MECHANISMS OF MYOBLAST TRANSFER IN TREATING HEART FAILURE

This application claims priority to U.S. provisional application serial number 60/402,050, filed August 9, 2002, the entirety of which is hereby incorporated by reference.

Field of the Invention

The invention relates generally to treatment of infarcted myocardium and more specifically to the use of cell therapy to repair myocardial infarction through concomitant angiogenesis and myogenesis.

Background of the Invention

Heart muscle degeneration is the leading cause of debilitation and death in humans. It cascades in losses of live cardiomyocytes, contractile filaments, and heart function. Cardiomyocytes do not regenerate significantly because the telomeric DNA repeats[1] in these terminally differentiated cells are minimal.

The degenerative heart transmits biochemical signals to recruit stem cells to repair the muscle damage. Being pluripotent, embryonic or adult stem cells exhibit uncontrolled differentiation into various lineages to produce bone, cartilage, fat, connective tissue, skeletal and heart muscles (Fig. 1). The damaged myocardium needs additional live myogenic cells to deposit contractile filaments to regain heart function, preferably before fibroblast infiltration which leads to scar formation. However, until scientists can accurately define the specific transcriptional factors and pathway to guide stem cell differentiation into cardiomyocytes, the use of stem cell injection into the human heart would have risk-benefit ratio higher than the use of myoblasts.

As young cardiomyocytes and myoblasts become committed and differentiate from stem cells, they are similar in that they are mononucleated cells without contractile filaments. In the presence of neurotrophic factors, myoblasts fuse to become myotubes that develop into myofibers. Under the influence of heart hormones, the young cardiomyocytes fuse to become mature cardiomyocytes. Cardiomyocytes and myofibers are myogenic cells that produce contractile proteins to provide contractility.

Like cardiomyocytes, myoblasts are differentiated cells that are destined to become muscles. Unlike cardiomyocytes however, myoblasts have long telomeric DNA subunits and are capable of extensive mitosis. The ability to undergo mitosis and to fuse are conserved in mononucleated satellite cells that are essentially myoblast reserves in adult muscles. Satellite cells are differentiated cells. They are not stem cells. Myoblasts survive and proliferate in intercellular fluid. Their survival does not depend on vascularization or nerve innervation.

Because of these desirable properties of myoblasts, attempts have been made to repair muscle tissue with them. As shown by the work of Law et al. an important feature in this regard is that the myoblast preparations used for cell transplantation should be relatively free of fibroblasts, an important point that seems to have been overlooked by many workers in this field. See for example, Law et al. Gene Therapy and Molecular Biology Vol. 1, 345-363, particularly page 350 to 351, describing myogenic cell transplant therapy for correction and amelioration of skeletal muscle disorders. Another point is that once introduced, the myoblasts are subjected to scavenger hunt by macrophages, typically for up to three weeks. Thus, a high number of myoblasts must be transplanted for the transplanted cells to be effective.

The first human myoblast transfer into the porcine heart revealed that it was safe to administer one billion myoblasts at $100x10^6$/ml through the Myostar catheter (Biosense Webster, Inc.) using 20 injections at different locations inside

the left ventricle.[2] It was determined that 0.3 ml to 0.5 ml would be the optimal volume per injection. This field of medicine has become very active. However, generally acceptable and successful results remain elusive. More needs to be done to obtain successful transfer of cells into damaged hearts in a manner that corrects the damage.

Summary of the Invention

Embodiments of the invention allow cell therapy of diseased heart tissue. In one embodiment a composition is provided that comprises isolated myoblasts that transgenically express VEGF. Another embodiment provides a composition wherein the number of myoblasts exceed the number of fibroblast cells by 100 to 1. Yet another embodiment provides a composition that comprises isolated myoblasts cotransfected with a gene that encodes an epithelial cell stimulator or angiogenesis stimulator and a second marker gene. Yet another embodiment provides a composition useful for alleviating congestive heart failure, comprising at least 1 billion myogenic cells that transgenically express at least one angiogenesis factor. In yet another embodiment the cells transgenically express VEGF 165. In yet another embodiment the cells further express VPF.

Another embodiment provides a method for treating congestive heart failure in an individual, comprising taking a biopsy of skeletal muscle from the individual to form a culture; transforming cells of the culture with at least one foreign gene that encodes an angiogenesis factor; forming a culture of cells suitably pure enough for repairing the heart of the individual; and introducing cells of the culture into a diseased heart of the individual.

Description of the Figures

Figure 1. Advantages of using myoblasts over stem cells in treating heart failure. MTT = myoblast transfer therapy

Figure 2. Human desmin immunostain for myoblast purity. (A) Positive control of leiomyosarcoma, desmin staining brown. (B) Negative control. (C) *Pure* human myoblasts immunostained with desmin. (D) *Pure* human myoblasts in culture.

Figure 3. (A) Brownish immunostain of human myosin in porcine myocardium 12 weeks after human myoblast injection. (B) Cardiomyocytes with Lac-Z positive nuclei and human myosin stain, indicative of donor or myoblastic in origin. (C) Negative immunostain (grey) of human myosin in porcine myocardium sham-injected without myoblasts.

Figure 4. (A) Heterokaryons derived from fusion of porcine cardiomyocytes and human myoblasts showing Lac-Z positive human myoblast nuclei (bluish green) and porcine cardiomyocyte nuclei (purple) in the heterokaryotic syncytium. (B) These heterokaryons expressed human myosin heavy chain.

Figure 5. Electron microscopy of the myoblast-injected porcine myocardium showing (A) myotubes with central nuclei and myfibril (ML) deposits, and (B) skeletal myofiber with satellite cell (SC) and nucleus (N). The satellite cell was located between the basement membrane (black arrow) and the plasma membrane (white arrow). Sarcomeres showed proper alignment of newly formed contractile filaments.

Figure 6. (A) Control myocardium immunostained for vWF VIII and counterstained with Eosin to show capillaries. (B) $VEGF_{165}$ transduced myoblasts produced increased vascular density. (C) As in B but without Eosin counterstain.

Detailed Description of the Invention

Myogenic cells according to embodiments of the invention preferably are autologous and obtained by tissue biopsy, as described for example in U.S.

Nos. 6,099,832, 5,833,978, 6,284,242 and 5,130,141. In preferred embodiments, a composition is prepared from a myogenic cell or precursor to myogenic cell having a minimum contamination by fibroblasts. The term "minimum contamination by fibroblasts means that on a total cell number basis, less than 5%, 2%, 1% 0.5% 0.2% or even less than 0.1% of the cells are fibroblast cells. This improved composition purity can be achieve a variety of ways. For example, in one way, fibroblast cells are preferentially inhibited or killed by the inclusion of one or more substances in the culture medium. In another way, a cell sorter is used that separate cells one at a time. In yet another way, a non-fibroblast specific promotor such as a muscle specific promoter is used to control expression of a gene that generates a product which allows a cell that makes that product survive in cell culture. In this way, transformed myogenic cells preferentially survive and the percentage of fibroblasts diminishes. In yet another way, the myogenic cells are cultured in the presence of a macrophage cytokine such as that described by Giurisato et al. in Basic Appl. Myol. 8(5): 381-388 (1998), which stimulates proliferation of myogenic cells but not fibroblast cells. The cytokine may be produced by culturing macrophages in a serum free medium and then harvesting the medium to obtain a crude preparation of cytokine. In a preferred embodiment basic cell transfer therapy techniques that utilize very purified (low fibroblast contamination) cultures are made possible by adding crude or partially purified preparations of a 50-10KDa cytokine secreted by macrophages to the culture and growing at least 2, 3, 5, 8 or 10 generations or more of the myogenic cells.

In each case some cell division may be used to increase cell number prior to use of the cells. Most desirably at least 1, 2, 5, 10, 25, 50 or even more than 100 billion cells are cultured and transferred. Where the cells are not autologous, it is
generally preferred to utilize an agent to minimize or eliminate xenograft rejection such as cyclosporine, in the animal or patient that receives the cells. Further agents may be added to the reintroduced cells such as viscosity

adjusting materials, adhesive agents and the like, to assist placement and positioning of the cells within myocardium during or after cell transfer.

Myogenic cells, or precursors to myogenic cells are activated or transformed to express one or more genes that stimulate endothelial cell growth and/or development of blood vessels. According to an embodiment of the invention the cells before transfer into a diseased heart are transformed with one or more genes under the control of a suitable promotor that expresses an endothelial cell growth and/or angiogenesis protein. For example, both acidic and basic fibroblast growth factor molecules are mitogens for endothelial cells and other cell types and desirably are stably incorporated into cells that are or become myogenic cells. Angiotropin and angiogenin can induce angiogenesis, as described by Folknan, J., Cancer Medicine, Lea and Febiger Press, pp. 153-170 (1993). A highly selective mitogen for vascular endothelial cells is vascular endothelial growth factor or VEGF (Ferrara, N. et al., Endocr. Rev. 13:19-32 (1992)), which is also known as vascular permeability factor (VPF). In most preferred embodiments VEGF is transgenically expressed. However, in some embodiments the desired gene is turned on by homologous recombination. Most preferably VEGF is transgenically expressed. In an embodiment at least two different genes are transgenically expressed such as VEGF with angiotropin or angiogenin. In another emobodiment more than two different genes are transgenically expressed. Multiple genes can be expressed within the same cell, or may be expressed by different cells within the same composition. In some circumstances expression of two different factors, such as two different angiogenesis factors synergistically results in greater establishment of the transplanted cells within a target diseased heart muscle.

Transformation of cells can be achieved by a variety of methods as will be appreciated by a skilled artisan. Generally a nucleic acid sequence encoding a desired polypeptide such as the VEGF165 gene is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter;

or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the .beta.-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

In one embodiment a retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, .psi.-2, .psi.-AM, PA12, T19-14X, VT-19-17-H2, .psi.CRE, .psi.CRIP, GP+E-86, GP+envAml12, and DAN cell lines as described in Miller, Human Gene Therapy 1:5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO.sub.4 precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host. In this embodiment the producer cell line generates infectious retroviral vector particles that include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce myogenic cells or precursors to myogenic cells. The transduced cells express the nucleic acid sequence(s) encoding the polypeptide.

In a preferred embodiment cells are transformed with VEGF. VEGF has four different forms of 121, 165, 189 and 206 amino acids due to alternative splicing as described, for example in U.S. No. 6,040,157. VEGF121 and VEGF165 are soluble and are capable of promoting angiogenesis, whereas VEGF189 and VEGF206 are bound to heparin containing proteoglycans in the

cell surface. The temporal and spatial expression of VEGF has been correlated with physiological proliferation of blood vessels (Gajdusek, C. M., and Carbon, S. J., Cell Physiol 139:570-579 (1989); McNeil, P. L., et al., J Cell. Biol. 109:811-822 (1989)). Its high affinity binding sites are localized only on endothelial cells in tissue sections (Jakeman, L. B., et aL., Clin. Invest. 89:244-253 (1989)). In an embodiment of the invention at least two types of cells are transplanted into differing regions of the heart. One type of cell expresses VEGF (and optionally another angiogenesis factor) and preferentially is transplanted into region(s) where blood vessel growth is most desired. A second type of cell is transplanted into region(s) where blood vessel growth is less needed. A cardiac specialist can readily determine optimized locations for transplanting the two (or more) types of cells.

Vascular permeability factor (VPF) has also been found responsible for persistent microvascular hyperpermeability to plasma proteins even after the cessation of injury, which is a characteristic feature of normal wound healing. This suggests that VPF is an important factor in myocardium wound healing. Brown, L. F. et al., J. Exp. Med.176:1375-1379 (1992). VEGF expression is high in vascularized tissues, (e.g., lung, heart, placenta and solid tumors) and correlates with angiogenesis both temporally and spatially. VEGF also has been shown to induce angiogenesis in vivo. Since angiogenesis is essential for the repair of normal tissues, especially vascular tissues, VEGF has been proposed for use in promoting vascular tissue repair (e.g., in atherosclerosis).

U.S. Pat. No. 5,073,492, issued Dec. 17, 1991 to Chen et al., discloses a method for synergistically enhancing endothelial cell growth in an appropriate environment which comprises adding to the environment, VEGF, effectors and serum-derived factor. Also, vascular endothelial cell growth factor C sub-unit DNA has been prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a heterodimer or homodimer. The protein is a mammalian vascular endothelial cell mitogen and, as such, is useful for the promotion of vascular development and repair, as disclosed in European

Patent Application No. 92302750.2, published Sep. 30, 1992. In embodiments of the invention co-expression of VEGF165 with VPF and/or vascular endothelial cell growth factor C is used, in the same cells or in the same composition of cells, for desirable synergistic effects.

Prepared transgenic myogenic cell compositions further may comprise cell stimulatory agents and other materials to facilitate deposition and affixation of cells to the myocardium. Generally, the cells are introduced as a thick suspension by injection into the damaged myocardium.

The example describes a clinical trial that demonstrates with unequivocal evidence that cGMP-produced *pure* human myoblasts and proof of concept for Heart Cell Therapy.

Human myoblasts survived and integrated into the porcine ischemic myocardium, allowing concomitant cell therapy and gene therapy. Whereas the newly formed myofibers harbor satellite cells and impart regenerative capacity to the heart muscle, the genetic transformation of cardiomyocytes *in vivo* to become regenerative heterokaryons through myoblast genome transfer constitutes the ultimate heart repair. The regenerative heart[3] also contains cardiomyocytes of myoblastic origin. In all three scenarios, new contractile filaments are deposited to improve heart contractility. This latter can be translated into the improvement in the quality of life of heart patients and in the prevention of heart attacks.

Accordingly *pure* (i.e. at least 95%, 97%, 98%, 99%, 99.5%, or even at least 99.7% pure) $VEGF_{165}$ myoblasts, when injected intramyocardially, are potential therapeutic transgene vehicles for concurrent angiogenesis and myogenesis to treat heart failure. Immunosuppression using cyclosporine for six weeks is effective for long term survival of xenografts or allografts.

Each document cited herein is specifically incorporated in its entirety by reference. The following example is presented by way of illustration and not by way of limitation.

Example

This example demonstrates cell therapy of myocardium damage using myogenic cells that transgenically express VEGF165. In this myogenesis example, cultured myoblasts derived from satellite cells of human rectus femoris biopsies were transduced with retroviral vector carrying Lac-Z reporter gene. Porcine heart model of chronic ischemia (n=9; control=3; myoblast implanted=6) was produced by clamping an ameroid ring around the left circumflex coronary artery. Four weeks later, each heart was exposed by left thoracotomy. Twenty injections (0.25ml each) containing 300 million myoblasts, or 5ml total volume of basal DMEM as control, were injected into the left ventricle intramyocardially. Left ventricular function was assessed using MIBI-Tc^{99m} SPECT scanning one week before injection to confirm myocardial infarction and at 6 weeks after injection.

Animals were maintained on cyclosporine at 5 mg/kg body weight from 5 days before, until 6 weeks after cell transplantation. The animals were euthanized at 6 weeks to 5 months post-operatively, and the heart was processed for histological, immunocytochemical and ultrastructural studies. Laser nuclear capture together with single nucleus RT-PCR was performed to delineate host and donor nuclei. *In situ* hybridization using fluorescent DNA probes specific for human Y-chromosome and chromosomes 1&10 for pig were used.

In the angiogenesis study, the human myoblasts were transduced with retroviral and adenoviral vectors carrying Lac-Z and human $VEGF_{165}$ genes, respectively. The cells were characterized for $VEGF_{165}$ transduction and expression efficiency by immunostaining, ELISA, immunoblotting and RT-PCR. A porcine heart model of infarction was created in eight female swines by left

circumflex artery ligation. The animals were grouped as control (n=3) and myoblast-implanted (n=5). Angiography was performed to ensure complete occlusion of the blood vessel. Infarction was confirmed with MIBI-Tc^{99m} SPECT scanning. Four weeks later, 5ml basal DMEM without or with $3x10^8$ human myoblasts carrying $VEGF_{165}$ and Lac-Z genes were injected into the left ventricle intramyocardially. The animals were maintained on cyclosporine (5 mg/kg body weight) for six weeks post-operatively. Hearts were then explanted and processed for immunocytochemical studies.

Human myoblasts of 99% purity determined by human desmin immunostaining were prepared. About 75% of the myonuclei were successfully transduced with retrovirus carrying Lac-Z gene. Trypan blue stain revealed >95% cell viability immediately before injection.

Histological examination of myoblast-injected myocardium showed cardiomyocytes containing Lac-Z positive nuclei (of donor origin) after 12 weeks (Fig. 3B). More than 80% of the Lac-Z positive cardiomyocytes immunostained positively for human myosin heavy chain (Fig. 3A). The control heart without myoblast injection did not show Lac-Z positive myonuclei nor human myosin (Fig. 3C). Triple stain of myoblast-injected myocardia demonstrated multinucleated heterokaryons containing human and porcine nuclei with expression of human myosin (Fig. 4). Electron microscopy demonstrated human myotubes and skeletal myofibers with satellite cells in the porcine myocardium (Fig. 5).

The transduction efficiency for Lac-Z and $VEGF_{165}$ was 75-80% and >95% respectively. The transduced myoblasts continued to secrete $VEGF_{165}$ for longer than 18 days, significantly higher (37 ± 3ng/ml) than non-transduced ones (200 ± 30pg/ml). Dye exclusion test reveals >95% cell viability at the time of injection. Histological examination showed extensive survival of the grafted myoblasts expressing Lac-Z gene in and around the infarct. The vascular density (mean ± SEM) counted in an average of 12 low power fields (x200) in

control animal hearts was (4.18 ± 0.42) as compared to the $VEGF_{165}$ myoblast-transplanted group (28.31 ± 1.84) (Fig. 6). The SPECT scans showed improved perfusion in the infarcted region. Discontinuation of cyclosporine after 6 weeks prompted no xenograft rejection for up to 20 weeks.

Further References

1 F Ishikawa, M J Matunis, G Dreyfuss, and T R Cech, "Nuclear Proteins that Bind the Pre-mRNA 3' Splice Site Sequence r(UUAG/G) and the Human Telomeric DNA Sequence d(TTAGGG)$_n$", *Molecular Cell Biology*, (1993), 13, 4301-4310.

2 P K Law, et al., "World's First Human Myoblast Transfer into the Heart", *Frontiers in Physiology*, (2000), p. A85.

3 P K Law, "The Regenerative Heart", *PharmaTech 2002*, 65-70.

Of course, changes and modifications to the embodiments presented herein are readily understood by the skilled artisan after reading this specification and furthermore, such changes and modifications may be practiced within the scope of the appended claims.

CLAIMS:

1. A patient matched cell composition, comprising myoblasts that:

a) transgenically express an epithelial cell stimulator or angiogenesis stimulator, said epithelial cell stimulator or angiogenesis stimulator being VEGF; and

b) transgenically express at least a second epithelial cell stimulator or second angiogenesis stimulator, wherein said second epithelial cell stimulator or second angiogenesis being Angiopoeitin-1;

wherein the composition is prepared by obtaining a muscle biopsy from the patient .

2. The composition of claim 1, wherein the number of myoblasts exceed the number of fibroblast cells by 100 to 1.

3. The composition of claim 1, wherein the epithelial cell stimulator or angiogenesis stimulator is VEGF.

4. The composition of claim 3, wherein the VEGF is selected from the group consisting of VEGF2, VEGF121, VEGF165, or a biologically active fragment thereof.

5. The composition of claim 1, wherein the myoblasts are cotransfected with a gene that encodes an epithelial stimulator or angiogenesis stimulator and a second marker gene.

6. The composition of claim 1, comprising at least 1 billion myogenic cells that transgenically express at least one angiogenesis stimulator.

7. A composition as described in claim 6, wherein the at least one angiogenesis factor comprises VEGF.

8. The composition of claim 1 useful for treating an individual, prepared by

a. forming an allogeneic or xenogeneic cell culture form a biopsy of skeletal muscle taken from a second individual;

b. transforming cells of the culture with at least one gene that encodes an angiogenesis stimulator; and

c. forming a culture of cells suitably pure enough for introduction into the first individual.

9. The composition of claim 8, wherein the cell culture from step 3 is at least 90% pure myoblast cells.

10. The composition of claim 8, wherein the cells are packaged into at least one container suitable for administration into the individual of at least 100 million cells per container.

11. The composition of claim 8, wherein the cells are packaged into at least one container suitable for administration into the individual of at least 1 billion cells per container.

12. The composition of claim 1, derived from:

a). providing an allogeneic or xenogeneic culture of myogenic cells, the cells comprising at least one gene that encodes an angiogenesis stimulator; and

b). culturing the transgenic cells into a pure enough form suitable for introduction into the patient.

13. The composition of claim 12, wherein the cell culture from step 2 is at least 90% pure myoblast cells.

14. The composition of claim 12, wherein the cells are packaged into at least one container suitable for administration into the individual of at least 100 million cells per container.

15. The composition of claim 12, wherein the cells are packaged into at least one container suitable for administration into the individual of at least 1 biillion cells per container.

16. The composition of claim 12, wherein the angiogenesis stimulator is VEGF.

17. The composition of claim1, claim 8, or claim 12 suitable for simultaneous, separate or sequential administration with at least one immunosuppressant agent.

18. The use of allogenic or xenogenic myoblasts that:

i. transgenically express an epithelial cell stimulator or angiogenesis stimulator, said epithelial cell stimulator or angiogenesis stimulator being VEGF; and

ii. transgenically express at least a second epithelial cell stimulator or second angiogenesis stimulator, wherein said second epithelial cell stimulator or second angiogenesis is selected from the group consisting of acidic fibroblast growth factor, basic fibroblast growth factor, angiotropin, angiopoeitin-1, angiogenin, and VPF;

resulting in the manufacture of a medicament for treating congestive heat failure in an individual.

19. The use of claim 18, wherein the number of myoblasts in the medicament exceed the number of fibroblast cells by 100 to 1.

20. The use of claim 18, wherein the epoithelial cell stimulator or angiogenesis stimulator is VEGF.

21. The use of claim 18, wherein the VEGF is selected from the group consisting of VEGF2, VEGF121, VEGF165, or a biologically active fragment thereof.

22. The use of claim 18, wherein the myoblasts further transgenically express at least a second epithelial cell stimulator or angiogenesis stimulator.

23. The use of claim 22, wherein the second stimulator is selected from the group consisting of acidic fibroblast growth factor, basic fibroblast growth factor, angiotropin, angiopoeitin-1, angiogenin, and VPF.

24. The use of claim 18, wherein the myoblasts are cotransfected with a gene that encodes an epithelial cell stimulator or angiogenesis stimulator and a second marker gene.

25. The use of claim 18, wherein the medicament is formulated for injection and comprises at least 100 million cells.

26. The use of claim 25, wherein the medicament is formulated for intramyocardial injection.

27. The use of claim 18, wherein the medicament is suitable for simultaneous, separate, or sequential administration with at least one immunosuppressant agent.

28. The use of claim 27, wherein the immunosuppressant agent is cyclosporine.

29. The use of claim 18, wherein the individual is a human.

30. The use of claim 29, wherein the human is undergoing treatment with an agent to minimize or eliminate xenograft rejection.