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In Situ Engineered Myocardial Tissue

by

Karen Leigh Christman

DISSERTATION

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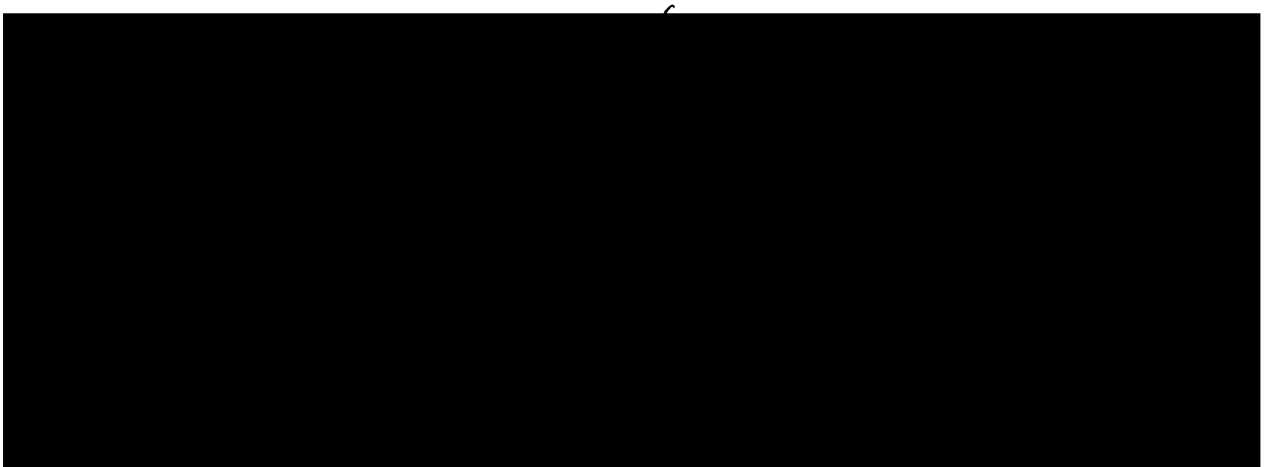

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Karen L. Christman, Hubert H. Fok, Qizhi Fang, Richard E. Sievers, Anne J. Kim, and Randall J. Lee. Injectable Fibrin Scaffold Preserves Cardiac Function and Left Ventricular Geometry in a Chronic Myocardial Infarction Model. S*ubmitted.* 2003.

Karen L. Christman, Qizhi Fang, Michael Yee, Richard E. Sievers, and Randall J. Lee. Delivery of Pleiotrophin Plasmid in Fibrin Glue Enhances Neovasculature Formation in Ischemic Myocardium. *Submitted.* 2003.

Karen L. Christman and Randall J. Lee. New Directions in Myocardial Tissue Engineering. Review article. *Submitted.* 2003.

Karen Christman developed and executed the experiments in the above papers. She also wrote all of the manuscripts. Co-authors only provided minimal assistance.

Abstract

In Situ Engineered Myocardial Tissue

by

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Doctor of Philosophy in Bioengineering

University of California, San Francisco

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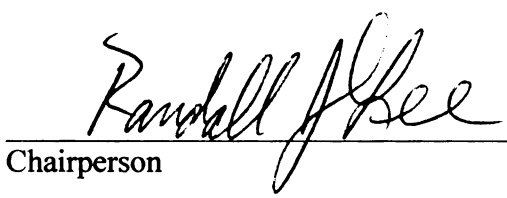
Heart failure following a myocardial infarction is currently the leading cause of death in the United States. Heart transplantation remains the only successful treatment for those patients suffering from end-stage heart failure despite the paucity of donor organs. Various alternative treatments have been examined, including angiogenic growth factor delivery, cell therapy, and left ventricular restraints. The emerging field of myocardial tissue engineering is also providing exciting possibilities. To date, tissue engineering approaches to the myocardium have involved cell injections or the implantation of *in vitro* engineered cardiac tissue. In this work, we present a novel approach to cardiac repair and reconstruction, which we have termed *in situ* engineered myocardial tissue. Tissue engineering involves the combination of growth factors, cell therapy, and biomaterial scaffolds to repair lost or damaged tissue. The aim of this work was to determine which combination of these three components was best suited for cardiac repair via *in situ* myocardial tissue engineering. The results of the following studies indicate that Pleiotrophin is an effective angiogenic agent in ischemic myocardium;

however, it is the combination of skeletal myoblasts and a fibrin glue scaffold that is the most effective treatment for cardiac repair. Our results also indicate that delivery of the fibrin scaffold alone may also be a potential treatment for those patients suffering from myocardial infarctions.



Chairperson

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These studies were approved by the Committee for Animal Research of the University of California San Francisco and were performed in accordance with the recommendations of the American Association for Accreditation of Laboratory Animal Care.

Chapter 1

Introduction

1.1 Coronary Heart Disease

Coronary heart disease is currently the leading killer in the United States for both men and women. In 1999, one of every five deaths in the U.S. was attributed to this disease. An estimated 1,100,000 Americans will suffer from a new or recurrent coronary attack (myocardial infarction) this year and over 45 percent of those will die of it in a given year[1,2]. Furthermore, about two-thirds of myocardial infarction patients do not make a complete recovery[3,4]. In addition to the loss of life and decline in quality of life, coronary heart disease results in a heavy burden on the health care system with estimated direct and indirect costs for 2002 in the U.S. of 111.8 billion dollars.

1.1.1 Pathology

The myocardium consists of three major components: cardiomyocytes, extracellular matrix, and microvasculature. The cardiomyocytes provide the contractile component. The extracellular matrix is a viscoelastic scaffold composed of type I and type III collagen. This collagen support system connects the cardiomyocytes in order to distribute force throughout the ventricles[5,6]. The capillary microvasculature supplies the necessary oxygen and nutrients to sustain the continually beating myocytes. It is well

documented that the myocardium is unable to significantly regenerate itself following injury[7,8]. Adult cardiomyocytes are terminally differentiated and are permanently withdrawn from the cell cycle. Therefore, the heart is unable to regenerate viable myocardium following a myocardial infarction. Subsequent heart failure following a myocardial infarction (MI) is often progressive. Following death of the cardiomyocytes, macrophages, monocytes, and neutrophils migrate into the infarct area, initiating the inflammatory response. Infarct expansion then begins to occur due to the activation of matrix metalloproteases (MMPs), which degrade the extracellular matrix and result in myocyte slippage. This weakening of the collagen scaffold results in wall thinning and ventricular dilatation. Following the initial inflammatory phase, there is an increase in fibrillar, cross-linked collagen deposition, which resists deformation and rupture. Evidence suggests that the death of cardiomyocytes results in negative left ventricular (LV) remodeling which leads to increased wall stress in the remaining viable myocardium. This process results in a sequence of molecular, cellular, and physiological responses that lead to LV dilation. Although the exact mechanisms of heart failure are unknown, it is suggested that LV remodeling may contribute independently to its progression[9].

1.1.2 Current Treatments

The most common first-stage treatment for MI is reperfusion therapy. Patients often receive thrombolytic therapy; however, after 20 years, its benefits are still controversial. Other reperfusion treatments include balloon angioplasty and coronary stent implantation. Advantages of these percutaneous coronary interventions include visualization of the coronary anatomy, greater success rates in restoring normal coronary perfusion, and

reduced risk of intracranial hemorrhage. There are also a variety of pharmacologic agents that can be administered to those who have suffered an MI. Aspirin is often used for its anti-platelet and anti-inflammatory capabilities. Heparin may also be administered to reduce the risk of venous thromboembolism for those patients not receiving thrombolytic therapy. Nitroglycerin, a vasodilator, has also been effective in treating ischemia, heart failure, and arterial hypertension following an acute MI. Newer classes of therapeutic agents include beta blockers, which are thought to attenuate negative remodeling, and ACE (angiotensin-converting enzyme) inhibitors, which attenuate LV dilation. Despite this vast array of treatment options, coronary heart disease continues to be the leading cause of death. Currently, the only successful treatment for end-stage heart failure following a myocardial infarction is cardiac transplantation; however, the ability to perform this procedure is limited by the availability of donated organs. Given the chronic shortage of donor hearts, alternate strategies are needed to improve the lives of those with heart failure.

1.2 Therapeutic angiogenesis

Therapeutic angiogenesis, cellular cardiomyoplasty, and LV supports have recently emerged as possible alternatives to cardiac transplantation. It is hypothesized that increased blood supply through the delivery of angiogenic growth factors will improve cardiac function following a myocardial infarction. In the initial stage of an infarct, the center portion consists of necrotic tissue while the edges, or border zone, contain viable cardiomyocytes that are at risk. These at-risk myocytes will eventually become necrotic and produce a larger infarct if a blood supply is not restored to them. Restoring blood

supply to the infarct regions through delivery of angiogenic growth factors, a concept known as therapeutic angiogenesis, will salvage the at-risk cardiomyocytes and reduce infarct expansion with the ultimate goal of improving cardiac function and decreasing the morbidity and mortality associated coronary heart disease.

1.2.1 Vascular Endothelial Growth Factor

The most widely studied growth factor in promoting angiogenesis is vascular endothelial growth factor (VEGF). It is necessary for both endothelial cell differentiation (vasculogenesis) and for angiogenesis[10,11]. Initially VEGF was thought only to increase vascular permeability; however, now it is understood that VEGF regulates several biological functions in endothelial cells including enhanced production of vasoactive mediators, increased expression of components of the thrombolytic and coagulation pathways, suppression of neointimal vascular smooth muscle cell hyperplasia, and inhibition of thrombosis, hypotension and vasorelaxation[12,13]. VEGF is also considered to have a key role in adult angiogenesis during pregnancy, wound healing, cancer, rheumatoid arthritis, ocular neovascular disorders, and cardiovascular disease[12,14,15]. Although VEGF is known to induce angiogenesis, delivery of both plasmid VEGF and skeletal myoblasts genetically engineered to express VEGF have had deleterious effects. Constitutive expression of VEGF by high doses of retroviral transduced skeletal myoblasts in murine hearts induced the formation of intramural hemangiomas[16] (unpublished data, Figure 1.1). Similarly, naked VEGF plasmid resulted in such vascular tumors when injected into either the myocardium or hind limb muscle[17,18].

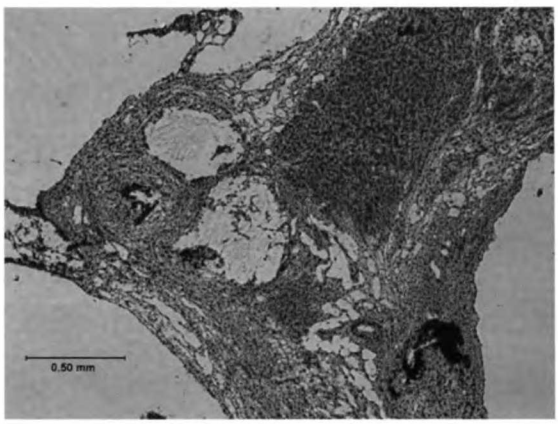

Figure 1.1 Cavernous hemangioma at site of VEGF expressing myoblasts injection in a murine heart.

1.2.2 Other Angiogenic Agents

Another growth factor, fibroblast growth factor (FGF) has also been implicated in the angiogenic process. FGF is also upregulated in response to ischemia in the myocardium[19]. Previous work has shown that injection of FGF plasmid into ischemic myocardium induces angiogenesis[20]; however, as with VEGF clinical trials, initial clinical trials for FGF have had mixed results[21-28]. More recently studied angiogenic agents include hepatocyte growth factor (HGF), which is upregulated following myocardial ischemia and has been implicated in tissue regeneration, wound healing, and angiogenesis[29,30], and hypoxia inducible factor 1 (HIF-1), which is also upregulated in the ischemic myocardium[31] and induces neovasculature formation in hypoxic myocardium[32].

1.2.3 Pleiotrophin

Another growth factor, pleiotrophin (PTN) may have the capability of inducing neovasculature formation in ischemic myocardium without resulting in vascular tumor formation. PTN was first described as a 18 kD growth promoting differentiation-inducing factor (Heparin Binding Growth Factor 8, HBGF 8,[33]. Additionally, PTN is also known as Hb GAM[34]. The *Ptn* gene is activated at sites of angiogenesis during development and in endothelial and inflammatory cells at sites of new vessel formation in ischemia-reperfusion injury in brain[35]. Its expression levels also are high and constitutive in many advanced human tumors with intense pathological angiogenesis. Furthermore, cells into which a constitutively activated *Ptn* gene is introduced develop extensively angiogenic xenografts when implanted into the flanks of nude mice[36]. Moreover, PTN has been shown to induce mitogenesis[35,37-40], angiogenesis,[37] and neurite and glial process outgrowth in vitro[41]. These different model systems that use an activated *Ptn* gene *in vivo* and *in vitro* provide a strong experimental basis to suggest that PTN signals angiogenesis *in vivo* and may be a potential therapeutic to stimulate angiogenesis in ischemic tissues.

1.3 Cellular Cardiomyoplasty

Cellular cardiomyoplasty involves the delivery of viable cells to replace lost cardiomyocytes. The idea behind this approach is to prevent the negative LV remodeling caused by the loss of myocytes in the myocardium. By preventing this remodeling, it may be possible to prevent heart failure.

1.3.1 Cell Types

A variety of cell types including fetal, neonatal, and adult cardiomyocytes[9], skeletal myoblasts[42,43], bone marrow progenitors cells[44] and embryonic stem cells[45,46] have been considered for this approach. Adult cardiomyocytes were unable to survive even when transplanted into normal myocardium. Both fetal and neonatal cardiomyocytes were able to form viable grafts as well as express cadherin and connexin43 (gap junction protein), which are necessary to form electromechanical junctions[47]. Although the fetal and neonatal cardiomyocytes were able to form these junctions, they are not a feasible source of transplant cells. There are many ethical and donor availability issues surrounding fetal cells which make clinical use of these cells unlikely. Skeletal myoblasts, on the other hand, are not subject to such ethical issues. These cells have been shown to survive and form intercalated discs in myocardium[42,43]. Although one group demonstrated that skeletal myoblasts improved cardiac performance in some animals, they do not form gap junctions and thus are unable to contract synchronously with the surrounding cardiomyocytes[43]. Bioheart, Inc. is currently in human clinical trials in Europe and phase I clinical trials in the U.S. with an autologous skeletal myoblast product called MyoCell™. Diacrin is also completeing phase I clinical trials in the U.S. and Genzyme Biosurgery is peforming clinical trials in Europe with similar products. Bone marrow stem cells have been shown to regenerate new myocardium. These cells have been shown to improve cardiac function and are able to differentiate into a cardiac phenotype. They were shown to express connexin43 and the cardiac specific transcription factor GATA-4. Muscle differentiation was also displayed with expression of the myocyte enhancer factor 2 (MEF2) and Csx/Nkx2.5, which is an early marker of myocyte

development. New endothelial cells and smooth muscle cells also developed from the stem cells which may have lead to the inclusion of coronary arteries, arterioles, and capillaries seen in the *de novo* myocardium[44]. There is also a lack of ethical and donor availability issues with bone marrow stem cells since they can be isolated from adult bone marrow and easily expanded in vitro. Embryonic stem cells have also been examined for myocardial repair despite the large ethical obstacles in human use. Many groups have isolated cardiac lineage specific embryonic cells which differentiate into a cardiomyocyte phenotype in vitro[48-57]. In addition, other studies have demonstrated engraftment[45] and improvement of cardiac contractile function[46] following injection of either cardiomyocytes derived from embryonic stem cells or undifferentiated embryonic stem cells. In addition to the ethical issues surrounding the use embryonic stem cells, there is also the concern of teratoma formation when implanting such immature cells into the myocardium.

1.3.2 Problems

Although a few studies have shown some improvement in cardiac performance by using cellular cardiomyoplasty in infarct rabbit models[43,58], there are several problems associated with this technique. The current transplantation technique involves the injection of cells afloat in saline, cell culture medium, or bovine serum albumin (BSA) and results in viable grafts; however, the technique is plagued by limited cell retention and transplant survival.[59-62] Furthermore, it was recently stated that the "basic protocol for cell grafting may need further optimization to prevent cell loss."[62] When reported, the number of animals receiving successful grafts is often low. It is also thought that the percentage of cells that remain in the myocardium following injection is small. Since the

heart is continually beating throughout the procedure and the cells are suspended in a very low viscosity liquid, many of the cells are squeezed out of the myocardium following injection. This is thought to be one of the major obstacles in delivering a large number of viable cells to the infarcted region. Given that the cells are injected in an ischemic region of the heart, there is also little to no vasculature to supply the implanted cells. Cell survival is thus limited by the lack of rapid vascularization. Another problem associated with the current technique is that the cells are poorly distributed. Cross sections of the infarcted region show clusters of the implanted cells between scar tissue. Conduction through the infarcted region should thus still be a problem since the cells are in isolated areas. Furthermore, the typical injection technique involves injection of cells in completely liquid solutions and does not give the transplanted cells a temporary matrix to attach to.

1.4 Left Ventricular Supports

Another alternative treatment to heart transplantation is the use of LV supports which are designed to prevent LV dilation and subsequent heart failure. One study demonstrated the feasibility of this approach using a sheep model. A polypropylene mesh was sutured onto the epicardial surface of the infarct wall. The Marlex mesh preserved LV geometry and prevented a decrease in pump function[63]. Several companies are also developing similar supports to restrain the LV. Acorn Cardiovascular, Inc. has developed the CorCap™ Cardiac Support Device, which is a "mesh-like" jacket that surrounds the ventricles. They have begun clinical trials worldwide. A major drawback, however, with Acorn's device is that it requires an open chest procedure for implantation. Paracor

Surgical is developing a similar restraining device consisting of a clamshell nitinol mesh. The mesh is designed to assist in systolic contraction by taking advantage of the super-elasticity properties of NiTi. Unlike Acorn's CapCor™, this device may be delivered using a minimally invasive thoracoscopic approach. Cardioclasp, Inc. is taking a slightly different approach by using a rigid ring around the heart to change the ventricular shape, to decrease the ventricular radius, which in turn decreases myocardial stress, and to prevent further LV dilation. The device is currently in the pre-clinical stage of development. Myocor, Inc. is developing a similar product called MyoSplint® System, which uses several splints or sutures implanted across the ventricle under tension. These splints are designed to change the shape of the LV and reduce cardiac wall stress. Both the Cardioclasp and Myocor devices require an open chest surgery.

1.5 Myocardial Tissue Engineering

The emerging field of tissue engineering may provide additional promising alternatives to heart transplantation. Tissue engineering approaches are designed to repair lost or damaged tissue through the use of cellular transplantation and biomaterial scaffolds.

1.5.1 *In Vitro* Engineered Myocardial Tissue

Several groups have produced cardiac tissue engineered constructs in vitro[64-79]. Other groups have also used this tissue engineering approach by implanting cells on the surface of the myocardium in a polymer scaffold[68,70,73,75,78-80]. To date, two different techniques have been used to combine cells and a scaffold for cardiac repair. The first involves the seeding of cells onto a pre-formed scaffold while the second involves the culture of cells in a soluble matrix. The first approach, albeit successful in other applications such as

bone, cartilage, intestine, liver and urologic tissues[81], has had limited success in regenerating the ailing myocardium. The following reasons have been hypothesized as possible mechanisms of failure for this more traditional tissue engineering approach[82]. The combination of diffusion limitations and the need for nutrient and oxygen supply constrains these constructs to less than 100 μm in thickness. Furthermore, current scaffolds do not promote the organization of cells into three dimensional cardiac tissue in vitro or the differentiation of fetal or neonatal cardiomyocytes into the adult phenotype. Upon mechanical analysis, the active forces generated by these engineered tissues are either fairly small or not detectable[65-68,70,71,74]. Fink, Eschenhagen, Zimmermann, and colleagues have utilized the second approach by combining collagen type I, extracellular matrix proteins and freshly isolate cardiac cells into to what they have termed "Engineered Heart Tissue (EHT)[64,69,72,77,79]." After addition of matrigel to the reconstitution mixture, culture under mechanical load and in a circular shape, and utilization of cell mixtures rather than only cardiomyocytes, they produced tissue engineered cardiac tissue in vitro which was highly differentiated and capable of generating a substantial contraction[82]. The EHT was contractile in vivo up to 8 weeks and was observed to be both vascularized and innervated[79]. Despite this apparent success, the single muscle bundles in the EHT did not increase above 100 μm[82] as seen with the first described approach to myocardial tissue engineering.

Shimizu, Okano and colleagues have recently developed a clever technique to create patches of cardiac tissue in vitro without the use of a material scaffold[76]. They utilized a temperature responsive polymer, poly(*N*isopropylacrylamide) (PIPAAm), which is slightly hydrophobic and cell adhesive at 37°C, but becomes hydrophilic and

non-cell adhesive at 32°C due to rapid hydration and swelling[83,84]. Tissue culture plates were coated with PIPAAm and subsequently seeded with neonatal cardiomyocytes. Once the cells have formed a monolayer, the temperature is dropped and the cell sheet may be removed intact. Both cell-to-cell junctions and adhesive proteins within the monolayers are preserved unlike with enzymatic digestion. Up to six sheets (100 μm) may be layered upon each other to create a three dimensional pulsatile cardiac tissue construct without resulting in a necrotic core. As seen with the other tissue engineering approaches, this *in vitro* engineered myocardial tissue is limited to a thickness of 100 μm or approximately six cardiomyocytes. Considering the maximum intercapillary distance in rat myocardium is a mere 20 μm[85] and a rat infarct wall is on the order of one to two millimeters, developing a sizable *in vitro* cardiac tissue construct using this or any of the approaches described above is currently a major obstacle. Upon application in humans, this presents even further size challenges. Additionally, implanting these *in vitro* engineered tissues would require a highly invasive procedure in humans.

1.5.2 *In Situ* Engineered Myocardial Tissue

Due to limitations of *in vitro* engineered myocardial tissue, we examined a novel approach to cardiac repair that uses an injectable biopolymeric scaffold to deliver cells directly into the infarct wall, which we have termed *in situ* engineered myocardial tissue. We hypothesized that injection of cells in a temporary scaffold would increase cell transplant survival within the infarct compared to the standard injection technique. We also hypothesized that this injectable scaffold may be used alone as an internal wall support to prevent LV dilation.

1.5.2.1 Injectable Polymers

There are currently a handful of injectable polymers that have been used as tissue engineering scaffolds and that were considered for this approach to heart repair. Several synthetic polymers, such as polyethylene oxide (PEO), PEO-poly-l-lactic acid (PLLA)-PEO block copolymer, poly(N-isopropylacrylamide-co-acrylic acid) (poly(NIPAAm-co-Aac)), pluronics, and poly-(N-vinyl-2-pyrrolidone) (PVP) have served as artificial extracellular matrices for transplanted cells. There are also a few biologic polymers such as alginate, collagen, and fibrin glue, which have been utilized as injectable scaffolds. The major benefit of each of these polymers is that they may be injected into the desired location without the need for more invasive implantation. More specifically, PEO is biocompatible and is known not to react with proteins and most biologic macromolecules [86,87]. It is injectable, however, larger needles such as 22 gauges must be used [88]. PEO-PLLA-PEO block copolymers are also biocompatible and biodegradable; however, they undergo gel solution transitions around 45 °C and thus require injections at temperatures above body temperature[89]. Poly(NIPAAm-co-AAc) gels also undergo gel solution transitions. These gels remain liquid at room temperature and solidify at body temperature[90]. In order to have a mechanically stable gel, larger gauge needles must be used. Pluronics are also known to be biocompatible, but are not biodegradable. They remain liquid at temperatures lower than 4 °C, which adds increasing complexity if the injection is to be delivered via catheter. PVP may be injected through smaller gauge needles such as 30 gauge. It is also non-antigenic and non-toxic; however, it is not biodegradable[91]. Alginate gels are linked together by calcium ions, which will dissociate and render the gel mechanically unstable. They are also non-biodegradable and have been

known to be immunogenic[92,93]. Collagen gels are biocompatible and biodegradable, but are not mechanically stable. Lastly, fibrin glue is biocompatible, non-toxic, and biodegradable. It may also be injected through 30 gauge needles at room or body temperature.

1.5.2.2 Fibrin Glue

Furthermore, fibrin glue is a biopolymeric gel that is formed by enzymatic polymerization of fibrinogen. Thrombin cleaves the dimeric fibrinogen molecule at two symmetric sites. Then a self-assembly step follows in which the fibrinogen monomers polymerize to form a noncovalently crosslinked polymer gel (Figure 1.2).

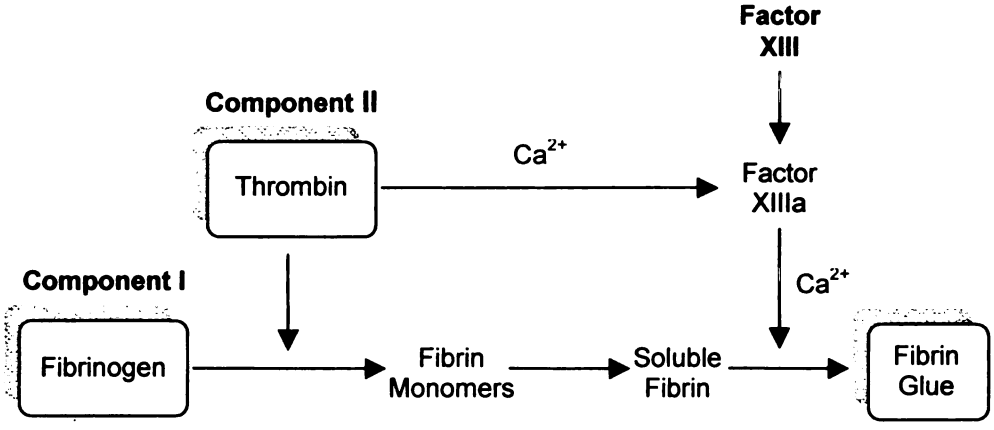

Figure 1.2 Formation of fibrin glue scaffold (adapted from Baxter's website on tisseel engineering: www.tisseelengineering.com)

The structure of fibrin glue may be modified by changing a number of factors including fibrinogen, thrombin and calcium ion concentrations, ionic strength, and pH, which can in turn alter the mechanical strength of the gel. The gel composition can range from small diameter fibrils and pores less than 2 μm, which is termed a fine gel, to large diameter fibers and pores greater than 2 μm, which is termed a coarse gel (Figure 1.3).

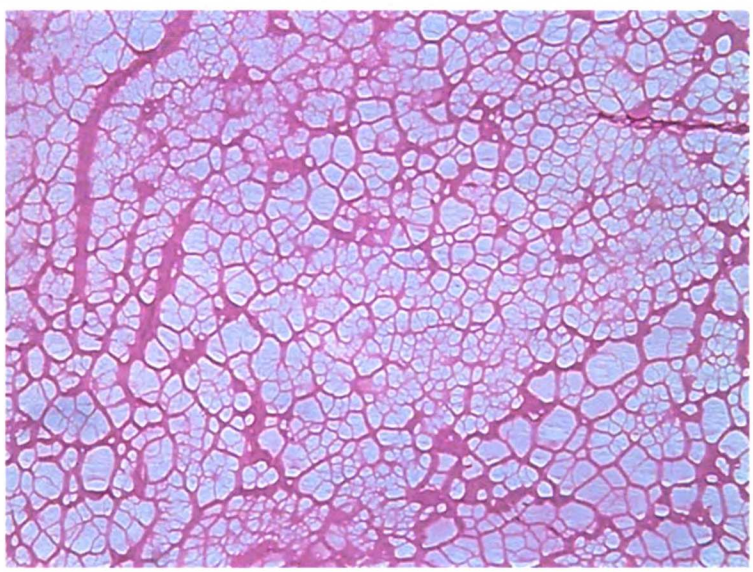

Figure 1.3 Hematoxylin and eosin stained cross section of fibrin glue. Note the fibril nature of the gel. ×4.

Fibrin glue is already FDA approved and is commercially available for use as a sealant and adhesive[94]. It is routinely used in cardiovascular, thoracic, gastrointestinal, neuro, plastic, orthopedic, oral, and cosmetic surgeries as well as ophthalmology, otorhinolaryngology, and urology[95]. It has also been shown that it does not induce

inflammation, foreign body reactions, tissue necrosis or extensive fibrosis[95]. In addition, it has been implicated in promoting angiogenesis[96], and local tissue growth and repair[97,98]. Immediately following mixing of its components, fibrin glue remains liquid for several seconds before polymerizing into a gel. It could therefore be injected via a dual chamber catheter into the human heart, requiring only a minimally invasive procedure.

Although fibrin glue's approved use is as a sealant and adhesive, the tissue engineering community has begun to study it as scaffold for delivering a variety of cells types and regenerating tissue. Fibrin has been examined for several tissue engineering applications including bone[99-101], blood vessels[102,103], cartilage[104-107], the nervous system[108,109], skin[110-115], and urethra[116,117]. Prior to the studies which are described in the chapters to follow, fibrin had not been examined as a tissue engineering scaffold in the myocardium

1.6 Objective

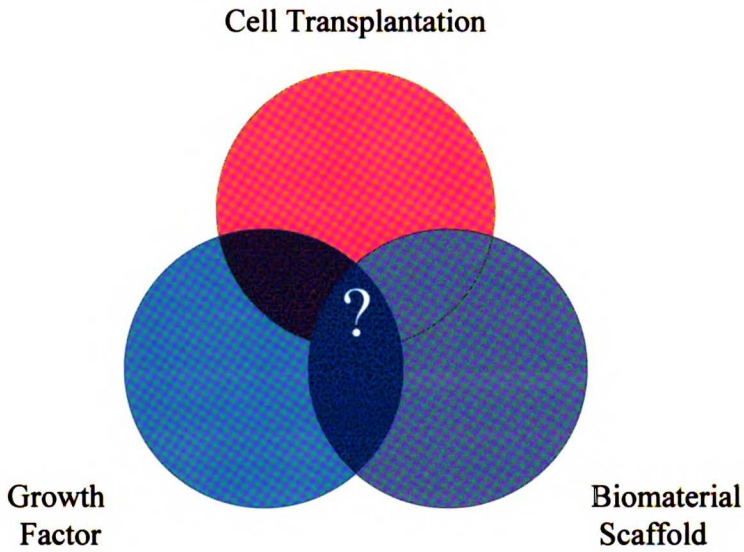

Figure 1.4 Classic tissue engineering triad. Tissue engineering approaches involve the combination of growth factors, cell transplantation, and biomaterial scaffolds for the repair of lost or damaged tissue.

The figure above illustrates the classic tissue engineering triad, containing cell transplantation, growth factors, and biomaterial scaffolds. In the emerging field of tissue engineering, it is the combination of these three factors which aid in tissue repair and regeneration. The objective of this work was to apply these tissue engineering principles to repair the myocardium following a myocardial infarction. The goal of the studies described in the chapters to follow was to determine what combination of these three

tissue engineering components was best suited for myocardial repair. Unlike current work in the field of cardiac tissue engineering, we examined a novel approach to cardiac repair using *in situ* engineered myocardial tissue. We utilized Pleiotrophin as the angiogenic growth factor, fibrin glue as the biomaterial scaffold, and skeletal myoblasts for cell transplantation.

Chapter 2

Pleiotrophin as a Therapeutic Angiogenic Agent

2.1 Introduction

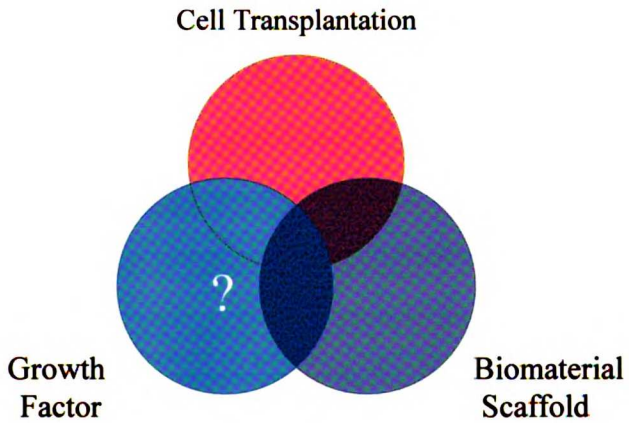

Figure 2.1 Classic tissue engineering triad. Examination of growth factor delivery for cardiac repair.

This study examines the use of a single component of the tissue engineering triad, an angiogenic growth factor (Figure 2.1). Due to the detrimental side effects of VEGF in pre-clinical studies, and mixed results of other growth factors in initial clinical trials[21-28], we examined the potential of another growth factor, PTN, for inducing neovasculature formation in ischemic myocardium. In each of the studies to follow, we elected to use a plasmid encoding the PTN gene rather than using the PTN protein. The delivery of

cytokines and growth factors in protein form has many limitations. Exogenous proteins are rapidly degraded and thus require a high dose, often in the milligrams[118], to achieve a therapeutic effect. Manufacturing such high doses of protein is also very costly. In addition, the delivery of such bulk doses has been associated with protein diffusion into the bloodstream and concerns over satellite tissue toxicity. Slow release devices using biomaterials have been developed in the attempt to overcome many of these disadvantages; however, the large size and delicate three dimensional structure of recombinant proteins has limited this approach[119,120]. The use of high temperatures and organic solvents in the fabrication of these devices has also resulted in diminished bioactivity of the cytokines.

To begin to determine the role of pleiotrophin as an in vivo angiogenic agent for the myocardium, we examined the expression of the PTN gene following ischemia in rat myocardium by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. We next constructed a pCMV plasmid encoding the pleiotrophin gene and transfected 293 human embryonic kidney cells with either this plasmid or a pCMV-β-galactosidase (β-gal) control plasmid. In order to show direct in vivo angiogenic activity of PTN in ischemic myocardium, we determined if myocardial injection of naked PTN plasmid would stimulate the growth of new blood vessels in a rat myocardial ischemia reperfusion model.

2.2 Methods

2.2.1 Induction of Myocardial Ischemia

Female Sprague-Dawley Rats (225-250 g) were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). A single stitch of 7-0 Ticron suture was placed under the left anterior descending (LAD) coronary artery. The suture was tightened to completely occlude the artery. A set of control animals underwent sham surgeries where the suture was not tightened. The animals were sacrificed either 30 min, 24 hr, 1 day, 3 days, 7 days, 14 days, or 30 days after placement of the suture (n=3 per group). The hearts were rapidly excised and the ischemic portion of the left ventricle (LV) or corresponding area of the controls was separated and flash frozen in liquid nitrogen.

2.2.2 RNA Isolation and RT-PCR

RNA from each sample was isolated using a Mini Rneasy Kit (Qiagen). RT-PCR was performed using 0.5 μg RNA from each sample, Qiagen's One-Step RT-PCR kit, and PTN sense (5'-CTGTGGAGAATGGCAATGGA-3') and antisense (5'-CGGCATTGTGCAGAGCTCT-3') primers. The PTN primers mapped nucleotides 406 to 656 of the rat PTN sequence. 22 cycles were run under the following conditions: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 10 min. The RT-PCR products were electrophoresed in a 2% agarose gel containing 0.005% ethidium bromide.

2.2.3 PTN Expression Constructs

The 580 base pair human PTN open reading frame (ORF) was isolated by RT-PCR from a human adenocarcinoma cell line (SW13), and was subcloned into the HindIII and XbaI

sites of pRc/CMV2 (Invitrogen) to generate pRC/CMV2-PTN. The CMV promoter/enhancer and PTN open reading frame were shuttled from the pRC/CMV2-PTN to pIRES (BD Biosciences Clontech) to generate pCMV-PTN-IRES. The β-gal ORF from CMVβ (BD Biosciences Clontech) was shuttled into pCMV-PTN-IRES to generate pCMV-PTN-IRES-β-gal-neo. The control plasmid for cell culture was pCMV-IRES-β-gal-neo, which is similar to the pCMV-PTN-IRES-β-gal-neo but does not contain the PTN open reading frame. The control plasmid for injections was a pCMV-β-gal plasmid (Invitrogen) which is a similar construct without the PTN gene.

2.2.4 ELISA

293 human embryonic kidney cells were transfected by calcium phosphate precipitation with pCMV-PTN-IRES-β-gal-neo or pCMV-IRES-β-gal-neo control plasmid. Stably expressing cell lines were selected using geneticin (G418, Gibco Invitrogen). Following selection cells were grown to confluency and an aliquot of cell culture medium was taken for ELISA analysis. Media and a PTN standard (R&D Systems) were serially diluted (in triplicate and duplicate, respectively) in coating buffer (50mM Na_2CO_3, pH 8.9) in a 96 well plate and incubated at RT for one hour. Wells were blocked with blocking buffer (1% BSA, 0.05% Tween in PBS) for one hour at RT. Wells were washed and a horse radish peroxidase-conjugated anti-human PTN antibody (900ng/ml; R&D Systems) was added and incubated for 1 hour at RT. Plates were washed and 100ul of TMB substrate was added to each well and incubated for 10 minutes. 50ul of 1% H_2SO_4 was added and the plate was read at 450nm.

2.2.5 Low Attachment, Anchorage Independent Growth Assay

A 96 well plate was coated with poly 2-hydroxyethyl methacrylate (Sigma), which prevents cell adherence. Growth factor (either PTN or bFGF) was serially diluted in triplicate in media (1% FBS/IMDM) and 2000 serum-starved SW13 cells (epithelial adenocarcinoma) were placed in each well. The plate was incubated for three days at which time MTS (Promega), a tetrazolium compound that is reduced by viable cells into a formazan product, was added to the wells. The amount of formazan product, which directly correlates with the amount of proliferation[121], was measured by reading absorbance at 490nm with a microplate reader (BioTek ELX 800).

2.2.6 Plasmid Injections

A previously described ischemia reperfusion model was used in this study[122]. Briefly, female Sprague-Dawley Rats (225-250 g) were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). Under sterile technique, the rats were placed in supine position and the chest was cleaned and shaved. The chest was opened by performing a median sternotomy. Keeping the landmarks of the base of the left atrium and the interventricular groove in view, a single stitch of 7-0 Ticron suture was placed through the myocardium at a depth slightly greater than the perceived level of the left anterior descending portion (LAD) of the left coronary artery while taking care not to enter the ventricular chamber. The suture was tightened to occlude the LAD for 17 minutes and then removed to allow for reperfusion. Ten minutes after occlusion, 250 μg of either a control pCMV-β-gal plasmid or a pCMV-PTN plasmid in 50 μl saline was injected into the ischemic LV through a 30-guage needle. The chest was then closed and the animals were allowed to

recover. Another set of animals received injections two months after LAD occlusion. This lab has extensive experience with this model and has previously demonstrated that this technique results in an acute infarct size of approximately 30% of the LV with reperfusion.

2.2.7 Microbead Perfusion.

A subset of rats was first anesthetized with an intraperitoneal injection of 200 μl of 50 mg/ml sodium pentobarbital. They were then injected with 700 μl of 50 μg/ml nitroglycerin in order to ensure vasodilation. After 10 minutes, the chest was opened and the abdominal aorta was cannulated with P-50 tubing. The left atrial appendage was cut to allow for drainage. 9 ml of saline was then perfused retrograde through the heart for approximately one minute. A 6 ml suspension of 0.2 μm blue fluorescent carboxylate-modified polystyrene beads (FluoSpheres, Molecular Probes) diluted 1:6 with PBS was then perfused through the heart. The hearts were immediately harvested, rinsed with PBS, and fresh frozen in O.C.T. freezing medium. They were then sectioned into 10 μm slices and examined under a Nikon TE 300 fluorescent microscope. Infarct areas were visualized by noting the lack of microbeads in the area. 5 sections evenly distributed through each heart were used for analysis. Under a 40x objective, 5 fields from the infarct area of each section were examined for microbeads.

2.2.8 Histology

The rats (n=6 per group) were euthanized with a pentobarbital overdose (200 mg/kg) five weeks after infarction at which point the remodeling process in the rat is complete.[123] Two additional animals injected with PTN plasmid for sacrificed after 3 months.

Another set of animals injected with PTN plasmid (n=5) were sacrificed five days after infarction in order to confirm PTN plasmid transfection in the rat myocardium. The hearts were rapidly excised and fresh frozen in Tissue Tek O.C.T. freezing medium (Sakura). They were then sectioned into 10 μm slices and stained with H&E. Five slides, equally distributed through the infarct area, were taken as a representative sample from each heart in those animals sacrificed after 5 days, and stained for β-gal activity. Following fixation in 4% paraformaldehyde, the slides were incubated with 40 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Sigma) diluted 1:40 in X-gal staining solution (5mM $K_3Fe(CN)_6$, 5mM $K_4(CN)_6 \cdot 3H2O$, 2mM $MgCl_2$ in PBS) at room temperature overnight. Transfected cells indicated by blue staining were noted to ensure *in vivo* activity of the PTN plasmid. Five slides were also taken from the animals sacrificed after 5 weeks and 3 months and stained with an anti-smooth muscle actin antibody (1:75 dilution; Dako, Carpinteria, California) to label arterioles.[124]. In order to visualize labeled arterioles, sections were incubated with a Cy-3 conjugated anti-mouse secondary antibody (1:100 dilution; Sigma). Arterioles in each section were quantified using the following criteria: 1) positive for smooth muscle labeling, 2) within the infarct scar, 3) having a visible lumen and 4) a diameter ≥ 10 μm. The scar area was measured using SPOT 3.5.1 software (Diagnostic Instruments, Sterling Heights, Michigan) and arteriole densities were calculated. 5 additional slides were taken from the same hearts and stained for capillaries. Capillaries were labeled with a biotinylated Griffonia simplicifolia lectin (GS-1; Vector Labs, Burlingame, California) and visualized using the LSAB2 System (Dako) as previously described[80]. The capillaries in five high

magnification fields within the infarct area of each slide were counted and vessel density was calculated.

2.2.9 Statistical Analysis

RT-PCR data was compared against time point controls using a student t-test while elevated time points (≥3 days) were compared against baseline time points using one-way ANOVA analysis. Both the capillary and arteriole densities were compared between groups using a student's t-test.

2.3 Results

2.3.1 Myocardial ischemia induced upregulation of PTN gene

Compared to sham operated controls, PTN mRNA becomes significantly elevated at 3 days following ischemia and returns close to the baseline level after 30 days (Figure 2.2). PTN mRNA levels did not significantly change at 30 minutes and 24 hours following ischemia. At 3 days, 7 days, and 14 days PTN mRNA was elevated approximately 230%, 330%, and 380% respectively compared to non-ischemic myocardium.

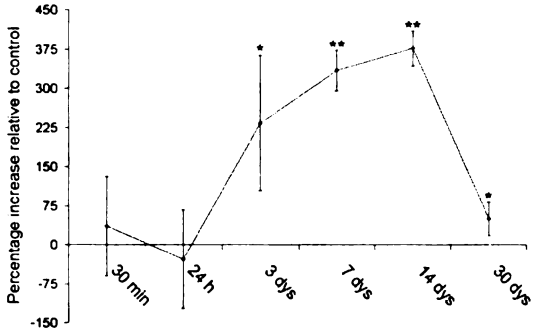

Figure 2.2 Time course of pleiotrophin (PTN) mRNA levels following ischemia in rat myocardium. PTN mRNA begins to elevate at day 3 and returns close to baseline by day 30. * $p<0.05$ vs. control, t-test; ** $p<0.05$ vs. control, t-test, and vs. respective groups at 30 min and 24 hrs, one-way ANOVA.

2.3.2 Expression of PTN

Using an ELISA assay, we determined whether the mammalian cells transfected with the pCMV-PTN plasmid were capable of producing and secreting PTN. PTN is secreted by cells transfected with the PTN plasmid but not by cells transfected with β-gal plasmid (Figure 2.3*a*). The decreasing slope seen in the lower dilutions of medium from PTN plasmid transfected cells is due to factors present in the media that interfere with the ELISA (data not shown). The aliquot of medium from PTN plasmid transfected cells contained approximately 2 μg/ml PTN. Mammalian cells transfected with the PTN plasmid are therefore capable of producing and secreting PTN.

We then determined whether the secreted PTN was biologically active using a low attachment, anchorage independent growth assay. PTN, isolated from the media of 293 cells transfected with PTN plasmid, and a tetrazolium compound that is reduced by

viable cells into a formazan product was added to each well containing SW13 cells. The amount of formazan, which directly correlates with the amount of proliferation, was measured by reading absorbance at 490nm. The presence of PTN increased the amount of proliferation in a dose-dependent manner similar to basic fibroblast growth factor (bFGF) (Figure 2.3*b*).

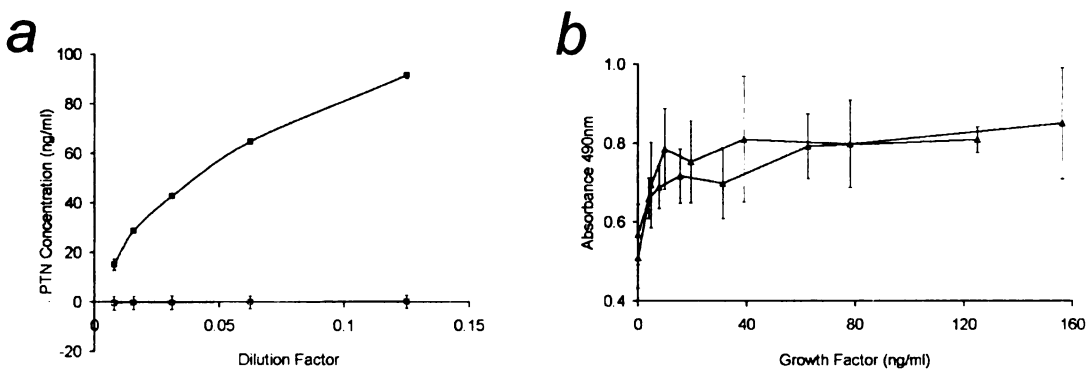

Figure 2.3 Pleiotrophin (PTN) expression and activity. ***a***, ELISA data of PTN secreted by 293 cells that were transfected with a pCMV-PTN plasmid (■) or a pCMV-β-gal plasmid (□). Cells transfected with the PTN plasmid synthesized and secreted PTN while those transfected with the β-gal plasmid did not. ***b***, Low attachment, anchorage independent growth assay demonstrating secreted PTN was biologically active. Increased absorbance relates to an increase in proliferation. (▲) PTN isolated from the media of 293 cells transfected with PTN plasmid increased proliferation of SW13 cells in a dose dependent manner similar to basic fibroblast growth factor (Δ), which was used as a positive control. Mammalian cells transfected with the PTN plasmid are therefore capable of producing and secreting biologically active PTN.

2.3.3 β-gal Transfection

β-gal stained cells indicating transfection with PTN plasmid for found in all hearts injected with the PTN plasmid (Figure 2.4).

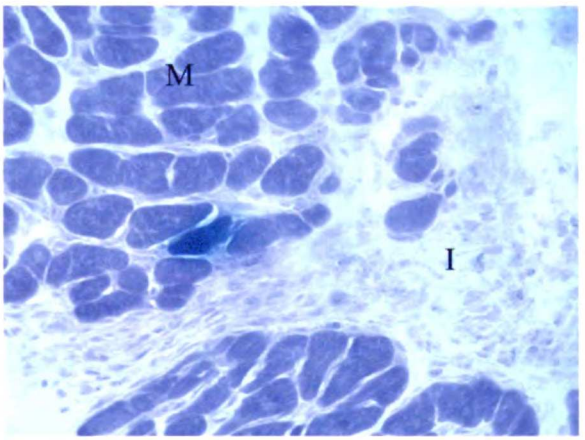

Figure 2.4 Transfection of PTN plasmid. Section is from heart injected with PTN plasmid in saline. (M) Normal Myocardium. (I) Infarcted Myocardium. Note β-gal staining in a cardiomyocyte bordering the infarct area.

2.3.4 PTN induced neovasculature formation

The capillary density was significantly higher in animals injected with PTN plasmid compared to those injected with the control β-gal plasmid (P=0.02). Capillary density increased to 1287±148 capillaries per mm^2 after PTN plasmid injection compared to 970±195 capillaries per mm^2 after β-gal plasmid injection (Figure 2.5). The capillary

density of normal, non-ischemic myocardium was 1665± 367 capillaries per mm^2 and thus, the treated areas of ischemic myocardium achieved nearly 75% the density of capillaries of the non-ischemic myocardium. Injection of PTN plasmid also significantly increased arteriole density compared to injection of β-gal plasmid (P=0.002) (Figure 2.6).

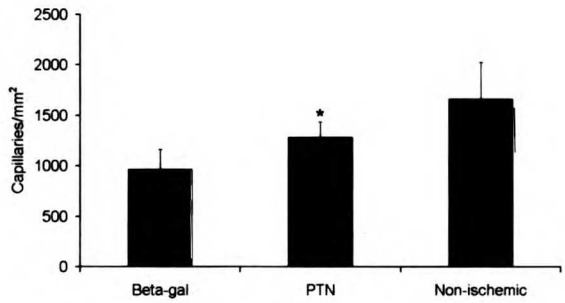

Figure 2.5 Infarct capillary density. Injection of PTN plasmid increased capillary density compared to injection of β-gal plasmid. * $p<0.05$ compared to β-gal control injection. Capillary density of normal, non-ischemic myocardium is provided as a reference.

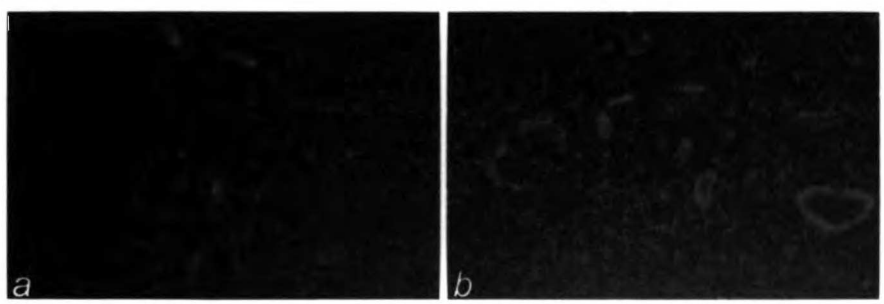

Figure 2.6 PTN plasmid induced arteriogenesis. Anti-alpha smooth muscle actin stained arterioles five weeks after plasmid injection into ischemic myocardium.

Arterioles formed as a result of (*a*) β-gal plasmid injection and (*b*) PTN plasmid injection. Note the increase in arteriole density following PTN plasmid injection

Arteriole density increased to 10±2 arterioles per mm^2 in the presence of PTN compared to 5±1 arterioles per mm^2 (Figure 2.7). Immuno-reactive smooth muscle cell actin was readily demonstrated within the larger vessels, confirming that the larger vessels were arterioles. Upon histological examination, the increase in vessels appeared to be localized to the infarcted tissue (Figure 2.8). Notably, there was also no macroscopic or histological evidence of angioma formation.

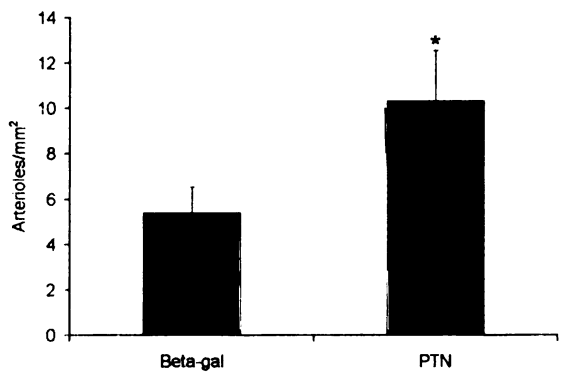

Figure 2.7 Infarct arteriole density. Injection of PTN plasmid increased arteriole density compared to injection of β-gal plasmid. * $p<0.05$ compared to β-gal control injection.

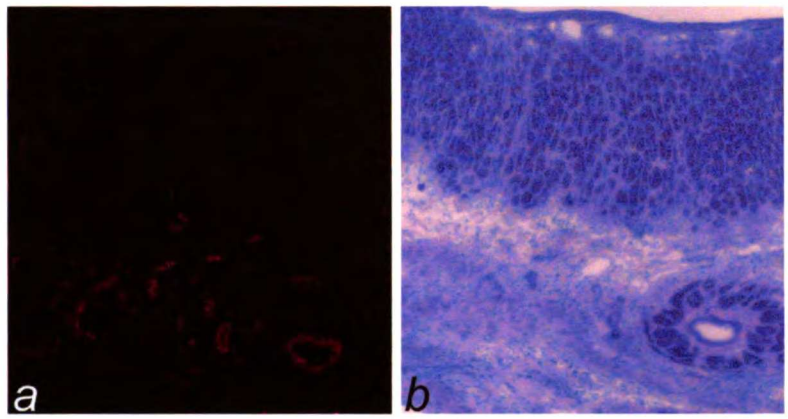

Figure 2.8 Localized increase in arterioles. ***a***, Anti-smooth muscle actin labeled arterioles. ***b***, Normal and infarcted myocardium can be visualized in the corresponding H&E labeled section. The increase in arterioles is localized to the infarct scar.

An additional two rats were injected with PTN plasmid and sacrificed after 3 months. The arteriole and capillary densities were similar to those 5 weeks after PTN plasmid injection. The arteriole density after 3 months was 10±2 arterioles per mm^2 while the capillary density was 1289±209 capillaries per mm^2. Again, there was no macroscopic or histological evidence of vascular tumor formation.

2.3.5 Microbead Perfusion

To determine if vessels in the PTN-treated ischemic myocardium are functionally connected with the pre-existent coronary circulation, microbeads were injected into the coronary vessels by retrograde infusion through the aorta and sections of PTN-treated ischemic myocardium were examined for perfusion. The injected microbeads were present both in new vessels within the PTN-treated ischemic rat myocardium and in the vessels from the adjacent normal myocardial tissue, demonstrating that the newly formed

vessels stimulated by PTN injection establish functional connections with existing coronary vessels (Figure 2.9).

Figure 2.9 Microbead perfusion. ***a***, Anti-smooth muscle actin labeled arterioles within PTN-treated ischemic mycardium. ***b***, Microbeads perfusion in the same section. ***c***, Overlay of *a* and *b* demonstrates neovasculature formed by injection of PTN is functionally connected to the coronary vasculature.

2.4 Discussion

During normal development, PTN gene expression peaks during late embryogenesis and in perinatal growth. With the exception of a subset of neurons, PTN gene expression is markedly lower in adult tissues[125-130]. However, in pathological states, such as response to ischemic injury in the brain, PTN is notably upregulated in areas of developing vasculature[35]. Our results also demonstrate that PTN gene expression is upregulated in response to ischemia in rat myocardium, indicating a possible universal role in angiogenesis in response to ischemic conditions. PTN mRNA in both brain and myocardium is elevated beginning by day 3 following induction of ischemia and

continues to be elevated in both tissues at two weeks. This temporal expression profile is noticeably different from the profile for VEGF and bFGF mRNA following myocardial ischemia. The VEGF mRNA expression profile follows a more acute response with elevated levels as early as 30 minutes after onset of ischemia[131-133]. Functionally, VEGF is thought to be involved in the initial phases of angiogenesis by affecting the permeability[134,135], recruitment and proliferation of endothelial cells[136-139]. There is also an acute response with bFGF which is upregulated within 6 hours following ischemia[19]. In contrast, the later expression profile of PTN indicates that it may play a more regenerative role influencing the maturation of the initial capillary beds into functional collateral arteries. Pleiotrophin may function more upstream than a number of different angiogenic factors and thus support several different signaling cascades. This view of PTN is supported by the pleiotrophic effects ascribed to PTN and consistent with the view that PTN is a differentiation factor. Thus, through activation of different cascades of signaling molecules, PTN may coordinate functional capillary and arteriolar new vessel formation in the ischemic myocardium.

Our results also indicate that injection of PTN plasmid induces neovasculature formation in ischemic myocardium, resulting in a higher capillary and arteriole density in the infarct scar. Furthermore, these vessels were interconnected with pre-existent coronary vessels. In vitro, PTN has been shown to induce angiogenesis in the rabbit corneal pocket assay[37]. It also stimulates proliferation of endothelial cells[38,39,140] and stabilizes endothelial cells tube formation in matrigel[140]. It is significant that injection of PTN plasmid results in both capillary and arteriole formation in ischemic myocardium since formation solely of capillaries does not necessarily result in a sustained increase in

blood flow[141]. In contrast, VEGF results solely in the formation of capillaries. Without the addition of other growth factors, VEGF is incapable of producing larger, more mature vessels such as arterioles and arteries[142]. Without the development of these vessels, the newly formed capillary bed will fail to properly perfuse the ischemic tissue. It has also been shown that the capillary bed formed as a result of VEGF injection, either in plasmid form or secreted from retroviral transduced myoblasts, is irregular and not connected to the coronary vasculature. High doses of VEGF also produced angiomatous structures[16,17]. In contrast, PTN plasmid was capable of inducing angiogenesis and arteriogenesis, without inducing vascular tumors. The vessel densities after both five weeks and 3 months following PTN plasmid injection were also similar, thus indicating that PTN has a sustained effect by forming long lasting, non-regressing vessels. The increase in vasculature as a result of PTN plasmid injection was also localized to the infarct tissue and not apparent in the surrounding normal myocardium. PTN may therefore only have effect where other ischemia induced factors are upregulated. When compared to other agents such as VEGF, which has had detrimental side effects in pre-clinical studies, and both bFGF and VEGF, which have had mixed results in initial clinical trials[21-28], PTN appears to consistently produce the appropriate vasculature localized within ischemic myocardium. PTN may therefore be a potential therapeutic angiogenic agent for use in ischemic myocardium.

Chapter 3

Effects of Fibrin Glue in an Acute MI Model

3.1 Introduction

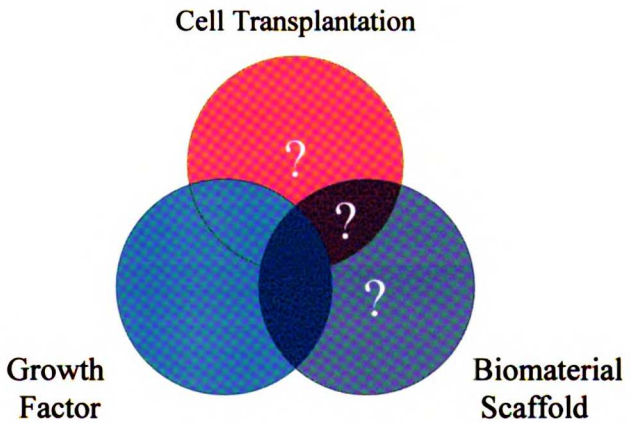

Figure 3.1 Classic tissue engineering triad. Examination of cell therapy and a biomaterial scaffold for myocardial repair.

In this study, we examine the use of cell transplantation and a biomaterial scaffold for myocardial repair (Figure 3.1). While cellular cardiomyoplasty has had some promising results, the technique suffers from low cell transplant survival[59-62]. Prior studies examined cell transplantation by injecting the cells in completely liquid solutions. We now present a novel method for delivering viable cells directly into the infarct wall. We utilized the injectable biopolymer, fibrin glue, to deliver the cells in an artificial extracellular matrix. We hypothesized that injection of cells in a fibrin scaffold would

increase cell transplant survival within the infarct compared to the standard injection technique. In addition, we hypothesized that the use of this injectable scaffold would result in an increase in infarct vasculature and a reduction of infarct expansion. In contrast to other attempts at myocardial tissue engineering which utilized patches of tissue created *in vitro*[70,80], this study is the first to examine the potential of *in situ* engineered myocardial tissue.

3.2 Methods

3.2.1 Rat Acute Myocardial Infarction Model

The rat ischemia reperfusion model described in Chapter 2 was used for this study. In this study, the rats were allowed to recover for one week before injection.

3.2.2 Skeletal Myoblast Isolation and Culture

Myoblasts from the hind limb muscle of Sprague-Dawley neonatal rats (2-5 days old) were isolated and purified according to the following described procedure.[143] Briefly, the hind limb was harvested under phosphate buffered saline (PBS)-Penicillin/Streptomycin (PCN/Strep) and mechanically minced. The tissue was enzymatically dissociated with dispase and collagenase (Worthington) in Dulbecco's PBS (Sigma) for 45 minutes at 37 °C. The resulting suspension was then passed through an 80 μm filter and the cells were collected by centrifugation. The cells were preplated for 10 minutes in order to isolate myoblasts from fibroblasts. The myoblast suspension was transferred to a collagen coated 100 mm polystyrene tissue culture dish and allowed to proliferate in growth media (80% Ham's F10C media, 20% fetal bovine serum, 1% PCN/Strep, 2.5 ng/ml

recombinant human basic fibroblast growth factor) at 37 °C in a humidified atmosphere of 95% air plus 5% CO_2. Cultures were allowed to reach a confluency of 70-75 % and passaged every 3-4 days (1:4 split). Cultures were routinely examined for fibroblast contamination and only populations of greater than 95% myoblasts were acceptable for injection. In order to verify the percentage of myoblasts in the population, cultured cells were stained with desmin (Sigma; 1:20 dilution) to label myoblasts and Hoechst 33342 (Molecular Probes) to label nuclei. Rat fibroblasts (ATCC) and L6 rat myoblasts (ATCC) were also stained as negative and positive controls respectively (Figure 3.2). All injections were from the same pool of cells. Prior to injecting the rats which were sacrificed 24 hours post-injection, the myoblasts were labeled with 4',6-diamidino-2-phenylindole (DAPI) for 25 minutes (3 μM; Molecular Probes).

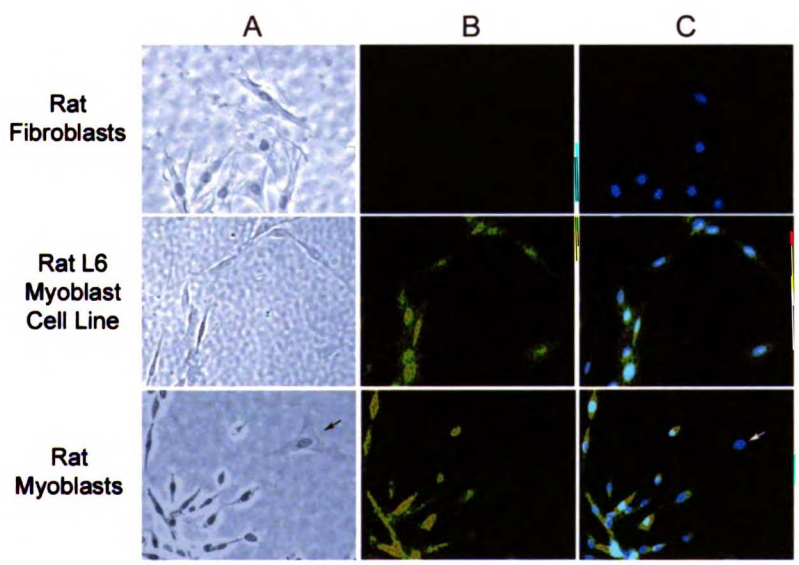

Figure 3.2 Transplant Myoblasts. (A) Phase contrast images. (B) Desmin labeled cells. (C) Hoechst 33342 labeled nuclei. Transplant rat myoblasts (bottom panel) were stained with desmin and hoechst to verify myoblast percentage. Transplanted cells contained greater than 95% myoblasts. One contaminating fibroblast is indicated by the

arrowheads. Control rat fibroblasts and rat myoblasts from the L6 cell line were stained with desmin and hoescht as negative and positive controls respectively.

3.2.3 Fibrin Glue

The fibrin glue used in this study was the commercially available Tisseel VH fibrin sealant (Baxter). It is a two component system which remains liquid for several seconds before solidifying into a solid gel matrix. The first component consists of concentrated fibrinogen and aprotinin, a fibrinolysis inhibitor. The second is a mixture of thrombin and $CaCl_2$. It is delivered through the supplied Duploject applicator, which holds the two components in separate syringes and provides simultaneous mixing and delivery (Figure 3.3). The ratio of fibrinogen to thrombin components was 1:1.

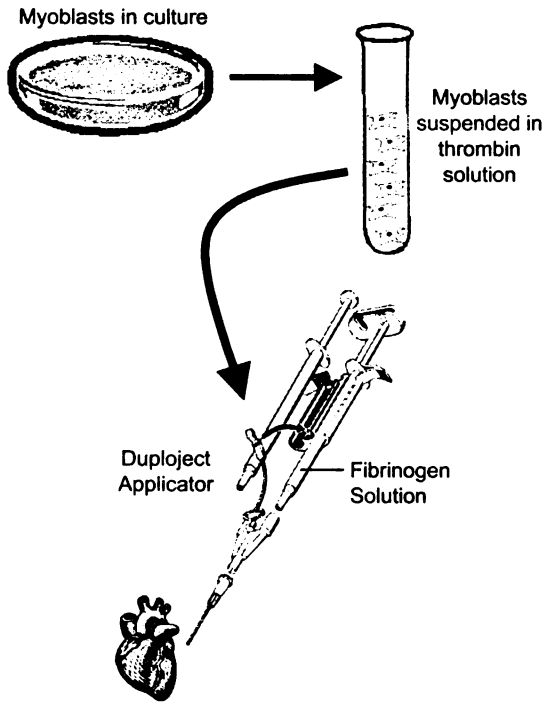

Figure 3.3 Schematic illustration of injection procedure for cells in fibrin glue.

3.2.4 Injection Surgeries

One week after myocardial infarction (MI), either 0.5% BSA in 50 μl PBS, 50 μl fibrin glue, 5×10^6 myoblasts in 50 μl 0.5% BSA, or 5×10^6 myoblasts in 50 μl fibrin glue was injected into the infarcted myocardium. Under sterile technique, the rats were anesthetized and the abdomen was opened from the xiphoid process to a left subaxillar level along the lower rib. The LV apex was exposed via a subdiaphragmatic incision, leaving the chest wall and sternum intact. Rats were randomized to either control or treatment groups and injections were made through a 30-guage needle into the infarcted area of the LV. One injection with a volume 50 μl was made for each animal. In the

cells group, 5×10^6 myoblasts were suspended in 50 µl 0.5% BSA and injected into the myocardium. In the cells in fibrin group, 5×10^6 myoblasts were suspended in 25 µl of the thrombin component of the fibrin glue. The thrombin-cell mixture was simultaneously injected into the myocardium with 25 µl of the fibrinogen component (Figure 11). 25 µl thrombin and 25 µl fibrinogen was simultaneously injected into ischemic myocardium in the fibrin group. The diaphragm was sutured closed after suction of the chest cavity and the abdomen was subsequently closed

3.2.5 Echocardiography

Transthoracic echocardiography was performed on all animals in conscious state[144] one week after MI (baseline echocardiogram). Then a follow-up echocardiogram was performed 5 weeks after injection. The study was concluded 6 weeks following infarction at which point the remodeling process in the rat is complete[123]. The methodology of echocardiography used in this laboratory has been previously described[2,4]. Other reports have demonstrated the accuracy and reproducibility of transthoracic echocardiography in rats with myocardial infarcts[145,146]. Briefly, the animals were shaved and placed in plastic DecapiCone restrainers (Braintree Scientific Inc.) in conscious state. A layer of acoustic coupling gel was applied to the thorax. Then the animal was placed in a prone or slightly lateral decubitus position. Echocardiography was performed using a 15-MHz linear array transducer system (Acuson Sequoia c256, Mountain View, CA). Care was taken to avoid excessive pressure on thorax, which could induce bradycardia. Two-dimensional images were obtained in both parasternal long and short axis views (at the papillary muscle level).

Enhanced resolution imaging function (RES) was activated with a region of interest adjusted to heart size whenever possible. The gain was set for best imaging, and the compression was set at 70 dB. Two criteria were used for adequate imaging. First, the short-axis view must demonstrate at least 80% of the endocardial and epicardial border. Second, the long-axis view must demonstrate the plane of mitral valve, where the annulus and the apex could be visualized. After adequate two-dimensional images were obtained, the M-mode cursor was positioned perpendicular to the ventricular anteroseptal wall (at the site of infarct) and the posterior wall, at the level of the papillary muscles. Wall thickness and left ventricular internal dimensions were measured according to the leading edge method of the American Society of Echocardiography. Fractional shortening (FS) as a measure of systolic function was calculated as FS (%) = [(LVIDd – LVIDs)/LVIDd] x 100%, where LVID was the left ventricular internal dimension, d was diastole and s was systole. An echocardiographer blinded to the treatment group acquired the images and performed the data analysis. The accuracy and reproducibility of the technique have been reported in a previous study from this laboratory[2,4].

3.2.6 Histology

Either 24 hours or 5 weeks following the injection surgeries, the rats were euthanized with a pentobarbital overdose (200 mg/kg). The hearts were rapidly excised and fresh frozen in Tissue Tek O.C.T. freezing medium (Sakura). They were then sectioned into 5 μm slices and stained with hematoxylin and eosin (H&E). Five slides, equally distributed through the infarct area, were taken from each heart as a representative sample and measured for infarct size as previously described to determine treatment effects on infarct expansion[147]. Briefly, the infarct and LV were traced and size was determined using

planimetry. Infarct scar size was determined as the infarct scar area divided by the total LV area as measured with SPOT 3.5.1 software (Diagnostic Instruments) and recorded as a percentage of the LV. Five additional slides from both the 24 hour cells in BSA group (n=5) and 24 hour cells in fibrin group (n=4) were examined for presence of DAPI labeled transplanted cells. The area covered by the myoblasts was traced using SPOT 3.5.1 and expressed as percentage of the infarct area. All H&E stained slides were also examined for any evidence of inflammation by our cardiac pathologist. Inflammation was assessed for evidence of an immune reaction.

3.2.7 Immunohistochemistry

Five slides, equally distributed through the infarct area, were also taken from each heart in the 5 week BSA group (n= 6), 5 week fibrin group (n=5), 5 week myoblasts in BSA group (n=5), and 5 week myoblast in fibrin group and stained with an anti-smooth muscle actin antibody (Dako; 1:75 dilution) to label arterioles.[124] 5 slides were also taken from each heart in the 5 week myoblasts group (n=5) and 5 week myoblasts in fibrin group (n=5) and stained with the MY-32 clone (Sigma; 1:400 dilution), which is directed against the skeletal fast isoform of myosin heavy chain (MHC)[148], in order to label transplanted cells. Sections of rat hind limb skeletal muscle were also stained with the anti-skeletal MHC antibody to serve as a positive control. In order to visualize labeled arterioles and skeletal myoblasts, sections were incubated with a Cy-3 conjugated anti-mouse secondary antibody (Sigma; 1:100 dilution). Sections which were only incubated with the secondary antibody were used as negative controls. Sections were mounted with Gel/Mount (Biomeda). Arterioles in each section were quantified and arteriole densities were calculated. Cell survival was determined by measuring the area covered by cells

that stained positive for anti-skeletal fast MHC in each section using Scion Image (Scion) and reported as percentage of infarct area. 5 additional slides were taken from each heart in all 5 week groups. Capillaries were labeled and quantified using the previously described procedure in Chapter 2.

3.2.8 Statistical Analysis

Data is presented as mean ± standard deviation. Cell density measurements were compared using a student's t-test. Infarct size and vessel measurements were compared using one-way ANOVA analysis with Holm's adjustment. Significance was accepted at $P< 0.05$. One week and 5 week post-MI echocardiography data was compared using a paired t-test.

3.3 Results

3.3.1 Cell Retention and Survival

After 24 hours, the myoblast density after injection in either BSA or fibrin glue was not significantly different (P=0.85). Myoblasts injected in BSA comprised 15.8±9.2% of the infarct while myoblasts injected in fibrin glue covered 17.3±14.6%. Myoblasts transplanted in fibrin glue were found both in clumps surrounded by the fibrin matrix and dispersed within its fibrils (Figure 3.4).

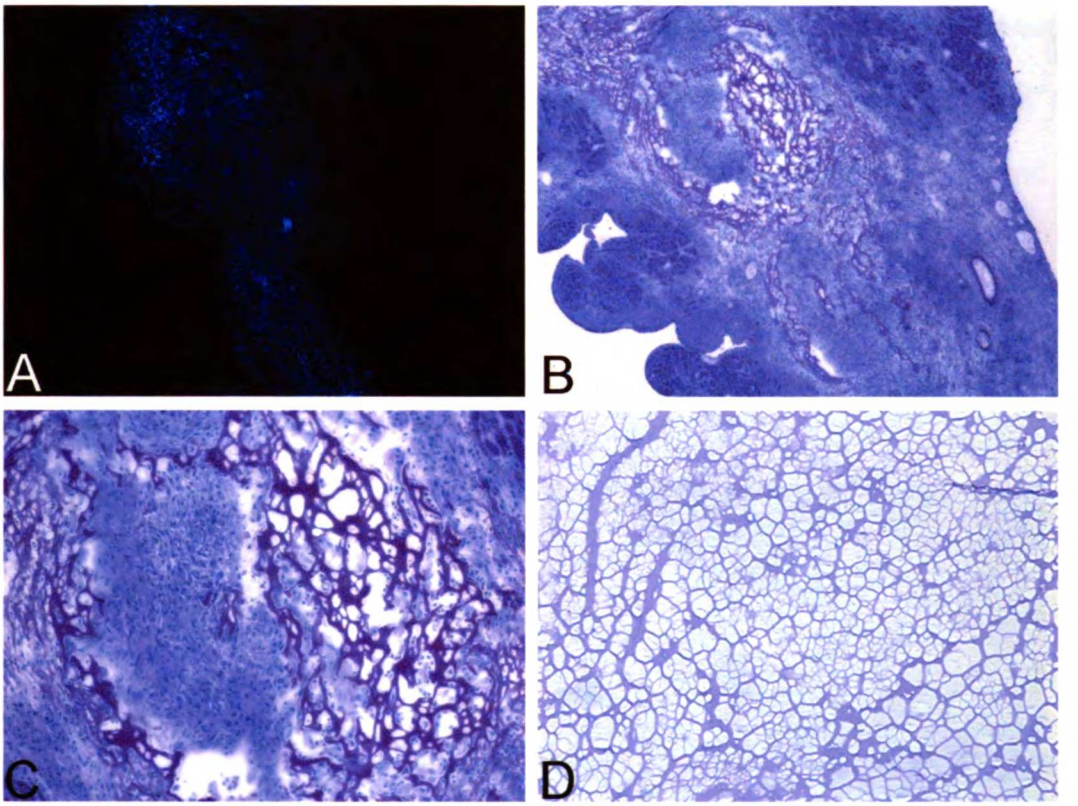

Figure 3.4 Myoblasts in fibrin glue 24 hours post-injection. (A) DAPI labeled myoblasts injected in fibrin glue are found in the infarct wall. ×4. (B) Corresponding hematoxylin and eosin (H&E) stained section. Transplanted myoblasts are surrounded by fibrin glue within the infarct scar. ×4. (C) Higher magnification H&E section displaying transplanted myoblasts in fibrin glue. ×10. (D) H&E stained section of fibrin glue ex vivo. ×10.

After 5 weeks, the myoblast density in the infarct area was significantly greater when the cells were injected in the fibrin scaffold compared to injection in BSA (P=0.03). Cells injected in fibrin glue covered 9.7±4.2% of the infarct area compared to 4.3±1.5% when injected in BSA. Transplanted myoblasts injected in BSA were most often found at the border of the infarct scar and not within the ischemic tissue five weeks post-injection

(Figures 3.5A and 3.5C). In contrast, myoblasts injected in fibrin glue were found both at the border and within the infarct scar (Figures 3.5B and 3.5D). Cells transplanted in fibrin glue were often surrounding arterioles within the infarct scar (Figures 3.5B and 3.5D, arrowheads). Figures 3.5C and 3.5D display the location of the normal and infarcted myocardium, thus allowing one to visualize the location of the anti-skeletal, fast MHC labeled myoblasts in Figures 3.5A and 3.5B respectively.

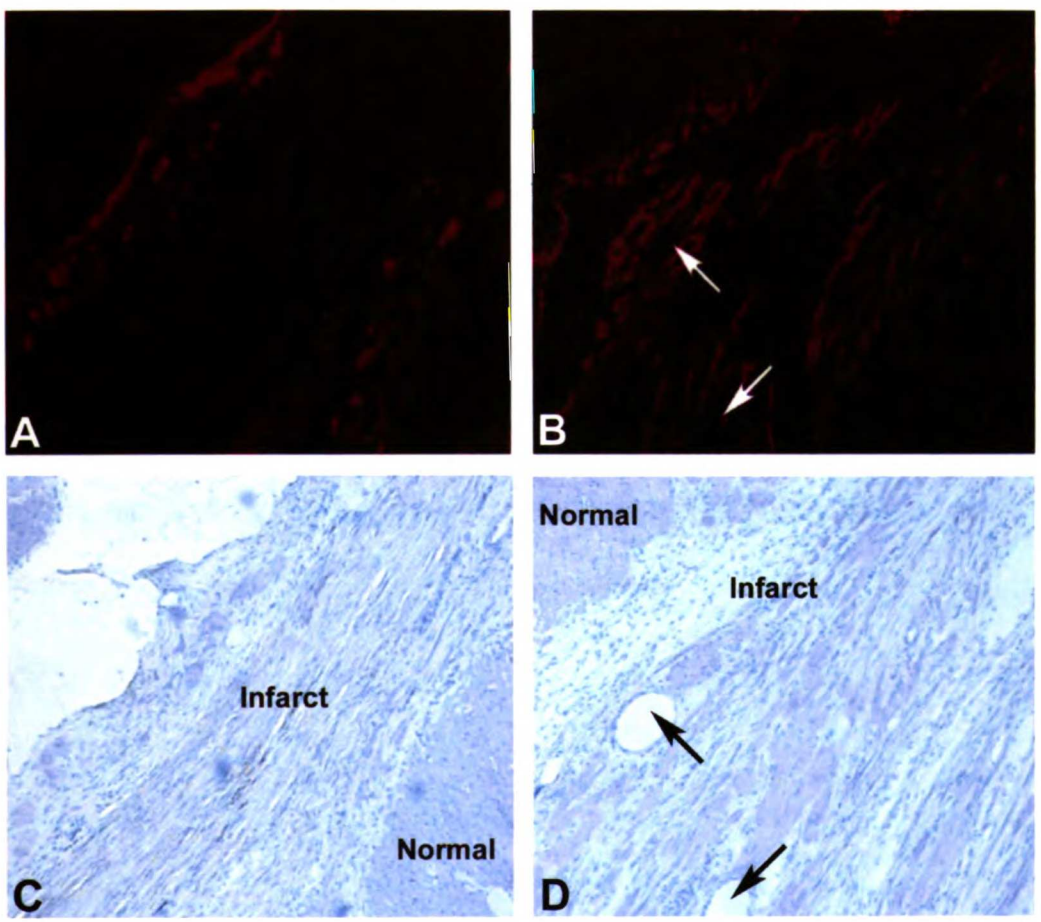

Figure 3.5 Myoblast survival and location within myocardium after 5 weeks. ×100. (A and B) Anti-skeletal, fast myosin heavy chain labeled myoblasts visualized with a Cy3 secondary antibody. (C and D) Hematoxylin and eosin stained sections that neighbor A and B respectively. (C and D) Normal and infarcted myocardium are labeled in order to

visualize the location of transplanted cells. (A) Transplanted skeletal myoblasts in bovine serum albumin are located at the border of the infarct scar. (B) In contrast, a greater number of skeletal myoblasts injected in fibrin glue are located both at the border and within the infarct scar. Surviving myoblasts are in clumps surrounding arterioles (arrowheads B and D) in the scar.

3.3.2 Histology

Infarct scar size as determined by percent of the LV was measured for each group. The infarct size in the control (BSA) group was 26.5±2.2 %. There was no significant difference in infarct size between treatment groups (P=0.45); however, both injection of fibrin glue and myoblasts in fibrin glue resulted in significantly smaller infarcts (P=0.03 and P=0.003 respectively) compared to a BSA control injection. Fibrin glue reduced the infarct size to 19.7±3.8 % while myoblasts in fibrin glue reduced the size to 17.5±3.4 %. In contrast, myoblasts injected in BSA did not produce a statistically smaller infarct than injection of BSA (20.9±5.2 %, P=0.24) (Figure 3.6).

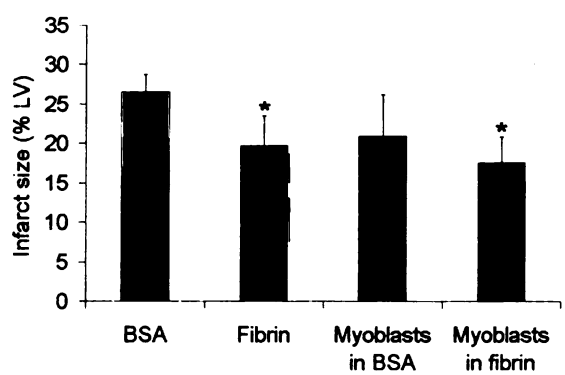

Figure 3.6 Infarct Size. Injection of fibrin glue and skeletal myoblasts resulted in statistically smaller infarcts than injection of bovine serum albumin (BSA). In contrast,

the infarct size of hearts injected with skeletal myoblasts in BSA was not statistically different than hearts injected with BSA. * $P<0.05$ compared to BSA (fibrin, $P=0.03$; myoblasts in fibrin, $P=0.003$).

Histological review of H&E stained sections from each group demonstrated that there was no significant inflammation. The scars did contain scattered hemociderin-laden macrophages, which are evidence of prior hemorrhage, and rare mononuclear cells; however, there was virtually no active inflammation.

3.3.3 Neovasculature Formation

To assess the angiogenic potential of fibrin glue in ischemic myocardium, infarcted rat hearts injected with fibrin glue and BSA were examined for capillary density five weeks after injection. There was no significant difference between groups ($P=0.64$). Arterioles were labeled with anti-smooth muscle actin in both the fibrin and BSA groups to determine if fibrin glue induces arteriogenesis after 5 weeks. Even without treatment, collateral arterioles are often seen bordering the scar after MI, thus separate arteriole counts were performed on vessels within the infarct and those bordering the scar. Arteriole density for the total infarct in the fibrin group was significantly greater than that in the BSA group ($P=0.004$). Arterioles in the fibrin group increased to 16 ± 1 arterioles/mm^2 compared to 10 ± 2 arterioles/mm^2 in the BSA group. There was no difference in arteriole density bordering the infarct between the two groups ($P=0.32$); however there was a significant difference in the arteriole density within the scar between the fibrin and BSA groups ($P=0.001$). Within the infarct scar, the arteriole density

following injection of fibrin glue was 13±1 arterioles/mm^2, compared to 10±2 arterioles/mm^2 for hearts injected with BSA. The arteriole density of the two groups including myoblasts was also calculated. Injection of myoblasts in fibrin glue significantly increased the total and within scar arteriole density compared to injection of myoblasts in BSA (P=0.007 and P=0.02 respectively). The total and within scar arteriole densities were increased to 12.9± 2.6 and 9.1±1.9 arterioles per mm^2 compared to 6.3±1.8 and 4.2±2.0 arterioles per mm^2 after injection of myoblasts in BSA. There was again no difference in arterioles bordering the infarct scar (P=0.21). We also compared the BSA group to the myoblasts in BSA group and the fibrin group to the myoblast in fibrin group to determine if the addition of myoblasts affected arteriole formation. Both addition of myoblasts in BSA and fibrin resulted in a significant or near significant decrease in the total arteriole density (P=0.04 and P=0.05 respectively). Addition of myoblasts also decreased the within scar arteriole density (P=0.02 and P=0.01 respectively) (Figure 3.7).

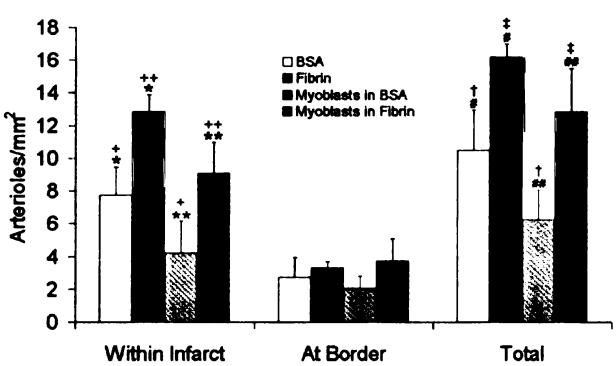

Figure 3.7 Arteriole Density. Injection of fibrin glue alone increased arteriole formation compared to injection of BSA within the scar (*, P=0.001) and in the total infarct area (#, P=0.004). Injection of skeletal myoblasts in fibrin glue also increased arteriole density within the scar (**, P=0.02) and in the total infarct area (##, P=0.007) compared to

injection of myoblasts in BSA. Addition of myoblasts to both fibrin glue and BSA partially inhibited arteriole formation for both the total and within scar densities (+, BSA within scar, P=0.02; †, BSA total, P=0.04; ++, fibrin within scar, P=0.01; ‡, fibrin total, P=0.05). There were no significant differences in arteriole density at the border of the infarct scar.

After fibrin glue injection, a large number of arterioles were found within the infarct scar (Figures 3.8A and 3.8B). Figure 3.8B has been stained with H&E and is the neighboring slide to Figures 3.8A. Normal, healthy myocardium, which is denoted by its darker staining, and the infarct scar, which is denoted by lighter staining, can both be visualized in Figure 3.8B. Figure 3.8B demonstrates that the large number of labeled arterioles in Figure 3.8A are in fact within the infarct scar.

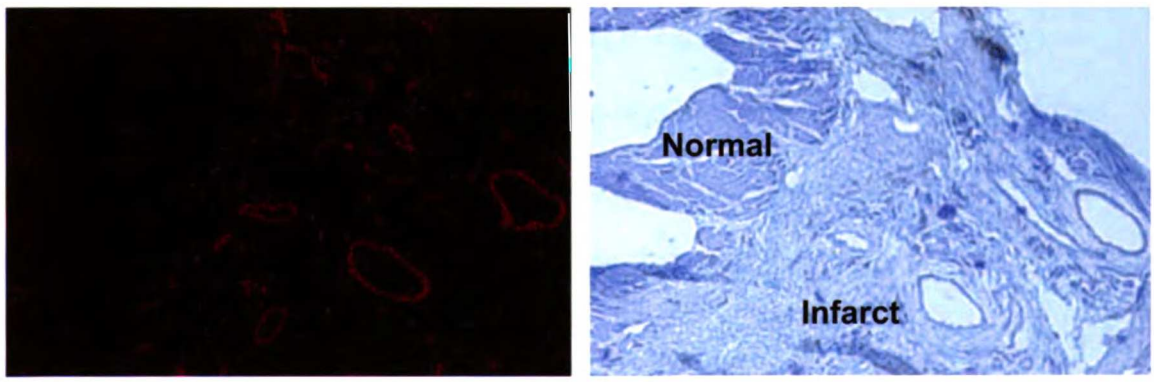

Figure 3.8 Fibrin induced arteriole formation. ×100. (A) Anti-smooth muscle actin labeled arterioles visualized with a Cy3 secondary antibody. (B) Hematoxylin and eosin stained section that neighbors A. (B) Normal, healthy myocardium is denoted by the

darker staining while the infarct scar has lighter staining. (A and B) Fibrin induces formation of several arterioles within the infarct.

3.3.4 Echocardiography

The echocardiographic data is summarized in the table below (Table 3.1). As typical of post-MI progression, the control group exhibited a deterioration of LV function and thinning of the infarct wall. After five weeks there was significant deterioration in FS (P = 0.0005) as well as a significant decrease in infarct wall thickness (P = 0.02) (Table 3.1, control group).

In contrast, injection of fibrin glue alone, myoblasts alone, and myoblasts in fibrin glue resulted in the preservation of FS and infarct wall thickness. FS for the fibrin group, cells group, and cells in fibrin group did not significantly decrease by P-values of 0.18, 0.89, and 0.19 respectively (Table 3.1). In addition, there was no significant difference in infarct wall thickness for all treatment groups (P = 0.40, 0.44, 0.43 respectively). Differences between before injection and post-injection FS and infarct wall thickness were compared among treatment groups. No significant difference was observed (P = 0.52 and P = 0.56 respectively), thus indicating that no single treatment was more effective than the others.

Table 3.1 Echocardiography Data*

	Before Injection	5 Weeks Post-Injection	*P*
Fractional shortening, %			
Control group	45±8	22±6	0.0005
Fibrin group	26±5	23±8	0.18
Cells group	29±14	28±2	0.89
Cells in fibrin group	42±10	33±6	0.19
Infarct wall thickness, cm			
Control group	0.29±0.08	0.24±0.04	0.02
Fibrin group	0.26±0.04	0.23±0.06	0.40
Cells group	0.30±0.08	0.26±0.06	0.44
Cells in fibrin group	0.30±0.04	0.32±0.02	0.43

*P values are for internal comparisons between measurements one week following infarction and five weeks following injection.

3.4 Discussion

Our results indicate that cell transplant survival, but not cell retention in infarcted myocardium is enhanced by injection of cells in fibrin glue. Injection of cells in fibrin glue did not affect the amount of myoblasts in the infarct after 24 hours. These results indicate that fibrin glue does not increase cell retention. Since fibrin glue remains liquid for a few seconds, cells may continue to be squeezed out of the beating myocardium upon injection. In contrast, the area of the infarct wall covered by transplanted myoblasts after

five weeks was significantly greater when the myoblasts were injected in fibrin glue, indicating that fibrin increases cell survival. Fibrin may increase cell survival by acting as a temporary extracellular matrix for the transplanted cells. Typically, cells have been injected in completely liquid formulations of saline, cell culture medium, or BSA; however, fibrin glue solidifies inside the myocardium, giving the cells a temporary semi-rigid scaffold. Fibrin glue also contains RGD motifs and binds to cell receptors (predominately integrins).[94] Upon injection in fibrin glue, the cells are entrapped within this temporary extracellular matrix. Fibrin glue is an injectable scaffold that allows delivery of more viable cells directly into the infarct wall. Another possibility for the increase in cell survival is that injection of fibrin glue into ischemic myocardium induced neovasculature formation. An increase in blood supply would provide a less ischemic region for the cells to thrive. It has been previously reported that injection of cells into vascularized myocardium increases survival compared to injection in ischemic myocardium.[60] This is further supported by the observation that myoblasts injected in fibrin glue were often found surrounding arterioles within the infarct scar. Fibrin may also be inducing proliferation of the transplanted myoblasts, resulting in an apparent increase in transplanted cells. Nevertheless, the fibrin scaffold increases the number of transplanted cells in ischemic myocardium and may be the necessary modification to the standard transplantation procedure.

We have also demonstrated that injection of fibrin glue alone as well as myoblasts in fibrin glue decreases infarct scar expansion. A decrease in the area covered by the scar indicates a reduction of late infarct expansion. As an indicator of negative LV remodeling, a decrease in late infarct expansion indicates that fibrin is capable of

preventing negative left ventricular remodeling following MI in rats. Fibrin may serve as an internal wall support by increasing stiffness. It may also simply affect remodeling by increasing wall thickness. Although there was no significant difference in infarct size among treatment groups, injection of skeletal myoblasts in BSA did not produce a statistically smaller infarct than the control, consistent with previous reports of transplantation survival problems within infarcted myocardium.[59-62] This trend indicates that fibrin and myoblasts in fibrin glue may have the potential to produce smaller infarcts compared to injection of myoblasts in BSA. Injection of myoblasts in BSA may not be capable of producing a large enough graft to reduce infarct expansion.

Fibrin glue also induced arteriole formation within the infarct scar. It is significant that fibrin glue results in arteriogenesis since formation of solely capillaries does not necessarily indicate an increase in blood flow.[141] Fibrin did not, however, increase capillary formation compared to injection of BSA. Injection into myocardium is known to induce some angiogenesis. Fibrin may therefore not be capable of producing a greater angiogenic response. Furthermore, the established method in the field for the counting of capillaries is currently limiting. Capillaries within the infarct tissue are not highly organized as they are in normal tissue, thus making it difficult to get an accurate capillary density. Natively, fibrin is highly involved in wound healing and acts as the body's natural matrix for angiogenesis. Endothelial cells migrate through the fibrin clot via $\alpha_v\beta_3$ integrin binding to RGD motifs in fibrin.[149] Production of plasmin at the location of migrating endothelial cells degrades the fibrin matrix. This decrease in fibrin density allows for capillary tube formation.[150] As the cells migrate through the less dense fibrin, they interact with residues on the β-chain of fibrin via vascular endothelial

cadherins and promote capillary morphogenesis.[151] In addition to providing a matrix for endothelial cell migration and capillary tube formation, fibrin also acts as a sustained release reservoir for several growth factors [152] and fibrinolytic enzymes.[153] A degradation product of fibrin, fibrin fragment E, has also been shown to induce angiogenesis in the chick chorioallantoic membrane assay [154], stimulate proliferation, migration and differentiation of human microvascular endothelial cells [155], and stimulate migration and proliferation of smooth muscle cells.[156] Administration of exogenous fibrin into the subcutaneous space of guinea pigs has also been shown to induce angiogenesis.[96]

The results of the present study also indicate that fibrin glue may be used as a support and/or tissue engineering scaffold to preserve infarct wall thickness and cardiac function following MI. Injection of fibrin glue alone as well as injection of skeletal myoblasts in fibrin glue attenuated any decrease in infarct wall thickness and fractional shortening following MI in rats. In accordance with other studies[43,157,158], we also found that injection of skeletal myoblasts alone was able to prevent wall thinning of the infarcted LV and deterioration of LV function. Although the exact mechanism by which myoblasts preserve LV function is unknown, it is unlikely that it is from active force generation during systole since implanted myoblasts do not form gap junction with surrounding cardiomyocytes[43]. As previously suggested[157], it is more likely the attenuation of negative left ventricular remodeling by the myoblasts that preserves cardiac function. The myoblasts may serve as a wall support by increasing stiffness, or may simply affect remodeling by increasing wall thickness. Our data further supports this hypothesis. Injection of fibrin glue alone did not produce statistically different

results from the injection of skeletal myoblasts, thus suggesting that the mechanism of action of the myoblasts is by preserving wall thickness and preventing deleterious ventricular remodeling, not from active force generation. Our results also suggest that fibrin glue alone could be used as an acellular approach to prevent a decrease in cardiac function following MI. Fibrin glue would be an "off the shelf" treatment that could be delivered at anytime without the need for cell collection and expansion.

A recent study reported that a polymer mesh was capable of acting as an external support to prevent LV dilation[63]. Fibrin glue may act similarly as an internal support to preserve cardiac function; however, the exact mechanism is currently unknown. During the initial stage in MI, matrix metalloproteases are upregulated[159] which results in degradation of the extracellular matrix (ECM). This ECM degradation leads to weakening of the infarct wall and slippage of the myocytes, leading to LV aneurysm[6,160]. In addition, it has been suggested that negative ventricular remodeling continues until the tensile strength of the collagen scar strengthens the infarct wall[161,162]. By administering fibrin glue during the initial stage of an infarct, it may prevent remodeling by increasing the mechanical strength of the infarct before the collagen scar has had to time to fully develop. Furthermore, fibrin glue adheres to various substrates including collagen and cell surface receptors (predominately integrins) through covalent bonds, hydrogen and other electrostatic bonds, and mechanical interlocking[94]. Therefore, it may prevent myocyte slippage and subsequent aneurysm by binding to the neighboring normal myocardium. Finally, fibrin glue may also improve cardiac function by inducing neovasculature formation. An increase in blood flow to the infarct is thought to salvage at risk cardiomyocytes and produce a smaller infarct.

In addition to providing an internal support, our data also suggests that fibrin may be used as an injectable scaffold in the myocardium. Injection of myoblasts in fibrin glue prevented infarct wall thinning and preserved cardiac function. The benefit of using fibrin glue as a scaffold is that it is injectable, thus requiring only a minimally invasive procedure in humans. In this study we provide a proof of concept for use of fibrin glue as an injectable scaffold in myocardium. In addition to the use of skeletal myoblasts, other cell types including fetal cardiomyocytes and adult bone marrow stem cells, which produce gap junctions in recipient hearts[44,47,163,164], could be delivered to the myocardium in fibrin glue with the aims of improving both contractility and preventing remodeling.

Fibrin glue is an already FDA approved biomaterial that is routinely used as a surgical adhesive and sealant. This biopolymer is formed by the addition of thrombin to fibrinogen. Thrombin enzymatically cleaves fibrinogen, which then forms the polymer fibrin. After combination of the two components, the solution remains liquid for several seconds before polymerizing. Fibrin glue could therefore be delivered to the myocardium via a dual chamber catheter in humans, thus requiring only a minimally invasive procedure. It is also biocompatible and non-toxic, without inducing inflammation, foreign body reactions, tissue necrosis or extensive fibrosis.[95] Further modification to fibrin glue may also be performed to enhance its functionality in the myocardium. As a support, fibrin glue may be modified to tailor its mechanical properties. An increase in thrombin or fibrinogen concentration results in an increase in tensile strength and Young's modulus. An increase in fibrinogen concentration will also decrease the degradation rate of the biopolymer. As a scaffold, fibrin glue is also capable

of delivering proteins[165-168] and plasmids[169] and could be used to deliver both growth factors, either in protein or plasmid form, and cells to the myocardium.

One limitation of the animals used in this study is that they were not an inbred strain, thus graft rejection is expected to be higher. Our preliminary results with fibrin glue and myoblasts indicated that viable grafts survive in Sprague-Dawley rats. Sprague-Dawley rats represent a "worst-case" scenario for cell survival due to the increased immune reaction. If fibrin glue is capable of increasing graft size in this "worst-case", we anticipate that we would find a more dramatic effect in an inbred strain.

In conclusion, our results indicate that fibrin glue may serve as an injectable internal support and/or scaffold to prevent infarct wall thinning and deterioration of cardiac function following a MI. Fibrin glue alone may be used as an acellular approach to treat MI with the benefit of being an "off the shelf" treatment. Fibrin glue may also be used as an injectable scaffold to deliver more viable cells directly into infarcted myocardium. Unlike previous attempts at cardiac engineering, this study introduces a novel approach by creating *in situ* engineered myocardial tissue.

Chapter 4

Effects of Fibrin Glue in a Chronic MI model

4.1 Introduction

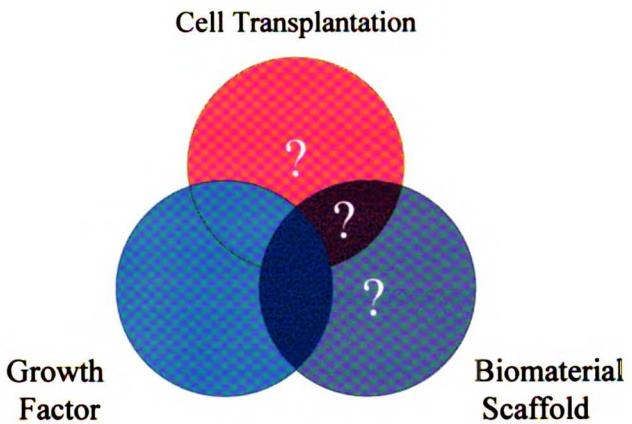

Figure 4.1 Classic tissue engineering triad. Examination of cell therapy and a biomaterial scaffold in a chronic MI model.

Approximately 550,000 new diagnosed cases of congestive heart failure (CHF) occur each year with the incidence approaching 10 per 1,000 people after age 65. Currently, there are approximately 4,790,000 Americans with heart failure. Survivors of myocardial infarctions comprise a large portion of patients with CHF. About 22 percent of male and 46 percent of female myocardial infarction patients will be disabled with heart failure within 6 years. Furthermore, the 5-year mortality rate for CHF is roughly 50% with the sudden cardiac death rate at 6-9 times greater than the general population[170].

Heart transplantation remains the only viable solution for end stage CHF[171]. Due to limited organ availability, alternatives to cardiac transplantation have been evaluated. Surgical left ventricular restoration[172], myocardial scar reduction[173], left ventricular assist devices[174], and biventricular pacing[175] are interventions suggested for the treatment of CHF. Several companies are also developing a variety of ventricular restrictive devices to prevent chronic heart failure; however, the majority of these devices require highly invasive procedures for implantation.

Initial results with our *in situ* engineered myocardial tissue suggested that the delivery of fibrin glue prevents deterioration of LV function following an acute myocardial infarction.[176,177] In this study, we investigate whether intramyocardial injection of fibrin glue can act to augment myocardial tissue volume and restore LV geometry and function in a chronic MI model. Additionally, we examine the effects of delivering skeletal myoblasts inside the fibrin glue scaffold in a chronic MI model to determine whether this treatment could be used once the remodeling process has already been completed. Moreover, we continue to examine the effects of cell transplantation and biomaterial scaffolds on cardiac repair (Figure 4.1).

4.2 Methods

4.2.1 Rat Chronic Myocardial Infarction Model

The ischemia reperfusion technique described in Chapter 2 was used in this study. After occlusion of the LAD, the rats were allowed to recover for five weeks before injection.

4.2.2 Skeletal Myoblast Isolation and Culture

Myoblasts were isolated and culture as previously described in Chapter 3.

4.2.3 Injection Surgeries

Following completion of the remodeling process (five weeks after MI) [123], either 0.5% BSA in 50 μl PBS, 50 μl fibrin glue, 5×10^6 myoblasts in 50 μl 0.5% BSA, or 5×10^6 myoblasts in 50 μl fibrin glue was injected into the infarcted myocardium as described in Chapter 3.

4.2.4 Echocardiography

Echocardiograms were performed as described in Chapter 3 five weeks after infarction (prior to injection) and five weeks after injection (10 weeks post-infarction).

4.2.5 Histology and Immunohistochemistry

Following the second echocardiogram (10 weeks post-MI), the rats were euthanized with a pentobarbital overdose (200 mg/kg). The hearts were rapidly excised and fresh frozen in Tissue Tek O.C.T. freezing medium (Sakura). They were then sectioned into 10 μm slices and stained with hematoxylin and eosin (H&E). All H&E stained slides were examined for any evidence of inflammation. Five slides, equally distributed through the infarct area, were also taken from each heart in the BSA group (n= 5) and fibrin group (n=7) and stained with an anti-smooth muscle actin antibody (Dako; 1:75 dilution) to label arterioles.[124] 5 slides were also taken from each heart in the myoblasts in BSA group (n=6) and myoblasts in fibrin group (n=5) and stained with the MY-32 clone

(Sigma; 1:400 dilution) in order to label transplanted cells. Arteriole density, cell survival, and infarct size were determined as previously described in Chapters 2 and 3. An additional three rats were sacrificed 24 hours after injection, in order to examine the location of fibrin glue in the heart. The hearts were sectioned and stained with H&E as described above.

4.2.6 Statistical Analysis

Data is presented as mean ± standard deviation. Cell density measurements were compared using a student's t-test and arteriole density was compared using one-way ANOVA analysis with Holm's adjustment. Animals served as internal controls by comparing the baseline echocardiography data (5 weeks post-MI) and the 5 weeks post-injection (10 week post-MI) echocardiography data with a paired t-test. As a comparison across groups, 10 week data was analyzed using one-way ANOVA with Holm's adjustment. Significance was accepted at $P< 0.05$.

4.3 Results

4.3.1 Echocardiography

The echocardiographic data is summarized in the table below (Table 4.1). As typical of post-MI progression, the BSA control group exhibited a deterioration of LV function and an expansion of LV size. After ten weeks there was a significant deterioration in FS (P = 0.04), a significant decrease in infarct wall thickness (P = 0.01), and a significant increase in LVID during both systole (P = 0.02) and diastole (P = 0.01) (Table 4.1, control group).

Similarly, expansion of the LV was also seen in animals that were injected with myoblasts in BSA. The LVID during systole (P = 0.02) and diastole (P = 0.009)

significantly increased five weeks after injection. The infarct wall thickness also significantly thinned ($P = 0.04$). Injection of myoblasts in BSA was, however, capable of preserving LV function ($P = 0.20$) (Table 4.1, cells in BSA group).

In contrast to the control BSA injections and injection of myoblasts in BSA, injection of fibrin glue alone preserved infarct wall thickness (P=0.86), systolic LVID (P=0.30), and LV function (P=0.68) (Table 4.1, fibrin group). Furthermore, injection of myoblasts in fibrin glue preserved infarct wall thickness (P=0.56), systolic LVID (P=0.31), diastolic LVID (P=0.05), and LV function (P=0.47) (Table 4.1, cells in fibrin group).

Although both fibrin glue alone and myoblasts injected in fibrin glue preserved LV geometry and cardiac function, at 10 weeks post-MI, animals which were injected with myoblasts in fibrin glue had significantly smaller systolic LVID (P=0.003) and significantly better fractional shortening (P=0.002) compared to injection of fibrin glue alone. At 10 weeks, animals in the cells in fibrin group also had statistically better systolic LVID (P=0.0496) and cardiac function (P=0.02) compared to animals injected with BSA. The infarct wall thickness (P=0.002), systolic LVID (P=0.01), and fractional shortening (P=0.001) of animals in the cells in BSA group were also significantly worse than those in the cells in fibrin group (Table 4.1).

As a control for each rat's level of excitement, the heart rate was also measured. There was no significant difference in heart rate between groups (P=0.92)

Table 4.1 Echocardiography Data

	Before Injection (5 Weeks Post-MI)	After Injection (10 Weeks Post-MI)	*P*
Fractional shortening, %			
Control group	52.8±9.8	38.2±13.2	0.04
Fibrin group	39.9±15.0	36.9±9.7	0.68
Cells group	41.2±18.9	34.2±9.2	0.20
Cells in fibrin group	67.0±8.0	63.4±6.8*†	0.47
Infarct wall thickness, cm			
Control group	0.17±0.04	0.13±0.03	0.01
Fibrin group	0.12±0.06	0.13±0.05	0.86
Cells group	0.14±0.04	0.10±0.03	0.04
Cells in fibrin group	0.17±0.02	0.18±0.02†	0.56
LVID systole, cm			
Control group	0.32±0.10	0.48±0.16	0.02
Fibrin group	0.42±0.14	0.48±0.10	0.30
Cells group	0.47±0.20	0.57±0.18	0.02
Cells in fibrin group	0.20±0.05	0.24±0.04*†	0.31
LVID diastole, cm			
Control group	0.66±0.11	0.75±0.13	0.01
Fibrin group	0.69±0.09	0.76±0.08	0.04
Cells group	0.76±0.13	0.85±0.15	0.009
Cells in fibrin group	0.61±0.06	0.64±0.03	0.05
Heart Rate (beats per min)			

Control group	480±90	459±65	0.29
Fibrin group	479±52	478±39	0.94
Cells group	490±34	473±52	0.27
Cells in fibrin group	499±35	474±26	0.13

* $P<0.05$ vs. 10 week post-MI BSA control
† $P<0.05$ vs. 10 week post-MI fibrin group and cells in BSA group

4.3.2 Histology and Immunohistochemistry

Transplanted myoblasts were labeled with anti-skeletal fast MHC to determine whether injection of cells in fibrin glue increased cell survival in the chronic MI model. After 5 weeks, the myoblast density in the infarct area was significantly greater when the cells were injected in the fibrin scaffold compared to injection in BSA (P=0.008). Cells injected in fibrin glue covered 11.5±4.3% of the infarct area compared to 4.7±2.3% when injected in BSA (Figure 4.2 and 4.3).

Figure 4.2 Myoblast survival within fibrin glue. ×40. Anti-skeletal, fast myosin heavy chain labeled myoblasts visualized with a Cy3 secondary antibody. Myoblasts delivered in fibrin glue are found within the infarct scar 5 weeks post-injection.

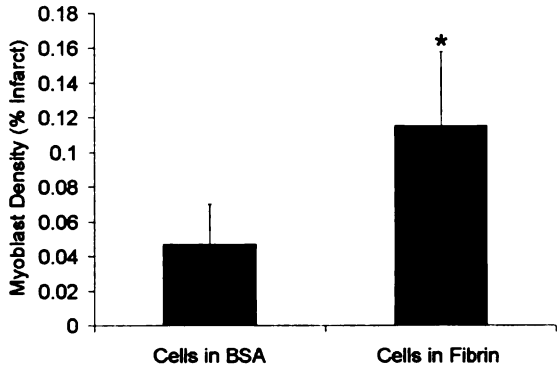

Figure 4.3 Myoblast Density. Injection of skeletal myoblasts in fibrin glue significantly increased myoblast density within the infarct compared to injection of myoblasts in BSA (*, $P<0.05$).

Arterioles were labeled with anti-smooth muscle actin in both the fibrin and BSA groups to determine if fibrin glue induces arteriogenesis when delivered 5 weeks after infarction. Fibrin glue significantly increased arteriole formation compared to injection of BSA ($P=0.04$). Arteriole density increased to 14.2±3.3 arterioles per mm^2 after fibrin injection compared to 10.2±1.6 per mm^2 after BSA injection (Figure 4.4 and 4.5).

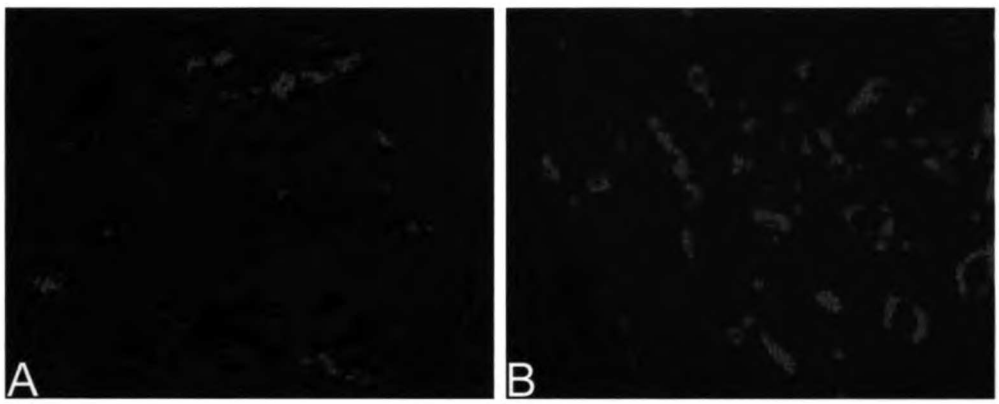

Figure 4.4 Anti-smooth muscle actin stained arterioles. ×40. (A) Arterioles within the infarct scar after injection of BSA. (B) Arterioles within the infarct scar after injection of fibrin glue. Note the increase in arterioles with the fibrin glue injection.

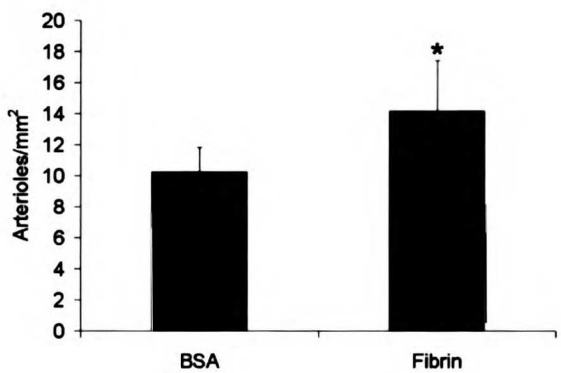

Figure 4.5 Arteriole Density. Injection of fibrin glue significantly increased arteriole formation compared to injection of BSA within the infarct (*, $P<0.05$).

Histological review of H&E stained sections from each group demonstrated that there was no significant inflammatory response. In those animals sacrificed 24 hours after

injection, fibrin glue was observed within the infarct wall. At locations of injection, the wall thickness was markedly increased compared to neighboring tissue without fibrin glue and BSA controls (Figure 4.6).

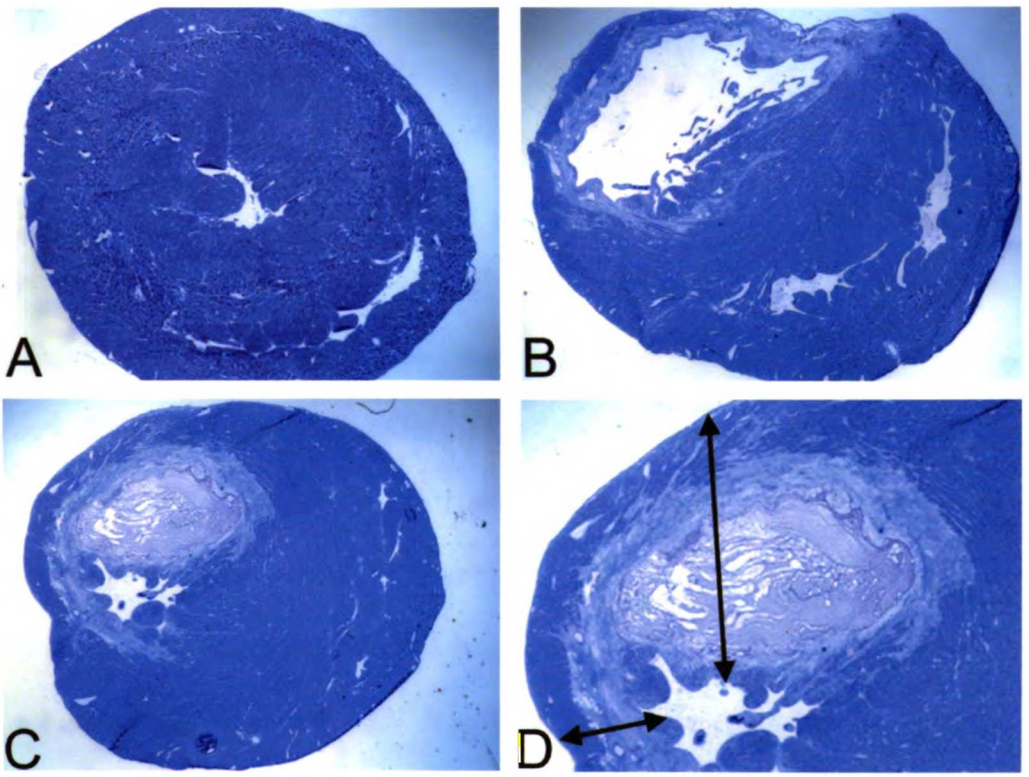

Figure 4.6 Fibrin glue *in situ* after 24 hours. (A) Normal, non-infarcted myocardium. (B) Infarcted myocardium treated with BSA. (C) Infarcted myocardium treated with fibrin glue. Fibrin glue is located within the infarct scar. It can be distinguished by its pink staining and fibril nature. Note the difference in LV geometry of the infarcted myocardium treated with BSA compared to that treated with fibrin glue. The LV geometry of the fibrin glue treated myocardium resembles that of the normal, non-infarcted myocardium. (D) Higher magnification of fibrin glue in (C). Note the increase

in infarct wall thickness at the location of fibrin glue compared to the non-affected infarct wall.

4.4 Discussion

This study introduces a new therapeutic modality for treatment of chronic heart failure. Prior to this study, cardiac tissue engineering approaches had been limited to *in vitro* engineered tissue. Additionally, the ability of biopolymers to restore LV geometry and function, and effect myocardial regeneration in a chronic MI had yet to be explored. We have now demonstrated that *in situ* engineered myocardial tissue improves cardiac function and cell transplant survival in the infarcted myocardium, thus aiding in myocardial regeneration. Moreover, our results demonstrate that injection of fibrin glue alone augments LV wall thickness and preserves LV geometry and function.

Fibrin may also preserve LV function by increasing blood flow to the ischemic tissue. Similar to when delivered in an acute MI[176], fibrin glue also increased neovasculature formation compared to injection of BSA in our chronic MI model. Natively, fibrin is highly involved in wound healing and acts as the body's natural matrix for neovasculature formation. A degradation product of fibrin, fibrin fragment E, has been shown to induce angiogenesis in the chick chorioallantoic membrane assay[154], stimulate proliferation, migration and differentiation of human microvascular endothelial cells[155], and stimulate migration and proliferation of smooth muscle cells.[156]

Fibrin also acts as a sustained release reservoir for several growth factors[152] and may result in an upregulation or release of certain factors that could recruit cells into the

infarct or inhibit the processes of LV expansion. Fibrin glue has been implicated in inducing fibroblast migration[178,179] and may cause recruitment and proliferation of fibroblasts in the infarct, resulting in a thicker infarct wall. Initially, the fibrin matrix itself augments LV wall thickness and geometry; however, as it degrades it may be this ingrowth of cells which continues to maintain a more normal geometry (Figure 4.7)

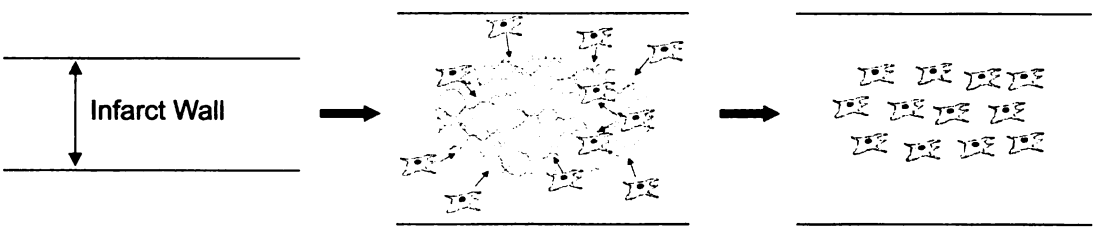

Figure 4.7 Suggested effects of fibrin glue injection. At five weeks following infarction the infarct wall has significantly thinned (left). Injection of fibrin glue augments LV wall thickness, creating an internal support (center). Cells begin to migrate into and proliferate inside the fibrin matrix as the matrix degrades. These cells eventually take the place of the matrix once it has degraded, thus maintaining the thicker infarct wall.

It is also possible that injection of fibrin glue results in recruitment of stem cells from the bone marrow, which may aid in cardiac repair and reconstruction.

In addition to the regenerative and restorative effects exhibited with injection of fibrin glue alone, injection of skeletal myoblasts in fibrin glue significantly improved cell

transplant survival compared to injection in BSA. It has been well documented that the standard technique suffers from limited cell survival[59-62] and it was recently stated that the "basic protocol for cell grafting may need further optimization to prevent cell loss[59-62]." The use of an injectable biomaterial scaffold may be the necessary enhancement to the protocol. Fibrin glue may increase cell survival by acting as a temporary extracellular matrix for the transplanted myoblasts. Instead of being injected in a completely liquid solution such as saline, cell culture medium, or BSA, fibrin glue solidifies inside the myocardium, giving the cells a temporary semi-rigid scaffold. Fibrin glue also contains RGD motifs and binds to cell receptors, predominately integrins[94], thus giving the cells a matrix to which they can attach. Fibrin glue may also increase cell survival by inducing neovasculature formation in ischemic myocardium. An increase in blood supply would provide a less ischemic region for the cells to thrive. There is also the possibility that fibrin is inducing proliferation of the transplanted myoblasts, resulting in an increase in skeletal, fast MHC labeled cells. The combination of skeletal myoblasts and the fibrin glue scaffold also significantly increased cardiac function and significantly decreased LV expansion compared to BSA, fibrin glue alone, and myoblasts in BSA. While skeletal myoblasts were used in this study, it is presently uncertain which cell type will be most effective for cardiac repair. Despite the enthusiasm surrounding stem cells, it was recently reported that transplantation of bone marrow stem cells increased regional systolic heart function, but was not statistically different from transplantation of skeletal myoblasts[180]. We anticipate that the fibrin scaffold could also be used to deliver and improve cell viability of other cell types.

In accordance with other studies, which show that fibrin glue is biocompatible, non-toxic, and that it does not induce inflammation, foreign body reactions, tissue necrosis or extensive fibrosis[95], we also found that there were no significant inflammatory responses in the myocardium. Another benefit of this injectable scaffold is that it is an already FDA approved material, which is routinely used as a surgical adhesive and sealant. Since it remains liquid before combination of its two components, it could also be delivered via a dual chamber catheter, thus requiring only a minimally invasive procedure in humans. In contrast, other biomaterial scaffolds that have been used in *in vitro* engineered myocardial tissue[68,70] would require highly invasive procedures to implant the engineered tissue in humans. LV support and assist devices also require invasive procedures for implantation.

In conclusion, our results suggest that an injectable biopolymer may be used to restore and regenerate infarcted myocardium. Injection of fibrin glue alone preserves LV geometry and cardiac function. Fibrin glue may also be used an injectable scaffold to improve cell transplant survival. *In situ* engineered myocardial tissue may therefore be a potential minimally invasive treatment for patients who suffer from chronic MI.

Chapter 5

Delivery of Pleiotrophin Plasmid in Fibrin Glue

5.1 Introduction

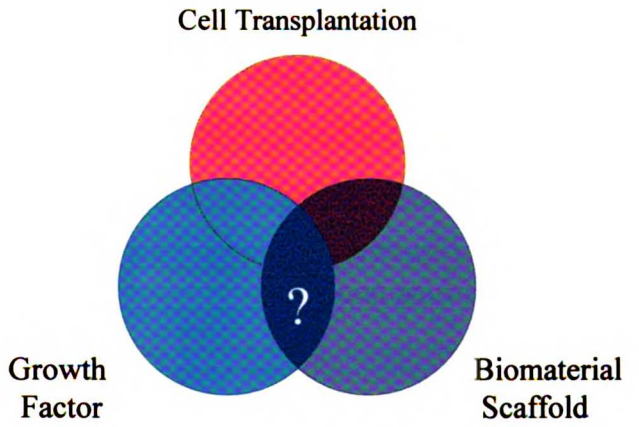

Figure 5.1 Classic tissue engineering triad. Examination of a growth factor and biomaterial scaffold for cardiac repair.

In this study, we examine the combination of an angiogenic growth factor and a biomaterial for cardiac repair (Figure 5.1). Although injection of the naked PTN plasmid in saline produced an angiogenic response, we examined delivering it inside a biomaterial matrix to determine if we could magnify this response. Typically, naked plasmid injections result in very low transfection efficiency, possibly due to limited exposure of cells to the plasmid[181]. In order to alleviate this problem, researchers have examined

delivering plasmid in a polymer matrix. By encapsulating the plasmid in a polymer, the DNA will be released more slowly as the polymer degrades, thus increasing chances of plasmid-cell interaction. This combination of plasmid DNA and a biodegradable structural matrix carrier has been termed a gene-activated matrix[182]. This technology takes advantage of the normal processes of wound healing. The gene-activated matrix immobilizes the plasmid, until endogenous wound healing fibroblasts arrive and migrate through the matrix. Once the fibroblasts have been transfected, they act as *in vivo* bioreactors within the matrix which secrete the therapeutic growth factor encoded by the plasmid. Unlike with the traditional drug delivery paradigm, in this approach, the target cells find the plasmid (or drug). Several studies have demonstrated the effectiveness of this approach[182-185]. *In vitro* studies have also demonstrated that use of fibrin glue as a gene-activated matrix improves transfection efficiency in keratinocytes up to 100-fold in vitro[169]. We hypothesized that inject of PTN plasmid in fibrin glue would increase neovasculature formation in ischemic myocardium compared to injection of PTN plasmid alone by acting as a gene-activated matrix.

5.2 Method

5.2.1 Rat Acute Myocardial Infarction Model

The rat ischemia reperfusion model described in Chapter 2 was used for this study. In this study, the rats were allowed to recover for one week before injection.

5.2.2 Plasmids

The pCMV-PTN-IRES-β-gal-neo and control pCMV-β-gal plasmid (Invitrogen), which are described in Chapter 2, were also used in this study.

5.2.3 Injections

One week after MI, either 250 μg PTN plasmid in 50 μl saline (n=6), 250 μg β-gal plasmid in 50 μl fibrin glue (n=5), or 250 μg PTN plasmid in 50 μl fibrin glue (n=7) was injected into the ischemic LV according to the procedure described in Chapter 2. In plasmid and fibrin glue injections, 250 μg of plasmid were suspended in 25 μl of the thrombin component of the fibrin glue. The thrombin-cell mixture was then simultaneously injected into the myocardium with 25 μl of the fibrinogen component. The animals were then euthanized with a pentobarbital overdose (200 mg/kg) five weeks after injection.

5.2.4 Echocardiography

Echocardiographic analysis was performed on each animal as described in Chapter 3. A baseline echocardiogram was performed one week after infarction (prior to injection) and a follow-up was performed five weeks after injection.

5.2.5 Microbead Perfusion

The hearts from a subset of rats were perfused with microbeads as described in Chapter 2.

5.2.6 Histology and Immunohistochemistry

The hearts were rapidly excised and fresh frozen in Tissue Tek O.C.T. freezing medium (Sakura). They were then sectioned into 10 μm slices and stained with hematoxylin and eosin (H&E) to determine the location of the infarct. Capillary and arteriole densities within the infarct were determined using the procedures described in Chapter 2.

5.2.7 Statistical Analysis

Data is presented as mean ± standard deviation. Differences in vessel densities were compared using a one-way ANOVA with Holm's adjustment. Significance was accepted at $P< 0.05$.

5.3 Results

5.3.1 Neovasculature Formation

Five weeks following injection, the arteriole density within the infarct scar of those animals injected with PTN plasmid in fibrin glue was significantly greater than those animals who were injected with PTN plasmid in saline (P=0.003) (Figure 5.2). Arteriole density increased to 18±4 arterioles per mm^2 when PTN plasmid was delivered in fibrin glue compared to 10±2 arterioles per mm^2 when the same plasmid was delivered in saline. The arteriole density in the PTN plasmid in fibrin glue group was also significantly higher than that of the β-gal plasmid in fibrin glue group (P=0.02). The arteriole density of those animals injected with β-gal plasmid in fibrin glue was 11±3 arterioles per mm^2. There was no significant difference between the animals injected

with PTN plasmid in saline compared to those injected with β-gal plasmid in fibrin glue (P=0.46). There were also no significant differences in capillary density within the infarct among the three groups (P=0.61).

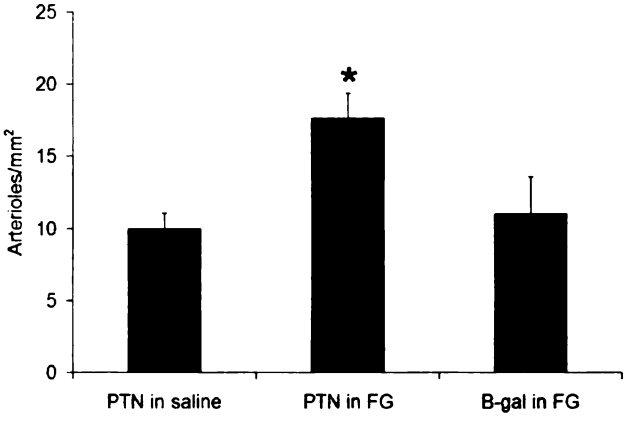

Figure 5.2 Arteriole Density. Arteriole density in the infarct following injection of PTN plasmid in fibrin glue (FG) was significantly increased compared to injection of PTN plasmid in saline and β-gal plasmid in fibrin glue. Note that naked PTN plasmid did not produce significantly more arterioles than injection of β-gal in fibrin glue. * $p<0.05$ compared to PTN plasmid in saline and β-gal in fibrin glue.

Microbeads were visualized in vessels within the infarct area of those hearts injected with PTN plasmid in fibrin glue, indicating functional vessels (Figure 5.3).

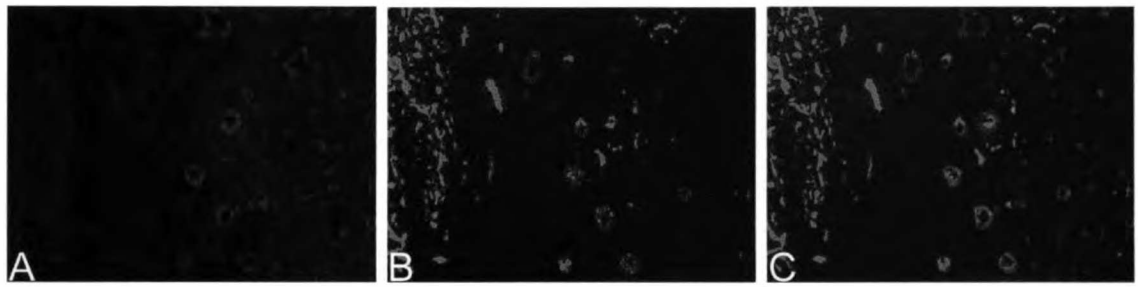

Figure 5.3 Microbead Perfusion. (A) Anti-smooth muscle actin stained arterioles in infarct area after injection of PTN plasmid in fibrin glue. (B) Microbead perfusion through the same section. High density of microbead perfused capillaries in normal myocardium can be observed at the far left of the section. Other perfused vessels are seen in the infarct area. (C) Overlay of A and C demonstrates that vessels formed as a result of injection of PTN plasmid in fibrin glue are functionally connected to existent coronary vasculature.

5.3.2 Echocardiography

The echocardiographic data is summarized in the table below (Table 5.1). PTN plasmid in saline and β-gal plasmid in fibrin glue were capable of preserving cardiac function; however, the fractional shortening of those animals injected with PTN plasmid in fibrin glue significantly deteriorated after five weeks. There were no significant differences in fractional shortening among groups at the end of the study.

Table 5.1 Echocardiography Data*

	Before Injection	5 Weeks Post-Injection	*P*
Fractional shortening, %			
PTN plasmid in saline	43±8	41±12	0.62
PTN plasmid in FG	34±12	23±12	0.005
B-gal plasmid in FG	31±8	25±5	0.18

**P* values are for comparisons between measurements one week following infarction and five weeks following injection.

5.4 Discussion

The results of this study indicate that delivering PTN plasmid in fibrin glue increases neovasculature formation compared to injection of either PTN plasmid or fibrin glue alone. Fibrin glue alone is also capable of inducing a similar response as PTN plasmid alone. This data further indicates that fibrin glue can be used as an effective gene-activated matrix in the heart. Previous work demonstrated that fibrin glue is an effective matrix for increasing transfection efficiency of keratinocytes *in vitro* and in rat skin *in vivo*[169]. Andree and colleagues demonstrated up to a hundred fold increase in transfection *in vitro* using hEGF (human endothelial growth factor) plasmid and fibrin glue. When this gene-activated matrix was implanted *in vivo*, they witnessed a 180 fold increase in EGF expression[169]. Gene-activated matrices are also known to be effective in plasmid gene transfer in other tissues including bone[182,183], skin[184], tendon and ligament[181], and skeletal muscle[185]. This technology allows for the passive targeting of endogenous repair cells, primarily fibroblasts. Bonadio and colleagues have reported that

30-50% of available wound cells are transfected within three weeks after gene-activated matrix implantation[181].

There is also the possibility that there is a synergistic effect with fibrin glue and the exogenous PTN protein produced from transfection cells. Addition of plasmid to the thrombin component may have also diluted the final thrombin concentration in the fibrin glue. A decrease in thrombin concentration would result in less dense fibrin gel with larger fibrils and pores[94], which would be more conducive to vessel infiltration[150,151]. In addition, fibrin glue acts as a sustained release reservoir for many growth factors which may contribute to increased production of vessels[152]. Future studies examining transfection efficiency shortly after injection will elucidate the exact mechanism, although it is likely that it is mediated through gene-activated matrix plasmid gene transfer.

Despite the increase in blood vessels that occurs following injection of PTN plasmid in fibrin glue, cardiac function continued to deteriorate following injection. The dramatic increase in vessels may have resulted in high-output cardiac failure. Having an overproduction of vessels could result in mechanical dysfunction by increasing the amount of blood that must be pumped in order to adequately supply tissues with oxygen. In contrast, both naked PTN plasmid in saline and β-gal plasmid in fibrin glue were capable of sustaining cardiac function five weeks after injection. The increase in neovasculature as a result of injection of PTN plasmid or fibrin glue was statistically similar, indicating only one treatment is necessary.

In conclusion, our results indicate that fibrin glue is an effective gene-activated matrix for increasing transfection of the PTN plasmid. By injecting the plasmid in a

gene-activated matrix we were successful in increasing the production of neovasculature in ischemic myocardium; however, this increase in vessels resulted in a deterioration of cardiac function, most likely a result of high output cardiac failure.

Chapter 6

Delivery of Pleiotrophin Plasmid and Myoblasts in Fibrin Glue

6.1 Introduction

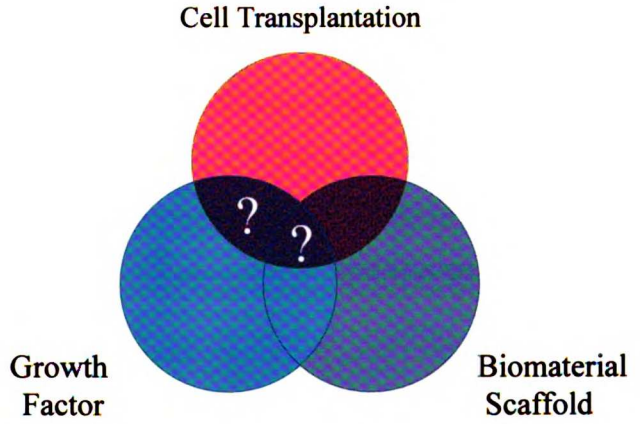

Figure 6.1 Classic tissue engineering triad. Examination of a growth factor, cell transplantation and a biomaterial scaffold for myocardial repair

Finally, we examined the last combinations of the classic tissue engineering components for myocardial repair. In this study we determine the effects of an angiogenic growth plus cell transplantation as well as the combination of all three components on neovasculature formation, cell survival and cardiac function (Figure 6.1).

Previous work has shown that the use of an angiogenic growth factors and cell therapy improves cardiac function compared to the each treatment individually. Miyagawa and colleagues demonstrated that co-injection of HJV-liposomes containing a plasmid encoding the hepatocyte growth factor gene and neonatal cardiomyocytes improved cardiac function following MI in a rat model compared to either treatment alone.[29]

Moreover, we hypothesized that the combination of an angiogenic growth factor and cell therapy would improve cell transplant survival by increasing the blood supply to the transplanted cells and providing a less ischemic region for them to thrive. We also anticipated that injection of PTN plasmid and skeletal myoblasts in a fibrin glue scaffold would further increase cell survival and neovasculature formation in ischemic myocardium and further improve cardiac function.

6.2 Methods

6.2.1 Rat Acute Myocardial Infarction Model

The rat ischemia reperfusion model described in Chapter 2 was used for this study. In this study, the rats were allowed to recover for one week before injection.

6.2.2 Skeletal Myoblast Isolation and Culture

Skeletal myoblasts were isolated from neonatal rats and cultured as previously described in Chapter 3.

6.2.3 Injection Surgeries

One week after myocardial infarction (MI), either 250μg β-gal plasmid and 5×10^6 skeletal myoblasts in 50 μl saline, 250μg PTN plasmid and 5×10^6 skeletal myoblasts in 50 μl saline, 250μg β-gal plasmid and 5×10^6 skeletal myoblasts in 50 μl fibrin glue, or 250μg PTN plasmid and 5×10^6 skeletal myoblasts in 50 μl fibrin glue was injected into the infarcted myocardium according to the injection procedure described in Chapter 3.

6.2.4 Echocardiography

Echocardiographic analysis was performed on each animal as described in Chapter 3. A baseline echocardiogram was performed one week after infarction (prior to injection) and a follow-up was performed five weeks after injection.

6.2.5 Histology and Immunohistochemistry

5 weeks following the injection surgeries, the rats were euthanized with a pentobarbital overdose (200 mg/kg). Capillary and arteriole densities within the infarct area were calculated as described in Chapter 2. Myoblast density within the infarct was determined using the procedure described in Chapter 3.

6.2.6 Statistical Analysis

Data is presented as mean ± standard deviation. Vessel densities were compared using one-way ANOVA analysis with Holm's adjustment. Transplant cell density was compared using a student's t-test. Animals served as internal controls by comparing the baseline echocardiography data (pre-injection) and the 5 weeks post-injection echocardiography data with a paired t-test. As a comparison across groups, the follow up

echocardiogram data was analyzed using one-way ANOVA with Holm's adjustment. Significance was accepted at $P< 0.05$.

6.3 Results

6.3.1 Vessel Densities

There were no differences in capillary densities among the four groups (P=0.94). As for the arteriole densities within the infarct, those animals which were injected with skeletal myoblasts and PTN plasmid in fibrin glue had significantly greater arteriole density compared to those animals injected with skeletal myoblasts and β-gal plasmid in saline (14±4 arterioles per mm^2 compared to 6±1 arterioles per mm^2; P=0.005). The arteriole density of animals injected with skeletal myoblasts and PTN plasmid in fibrin glue was nearly significantly greater compared to injection of skeletal myoblasts and PTN plasmid in saline (14±4 arterioles per mm^2 compared to 8±2 arterioles per mm^2; P=0.05). There was also a similar trend when comparing animals injected with skeletal myoblasts and PTN plasmid in saline to animals injected with skeletal myoblast and β-gal plasmid in saline (8±2 arterioles per mm^2 compared to 6±1 arterioles per mm^2; P=0.056) (Figure 6.2).

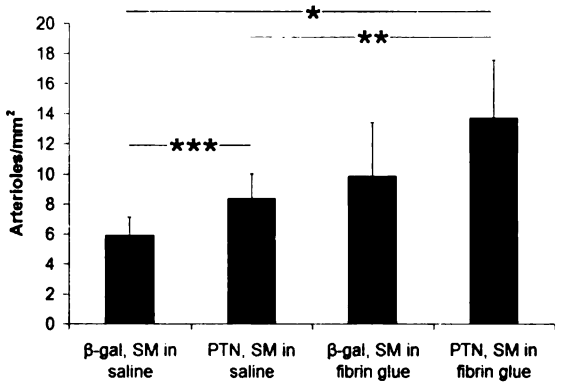

Figure 6.2 Arteriole Densities. Arteriole density within the infarct following injection of PTN plasmid and skeletal myoblasts (SM) in fibrin glue was significantly greater compared to injection β-gal plasmid and SM in saline (*, P=0.005) and nearly significantly greater compared to injection of β-gal plasmid and SM in fibrin glue (**, P=0.05). Injection of PTN plasmid and SM in saline also followed a trend that indicated it produced more arterioles than injection of β-gal plasmid and SM in saline (***, P=0.056).

6.3.2 Cell Survival

In order to determine if exogenous PTN could increase cell transplant survival, the myoblast density within the infarct of those animals injected with skeletal myoblasts and PTN plasmid in saline was compared to those injected with skeletal myoblasts and β-gal plasmid in saline. There were no difference between the two groups (P=0.46)

6.3.3 Echocardiography

The echocardiographic data is summarized in the table below (Table 6.1). All groups were capable of preserving cardiac function; however, there were no differences in fractional shortening among groups at the end of the study (P=0.70).

Table 6.1 Echocardiography Data*

	Before Injection	5 Weeks Post-Injection	*P*
Fractional shortening, %			
B-gal and myoblasts in saline	42±18	37±15	0.05
PTN and myoblasts in saline	39±13	44±18	0.32
B-gal and myoblasts in FG	36±12	35±6	0.91
PTN and myoblasts in FG	41±6	40±13	0.94

**P* values are for comparisons between measurements one week following infarction and five weeks following injection.

6.3 Discussion

Although all groups were capable of preserving cardiac function, the results of this study indicate that co-injection of PTN plasmid and skeletal myoblasts does not preserve cell survival. Similarly, this combination delivered in fibrin glue does not affect cell transplant survival, although it does increase neovasculature in ischemic myocardium.

It was anticipated that an increase in blood supply caused by delivery of an angiogenic growth factor would enhance cell transplant survival; however, PTN did not affect survival of the transplanted myoblasts. The angiogenic process is known to take several days while more mature vessels may take weeks to develop[186]. While PTN may

be an effective angiogenic agent (Chapter 2), its ability to affect cell survival may be correlated with the time at which it is delivered. In this study, the plasmid was delivered simultaneously with the transplanted cells; however, if the plasmid was delivered 1-2 weeks prior to cell implantation, allowing time for neovasculature formation, delivery of PTN plasmid may have been able to enhance myoblast survival.

Both PTN plasmid and β-gal plasmid delivered with myoblasts in fibrin glue did not improve cell survival compared to plasmid and myoblasts delivered in saline, indicating that addition of plasmid affects fibrin glue's ability to improve cell transplant survival. As stated in Chapter 5, addition of plasmid may dilute fibrin glue. While a more dilute fibrin glue will create a less dense fibrin matrix which may aid in vessel ingrowth and formation[150,151], a less dense fibrin matrix may not provide an adequate extracellular matrix for the transplanted cells. A decrease in the thrombin concentration results in a coarser gel, containing larger fibrils and pores, which may not be promote cell attachment and growth.

The results of this study indicate that the combination of all three components (PTN plasmid, skeletal myoblasts, and fibrin glue) is not necessary for myocardial repair. Myoblasts in fibrin glue produced a similar number of new vessels as PTN plasmid and myoblasts in fibrin glue. Furthermore, the addition of PTN plasmid did not further improve cell survival or cardiac function.

Chapter 7

Conclusions

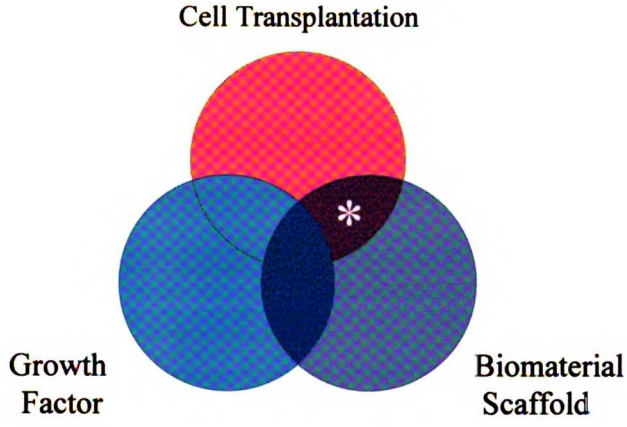

Figure 7.1 Classic tissue engineering triad. Cell therapy and a biomaterial scaffold is the most effective combination for *in situ* myocardial tissue engineering.

The studies described above indicate that only two out of the three tissue engineering components are beneficial in myocardial repair and reconstruction (Figure 7.1). In this model, only skeletal myoblasts and fibrin glue were necessary to create *in situ* engineered myocardial tissue. Although the addition of the angiogenic growth factor Pleiotrophin did not provide any added benefits, it may be necessary with the use of another more inert biomaterial scaffold. Fibrin is a bioactive polymer that is known to have angiogenic properties[149-156]; therefore, in this model, only cell therapy and a biomaterial scaffold are necessary.

Fibrin glue appears to be a near ideal scaffold for *in situ* myocardial tissue engineering. As a sealant and adhesive in surgeries, it is known to be biocompatible and non-toxic, without inducing inflammation, foreign body reactions, tissue necrosis or extensive fibrosis.[95] In the myocardium, such adverse effects were also not apparent. In addition, the fibrin scaffold is biodegradable, thus allowing the transplanted cells to produce their own matrix as the artificial one degrades. Furthermore, the fibrin scaffold is a bioactive material which we have shown to induce neovasculature formation in ischemic myocardium that likely promotes cell transplant survival. Lastly, fibrin glue allows for the *in situ* approach to cardiac tissue engineering by being an injectable biopolymer. Both components of the glue, thrombin and fibrinogen, are low viscosity liquids which can be injected through very small apertures including 27 and 30 gauge needles. Despite the use of non-inbred strain and non-immunosuppresed rats, fibrin glue increased cell transplant density within the infarct. We consider this a "worst-case" scenario for cell survival due to the increased immune reaction and anticipate that we would find a more dramatic effect in an inbred strain. Skeletal myoblasts delivered in a fibrin scaffold may therefore be an alternative to heart transplantation.

Although myoblasts in fibrin glue was the most effective treatment group in the chronic MI model, fibrin glue alone was equally effective at preserving cardiac function as the combination of this scaffold with cell therapy in the acute MI model. Fibrin glue alone was also capable of preserving cardiac function in the chronic MI model. Possibly, one of the most important findings of this work is that injection of this biopolymer was capable of inducing neovasculature formation and preserving cardiac function and left ventricular geometry on its own, without the addition of cells. This is the first indication

that a polymer may be used to restore left ventricular volume and function. Fibrin may act as an internal support which affects the mechanical properties of the myocardium. By injecting this semi-rigid material, the wall may effectively become stiffer and, in turn, modify the progression of negative left ventricular remodeling. These results indicate that injection of solely fibrin glue may also be a potential treatment for those suffering from MI. Treatment with fibrin glue without the addition of cell therapy would have several advantages. This type of therapy would be an "off the shelf" treatment. Moreover, clinicians would be able to administer this treatment immediately since the fibrin glue only takes approximately 30 minutes to prepare. In contrast, the addition of cell therapy presents several complications. The use of allogeneic cell sources results in concerns over disease transmission, graft rejection, and immune suppression, while autologous cell sources require additional procedures for harvesting and additional time for isolation and culture. Furthermore, the appropriate cell type for cardiac repair is currently unknown and continues to remain a major obstacle in myocardial tissue engineering. Initial clinical studies have examined transplantation of skeletal myoblasts and found many patients suffer from arrhythmias[187], most likely due to the electrical mismatch between the skeletal muscle cells and cardiomyocytes. Sole injections of fibrin glue may decrease the likelihood of these electrical abnormalities.

Cardiac function in the above described studies was evaluated at five weeks following treatment injections. In both the acute and chronic MI models, fibrin glue alone and skeletal myoblasts in fibrin glue preserved cardiac function. Future studies will determine whether this effect is sustained for longer durations. Following injection of skeletal myoblasts in the fibrin scaffold, the transplanted myoblasts as well as other

recruited cell types remain once the fibrin scaffold has degraded, allowing for long-term preservation of cardiac function. Following injection of fibrin glue alone, it may be the migration and proliferation of cells in the matrix which sustains cardiac function; however, it is also possible that the effect we are seeing is a result of the structure of the polymer matrix itself and its ability to augment LV wall thickness and geometry. In this latter case, we may not continue to see an improvement in cardiac function at time points significantly greater than five weeks. If it is in fact the structural support which preserves cardiac functions, other injectable polymers, which do not degrade, may be more applicable. Future studies will elucidate the exact mechanisms of fibrin glue induced improvement as well as examine other polymers for *in situ* myocardial tissue engineering.

Until recently, the idea of creating an *ex vivo* heart from *in vitro* engineered myocardial tissue seemed to be the goal for which many were striving; however, our findings on *in situ* engineered myocardial tissue suggest that other approaches may also be beneficial. Although *in vitro* engineered tissue is the more classical tissue engineering approach, using the body as a type of bioreactor to engineer tissue may provide the ideal environment, which we attempt to mimic *in vitro,* as well as other unforeseen advantages. We are the first to examine the application of *in situ* tissue engineering in the myocardium and the results of our studies have opened a new path in the field of myocardial tissue engineering. This novel approach to cardiac repair may redefine treatments for those suffering from MI and heart failure.

While these studies have advanced the field of cardiac tissue engineering, there are still many studies that need to be performed. Future work on *in situ* engineered

myocardial tissue will include examining the exact mechanisms by which this approach improves cardiac function. In addition, modifications of the fibrin scaffold will be examined for their affect on neovasculature formation, cell survival, and cardiac function. To date many cell types and tissue engineering approaches have improved cardiac function in animal models; however, the exact mechanisms of each approach are currently unknown. We therefore agree with Zimmerman and Eschenhagen who stated that is not yet the time for clinical applications of myocardial tissue engineering[82]. There are still many questions and issues to be addressed before this technology can be safely applied to patients. We are however optimistic that future studies will continue to provide more insights and that the field of myocardial tissue engineering will bring new treatments for those patients suffering from myocardial infarctions.

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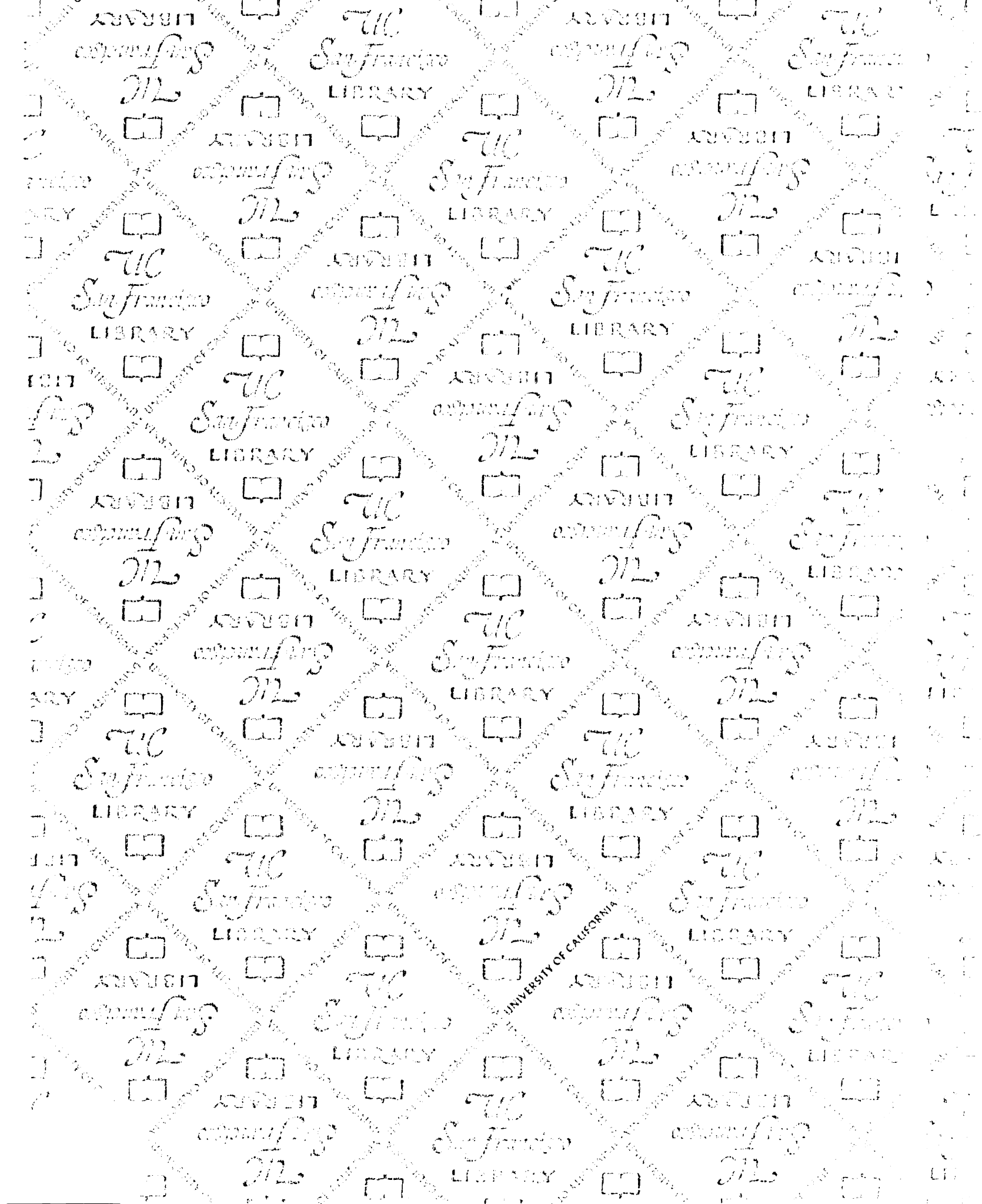
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