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Myoblast-Mediated VEGF Gene Delivery to the Acutely Infarcted Murine Myocardium

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by

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Introduction

Cardiovascular disease is the leading cause of death in the US, with 958,775 million Americans dying of CVD in 1999. Cardiovascular disease includes various disease categories including myocardial infarction and congestive heart failure. In terms of incidence, approximately 1.1 million American have a new or recurrent coronary attack (myocardial infarction of fatal coronary heart disease), and 55% of those who experience an MI in a given year survive. In terms of prevalence, approximately 7.5 million people over age 20 have a history of myocardial infarction. Approximately 25% of men and 38% of women will die within one year after having an initial recognized MI. Thus, a significant number of Americans are living today with a history of MI and have a decreased quality of life due to aftermath of their heart disease. According to the Framingham Heart Study, CHD (which includes MI) accounts for 19% of disability allowances by the Social Security Administration and is the leading cause of premature, permanent disability in the US labor force.[1]

A known complication of MI is congestive heart failure (CHF). Approximately 22% of male and 46% of female myocardial infarction victims are disabled with heart failure within 6 years. Approximately 4.9 million Americans are living with congestive heart failure. Annual mortality from CHF is about 50,824 with 18,987 men (37.4% of total deaths from CHF) and 31,837 women (62.6% of total deaths from CHF) dying from CHF. About 550,000 new cases of

CHF occur each year (based on the Framingham Heart Study), and the incidence approaches 10 per 1,000 population after age 65. After diagnosis with CHF, survival is worse in men; however, less than 15% of women survive more than 8-12 years, given their 1-year mortality rate is much higher (1 in 5 women diagnosed with CHF die each year).[1]

The majority (44.6%) of US heart transplants are due to ischemic cardiomyopathy,[2] and heart transplantation remains the only viable solution for severely injured hearts. The majority of heart transplants outside the US are performed on patients with idiopathic cardiomyopathy (50.5%). In the US, heart transplant recipients are predominantly male (73%), between 50-64 years of age (51%), and white (77%).[2] The number of heart transplant operations in the US has reached a plateau in recent years with about 2,198 heart transplants performed in 2000 (2107 transplants were performed in 1990, and 2359 transplants were performed in 1995).[1] One-year survival for heart transplants in the US is 84%, three-year survival is about 77%, and five-year survival is 69% based on heart transplants performed from 1994 to May 2000.[1]

Due to the disparity between the number of acceptable donor organs and the number of potential transplant recipients, the opportunity for heart transplantation is limited to a small percentage of eligible patients.[3] Indeed, the donor shortage, which limits US heart transplants to less than 2500 procedures per year, demonstrates the discrepancy between the number of patients with end-stage heart failure who might benefit from transplantation

(about 25,000) and those able to receive a donor organ.[4] This raises the proportion of potential recipients who die while waiting for a heart transplant. Extending the current criteria for organ donation has been proposed as a solution to the donor heart shortage. However, extending the donor age carries the risk of accepting hearts with coronary artery disease. Such hearts, if transplanted may be associated with increased morbidity and mortality and poor long-term transplant survival.[3] Given the limited number of donor hearts, alternative strategies to treat severely damaged hearts are necessary and are currently being developed including the artificial heart and the bioengineered heart. The sections below describe current approaches to the repair of damaged myocardium, including cell transplantation and therapeutic angiogenesis.

Repair of Damaged Myocardium: Current approaches

I. Introduction

Heart transplantation is the only current treatment for severely damaged hearts. Given the scarcity of donor hearts, alternate strategies are needed to improve the lives of those with end-stage heart failure. Cardiac tissue engineering is an exciting field that combines elements of cell transplantation and therapeutic angiogenesis in an attempt to repair damaged myocardium. Transplantation of a variety of cell types from allogeneic, transgeneic, and autogeneic sources have been proposed as possible cells for use in myocardial repair. Additionally, a large body of evidence demonstrates that VEGF (Vascular Endothelial Growth Factor) administration follows a dose-response relationship with angiogenic growth factor delivery resulting in increased neovascularization. This review outlines the repair of myocardial tissue and summarizes cell transplantation, therapeutic angiogenesis, and current efforts in cardiac tissue engineering.

II. Cell Transplantation

Cell transplantation is the most researched approach to cardiac repair. Various cell types have been transplanted into heart tissue including cells from allogeneic, transgeneic, and autogeneic sources. Allogeneic cells are those taken from a genetically different individual of the same species. Transgeneic cells are

those taken from a genetically altered individual of the same species. Autogeneic cells are those taken from self. The term cardiomyoplasty is defined as an experimental therapy for heart failure in which skeletal muscle is wrapped around the heart to serve as a ventricular assist device.[5] According to Kessler and Byrne, the basic issues common to all cell types used for cardiac repair are identification and survival of graft cells, cell differentiation, host tissue-transplant cell interaction, and electromechanical coupling.[6] Cellular cardiomyoplasty (CCM) is often used synonymously with cell transplantation and cell injection, however CCM itself is defined as the transplantation of autologous skeletal myoblasts into injured myocardial regions.[7] At the present, it is uncertain which cell type will prove most rewarding for cardiac repair, especially when considering the possibility of genetic engineering to improve cell survival and integration into recipient myocardium. While embryonic stem cells and mesenchymal cells seem promising in the future, fetal cardiac myocytes, though ethically controversial, appear the most rewarding at the present given their ability to both structurally and functionally integrate into myocardium.

Allogeneic Sources

Allogeneic cell transplants are cells transplanted into an individual that is genetically different but of the same species. Thus, the donor is a member of the same species, yet genetically distinct from the recipient.

Human Embryonic Stem Cells

Derived from the inner cell mass of blastocysts,[8] embryonic stem (ES) cells are pluripotent cells with the ability to differentiate into multiple cell lineages in vitro.[9] Given that ES cells can be removed from the blastocyst for preimplantation diagnosis without harming fetal development, ES cells are not equivalent to embryonic cells and do not form a specific part of the embryo.[10] Bongso et al first isolated and cultured ES cells from the ICM of blastocysts (human preimplantation embryos).[10] Moreover, ES cells are distinct from embryonic germ (EG) cells, also a pluripotent cell type but derived from primordial germ cells (PGCs) rather than the ICM. [11] [12] While EG cells have been isolated and cultured from primordial germ cells of aborted human fetuses, it remains uncertain whether EG cells have the same capacity to differentiate as ICM-derived ES cells.[13]

Klug et al demonstrated the ability of genetically selected cardiomyocytes from differentiating murine ES cells to form stable intracardiac grafts in adult dystrophic mice.[14] ES-derived cardiomyocytes express α-cardiac myosin heavy chain (MHC) and β-cardiac MHC,[15] atrial natriuretic factor and myosin light chain 2v (MLC-2v),[16] α-tropomyosin,[17] type B natriuretic factor,[18] phospholamban,[19] show normal contractile sensitivity to calcium,[20] and demonstrate atrial, ventricular, and conduction system cardiomyocyte type action potentials.[21]

Klug and colleagues transfected pluripotent murine ES cells with a fusion gene consisting of the α-cardiac MHC promoter and a cDNA encoding aminoglycoside phosphotransferase. The expression of this fusion gene in ES-derived cardiomyocytes (EA3 ES cells) enabled their selection with G418 after in vitro differentiation. According to light and electron microscopic analysis of cells, the authors conclude that G418 selection did not negatively impact cardiomyocyte differentiation. Moreover, selected cardiomyoctes were directly injected into the left ventricular free wall of dystrophic mdx recipient mice, and were able to form stable intracardiac grafts with transplanted cells aligned with host cardiomyocytes.

Reubinoff et al derived two diploid pluripotent ES cell lines from human blastocysts: HES-1 and HES-2.[22] The cell lines were cultivated for 45 generations (HES-1) and 25 generations (HES-2) while maintaining genetic markers characteristic of pluripotent primate cells. The cell lines were then grafted into SCID mice, with both lines giving rise to teratomas containing derivatives of all three embryonic germ layers. Selected cardiomyocytes from these cell lines may provide an alternative source of donor cells for cell transplantation.

Fetal or Neonatal Cardiomyocytes

The successful transplantation of fetal and neonatal cardiac myocyte suspensions (allogeneic cardiac myocytes) into the myocardium has been demonstrated by various researchers.[14, 23-28] Fetal cardiac myocytes were the

first cell type used in a published study that demonstrated improved hemodynamics after cell transplantation.[25] The inherent electrophysiologic, structural, and contractile properties of fetal cardiac myocytes enabling functional integration into host myocardium, suggests that fetal cardiac myocytes are the ideal cell-type for myocardial repair.[14]

Transgeneic Sources

Transgeneic cells are taken from a genetically altered (transgeneic) individual of the same species and transplanted into another member of the same species. Thus, the donor is a genetically altered member of the same species while the recipient is of the same species but genetically different from the donor.

Researchers isolated atrial tumors from transgenic mouse tumors caused by atrial expression of SV40 large T antigen (ANF-TAG). When implanted into a syngeneic animal, a cell line with characteristics of differentiated atrial cells was established (AT-1 cells).[29, 30] Koh et al tested the long-term survival of AT-1 cardiomyocyte grafts in syngeneic murine myocardium and reported that while the grafts survived for as long as four months postimplantation with the presence of abundant gap junctions within the grafted tissue, the grafts themselves failed to form gap junctions with host cardiomyocytes.[31] A subsequent study by the same research group using AT-1 transgenic fetal myocytes showed the presence of gap junctions between recipient tissue and grafted myocytes.[32] When AT-1 cells were implanted into normal porcine

myocardium, adherens and gap junctions formed;[33] however, the cells failed to survive in a porcine model of myocardial infarction.[23]

Autogeneic Sources

Autogeneic cells are those taken from self and transplanted to self. Given the autologous source of the transplant cells, complications involving the immune system are avoided.

Satellite Cells

While cardiac cells are unable to proliferate after birth, skeletal muscle is able to regenerate due to the existence of satellite cells. Satellite cells enable regeneration of muscle fibers after injury or loss. Using experimental animals, satellite cells have been harvested and passaged in culture as a population of myogenic cells.[34] Autologous satellite cells transplanted into cryoinjured myocardium in dogs formed skeletal muscle grafts in healing tissue.[35] In response to changes in tissue culture milieu, the established myogenic cell lines C2C12 (murine) and L6 (rat) are able to differentiate into myotubes.[34] C2C12 cells transplanted into the myocardium of normal syngeneic mice fused and formed multinucleated myofibers.[36] C2C12 cells transfected with a plasmid encoding TGF-β induced angiogenesis around the implantation site.[37] Given the immunogenicity of xenotransplantation, harvesting a patient's own satellite cells and then genetically modifying these cells and multiplying them in vivo before injection remains a potential method of cell transplantation as therapy for damaged myocardium.

Skeletal Myoblasts

According to Murry et al,[38] two aspects of skeletal muscle theoretically make it superior to cardiac muscle for infarct repair: skeletal muscle is more resistant to ischemia than cardiac muscle (while irreversible injury occurs in myocardium within 20 minutes, skeletal muscle can withstand many hours of ischemia without developing irreversible injury,[39]), and skeletal myoblast grafts may establish satellite cells which can replace tissue damaged by ischemia.

Despite skeletal muscle's theoretical increased resistance to ischemic damage and ability to regenerate via satellite cells, current research suggests two fundamental structural differences exist between skeletal and cardiac myocytes that prevent normal skeletal muscle cell transplants from integrating into host myocardium.[40] The first difference is the absence of gap junctions, called connexins, which provide low resistance electrical communication and the exchange of metabolites between adjacent cardiac myocytes. The second difference is the two isoforms of the dihydropyridine receptor (DHPR)[41], which control excitation-contraction (EC) coupling, or the mechanism in which electrical impulses are transformed into muscle contraction (kinetic energy).[42-44] The DHPR receptor in skeletal muscle is thought to function as both a voltage sensor that directly controls Ca^{2+} release from sarcoplasmic reticulum and as a slow calcium channel. The cardiac DHPR functions as a fast-calcium channel. When depolarized, DHPR allows rapid influx of extracellular calcium which leads to intracellular calcium release from sarcoplasmic reticulum stores.[40]

Autogeneic cells can be transformed. Injecting cDNA encoding cardiac DHPRs (cDHPRs) into dysgenic myotubes enabled cardiac-type EC coupling and a rapidly activating calcium current.[41, 45] This supports the use of genetic transformation of skeletal myoblasts with cDHPRs to establish EC coupling with host tissue after cell transplantation.[40] Various methods can be used to transfer specific genes into the genome of target cells. Retroviruses and adenoviruses are two types of gene transfer systems. The retrovirus vector MFG, a nonhuman pathogen, is the most prevalent research vector in clinical and experimental gene transfer studies.[46]

Fibroblasts

Murry et al used adenoviral-mediated MyoD gene transfer to convert cultured rat cardiac fibroblasts to skeletal myocytes and applied these findings to convert cells in cardiac granulation tissue (fibroblast-rich) to differentiate into skeletal muscle in vivo when using high doses of MyoD adenovirus.[47] Double immunolabeling showed coexpression of myogenin and embryonic MHC in the same cells, demonstrating these cells were new muscle induced by adenoviral-mediated MyoD gene transfer.

The same vector was used to convert murine fetal dermal fibroblasts to primary myocyte-like cells that were able to form normal fibers when injected into skeletal muscle of an immunodeficient mouse.[48] These studies further demonstrate the possibility of using genetic technology to transform the post-ischemic cardiac granulation tissue directly into functional muscle (in vivo) or to

transform fibroblasts from dermal sources into functional muscle that can couple both electrically and mechanically with cardiac muscle. Cell transplantation can then be used to introduce in vitro transformed cells into the healing myocardium.

Adult Cardiomyocytes

While transplanted fetal cardiomyocytes improved heart function after cardiac injury,[49] the cells were eventually eliminated by rejection.[24] Sakai et al tested autologous adult heart cell transplantation by harvesting cells from rat left atrial appendage and increasing their number with cell culture.[50] Cells were then injected into the center of LVFW scar tissue three weeks after cryoinjury, and the authors concluded that transplanted autologous cultured adult atrial cells limited scar thinning and dilation and improved myocardial function when compared with controls. However, transplanted cells were in disarray, unlike the consistent in vivo arrangement found in atria or ventricles. Thus, while transplanted adult cells formed tissue within damaged myocardium, this tissue was not the same as true cardiac muscle.

Researchers further showed that autologous porcine heart cell transplantation improved regional perfusion and global ventricular function after an MI.[51] Porcine cells (a mixture of cell types including cardiomyocytes, smooth muscle cells, vascular endothelial cells, and fibroblasts) were biopsied from the interventricular septum at time of occlusion of the distal LAD coronary artery (infarction) and cultured for four weeks before injection into the infarct region. Pigs receiving cell injection had greater wall motion scores on ^{99m}TC-

MIBI SPECT, improved perfusion scores, greater preload recruitable stroke work and end-systolic elastance, and greater scar thickness than control animals. The authors state that the advantage of transplanting autologous cells is the elimination of the immunosuppressive regimen required for maintenance of allotransplants. Moreover, their study was the first to demonstrate that transplantation of autologous heart cells into an MI results in survival of the transplanted cells. They were, however, unable to definitively determine the cell type of the transplanted cells persisting in the infarct zone after transplantation.

Bone Marrow Cells (Hematopoeitic & Mesenchymal Cells)

Makino et al isolated a cardiomyogenic (CMG) cell line from murine bone marrow stromal cells demonstrating the ability of bone marrow stromal cells to differentiate into cardiomyocytes in vitro after treatment with 5-azacytidine (5-aza).[52] [53] The differentiated CMG cells had various phenotypic characteristics of cardiomyocytes. On electron microscopy, they demonstrated a cardiomyocyte-like ultrastructure with sarcomeres, a centrally located nucleus, numerous glycogen granules, mitochondria, and atrial granules. The CMG cells stained with antimyosin, antidesmin, and antiactin antibodies and expressed both atrial natriuretic peptide and brain natriuretic peptide. The differentiated CMG cells formed myotubes, connected to adjacent cells with intercalated discs, formed myotubes, and beat spontaneously in vitro.

Differentiated CMG cells expressed cardiomyocyte-specific genes such as ANP, BNP, GATA4, and Nkx2.5/Csx. However, differentiated CMG cells

expressed mainly ß-MHC and α-skeletal actin, both of which are fetal forms, and expressed low levels of α-MHC and α-cardiac actin, adult forms in ventricular tissue. Thus, differentiated CMG cells resembled fetal ventricular tissue. Moreover, CMG cells expressed Nkx2.5/Csx, GATA4, TEF-1, and MEF-2C genes before final 5-azacytidine treatment and expressed MEF-2A and MEF-2D genes after final 5-azacytidine treatment, a pattern of gene expression similar to in vivo developing cardiomyoctes at a stage between cardiomyocyte-progenitor and truly differentiated cardiomyocyte.

The electrophysiologic properties of differentiated CMG cells may further reflect a stage of developing cardiomyocytes between progenitor and fully differentiated cardiomyocyte. CMG myotubes have either sinus node–like or ventricular myocyte–like action potentials with a relatively long action potential duration or plateau, a relatively shallow resting membrane potential, and a pacemaker-like late diastolic slow depolarization. While action potentials are seen in skeletal muscle or nerve cells, the shape of the action potential in CMG cells was very similar to in vivo ventricular cardiomyocytes.

CMG cells differ from embryonic stem and embryonal carcinoma cells (embryonal carcinoma cells are pluripotent stem cells of primordial germ cell-derived tumors: teratomas and teratocarcinomas [12]) in several ways. CMG cells are obtained from adult bone marrow, do not require endoderm for differentiation and differentiate only into mesoderm. CMG cells have a high growth rate, are adherent like fibroblasts, and do not require expensive cytokine

supplement (leukemia inhibitory factor), making them easy to culture. Moreover, differentiation is easily induced by 5-azacytidine treatment.

Tomita et al demonstrated that bone marrow cells cultured in 5-aza differentiating into cardiomyocyte-like cells in vitro and in vivo, and improved myocardial function when injected into damaged rat myocardium created by cryoinjury.[54] While all BMC transplants showed similar increases in capillary density, only 5-aza–derived BMC transplants inhibited ventricular scar thinning and expansion and minimized left ventricular chamber dilatation when compared with control hearts, demonstrating that neovascularization alone did not contribute to the improved myocardial function. The researchers suggest that 5-aza–treated BMCs contained more contractile structures that provided the cells with more elasticity than the untreated BMCs.

Jackson et al transplanted side population (SP) cells (a side population of $CD34^-$ hematopoietic stem cells) marked with the lacZ gene into lethally irradiated mice that, 10-12 weeks post-transplantation, received left anterior descending coronary artery ligation/reperfusion to determine whether this highly enriched hematopoietic stem cell population that can give rise to all hematopoietic lineages in the mouse can contribute to the repair of nonhematopoietic tissue, namely damaged myocardium.[55] The study demonstrated that engrafted SP cells or their progeny migrated into ischemic myocardium and blood vessels, and contributed to the formation of functional tissue by differentiating to cardiomyocytes and endothelial cells, primarily at the

edge of the myocardial scar (the borderzone or region described of as being" at-risk").[56]

Using adult human bone marrow, researchers isolated endothelial precursor cells with phenotypic and functional characteristics of embryonic hemangioblasts (G-CSF mobilized adult-human $CD34^{+}/CD117^{Bright}/GATA\text{-}2^{Hi}$). Intravenous cell injection into a rat model of myocardial infarction demonstrated vasculogenesis in the infarct area and angiogenesis (proliferation of preexisting vasculature). The new blood vessel formation resulted in decreased apoptosis of hypertrophied cardiomyocytes in the border zone (peri-infarct region) which enabled long-term salvage and survival of viable myocardium, reduction in collagen deposition, and sustained improvement in cardiac function.[57]

Bone marrow stem cells have tremendous potential to repair damaged myocardium. Most noteworthy, Orlic et al showed that injection of male $Lin^{-}c\text{-}kit^{POS}$ bone marrow cells from transgenic mice expressing enhanced green fluorescent protein (EGFP) into the peri-infarct regions (anterior and posterior aspects of viable myocardium bordering the infarct) of infarcted left ventricles of female mice generated de novo myocardium that included three main cell types of the heart (myocytes, endothelial cells, and smooth muscle cells) and resulted in decreased infarct area and improved cardiac hemodynamics.[58] The transplanted $Lin^{-}c\text{-}kit^{POS}$ cells responded to signals from the necrotic area to migrate from the peri-infarct injection site and to proliferate and differentiate within the damaged ventricular wall. EGFP expression was combined with

labeling of proteins specific to cardiomyocytes, endothelial cells, and smooth muscle cells, enabled identification of cardiomyocytes and endothelial and smooth muscle cells organized in blood vessels. Connexin 43 was apparent in the cytoplasm and at the surface of closely aligned differentiating cells, indicating the expected cellular coupling and functional competence of the restored myocardium. The authors noted that the cardiomyocytes constituted the predominant and most actively growing cell-type of the regenerating myocardium, followed by the endothelial and smooth muscle cells, which were also fast growing but a smaller percentage of the developing tissue. Indeed, these findings demonstrate the application of hematopoeitic $Lin^{-}c\text{-}kit^{POS}$ cell transplantation in repair and reconstruction of healthy myocardium.

Though cell transplantation is an integral part of repair of damaged myocardium and cardiac tissue engineering, Thurmond et al explain that three goals must be achieved with cell transplantation/cardiac tissue engineering: the transplant must have enough surviving cells to have biological/physiological significance, the transplanted cells must differentiate and mature to the adult cardiomyocyte phenotype, and the pattern of tissue transplantation must restore three dimensional architecture with respect to individual cell contacts and alignment of cells in relation to the cardiac tissue planes in which contractile force is generated.[59] Indeed, mesenchymal and other cells located in bone marrow show great promise to achieve these goals.

III. Angiogenesis

Angiogenesis is often used in scientific literature as a general term describing new blood vessel formation. Specifically, angiogenesis is one of three types of blood vessel formation, and is defined as the growth of capillaries,[60] the sprouting of new capillaries from preexisting vessels,[61][62] or the growth and remodeling of the primitive vascular network into a complex network of vessels.[63] Arteriogenesis is defined as the growth of preexisting collaterals[60] or the acquisition of a smooth muscle coat on developing vessels.[63] Vasculogenesis is defined as in situ differentiation of undifferentiated precursor cells (angioblasts) to endothelial cells that assemble into a vascular labyrinth.[61][63] Though new blood vessels in the adult form through angiogenesis, vasculogenesis may also occur. In summary, vascular growth includes vasculogenesis (the formation of a primitive network of vessels via angioblast mobilization), angiogenesis (sprouting of new vessels), or arteriogenesis (collateral growth).[63]

Various molecules are involved with angiogenesis, arteriogenesis, and vasculogenesis. Two extensively studied angiogenic molecules are fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). The following description outlined by Carmeliet summarizes the sequence of events that occur during angiogenesis, the most extensively studied mechanism of blood vessel formation. In response to VEGF, vascular permeability increases and allows extravasation of plasma proteins that create a scaffold for migrating

endothelial cells. Proteinase, matrix metalloproteinase, chymase, or heparanase families degrade matrix molecules and activate/liberate growth factors within the extracellular matrix enabling endothelial cell proliferation and migration. Once in the matrix, endothelial cells form solid cords that later acquire a lumen through the actions of VEGF and other factors. The concerted actions of various molecules in the chemical microenvironment, such as angiopoietins, ephrins, and transforming growth factor β, work to mature, maintain, and regulate the new vessels.[62][63]

Fibroblast Growth Factor

FGF Molecule

FGF molecules act as angiogenic factors and function as regulatory proteins to induce proliferation of epithelial, mesenchymal, and neural cells while also promoting growth and regeneration of organs and tissues.[64] There are two main types of FGF: acidic FGF (aFGF) also known as FGF-1 and basic FGF (bFGF) or FGF-2. Basic FGF was the first pro-angiogenic molecule identified.[65] Both molecules are found in endothelial cells and cardiac myocytes.[66] The FGF family currently has about 20 molecules, which are 30-70% identical in primary amino acid sequence.[62] Given their affinity for heparin, FGFs are also called heparin-binding growth factors (HBGFs).

What is unique to FGF as a growth factor is that the two prototype isoforms, FGF-1 and FGF-2, lack cytoplasmic sequences (consensus N-terminal signal sequence) for extracellular transport,[62] and the precise mechanism for

its release is not known. Most growth factors, such as VEGF, are actively secreted from their producer cells. There are alternate forms of extracellular transport for FGF. It has been suggested that FGF binds to heparan sulfate proteoglycans (HSPGs) on the surface of cells, and these molecules function as a reservoir of growth factors that can be released in a regulated manner by heparanases.[67] Moreover, HSPGs function as co-receptors for FGF and modulate its effects.[68]

FGF Receptors

Currently, there are four known structurally related receptor tyrosine kinases called FGFR-1, -2, -3, and -4, which mediate the biological effects of FGF molecules.[62] Alternative splicing of the FGFR mRNA creates the four receptor variants.[69]

FGF Signal Transduction Pathways

Various signaling molecules and intracellular signaling cascades occur with FGFR signal transduction including the Ras pathway, Src family tyrosine kinases, phosphoinositide 3-kinase (PI3K), and the PLC pathway.[62] Several autophosphorylation sites (receptor tyrosine kinases dimerize resulting in intermolecular autophosphorylation of specific tyrosine residues) have been identified in FGFR-1,[62] and some of these sites have been assigned particular functions such as binding the small adaptor molecule Crk [70] and binding phospholipase C-γ (PLC-γ).[71]

FGF & Repair of Damaged Myocardium

Through angiogenic action, basic fibroblast growth factor (FGF-2) has been shown to improve cardiac function by salvaging ischemic myocardium from necrosis.[64] After intracoronary injection of bFGF into a canine myocardial infarct model, researchers demonstrated improved systolic function and reduced infarct size. While bFGF treatment increased the number of arterioles and capillaries in the infarct region, the study did not compare function of these new vessels to existing vessels.

FGF-2 binds to heparan sulfate proteoglycans, components of extracellular matrix, and is gradually released from the extracellular matrix by heparinase and proteolytic enzymes.[72] Watanabe et al explored the effects of FGF-2 (bFGF) injection alone or in complex with heparin, heparan sulfate, or heparin agarose beads on new blood vessel formation in the infarcted porcine heart at 4-5 weeks post infarction.[66] Though cardiac function and collateral blood flow was not assessed, the researchers concluded that FGF-2 delivery with heparin most effectively enhanced angiogenesis with a 2-fold increase in density of arterioles as compared to FGF-2 delivery alone.[66]

Vascular Endothelial Growth Factor (VEGF)

VEGF Molecule

VEGF is vital for both vasculogenesis and angiogenesis.[73][61] Vasculogenesis is defined as endothelial cell differentiation and angiogenesis is defined as the sprouting of new capillaries from pre-existing vessels during

development. VEGF has an important role in both human health and disease: wound healing, postnatal angiogenesis during pregnancy, cardiovascular disease, rheumatoid arthritis, cancer, and ocular neovascular disorders.[74-76]

VEGF is a cytokine that effects many cell types and tissues,[77-79] but is more known for its affect on endothelial cell function. VEGF was originally called vascular permeability factor (VPF) because of its ability to induce vascular leakage.[62] VEGF regulates various functions in endothelial cells including enhanced production of vasoactive mediators, increased expression of thrombolytic and coagulation pathway components, suppression of neointimal vascular smooth muscle cell hyperplasia, inhibition of thrombosis, hypotension, and vasorelaxation.[74, 80]

There are six current members of the VEGF cytokine family: VEGF-A (or VEGF-1, hereafter termed VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF),[73, 81, 82] all of which are endogenously expressed in mammals except VEGF-E which is encoded by the double stranded DNA orf parapox virus.[62] VEGF-B and PlGF are both expressed as two isoforms produced from alternative splicing.[83, 84] VEGF-C and VEGF-D are produced in precursor forms and undergo processing to various partially processed and mature forms with different receptor-binding specificities.[85]

The role of VEGF-B, -C, -D, and -E, are currently not well understood. VEGF-C may be involved in lymphangiogenesis (as a GF for lymphatic vessels).[86, 87] VEGF-B may be involved in cardiac development. For example,

VEGF-B knockout mice remain healthy but have smaller hearts and exhibit vascular dysfunction after coronary occlusion and impaired recovery following experimental cardiac ischemia.[88, 89]

Alternative exon splicing of human VEGF mRNA (8 exons) forms at least 5 different isoforms called $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ based on the number of amino acid residues.[90, 91] $VEGF_{121}$ and $VEGF_{165}$ have an absent exon 6. $VEGF_{121}$, and $VEGF_{145}$ have an absent exon 7. $VEGF_{121}$, $VEGF_{145}$, and $VEGF_{165}$ are secreted and form dimeric proteins. In contrast, $VEGF_{189}$ and $VEGF_{206}$ are secreted and thought to remain in the pericellular matrix, and $VEGF_{189}$ undergoes proteolytic cleavage by urokinase to generate a smaller biologically active isoform.[92] All isoforms except $VEGF_{121}$ bind heparin to varying degrees via a region rich in basic amino acid residues encoded by exon 6 and or exon 7, making $VEGF_{121}$ the most soluble.[90]

Human $VEGF_{165}$ is the most predominant form. It is glycosylated at Asn75 and is expressed as a 46-kDa homodimer of 23-kDa monomers.[90] Other biologically active isoforms in endothelial cells are $VEGF_{121}$ and $VEGF_{145}$.[93] Moreover, the VEGF family of cytokines have a secretory signal sequence which permits their active secretion from intact cells containing the VEGF gene obtained through transfection.[94]

VEGF Receptors

Currently, there are three known VEGF receptor tyrosine kinases (VEGFR1, VEGFR2, and VEGFR3) that mediate biological effects.[62] VEGF (VEGF-A) binds

two receptor tyrosine kinases, which have approximately 44% amino acid homology: VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1).[81, 82] While the affinity for VEGF binding is about tenfold higher for VEGFR1 than VEGFR2, activation of VEGFR2 generates the VEGF-mediated biological effects in endothelial cells.[62]

VEGF-C and VEGF-D bind to a third receptor called VEGFR3 (Flt-4). VEGFR3 does not bind VEGF-A. VEGF-C and VEGF-D also bind VEGFR2, but with a lower affinity than they bind VEGFR3.[95, 96] PlGF and VEGF-B bind with high-affinity only to VEGFR1.[97, 98] VEGF-E binds to VEGFR2.[99]

The three known VEGF receptor tyrosine kinases are structurally related to the PDGF family of receptors (class III) and have cytoplasmic regions with an insert sequence within the catalytic domain, a single hydrophobic transmembrane domain and seven immunoglobulin-like domains in the extracellular regions.[93] VEGF has 16-114 pM affinity for VEGFR1 and 0.4-1 nM affinity for VEGFR2.[81, 82]

Neuropilin-1 (NP-1), a non-tyrosine kinase transmembrane protein, was identified as a receptor for $VEGF_{165}$ with a Kd 0.3 nM and increases the effectiveness of VEGF-induced endothelial cell migration.[100] It also recognizes VEGF-B and the PlGF-2 splice form but not $VEGF_{121}$.[100, 101] With a short cytoplasmic tail with no known signaling function, it is thought that NP-1 is not a functional receptor but rather acts as a "docking" co-receptor.[93] NP-1 may function as a co-receptor for VEGFR2.[93]

VEGF Signal Transduction Pathways

Receptor signal transduction for VEGF remains poorly understood.[62] The protein kinase C and Akt pathways have an important role in mediating the various functions of VEGF such as cell survival, proliferation, angiogenesis, and the generation of nitric oxide and prostacyclin. Moreover, nitric oxide and prostacyclin are implicated in linking the signaling networks downstream from receptor-ligand interaction of VEGF to actual biological effects and endothelial functions.[93]

VEGF & Repair of Damaged Myocardium

Various injection sites have been attempted for VEGF delivery to the heart. Injection sites have included intravenous, intracoronary, transepicardial into the myocardium (occurring during bypass surgery or via thoracotomy), transendocardial into the myocardium (using electromechanical catheter), and intrapericardial, among others. The intracoronary (adenovirus) and intramyocardial (naked DNA or adenovirus) routes have been most favored in clinical trials given that local delivery of recombinant protein or gene is considered ideal.[94]

In animal models, angiogenic proteins or encoding genes have been administered through a variety of sources, and the effectiveness of angiogenesis has been well documented.[94] Various outcomes of the effectiveness of angiogenesis have been measured. Histological assessment has been made of capillary/vessel size and number. Perfusion measures have been made including

resting and vasodilating coronary blood flow. Blood vessels have been measured with angiography. Left ventricular function has been assessed at rest and during stress post-angiogenic therapy.

Nonviral, plasmid DNA encoding VEGF (phVEGF) was first shown to produce angiogenesis in a rabbit hindlimb ischemia model [102] and subsequently applied to the heart. Nonviral, plasmid DNA encoding $VEGF_{165}$ ($phVEGF_{165}$) was injected into porcine myocardium showing that direct intramyocardial gene transfer of $VEGF_{165}$ is safe and capable of producing sufficient levels of VEGF protein to improve myocardial perfusion and enhance collateral development.[103] This was despite the lower transfection efficiency of plasmids when compared to use of adenoviral vectors. Improved myocardial blood flow in ischemic areas was shown as well as improved collateral filling of the occluded circumflex artery used to create the model of focal myocardial ischemia.

VEGF gene application in skeletal muscle tissue has supported a dose-dependent effect of VEGF. Using intramuscular injection of primary murine myoblasts expressing both murine VEGF gene and B-gal gene from a retroviral promoter, Springer et al reported that while muscle exposed to low levels of VEGF did not develop vascular malformations, high serum levels of VEGF caused vascular tumor formation in non-ischemic adult skeletal muscle.[104] At low VEGF concentrations, angiogenesis occurs, while at high concentrations, vasculogenesis dominates. Springer et al explain a multiple threshold model in

which a specific level of VEGF is required for angiogenesis in ischemic muscle with a higher level stimulating vasculogenesis in non-ischemic muscle. Takeshita et al showed that low levels of serum VEGF (~35pg/ml) after transfection of phVEGF isoforms into the iliac artery of a rabbit hindlimb ischemia model induced angiogenesis, but occurred in ischemic rather than normal skeletal muscle.[105] In the study by Springer et al, the low levels of serum VEGF (~40 pg/ml) in the systemic circulation had no angiogenic effect in adjacent or contralateral muscles which were non-ischemic.[104]

Other studies have demonstrated hemangioma formation with intramyocardial gene therapy for VEGF, further supporting the dose-response effect of VEGF expression. Intramyocardial injection of $5x10^5$ primary murine myoblasts expressing both the murine VEGF gene and the β-gal gene from a retroviral promoter caused intramural vascular tumors resembling hemangiomas and failure to thrive in a nonischemic murine model, demonstrating the importance of regulating VEGF expression for therapeutic angiogenesis.[106] In a rat model of myocardial infarction, direct intramyocardial injection of $phVEGF_{165}$ (500 μg DNA) resulted in angiogenesis with macroscopic angioma-like structures.[107] The angioma formation failed to contribute to regional myocardial blood flow. The dose-dependent effect of VEGF was also reported in clinical trials using recombinant human VEGF (rhVEGF), in which patients receiving higher doses of rhVEGF showed improved resting myocardial perfusion at 60 days.[108]

While high levels of VEGF expression produce vascular tumors, a minimal threshold concentration of VEGF expression is required to produce therapeutic angiogenesis. Kloner et al investigated whether a lower dose of $phVEGF_{165}$ would enhance collateral flow and vascularity to ischemic tissue without inducing angioma formation. Rats receiving intramyocardial injections of 125 ug $phVEGF_{165}$ DNA at four separate sites within the anterior LV free wall showed no angiomatous structures.[109] The rat myocardium was first injected with $phVEGF_{165}$, followed by proximal left coronary artery occlusion (most studies, including their previous study, first occlude and then inject). This therapy failed to reduce infarct size and improve neovascularization and regional myocardial blood flow in this model of acute myocardial infarction. Given the limitations in this study, additional research must be done to further explore the dose-response relationship of VEGF gene expression and therapeutic angiogenesis for ischemic myocardium. This is particularly important given current efforts in myocardial tissue engineering to combine angiogenesis and cell transplantation for repairing damaged myocardium.

IV. Bioengineered Cardiac Tissue: The Combined Approach

Acute myocardial infarction can lead to numerous complications due to decreased contractility, electrical instability, and tissue necrosis. Decreased contractility can lead to cardiogenic shock, thromboembolism, and congestive

heart failure. Electrical instability can lead to arrhythmias, while tissue necrosis can lead to pericarditis, cardiac tamponade, and congestive heart failure.[110]

Congestive heart failure results in increased morbidity and mortality with tremendous direct and indirect costs such as medical expenditures, loss of quality of life, and loss of productivity. Currently the only successful treatment for severe CHF patients (those with New York Heart Association functional class III or IV symptoms) is cardiac transplantation. Cardiac transplantation has achieved noteworthy short and medium term results; however, its longevity has been limited by development of transplant coronary artery disease, recurrent episode of rejections, and complications of immunosuppression.[111] In addition to cardiac transplantation, dynamic cardiomyoplasty and the implantable total artificial heart remain the only alternate options for patients with severe heart failure. However, these alternate strategies remain controversial, necessitating the development of a novel approach for treating patients with severe heart failure.

One approach to improve the failing heart is surgical placement of a non-stretchable nylon patch over infarcted ventricular wall. Cell transplantation has demonstrated improved cardiac function despite the small number of remaining viable transplanted cells. Indeed, the mechanism by which cell transplantation improves cardiac function remains uncertain, and it has been proposed that cell implantation increases myocardial wall stiffness resulting in improved border zone function and improved global ventricular function. Lee et al (unpublished

data) applied a non-stretchable nylon patch in infarcted rats and examined change in contractile properties using echocardiography. Preliminary results suggested the patch limited dyskinesis of left ventricle aneurysm. While further research is underway to validate the beneficial effects of an LV patch, current LV aneurysm patch data suggests that increasing LV stiffness and limiting formation of LV aneurysm may possibly improve post-MI cardiac function and tissue remodeling.

Various researchers are now working to combine angiogenesis and cell transplantation to repair damaged myocardium. The preceding text has described two basic components of bioengineered myocardial tissue: living cells and angiogenic factors. The cells utilized for myocardial tissue engineering require the ability to electromechanically couple with functional recipient heart tissue via N-cadherin and connexin43, the adhesion and gap junction proteins of the intercalated disk, respectively. Electromechanical coupling is essential for the new tissue to function in syncytium with the native myocardium. Moreover, blood vessel formation is required to ensure a functional blood supply to the transplant tissue.

Among tissue engineering strategies to repair damaged myocardium, bioengineered cardiac grafts is an exciting area of investigation. Leor et al grew fetal cardiomyocytes within 3D porous alginate scaffolds that were implanted into infarcted rat myocardium.[112] The fetal cardiomyocytes formed multicellular beating aggregates within 24 hours of seeding of alginate scaffolds. After

implantation into infarcted myocardium, some seeded cells differentiated into mature myocardial fibers with near complete disappearance of the scaffold. The implanted biograft received intensive neovascularization from neighboring coronary vessels, contributing to prolonged graft cell survival. Moreover, the biograft improved remodeling indices and LV function in the infarcted hearts. The mechanism of this beneficial effect was unclear, but unlikely due to the direct contribution of the biograft to contractility. Only a small portion of the biograft was composed of myocardial tissue, and full integration of these graft fibers with host myocardium was not observed.

Tissue engineering of a biograft has various advantages over standard cell implantation/transplantation in that it allows control over the tissue formation process, the shape and size of the graft, and the ability to determine the consistency of the graft including parameters such as number of cells, and cell-to-cell ratio of functional cardiomyocytes and supportive cell types.[112] Polymers and molecular scaffolds can be used to organize cells spatially in 3D for transplantation. A most promising area of active investigation is the use of biodegradable hydrogels for myocardial tissue engineering. These hydrogels support tissue formation in vitro and have the potential to be used as injectable scaffolds for repair of damaged myocardium. At room temperature, the hydrogels are transparent and extremely pliable while at 37 degrees Celsius, the matrices become opaque and rigid. Moreover, biodegradable hydrogels can be injected through a small-diameter aperture (such as a 30-gauge needle) and

offer the benefit of in situ stabilization without the possible harmful effects of in situ polymerization.[113]

V. Conclusion

Though cardiovascular disease is the leading cause of death in the USA, efforts are underway to reduce morbidity and mortality caused by congestive heart failure and complications of myocardial infarction. The combined approach of cell injection and delivery of angiogenic molecules has tremendous promise to improve the quality of life of those suffering from heart failure. The possibility remains that tissue engineering to restore damaged myocardium will supplant the need for cardiac transplantation for treatment of heart failure.

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Myoblast-mediated VEGF Gene Delivery to the Acutely Infarcted Murine Myocardium

I. Overview

Background:

Myoblast-mediated gene delivery of Vascular Endothelial Growth Factor (VEGF) to the ischemic myocardium is a potential treatment modality for myocardial infarction. VEGF is a ubiquitous growth factor promoting angiogenesis in many tissue types, including skeletal and cardiac muscle. Moreover, skeletal myoblast transplantation has the potential to regenerate damaged myocardium caused by chronic myocardial ischemia. This study combines angiogenic growth factors and cells by implanting retroviral-modified myoblasts producing VEGF into the acutely infarcted murine myocardium. It is hypothesized that delivery of VEGF-producing myoblasts will improve myocardial function post-acute MI and promote reduced infarct size by both VEGF-mediated angiogenesis and myoblast mediated restoration of damaged tissue.

Methods & Results:

One-hundred thousand modified primary murine myoblasts containing the murine $VEGF_{164}$ gene (homologue to human $VEGF_{165}$), control myoblasts, or

saline alone were injected into the left and right border zones (fifty-thousand cells at each site) of the infarcted murine myocardium, two minutes post-ligation of the LAD. At one week and four weeks post-MI, echocardiographic assessment of cardiac function was made. Following echocardiography at week four, the animals were euthanized and histologic analysis was made to assess infarct size and angiogenesis within the infarcted LV free wall region. Increased angiogenesis occurred in the VEGF-producing myoblast group (9.11±0.491 capillaries per high-power field; P=0.003 versus saline treated mice 5.5±0.528 and P=0.006 versus myoblast controls 5.96±0.734). Incidentally, cavernous hemangioma formation occurred in mice receiving injections of VEGF-producing myoblasts. Four-week and seven-day Ejection Fraction % and Fractional Shortening % was not statistically significant between groups, nor was Mean Ventricle Infarct%. However, the VEGF myoblast injected mice revealed greater mean LV Mass at 4-weeks when compared to mice injected with control myoblasts or with saline alone (0.0935±0.0118 grams; P=0.044 versus myoblast injected mice 0.0628±0.0049; versus P=0.036 saline controls 0.0612±0.00679).

Conclusions:

The combined approach of implanting cells producing angiogenic agents is a valid concept that can be applied for treatment of myocardial infarctions. Moreover, regulation of VEGF expression is necessary to avoid cavernous hemangioma formation.

II. Introduction

Vascular Endothelial Growth Factor (VEGF) is an established growth factor integral to both vasculogenesis and angiogenesis.[1, 2] VEGF affects many different cell types[3-5] but is known more for its direct action on endothelial cells promoting endothelial cell production of nitric oxide synthase, endothelial cell survival via activation of anti-apoptotic cell signaling, and endothelial cell migration via induced expression of matrix-degrading metalloproteinases.[6] VEGF is a promising therapeutic option for severe ischemic heart disease and myocardial infarction, and research is underway to determine the optimal delivery strategy to the damaged myocardium. Human clinical trials have pursued intravenous and intracoronary injection of recombinant VEGF as well as intracoronary and intramyocardial injection of the VEGF gene using naked plasmid or an adenoviral vector.[7] Additionally, myoblast transplantation is a potential treatment modality for the severely damaged myocardium. Myoblasts have been shown to survive within the normal[8] and cryoinjured myocardium,[9] though myoblast transfer to the acutely infarcted myocardium is a relatively new area of investigation.

In this study, we combine VEGF gene delivery with myoblast transplantation by implanting modified myoblasts producing VEGF into the acutely infarcted murine myocardium. The effect of implantation of various cell

concentrations of VEGF-producing myoblasts into the noninfarcted murine LV is first examined to identify low-risk cell amounts that will produce angiogenesis without hemangioma formation (dose-response). This study then applies the optimal VEGF-producing myoblast concentration to the acutely infarcted murine myocardium to demonstrate whether continuous localized expression of VEGF is an effective strategy for treatment of acute MI.

III. Methods

The study protocol was approved by the Committee for Animal Research of the University of California at San Francisco and University of California at Berkeley, and was performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Genetic engineering of primary mouse myoblasts

The murine $VEGF_{164}$ producing myoblasts were the generous gift of Drs. Matthew L. Springer and Helen Blau (Department of Pharmacology, Stanford University School of Medicine). The murine $VEGF_{164}$ used is the homologue to human $VEGF_{165}$, the predominant form in humans, which binds to heparin and remains more closely bound to the cell surface or to heparin sulfate in the ECM.[6] The genetic engineering of the primary mouse myoblasts was previously described by Springer et al.[10] Briefly, primary myoblasts expressing

the marker enzyme B-galactosidase (B-gal) from a retroviral promoter[11] were transduced with four successive exposures to MFG-VEGF virus, resulting in populations of cells that were 85-90% VEGF-positive (determined by immunofluorescence).[10] An average 214 ng VEGF protein/10^6 cells/day was secreted into the culture medium as determined by ELISA.[10] The control murine myoblasts used were primary myoblasts expressing LacZ from a retroviral promoter as described by Rando and Blau.[11]

Culture Conditions

Tissue culture plastic dishes (60 ml and 100 ml) were coated with 0.01% type I collagen (Sigma Chemical Co., St. Louis, MO). Growth medium for primary mouse myoblasts consisted of Ham's F-10 nutrient mixture (GIBCO BRL, Gaithersburg, MD) supplemented with 20% FBS (HyClone Laboratories, Inc., Logan, UT), 2.5 ng/ml bFGF (Promega Corp., Madison, WI), penicillin G (200 U/ml) and streptomycin (200 µg/ml). Cells were growth in 5% CO_2 in a humidified incubator at 37ºC.

Cell Preparation for Injection

Tissue culture plastic dishes with primary mouse myoblasts were aspirated, rinsed with PBS, and reaspirated to remove debris. Trypsin was used to release cells from collagen-coated plates. Myoblasts were suspended in 10 ml growth medium. A cytometer was used to count total number of myoblasts.

Myoblasts in growth medium were centrifuged to create a cell pellet. The pellet was resuspended in PBS with 0.5% BSA to create specific concentrations of cells for 10 µl injections. Cell concentrations used in dose-response part of experiment were as follows: $1x10^4$, $5x10^4$, $1x10^5$, $5x10^5$, $1x10^6$, $5x10^6$, $1x10^7$. Cell concentration used per injection during intervention experiment was $5x10^4$ cells (total cell amount was $1x10^5$).

Surgeries and Myoblast Transplantation

Eight-week-old female SCID CB-17 mice (Taconic, Germantown, NY) were anesthetized with an intraperitoneal injection of ketamine (90-120 mg/kg) and xylazine (5-10 mg/kg). The mouse was placed on a heating pad at the surgical workstation, and was intubated with size 50 polyethylene tubing (PE-50) and placed on a ventilator (Harvard Rodent Ventilator, Model 683, South Natick, Massachusetts) with a 0.5 ml tidal volume and 120-130 respirations per minute. At all times during surgery and recovery from anesthesia, the animal's core body temperature was measured with a rectal thermometer, and heating lamps were used to maintain the core temperature between 36.5º and 37ºC.

An aseptic technique was maintained. The mouse was placed in the supine position under a dissecting microscope, and a median sternotomy was made. The skin along the sternum was incised and muscular attachments to the sternum were dissected off using electrocautery. The sternum was split sharply opening the chest cavity. A retractor was used to expose the heart. The heart

was manipulated using cotton-tipped swabs so that the junction between the left and right ventricles was visible. Keeping the landmarks of the base of the left atrium and interventricular groove in view, a single stitch of removable 7-0 Ticron (Davis and Geck, Wayne, NJ) suture was inserted into the myocardium at a depth slightly greater than the perceived level of the left coronary artery, without entering the ventricular chamber. The suture ends were doubled over, and a small flexible plastic tube was inserted over the doubled-over suture, to provide a hold from which to temporarily occlude the LCA at the time of injection. Occlusion occurred by pulling plastic tubing along doubled-over suture while holding the free ends of the suture. Through numerous surgeries with injections, our lab group has ascertained that an optimal cell injection occurs when the LCA is occluded during injection.

For control animals that received cell transplantation alone without infarction of the myocardium, occlusion of the LCA was made at the time of cell injection. Cell transplantation into the myocardium was conducted using a 30-guage needle to inject myoblasts into the free wall of the left ventricle. The needle was inserted into the left ventricular free wall with caution not to break into the left ventricular chamber. During injection, blanching of the left ventricular wall around the injection site was seen. Experience with myocardial injections has enabled our lab group to assert that blanching of the superficial myocardial wall during cell injection indicates a successful cell injection, such that the injection occurred within the myocardium and the injection contents were not

implanted into the ventricular chamber or onto the superficial surface of the heart. The needle was held in the myocardium for about 30 seconds after completion of cell injection, to ensure maximal transplantation of myoblasts. The needle was then slowly removed from the myocardium. Given this technique of cell transplantation, it is inevitable that a small fraction of injected cells seep out through the hole made by the needle.

During surgery, assessment of anesthetic depth using reflex tests was made at regular intervals of 5-10 minutes. If the mouse responded to stimulus, it received a first supplemental dose of ketamine alone that was 50% of the original dose of ketamine. If additional doses were needed, 1/3 to 50% of the original combination was used. After completion of cell transplantation, the sternum was closed in three layers (ribs, muscles, and skin).

The mice were kept on the ventilator for at least 35 minutes. Once it was clear that the mice were breathing reliably and they were emerging from anesthesia as determined by withdrawal of limbs from noxious stimuli, they were extubated, and kept on the heated pad until they regained their ability to turn upright. The mice were then placed in a heated cage until they awakened more fully, after which they were placed in their own cages. The mice were given the first dose of buprenorphine (0.05-0.1 mg/kg SQ) for analgesia after wound closure, and three times post procedurally.

Acute Myocardial Infarction Model

The acute myocardial infarction model was created by using the single stitch of the 7-0 Ticron suture described above to permanently ligate the left anterior descending (LAD) branch of the left coronary artery. The suture ends were tied together tightly around the LAD to cause ligation and permanent occlusion. After 5 minutes of ligation, cell injections using a 30-gauge needle as described above was used to transplant myoblasts or to inject saline. Surgical wounds were promptly closed as described above. The Ticron suture remained permanently around the LAD.

Histology

The mice were anesthetized with pentobarbital (50-90 mg/kg IP), followed by rapid sternotomy and physical removal of the heart for histology. The hearts were rapidly rinsed in cold saline for 1 minute and excess tissue around the base was trimmed under a dissecting microscope. The hearts were then flash frozen in OCT compound at low temperature. 10 μm sections were cut on a Reichert-Jung cryostat and frozen sections were stained with hematoxylin-eosin (HE) or X-gal solution (to stain for β-galactosidase).

For HE and X-gal stained sections with unique pathology and for capillary counts, adjacent sections were stained with rat anti-mouse CD31/PECAM (PECAM is platelet endothelial cell adhesion molecule; Pharmingen; working dilution 1:25) and Cy™ 3-conjugated affinipure mouse anti-rat IgG secondary (Jackson

ImmunoResearch; working dilution 1:200). Regions with unique pathology were also stained with primary antibody monoclonal mouse anti-alpha smooth muscle actin (ICN Biomedicals; working dilution 1:400) and Alexa Fluor™ 488 goat anti-mouse IgG fluorescent secondary (Molecular Probes; working dilution 1:200) according to standard immunohistochemical protocols. The X-gal solution enabled detection of transplanted myoblasts carrying the beta-galactosidase reporter gene. Angiogenesis and tumorgenesis was visible using the special stains for PECAM with and without smooth muscle actin stain under the fluorescent microscope.

Capillary Counts per High-Power Field

Five separate 10 μm sections containing scar tissue were randomly selected and stained with CD31 primary (marking endothelial cells) and Cy™3 florescent secondary to visualize capillaries. Spot RT™ camera and software (Diagnostic Instruments Incorporated, Sterling Heights, Michigan) was used to image three randomly chosen high-power fields (40x visualized using a Nikon™ NT-88NE microscope) per stained section within the left ventricular free wall scar region. The number of capillaries per high-power field was counted.

Mean Ventricle Infarct Percent

Ten HE stained sections were randomly chosen per mouse. Using a Nikon™ NT-88NE microscope, ten randomly selected sections containing

infarcted myocardial tissue were imaged at 1x using Spot RT™ camera and software. Spot RT™ software was used to calculate total left ventricle wall area and infarcted wall area. Mean Ventricle Infarct Percent was calculated by dividing infarcted wall area by total left ventricle wall area and taking the mean of ten HE sections.

Echocardiography

Echocardiographic examination was performed using a commercially available 15-Mhz transthoracic linear-array transducer system (Acuson Sequoia c256 System, Mountain View, CA). Briefly, the mouse chest was shaved and the mouse was placed in an open ended cone-shaped plastic animal holder. A layer of acoustic coupling gel was applied through the plastic cone to the thorax, and echocardiographic studies (2D and 2D guided M-mode) were performed. To avoid induced bradycardia, care was taken to avoid excessive pressure on the thorax.

Parasternal long- and short-axis views were obtained by 2D echocardiography as described by HJ Youn et al.[12] Before doing so, angulation and rotation of the transducer and gain settings were optimized to ensure imaging of both endocardial and epicardial surfaces. Compression was 60 dB. Animals were imaged in a shallow left lateral decubitus position. A frame-rate (sweep-speed) of 200 mm/s was used to record views. Additionally,

two-dimensionally guided M-mode of the left ventricle at the papillary level was obtained from the short-axis 2D view through the anterior and posterior LV walls.

Measurements from Echocardiographic Images

Specialized analysis software present in the Acuson Sequoia c256 machine was used to make primary measurements from the digital images captured on cineloops at week one and week four of the study. Images were digitally stored on magnetooptical disk (SONY EDM-230C, Sony; Tokyo, Japan). Images were considered technically adequate according to criteria described by HJ Youn et al.[12] To summarize, two criteria were met for each image used: 1) the long-axis view demonstrated the plane of the mitral valve with visualization of the mitral valve annulus and the apex of the heart; and 2) the short-axis view demonstrated greater than or equal to 80% of the epicardial border and greater than or equal to 80% of the endocardial border. For each mouse, measurements were made from 5 beats.

Two methods[13, 14] were used to calculate estimated echocardiographic LV mass (in milligrams) according to a previously determined formula for Area-Length method: LV Mass = 1.05 (5/6 x A x L), in which A = epicardial – endocardial short-axis cross-sectional area in end-diastole (planimetered short-axis area obtained at the papillary muscle level). L = the LV length (apex to plane of midmitral annulus) obtained from parasternal long-axis view at end-diastole.

Ejection fraction percentage was calculated using 2D single-plane ellipsoid method in which the parasternal long axis was used (adhering to criteria aforementioned) providing data for the length from apex to mitral value annulus, and area of the ventricular chamber. Fractional shortening percentage was calculated using long-axis M mode.

VEGF Gene Delivery Dose-Response Experiment using Retrovirus-Modified Myoblasts Implantation

Using two mice per cell concentration, seven cell concentrations were injected into non-infarcted murine left ventricle free wall (as described above) to establish a dose-response relationship for VEGF gene delivery using modified murine myoblasts containing both the $VEGF_{164}$ gene and the LacZ reporter gene. The following cell concentrations were used: $1x10^4$, $5x10^4$, $1x10^5$, $5x10^5$, $1x10^6$, $5x10^6$, $1x10^7$. Cells were injected in 10 µl PBS with 0.5% BSA. Two weeks after cell implantation, animals were euthanized and examination of the histology sections was made to assess angiogenesis and tumorgenesis. Sections stained with X-gal were examined for presence of VEGF/LacZ producing myoblasts, indicating successful transplantation of cells. Adjacent sections were stained with CD31 (as previously described) and examined along with corresponding HE sections for gross presence of tumorgenesis.

VEGF Gene Delivery Intervention for Infarcted Myocardium using Retrovirus-Modified Myoblasts Implantation

At time = 0, two separate injections of $5x10^4$ cells/injection or PBS were made into the left and right border zone of the infarct region created by the acute myocardial infarction model (Figure 1-2). Total cell amount injected per mouse was $1x10^5$. The LV free wall infarct border zone, rather than infarct region itself was chosen for injection to maximize implanted myoblast survival. It was felt the border of the ischemic/necrotic infarction would maximize transplanted myoblast survival, thereby maximizing therapeutic effect on damaged myocardial tissue. It is established that injected cells spread a variable distance from the injection site, and that injecting cells at the border zone permits cell implantation into both normal and infarcted myocardium. At seven days and 4 weeks, echocardiography was performed as described. Following echocardiography at week 4, the mice were euthanized and examination of histology sections was performed to assess capillary counts per high-power field and mean ventricle infarct percent.

Statistical Analysis

SPSS v 10.1 was used for statistical analysis. Statistical comparison between groups was performed using one-way ANOVA followed by Tukey post hoc testing with $P<0.05$ considered significant. All values are expressed as mean±SEM.

IV. Results

VEGF Gene Delivery Dose-Response Experiment

To establish a dose-response gradient for retrovirus-mediated VEGF/LacZ expressing myoblasts, seven concentrations of transformed myoblasts were injected into noninfarcted left ventricle free wall with two animals per cell injection concentration. It has been shown that high dose implantation of modified-modified primary murine myoblasts produces cavernous hemangiomas when injected substernally into murine myocardium.[15]

Gross assessment of tumorgenesis and angiogenesis was made using alternate histology sections stained with HE and antibody to CD31 (endothelial cell marker) and secondary florescent conjugate. Cavernous hemangioma formation was grossly visible at higher doses ($10x10^6$ and $5x10^6$) while angiogenesis prevailed at lower doses ($1x10^5$, $5x10^4$, and $1x10^4$). Low-risk doses can be applied in subsequent experiments to assess changes in ventricular function and infarct size using the acute myocardial infarction model.

VEGF Gene Delivery to the Acutely Infarcted Murine Myocardium

Two research questions were addressed by implanting VEGF producing myoblasts in the infarcted murine myocardium. The first question addresses whether low-risk, low-dose constitutive myoblast-mediated delivery of VEGF promotes angiogenesis in the infarcted myocardium. The second question

examines whether or not implantation of VEGF producing myoblasts improves myocardial function in the acutely infarcted murine heart. Methods used for injection of VEGF producing retrovirus-modified myoblasts have been previously described (Figure 2).

Mean Ventricle Infarct Percent and Echocardiographic Assessment of LV Function

Using calculations on histology sections, Mean Ventricle Infarct Percent values were not statistically significant between groups (Figure 3). Moreover, histologic calculation of ventricle infarct percent did not necessarily correlate with echocardiographic assessment of ejection fraction percent.

Data obtained using echocardiographic assessment of ventricular function demonstrated no statistical significance between groups for Ejection Fraction % (EF%) at four-weeks. Four-week minus seven-day EF% difference was not statistically significant between groups. Moreover, the four-week minus seven-day Fractional Shortening % (FS%), calculated using M-mode, was not statistical significant between groups.

However, LV Mass (milligrams) calculated using 2D echocardiography revealed greater mean LV Mass at 4-weeks in mice receiving the VEGF myoblast injections when compared to mice injected with control myoblast or with saline (93.5±11.8 milligrams; P=0.044 versus myoblast injected mice versus P=0.036 saline controls; Figure 3). LV Mass calculated using 2D echocardiography is an

estimate of the dry mass of the LV if it was dissected and weighed. There was no increase in LV Mass at 4-weeks in the myoblast control group when compared to mice injected with saline alone (62.8±4.9 mg versus 61.2±6.79; P=0.004; Figure 3). The four-week minus seven-day LV Mass difference was not statistically different between groups.

Angiogenesis and Hemangioma Formation

Capillary counts per high-power field (Figure 4) demonstrates significantly increased capillary counts in infarcted regions of mice treated with VEGF-producing myoblasts when compared to infarcted regions of mice receiving control myoblasts and saline (9.11±0.491 capillaries per high-power field; P=0.003 versus saline treated mice and P=0.006 versus myoblast controls; Figure 5). There was no increase in capillaries per high-power field in the myoblast control group when compared to mice injected with saline alone (5.96±0.734 versus 5.5±0.528; P=0.857; Figure 5).

In addition to angiogenesis in the scar region of the LV free wall, cavernous hemangioma formation also occurred in the ventricular wall of mice treated with VEGF-producing myoblasts (Figure 6). Hemangioma formation did not occur in mice injected with control myoblasts or saline. Interestingly, the cavernous hemangioma formation occurred on the epicardial surface of VEGF-producing myoblast injected hearts, rather than exclusively within the

myocardium, as occurred during dose-response VEGF-gene delivery using retrovirus-modified myoblasts.

Implanted Myoblasts and LV Wall Thickness

Gross examination of histology sections from hearts injected with VEGF-producing myoblast and control myoblasts demonstrates that cell implantation using the injection technique enabled cell survival and formation of a tissue-like graft of myoblasts within the myocardium. The left image in Figure 7 shows an HE section that demonstrates myoblasts within the ventricle wall. The image to the right demonstrates the location of myoblasts using the X-gal stain for beta-galactosidase. While it is difficult to ascertain whether or not the implanted myoblasts aligned themselves with major cardiac fiber axis within the host myocardium, the positive staining with X-gal indicates the cells survived implantation and were maintained within the native myocardium.

An incidental finding with examination of histology sections was that myoblast implantation resulted in preservation of ventricular wall thickness. Figure 8 compares the ventricular wall of saline injected myocardium versus control myoblast injected myocardium. The LV Free Wall of the saline injected myocardium demonstrates considerable thinning when compared to the myoblast-injected heart to the right in Figure 8. The inset represents the X-gal stain of the myoblast injected section, demonstrating that preservation of myocardial wall thickness occurred in the region actually implanted with

myoblasts. Figure 9 further represents preservation of LV wall thickness in LV free wall regions successfully implanted with myoblasts. The HE image shows thinning and dilation of the infarcted LV free wall in the region that was not implanted with myoblasts (arrow), whereas wall thickness was preserved in the regions to the left and right (arrowheads), which received myoblasts. The X-gal stained image to the right demonstrates blue staining myoblasts (arrowheads) corresponding to regions of preserved myocardial wall thickness.

V. Discussion

This study demonstrates concept validity such that it is possible to combine cells with an angiogenic agent to promote angiogenesis within the infarcted myocardium. Cell implantation of retrovirus-modified murine myoblasts produced angiogenesis within the scar region of acutely infarcted murine left ventricle in comparison to control-myoblast implanted hearts and saline injected hearts. Moreover, the injected myoblasts survived implantation and were maintained with the ventricular wall. These cells formed a tissue-like graft within the ventricular wall; however, it is unclear whether the cells were aligned along the major axis of cardiomyocytes within the ventricular wall and whether or not the cells formed gap junctions with native cardiomyocytes, which is quite technically difficult to assess in vivo. Indeed, gap junctions (identified by connexin 43) are an important component of cell implantation into the

myocardium, which reflects the three main criteria according to Thurmond et al[16]: cell survival, differentiation/maturation to an adult cardiomyocyte phenotype, and recreation of the normal three-dimensional architecture of native myocardium.

While angiogenesis was demonstrated in the infarct region of VEGF-producing myoblast implanted hearts, improved function on echocardiographic assessment was not demonstrated using the four-week and seven-day difference in EF% (calculated with 2D echocardiography) and FS% (calculated with M-mode). The probable reason for lack of improved function in the VEGF-producing myoblast injected hearts in comparison to control-myoblast and saline implanted hearts is the cavernous hemangioma formation, which inadvertently occurred in the VEGF-producing myoblast treated hearts.

The formation of cavernous hemangiomas, though not the intended outcome for the mice receiving injections with VEGF-producing myoblasts, demonstrated that the VEGF-myoblast cell concentration used in this study was too high, despite being categorized as low-risk in the preliminary dose-response experiment. Hemangioma formation may be prevented in future experiments by using an even lower risk dose of VEGF-producing myoblasts. Alternatively, regulatable promoters may be used to limit VEGF production by retrovirus-modified murine myoblasts.

This study also demonstrates a statistically significant increase in LV Mass using 2D echocardiography in the VEGF-myoblast treated group versus the

control-myoblast and saline injected groups. The increased LV Mass in the VEGF-myoblast implanted hearts may be attributed to the cavernous hemangioma formation and increased angiogenesis that occurred in this group, thereby increasing the mass of the left ventricle. Given the non-statistically significant increase in LV Mass in the myoblast-control group versus mice injected with saline alone, it is reasonable to conclude that the implanted cells in the VEGF-myoblast group were not the cause of the increased left ventricular mass, unless the VEGF secondarily promoted myocyte growth & development.

Importantly, the myoblast implanted regions of the infarcted hearts (both VEGF-producing myoblasts and control-myoblasts) demonstrated preservation of myocardial wall thickness. Preservation of myocardial wall thickness is an important therapy post-MI, given that ventricular remodeling post-MI can result in considerable dilation and thinning of the ventricular free wall predisposing the heart to rupture through a tear in the necrotic/damaged myocardium (typically occurring within two weeks of an acute MI) or to ventricular aneurysm which results in a segmental outward bulge of the heart muscle during contraction (typically occurring weeks to months post-MI). Complications of ventricular aneurysm include thrombus formation, arrhythmia, and heat failure. The optimal number of transplanted cells to improve myocardial wall thickness must be further explored. Indeed, subsequent studies can examine preservation of myocardial wall thickness by quantifying changes in wall thickness using both echocardiography and histology.

Future experiments are needed to further elucidate the role of retrovirus-modified myoblasts producing VEGF in treatment of the acute MI. Indeed, an important question concerning implantation of VEGF-producing myoblasts for treatment of MI is the timing issue. When is optimal time to implant a therapeutic cell-line? It is possible that cell injection directly following an acute-MI as performed in this project can result in a greater infarct region. Acute ligation of the LAD produces ischemia and necrosis in the LV free wall. It is possible, that cardiomyocytes initially surviving the ischemia caused by LAD ligation are pulled apart by the injection of myoblasts. The viable gap junctions, which normally would ensure native cardiomyocyte survival, are pulled apart by the injected cells due to disruption of the indigenous three-dimensional architecture of the ischemic/necrotic myocardium. However, if a similar injection of myoblasts occurs at one-week post-LAD ligation, the injected myoblasts may less likely disrupt gap junctions between surviving native cardiomyocytes, but rather target areas of coagulative necrosis and granulation tissue formation, regions in the heart permanently damaged by ischemia.

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Conclusion

Given that heart transplantation is the only current treatment for patients with end-stage heart failure, alternate strategies to treat severely damaged hearts are necessary. Currently the mechanical heart is being tested in clinical trials, and is intended for use in patients who are transplant candidates, but who cannot receive donor hearts due to the organ shortage. In the future, the mechanical heart may supplant the donor-heart for transplantation, however, major invasive surgery will still be required to conduct the transplant procedure. Regeneration of myocardial tissue using a combined angiogenic growth factor and cell implantation approach may obviate the need for major invasive surgery, and enable cells and molecules to mend the broken heart.

Time = 0	**Cell Injections into Left & Right Border Zones**
Time = 7 days	**Echocardiography**
Time = 4 weeks	**Echocardiography Histology**

Figure 1: Experimental Protocol

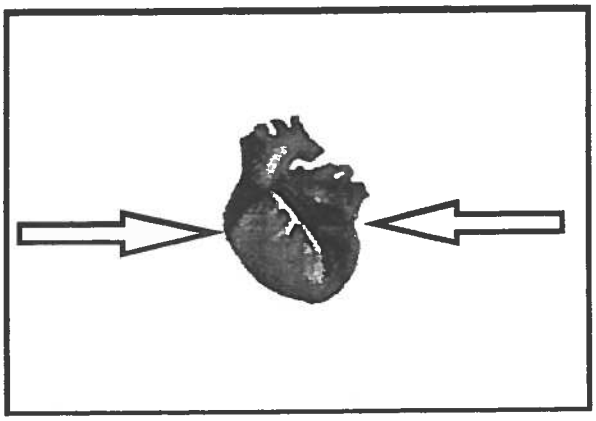

Figure 2: Cell Injections into Left and Right Border Zones (arrows)

Group	Mean Ventricular Infarct %	4-week, 7-day EF%	4-week EF %	4-week FS %	LV Mass (mg) at 4 weeks
Saline (n=6)	14.37±3.72	4.17±10.31	56.83±4.39	43.15±4.41	61.2±6.8
control myoblast (n=7)	31.41±5.18	-6.86±4.59	59.71±2.06	46.69±3.35	62.8±4.9
VEGF myoblast (n=6)	20.27±6.29	-6.5±6.01	68.00±4.24	41.74±6.36	93.5±11.8*

Figure 3: Data are presented as mean±SEM. EF = Ejection Fraction; FS = Fractional Shortening; LV = left ventricle; *P<0.05 vs both control myoblast and saline groups

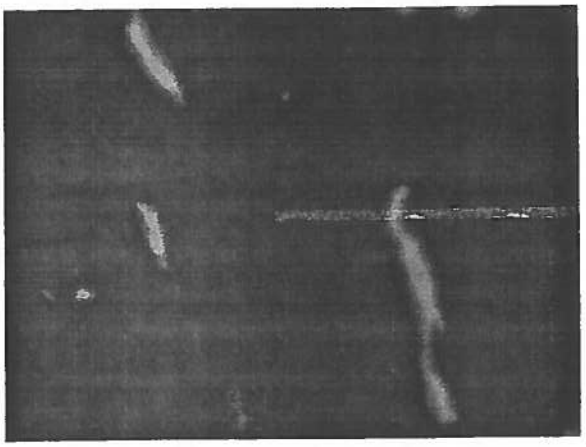

Figure 4: Capillary Counts Per High-Power Field were performed after labelling endothelial cells with antibody to CD31, and Cy3-conjugated florescent secondary. 40x Magnification

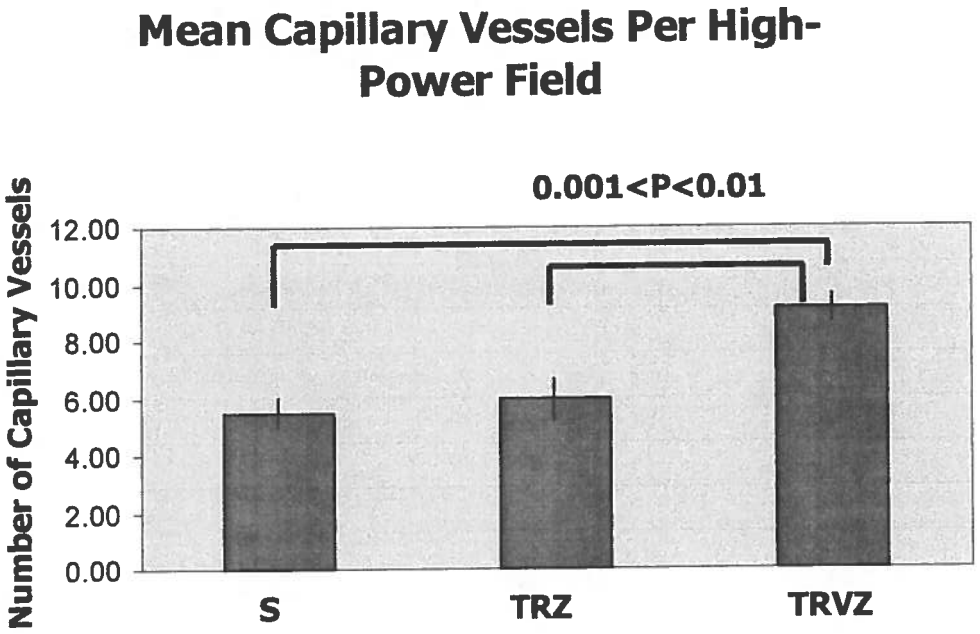

Figure 5: The VEGF-myoblast group had significantly increased angiogenesis in scar regions (9.11±0.491; P=0.003 versus saline treated mice and P=0.006 versus myoblast controls). There was no increase in capillaries per high-power field in the myoblast group when compared to the saline group (5.96±0.734 versus 5.5±0.528; P=0.857); S = saline; TRZ = control myoblast; TRVZ = VEGF myoblast

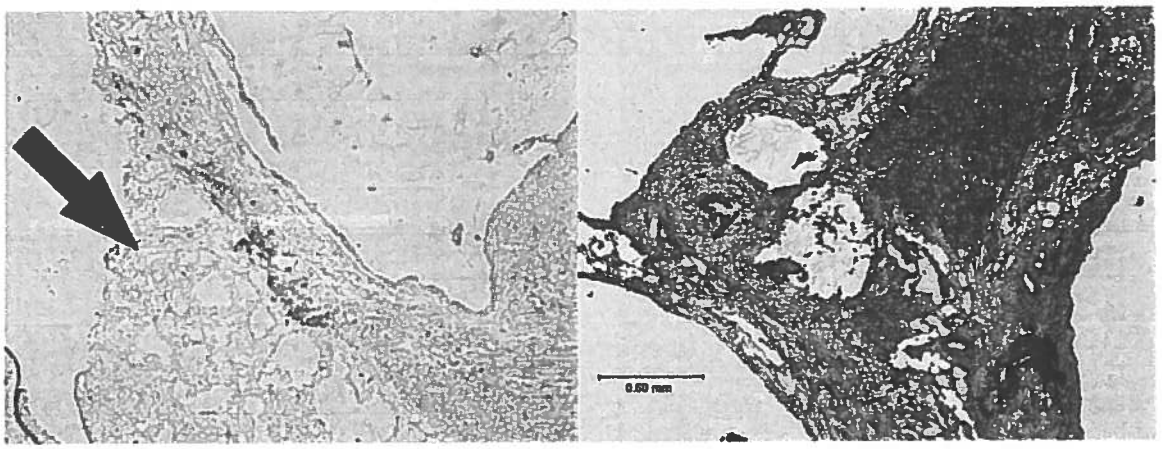

Figure 6: Image on left demonstrates positive X-gal stain (blue) of VEGF-producing myoblast and adjacent hemangioma on epicardium (arrow); Image on right demonstrates HE of cavernous hemangioma formation in hearts treated with VEGF myoblasts; 4x magnification

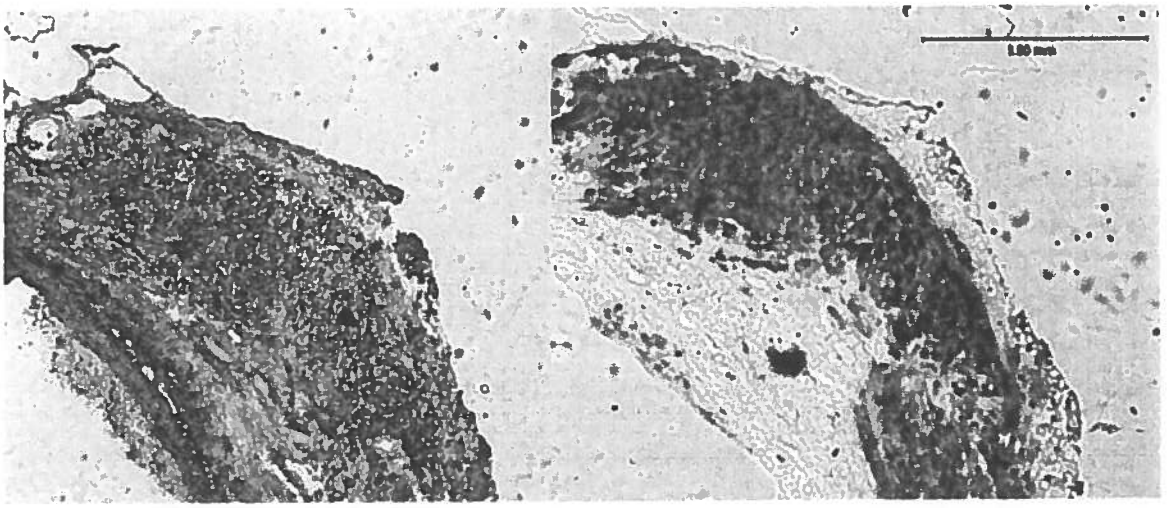

Figure 7: Transplanted myoblasts survived and formed a stable-graft within the myocardial wall. Image on left is HE demonstrating graft-like tissue formation by injected myoblasts; Image on right is adjacent X-gal section showing location of myoblasts within ventricular wall. 10x magnification.

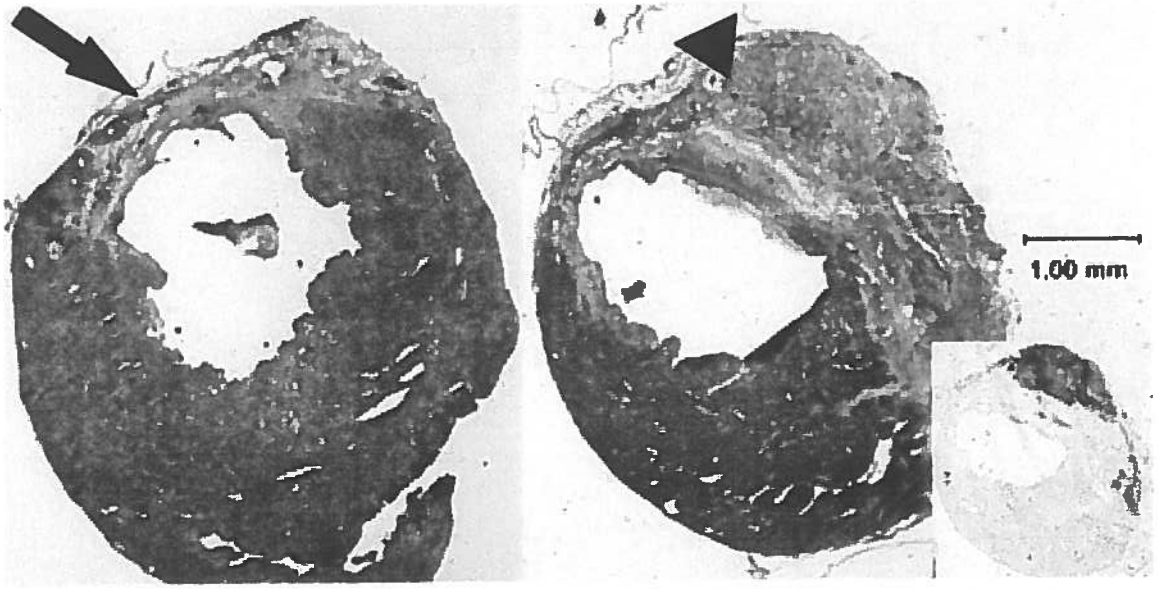

Figure 8: Preservation of myocardial wall thickness with cell implantation. Image on left is HE of saline injected heart with thinning of free wall (arrow). Image on right is HE of myoblast-injected heart, with corresponding X-gal inset, with preservation of myocardial wall thickness (arrowhead). 1x magnification

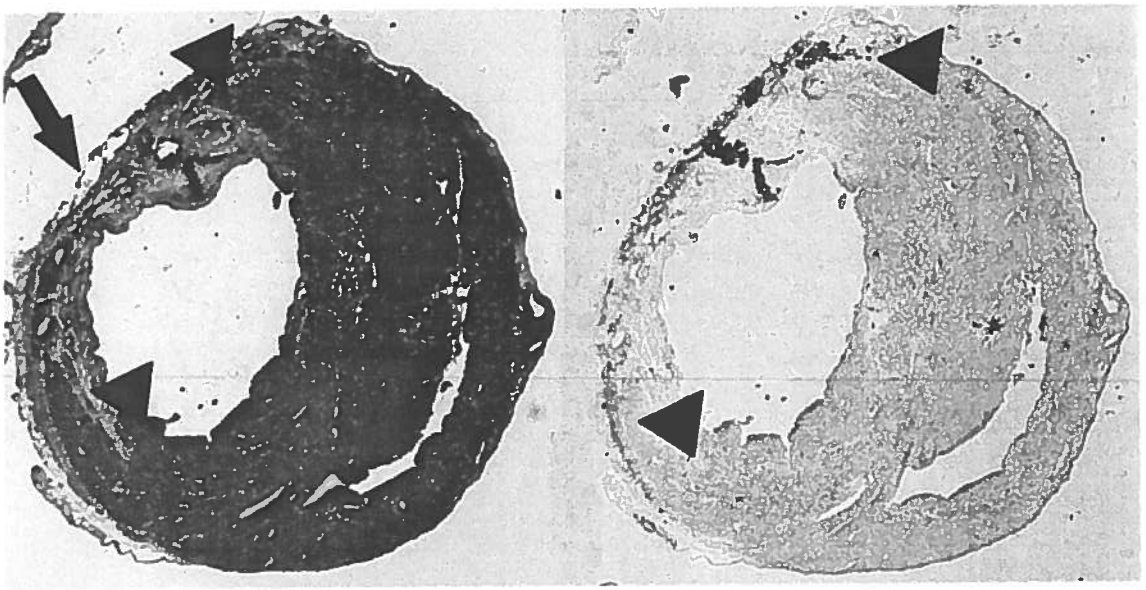

Figure 9: Image on left is HE of myoblast injected heart, showing thinning of infarcted LV free wall in region no implanted with myoblasts (arrow). Wall thickness was preserved in regions to left and right (arrowheads) which received myoblast injections. Image on right is corresponding X-gal (arrowheads indicate blue-staining regions).