vessels requires a latency period of vessel formation in which the transplanted tissue would remain without a vascular supply. The ideal strategy for tissue engineered cardiovascular tissue relies on the rapid inosculolation of the host vasculature with the pre-existing vascular elements of the implanted tissue. Furthermore, patients who present with existing microvascular dysfunction, may be non-responsive to therapies requiring new microvascular growth and maturation.

In this study we present successful implantation of a tissue engineered microvascular graft (MG) in both a rat syngraft model of ventricular cryoinjury and a murine model of myocardial infarction. Rats undergoing syngeneic MG transplantation did not display any signs of acute rejection and grafted MGs were well integrated with underlying host tissue and well perfused by a microvasculature comprised of arterioles, venules, and capillaries. Severe combined immunodeficient (Scid) mice underwent left coronary artery ligation followed by MG implantation. The grafted MG was positioned overlaying perfused tissue and blanched, ischemic tissue distal to the occlusion. Mice treated with MG demonstrated decreased infarct size and increased hemodynamic function versus
control groups of infarct alone, or infarct treated with acellular collagen gels. These results indicate the potential for tissue engineered microvascular grafts to serve not only as a platform for large, three-dimensional tissue engineered constructs, but as a stand-alone treatment for myocardial microvascular dysfunction.
Materials and Methods

All studies were approved by the University of Arizona animal review committee, and animals housed in American Association of the Accreditation of Laboratory Animal Care approved facilities following procedures outlined by the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985).

Microvessel Isolation Microvascular Graft Formation

Microvessels were isolated from epididymal fat pads of retired-breeder Fischer 344 rats using previously published methods (Hoying, et al., 1996). Microvessel fragments between 30 and 500 μm were isolated from adipose tissue by mincing, enzyme digestion, and filtration using nylon screens. Microvessels were suspended (12,000 – 15,000 fragments/ml) in cold rat tail type I collagen (BD BioSciences, Bedford, MA) prepared with DMEM and pH neutralized with NaOH (1M). The microvascular grafts (MG) were plated into individual wells (0.125 ml/well) of 96 or 24 (0.250 ml/well) well plates, with an equal volume of 10% FBS-DMEM added after polymerization. Culture time for graft formation was 7 days.

Rat Left Ventricular Cryoinjury and MG Implantation

On the day of surgery anesthesia was induced by placing the rats in an induction chamber with gaseous Isoflurane. Animals were intubated with a 16G catheter for ventilation and anesthesia was maintained with 2.5% Isoflurane. The left chest was shaved and
aseptically prepared. The heart was exposed through a lateral thoracotomy. Cryoinjury was performed by cooling a metal probe in liquid nitrogen and application directly to the epicardial surface of the left lateral wall for one minute and repeated ten times. Immediately following cryoinjury a cultured MG, fresh isolate MG, or acellular control collagen gel (n=6,6,6), was grafted directly to the area of observed damage, using two 7-0 prolene stitches. Cryoinjury only controls received no suture or gel placement (n=5). The chest was closed in layers and the animal extubated.

**Murine Coronary Artery Ligation and MG Implantation**

On the day of surgery mice were anesthetized with 2.5% Avertin (2.5% 2,2,2-tribromoethanol, 2.5 tert-amyl alcohol in PBS: Aldrich) by intraperitoneal injection. The neck and chest shaved, and aseptically prepared. Airway access was obtained through a tracheotomy and a 22-gauge catheter was inserted into the trachea for ventilation. The heart was exposed through a lateral thoracotomy. The LCA was ligated with 8-0 nylon suture (Ethicon). Immediately following coronary ligation, a MG or acellular control collagen gel (n=6,6), was grafted to the area of observed blanching, using a single 8-0 nylon stitch. Ligation only controls received no suture or gel placement (n=6). The chest was closed in layers, and the trachea and neck closed.

**Explant and Evaluation**

On the day of explant the animals were anesthetized as described above. The abdominal aorta was isolated and cannulated with a 16G (rat) or 24G catheter (mouse). The heart
was arrested in diastole and vasodilated with PBS containing Sodium Nitroprusside (10\textmu M). Hearts were excised and placed in Histochoice® fixative (Ameresco). Tissue sections (6\textmu m) were prepared from paraffin embedded tissue and stained using Hematoxylin and Eosin, Masson’s Trichrome, and lectin cytochemistry.

**Vessel Density and Characterization**

For vessel characterization studies, five high power fields (HPF) were examined within the implant for each animal in both the MG and control implant groups. Capillaries were identified as a single layer of flattened endothelial cells. Arterioles were identified by an ID $\geq 10\mu$m and by the presence of endothelium, tunica media, and tunica adventitia. Control infarct animals without implants were only evaluated within the myocardial scar (n=5). Venules were differentiated from arterioles by an ID $\geq 10\mu$m with a thinner or absent smooth muscle layer. Vessel density studies were done using $G_s$-$I$ reacted sections. Ten HPF were identified within the infarcted myocardium for animals in each group. In each HPF the number of vessels that were positive for $G_s$-$I$ staining were counted.

**Determination of Infarct Size**

Tissue sections (6\textmu m) from the mid-infarct stained using Masson’s Trichrome area were used to determine the percent fibrosis of the left ventricle. Digital images of the tissue sections were captured and the area of infarct was determined using a planimetric analysis program (Spot).
**Left Ventricular Function Evaluation**

The functional analysis of each animal was performed prior to animal sacrifice, 14 days following coronary ligation and MG implantation. Left ventricular function analysis was performed as described (Yang et al., 2001), (Nemoto et al., 2002). At 14 days, mice were anesthetized with urethane (1,000 mg/kg) and α-chloralose (50 mg/kg), IP (Kass et al., 1998), (Dalkara et al., 1995). The right carotid artery was cannulated with a 1.4 Fr. Millar Conductance Catheter. The catheter was advanced and positioned along the cardiac longitudinal axis. Ventricular placement of the catheter was confirmed by pressure-volume loop profile. The jugular vein was cannulated for volume administration of 300 μl of saline:albumin (50:50). Pressure and volume data were collected for 5 to 10 seconds. Data collection was done with the Millar Aria-1 system with AD Instruments Powerlab software. Data analysis was performed using Millar PVAN Cardiac Pressure-Volume Analysis software, version 2.9.
Results

Microvascular Grafts

The enzymatic digestion and screen filtration of the excised rat adipose tissue resulted in the isolation of intact, microvascular fragments. The fragments were comprised of the full complement of microvascular cell types including endothelial cells, vascular smooth muscle cells, and vascular pericytes (Hoying et al., 1996). Upon suspension in a 3-dimensional collagen matrix, the vascular elements underwent spontaneous angiogenic sprouting when placed in culture. Figure 1a illustrates a culture-expanded MG prior to implantation on the epicardial surface of a mouse heart. The implanted MG were found to be well integrated with the underlying cardiac tissue at post implant day 7 (Fig. A.1b) and post implant day 14 (Fig. 1c). Control grafts that were constructed of collagen gel without microvascular fragments were implanted and found to remain avascular, regardless of implant duration (Fig. A.1d).

Vascularization of Microvascular Grafts

Hematoxylin and Eosin staining of 6 μm sections of acellular control grafts and underlying cardiac tissue confirmed the absence of vascular profiles throughout the graft (Fig. 2a). Histologic evaluation of MG that were implanted in the epicardial position of mice immediately following left coronary artery ligation revealed the presence of a mature microvasculature throughout the graft. Vascular heterogeneity was observed including arterioles, venules, and small caliber capillaries. Additionally, the presence of artery-vein pairs was noted in the MG (Fig. A.2b).
When MG were grafted as a syngraft on the epicardial surface of cryoinjured rat hearts, histological evaluation confirmed the presence of an extensive microvascular network, and minimal inflammation. In this model of a tissue engineered allograft, both MG that underwent microvascular culture expansion for seven days (cMG), as well as MG that were implanted immediately, without in vitro expansion (fMG), were evaluated. Both the cMG and fMG inosculated with the host cardiac vasculature and the presence of erythrocytes within the vessels of the graft confirmed graft perfusion (Fig. A.2c,d). The presence of an endothelial cell lining of the vascular profiles seen with H&E staining was confirmed by cytochemistry with the lectin Gs-1 (Fig. A.2e,f).

**MG Vessel Density**

The number of vessel profiles per unit area, vessel density, was evaluated in cardiac tissue in normal myocardium, normal epicardium, cryoinjured epicardium, and epicardial tissue under control grafts, cultured MG and fresh MG (Fig. A.3a). The vessel density of epicardial tissue underlying grafted MG was found to be higher than control grafts or cryoinjury alone. The vessel density within grafts implanted in a syngraft fashion on rat epicardium following cryoinjury was found to be 1097 +/- 204 vessels/mm² in cultured grafts and 761 +/- 120 vessels/mm² in grafts made from freshly isolated fragments (Fig. A.3b). The presence of arterioles, venules and capillaries as a percent of all vessels within fresh MG and cultured MG were also evaluated, and found to be 4.4%, 33.6%, and 62.8%, respectively in fresh grafts, and 6.4%, 52%, and 42%, respectively in cultured grafts (Fig. A.3c). Microvessel density in MG implanted in the murine model of
coronary ligation for 14 days (n=5) was 802 +/- 244 vessels/mm² and after 28 days (n=5) was 1419 +/- 337 vessels/mm² (Fig. A.3d).

**Left Ventricular Infarct Size**

Groups of animals treated with MG were evaluated against control groups of infarct only and infarct with control grafts to determine the extent of ventricular fibrosis at mid-infarct level. MG treated animals as a group were found to have a smaller infarct compared to control groups (Fig. A.3e).

**Left Ventricular Function**

The ability of an implanted MG to confer functional or physiologic advantage to acutely infarcted hearts was also investigated. Pressure volume loops of animal groups that had undergone experimental coronary ligation alone, ligation followed by implantation of control grafts, or ligation treated with implantation of MG were analyzed and compared with a control group of Scid mice that had not previously undergone surgery. The animal groups that received either ligation alone or ligation treated with control grafts demonstrated PV loops indicative of the development of heart failure, characterized by a pronounced upward, rightward shift of the PV loop. By contrast, those animals from the group that was treated with MG following coronary ligation appeared to have an attenuated development of LV failure (Fig. A.4a) and less pronounced right PV shift. A number of hemodynamic parameters can be evaluated with the PV loop system used in this study and parameters often associated with development of heart failure were found to be significantly improved in the MG treatment group versus control (Fig. A.4b).
Discussion

The introduction of a pre-formed microvascular network as a primary therapeutic option or for vascularizing tissue engineered constructs will greatly progress future medical management of cardiovascular disease patients. The presence of a functional microcirculation in tissue engineered constructs will be critical to not only the viability of the construct but also the tissue perfused by the construct (Murphy et al., 2000). A number of laboratories have previously reported models to vascularize ischemic or avascular tissue, primarily through the use of angiogenic growth factors and administration, or utilization of single cell transplantation (Elcin et al., 2001), (Lee et al., 2002), (Yang et al., 2001). We present a novel strategy to rapidly establish perfusion through a surgically grafted tissue engineered microvascular graft comprised of intact, microvessels.

The vascularizing potential of the tissue engineered microvascular graft utilized in the experiments described here has been previously characterized by our laboratory following grafting in the subcutaneous position of mice (Shepherd et al., 2004). In this case, grafts were found to undergo spontaneous inosculation with the host circulation as soon as one day post implantation, with evidence of extensive vascularization occurring within the gel by day two. The presence of large caliber vessels increased as a function of time, as did the relative level of smooth muscle cell coverage of vessels within the graft, a measure indicative of a mature heterogenous microvasculature.

These previous studies led us to the present hypothesis, that a tissue engineered microvasculature in the epicardial position, capable of vascular remodeling following
implantation, would confer a physiological benefit to cardiac tissue following acute myocardial injury.

The study presented here aimed to critically evaluate the potential benefits of such a therapeutic strategy by utilizing a model of acute ischemia and infarction that would prove a stringent test of the capability of graft-host inosculation in an area of pathologic hypoperfusion (coronary ligation) and localized injury (cryoinjury). To appropriately model and extrapolate potential clinical uses and benefits of such a graft, a number of grafting models were evaluated including a syngraft model of left ventricular cryoinjury, using culture expanded and non-culture expanded grafts, and coronary artery ligation followed by epicardial engraftment of the experimental construct.

The ideal tissue engineered microvascular graft would serve not only as a source of microvessels for the graft or the host tissue, but would confer a physiologic benefit through the addition of functional microvascular units to the host vasculature. As predicted, the tissue engineered microvascular graft presented in this study was capable of inosculation with the host vasculature. Furthermore, the graft conferred a critical physiologic benefit to the tissue site treated, by limiting left ventricular infarct size and attenuating the development of heart failure following coronary artery ligation.

The process of vascular inosculation was not negatively affected by the syngraft transplantation scenario. While there did appear to be a low level of extravascular inflammatory cells associated with the implant, the surgical procedure and cryoinjury undoubtedly played a major role in the inflammatory stimuli present. There was neither the presence of microvessels or inflammatory infiltrate seen in the control grafts. The
lack of blood flow, and the subsequent introduction of blood-borne inflammatory cells may account for the lack of inflammatory cells in response to the grafted acellular collagen gels.

Tissue engineered microvasculature can be potentially used in either the setting of microvascular dysfunction as a grafted microvasculature, or as a microvascular network responsible for blood perfusion of a large tissue engineered construct. The former of these two options will often require a tissue supply that is immediately available, while the latter option may more easily accommodate *in vitro* expansion. Therefore, MG that were comprised of microvascular elements having undergone seven days of in vitro culture expansion, and MG constructed with freshly isolated fragments were tested. The histologic comparison of these two model systems did not reveal any remarkable differences. Microvascular characterization of the vessels within the grafts suggests that the process of vascular remodeling may be accelerated in those grafts allowed to undergo culture expansion. At explantation, the microvascular density within cultured MG was higher than fresh MG, and there were a greater proportion of large caliber vessels in those grafts. At the time of surgical grafting the vessel density within the cultured MG is higher than that of the fresh MG, and this increase in vessel density at the time of implantation may accelerate the process of vascular remodeling in the graft. However, the *in vitro* culture expansion of vascular elements within the graft is not necessary for graft-host inosculation and the establishment of perfusion through the graft. When the tissue site under the implanted MG in the cryoinjury model was evaluated for microvascular density, it was noted that the cMG group had a higher epicardial vascular
density than the fMG group, and both were higher than control grafts or cryoinjury alone. These results combined with the previous characterizations add further evidence to an active vascular remodeling that is occurring as a result of shared signals between the host and grafted tissue.

The microvascular tissue engineering model that we present appears to not only exhibit vascular remodeling within the graft, but there is evidence to support the conclusion that there is tissue specific, perhaps tissue regulated, adaptation occurring. The microvascular density within the grafts implanted in the rat syngraft study was greater (cMG = 1097/mm², fMG = 761/mm²) than those observed in our previous study involving subcutaneous placement of the MG (subQ MG = 610/mm²). Furthermore, when characterization of vessel type was performed, it revealed the presence of a vessel-type distribution in the cMG grafts similar to native epicardium. That is, there were relatively few arterial structures, and an approximately equal representation of venules and capillaries. These results suggest a tissue-specific response that is dependent on the local grafting environment or pathologic condition. While these differences were not statistically different, they demonstrate the same trend in host tissue response seen in the rat model of cardiac injury and MG engraftment. Additionally, peri-infarct regions demonstrated this same trend of mild increase in vascular density (data not shown), while distant myocardial control tissue in all groups had a vascular density between 3500 and 4000 vessels/mm² – well within previously reported results in the literature (Unger 200). Immediately following left coronary artery ligation in mice, MG were sewn directly to the epicardial surface such that the graft lay directly over perfused tissue as well as the
acutely ischemic tissue distal to the site of coronary occlusion. The MG transplantation resulted in reduced LV infarct size and the attenuation of LV dysfunction and heart failure pathogenesis. While the exact site of vessel inosculation cannot be determined, the portion of the graft that is in contact with perfused tissue is likely where perfusion into and through the MG is initiated. Through expansion of microvessel elements in the graft and inosculation with adjacent heart tissue, the graft appears to act as a microvascular bypass graft facilitating perfusion from proximal, perfused tissue to distal ischemic tissue. The increased distal perfusion generated by the MG prevented left ventricular infarct expansion and attenuates the development of left ventricular dysfunction.

Other groups performing cellular transplantation and tissue engineering studies have reported efficacy in the murine myocardial infarction model (Kellar et al., 2001), however grafting of an intact microvascular network has not been previously reported. While the approach of adding single cells to a hypoxic tissue site either with or without a tissue scaffold has been tried previously (Chekanov and Kipshidze, 2003), (Schechner et al., 2003), (Whittaker et al., 2003), (Watanabe et al., 1998), the addition of intact microvascular segments for physiologic benefit has not. For patients suffering from microvascular dysfunction or global ischemic conditions, treatment modalities that rely on the formation of new vascular conduit through remodeling of existing vessels or the de novo generation of vessels from transplanted cellular units, may be destined to fail due to the pre-existing vasculopathic condition of the native tissue. A treatment strategy whereby an allogeneic microvascular network, or autologous microvascular segments
isolated from a healthy tissue site is implanted, may demonstrate more clinical efficacy in areas of chronic microvascular dysfunction and tissue hypoxia. Furthermore, due to the inherent capability of adipose-derived microvessel fragments to readily inosculate with the host vasculature, even in the setting of acute, global ischemia, this approach of microvascular isolation and incorporation into bioengineered tissue may be an ideal method of prevascularizing large tissue engineered constructs.
Figure A.1 Microvascular Grafts were formed by the resuspension, in Type I collagen, of microvessel fragments isolated from adipose tissue of male donor Fischer 344 rats. a MG prior to grafting on the epicardial surface of a mouse having undergone coronary ligation. b and c Following 7 (b) and 14 days of engraftment, the presence of extensive vasculaization is readily apparent. d Control grafts comprised of Type I collagen without microvessels remains avascular at 14 days.
Figure A.2 Six micron sections were cut from paraffin embedded tissue samples of hearts that had been arrested in diastole and perfused-fixed with Histochoice®.  

a Sections of control grafts demonstrate a completely avascular graft following 14 days of implantation.  
b H&E staining of MGs implanted in mice demonstrated heterogeneous distribution of vascular profiles within the graft.  
c and d Surgical engraftment of MGs that had not undergone culture expansion of the microvessels within the graft, as well as those that had been expanded in culture, were utilized in a rat model of LV cryoinjury, with both demonstrating the presence of a mature microcirculation.  
e and f Gs-1 lectin staining was utilized in MGs to confirm endothelial cell lining of the vascular profiles observed in cultured and non-cultured MG.
Figure A.3 Microvascular characterization of the vessels within the MG demonstrates active vascular remodeling. *a* Grafts implanted on the epicardial surface of cryoinjured rat hearts were used for a characterization of the vascular densities of the graft proper as well as surrounding cardiac tissue. *b* The vascular density was increased in the grafts constructed of cultured vessels (cMG) compared to those containing fresh vessels (fMG). *c* The process of vascular remodeling and the establishment of vascular heterogeneity was accelerated in the cMG, giving rise to a vascular bed comprised of more large vessels than fMG. *d* The vascular density of MG implanted in mice following coronary ligation increased from 28D to 14D. *e* MG treated mice were found to have smaller infarcts than infarct alone or infarct with control graft, p<.05.
Figure A.4 Cardiac function and hemodynamic analysis of mice following myocardial infarction and MG engraftment. *a* Normal *Scid* mice were used as control animals. Animals that were treated with MG following coronary ligation demonstrated attenuation of LV failure and improved cardiac function. Animals undergoing coronary ligation alone or followed by control graft implantation demonstrated a right-upward shift of the PV loop, indicative of heart failure development. *b* Selected hemodynamic parameters measured with the Conductance Catheter System, * indicates p<.05 versus controls.
Figure A.5 Graphical representation of selected functional parameters of cardiac function that were improved in the MG-treated animals. MG treatment was compared against infarct alone and infarct treated with acellular control grafts. These values were not compared to normal Scid mice. * indicates p<.05 versus controls.
Appendix B

Methods for Making Avertin Anesthetic

Stock (100%)

10 g 2,2,2-tribromoethanol
10 ml tert-amyl alcohol

Tribromoethanol is stirred into tert-amyl alcohol for 12 hours in either an amber bottle or aluminum foil wrapped glass bottle. Stock Avertin is stored at 4°C in the dark.

Working Solution (2.5%)

Mix stock solution by stirring on a warm plate at 37°C. Add 1 ml Stock Avertin to 39 ml of warm PBS. Mix for 2-4 hours and add filter sterilize before use. Store at 4°C in the dark.

Avertin is administered via intraperitoneal injection and can be used at approximately .22ml/15g in mice for 40 minutes. Following injection, a plane of anesthesia should be achieved within 30 seconds. Booster injections can be given in 0.15ml increments as needed.

Mouse Weight

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Appendix C

Left Ventricular Cryoinjury Surgical Procedure

Anesthesia is induced by placing the animal in an induction chamber that is infused with Isoflurane gas. When the animal has achieved a light plane of anesthesia the rat is then intubated with the use of an otoscope and a short guidewire. The otoscope is used to visualize the vocal cords and the guide wire is then placed through the hole of the otoscope and into the trachea through the vocal cords. The endotracheal tube (a modified 18G catheter) is then advanced into the trachea over the wire. The rat is then connected to the anesthesia machine and 2.5% Isoflurane is used to maintain anesthesia. The left chest is shaved and scrubbed for sterile procedure. The endotracheal tube is attached to a small animal ventilator and the ventilatory rate set at approximately 70 breaths/min. The isoflurane is administered through the ventilator at 2.5% throughout the duration of the procedure. The animal is then placed on its right side under the operating microscope and draped in sterile fashion. A left lateral thoracotomy is performed and the chest entered through the intercostal space between the fourth and fifth ribs. The ribs are then separated with a retractor. The lung is retracted with a moistened piece of gauze. The heart is then exposed through a pericardotomy and the pericardium moved to expose the anterolateral surface of the heart. A small metal probe with a round end is submerged in liquid nitrogen for approximately 90 seconds to chill the probe. The probed is then placed on the epicardial surface in the region bordered by the first diagonal of the left coronary artery and the first obtuse marginal branch of the left circumflex artery. The probe is left for 60 seconds and then re-chilled. This procedure is repeated 10 times to
achieve left ventricular injury. Following injury formation, an MG is grafted to the epicardial surface over the area that has been damaged. The graft is attached to the surface with the use of two 7-0 prolene or nylon stay sutures, placed on opposing sides of the graft. The ribs are approximated with 4-0 prolene and the chest and skin closed in three layers using 5-0 vicryl. The chest is evacuated by aspiration with a 23G needle. The Isoflurane administration is stopped and the animal is weaned off the ventilator. Following surgery, the animal is given Buprenorphine and saline, and the animal is allowed to recover under a heat lamp until ambulation is noted.
Appendix D

Murine Coronary Ligation Surgery

Anesthesia is induced with 2.5% Avertin. Once a planed of anesthesia is reached, the chest and neck are shaved, a depilatory agent (Nair) used to remove remaining hair from the left chest and neck, and the shaved areas scrubbed with Nolvasan. The animal is then placed in the supine position with the neck exposed. A thoracotomy is performed and the trachea cannulated with a 24G catheter. The catheter is attached to a small animal ventilator with the tidal volume set at 0.5ml and the respiratory rate set at 110 breaths/min. Anesthesia is maintained with 2.5% Isoflurane administered through the ventilator. Following isolation and cannulation of the trachea, the animal is placed on the right side for thoracotomy. The chest is entered through a left lateral thoracotomy. The heart is exposed through a pericardotomy and with the pericardium being moved to the side. Using a moistened piece of gauze, the left lung is retraced to expose the left anterolateral heart. The left coronary artery is then ligated at the proximal third using 8-0 or 9-0 prolene or nylon. Arterial ligation is confirmed by visualization of tissue blanching distal to the site of ligation. For animals receiving grafting, the MG is sutured to the epicardial surface using a single 8-0 tack suture through the middle of the graft. The graft is positioned so as to overlay visibly perfused tissue and visibly ischemic tissue. Following graft placement, the gauze packing over the left lung is removed and lung allowed to fully re-inflate. The ribs are approximated with 6-0 prolene. The chest is closed in two layers using 6-0 prolene. Evacuation of air from the chest is achieved by aspiration using a 1cc syringe and a 27G needle until negative pressure has been
reestablished. The trachea is then closed with a single 8-0 prolene stitch. The skin is then closed with interrupted sutures with 6-0 prolene. Bruprenex, 2ng/g, is given as an analgesic, with normal saline for volume replacement. The animal is then allowed to recover under a heat lamp until ambulatory.
Appendix E

India Ink Casting

The animal is euthanized with 1ml of Avertin and the abdominal aorta is rapidly isolated and cannulated with a 24G i.v. catheter. Following placement of the aortic catheter, the vena cava is cut for fluid drainage. The aorta and heart are then perfused in a retrograde fashion with 10ml warm saline containing 10U/ml Heparin, Sodium Nitroprusside, and Papaverine. The chest was then opened to expose the heart and implanted MG. The animal is then perfused in a retrograde fashion with India Ink (Speedball #3398; Hunt Manufacturing Co., Statesville, NC) that has been dialyzed against PBS and filtered through #1 Whatman paper. India ink is perfused at a pressure of 90-100 mmHg until the graft is visibly filled with ink (usually within 2 mls, if not sooner). The animal is then placed at 4C for 12-24 hours to allow for the India ink to set up. The heart is then excised and dehydrated in graduated concentrations of ethanol (25, 50, 75, 95, 100%) and finally placed in methyl salicylate for tissue clarification. The vasculature filled with India ink is then visualized by placing the organ in a glass dish and underlighting the tissue.
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