

Original article

Recombinant human growth hormone secreted from tissue-engineered bioartificial muscle improves left ventricular function in rat with acute myocardial infarction

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Keywords: heart failure; muscles; growth hormone; gene therapy

Background Experimental studies and preliminary clinical studies have suggested that growth hormone (GH) treatment may improve cardiovascular parameters in chronic heart failure (CHF). Recombinant human GH (rhGH) has been delivered by a recombinant protein, by plasmid DNA, and by genetically engineered cells with different pharmacokinetic and physiological properties. The present study aimed to examine a new method for delivery of rhGH using genetically modified bioartificial muscles (BAMs), and investigate whether the rhGH delivered by this technique improves left ventricular (LV) function in rats with CHF.

Methods Primary skeletal myoblasts were isolated from several Sprague-Dawley (SD) rats, cultured, purified, and retrovirally transduced to synthesize and secrete human rhGH, and tissue-engineered into implantable BAMs. Ligation of the left coronary artery or sham operation was performed. The rats that underwent ligation were randomly assigned to 2 groups: CHF control group ($n=6$) and CHF treatment group ($n=6$). The CHF control group received non-rhGH-secreting BAM (GFP-BAMs) transplantation, and the CHF treatment group received rhGH-secreting BAM (GH-BAMs) transplantation. Another group of rats served as the sham operation group, which was also randomly assigned to 2 subgroups: sham control group ($n=6$) and sham treatment group ($n=6$). The sham control group underwent GFP-BAM transplantation, and the sham treatment group underwent GH-BAM transplantation. GH-BAMs and GFP-BAMs were implanted subcutaneously into syngeneic rats with ligation of the left coronary artery or sham operation was performed. Eight weeks after the treatment, echocardiography was performed. hGH, insulin-like growth factor-1 (IGF-1) and TNF- α levels in rat serum were measured by radioimmunoassay and ELISA, and then the rats were killed and ventricular samples were subjected to immunohistochemistry.

Results Primary rat myoblasts were retrovirally transduced to secrete rhGH and tissue-engineered into implantable BAMs containing parallel arrays of postmitotic myofibers. *In vitro*, they secreted 1 to 2 μ g of bioactive rhGH per day. When implanted into syngeneic rat, GH-BAMs secreted and delivered rhGH. Eight weeks after therapy, LV ejection fraction (EF) and fractional shortening (FS) were significantly higher in CHF rats treated with GH-BAMs than in those treated with GFP-BAMs ((65.0 \pm 6.5)% vs (48.1 \pm 6.8)%, $P < 0.05$), ((41.3 \pm 7.4)% vs (26.5 \pm 7.1)%, $P < 0.05$). LV end-diastolic dimension (LVEDD) was significantly lower in CHF rats treated with GH-BAM than in CHF rats treated with GFP-BAM ($P < 0.05$). The levels of serum GH and IGF-1 were increased significantly in both CHF and sham rats treated with GH-BAM. The level of serum TNF- α decreased more significantly in the CHF treatment group than in the CHF control group.

Conclusions rhGH significantly improves LV function and prevents cardiac remodeling in rats with CHF. Genetically modified tissue-engineered bioartificial muscle provides a method delivering recombinant protein for the treatment of heart failure.

Chin Med J 2009;122(19):2352-2359

Chronic heart failure (CHF) is often observed in patients with end-stage cardiovascular disease. It is considered to be an irreversible and progressive process characterized by ventricular dilatation and hypertrophy, a process known as remodeling, by diminished pump performance, and by a number of neurohormonal perturbations, including abnormalities in the growth hormone (GH)/insulin-like growth factor-1 (IGF-1) signaling axis.¹ GH and its mediator, IGF-1, are anabolic hormones that are essential to skeletal and myocardial growth and metabolic homeostasis. Studies showed that patients with heart failure exhibit the low levels of serum

DOI: 10.3760/cma.j.issn.0366-6999.2009.19.030

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This work was supported by grants from the National High-Technology Research and Development Program of China (863 Program) (No. 2002AA745070), Shanxi Province Natural Science Foundation (No. 2009011055-4), and Scientific Research Foundation of High Education Institutions of Shanxi Province, China (No. 200811034).

GH and IGF-1. GH administration might improve ventricular function, hemodynamics and exercise tolerance in patients with heart failure secondary to postischemic left ventricular dysfunction. These findings indicate that GH/IGF-1 axis plays a role in cardiac structure and function in patients with heart failure.²⁻⁵

But in a number of clinical studies, rhGH did not result in the expected benefits in many patients and this was attributed to the inability to deliver the protein in a consistent manner. In any event, for clinical use, lack of patient compliance, side effects, and high cost make multiple daily injection of rhGH unlikely to find widespread use.⁶⁻⁸ However, the delivery of genes encoding the sort of growth factor has the potential to provide suitable concentration. A study showed that local myocardial overexpression of human GH by adenoviral-mediated gene transfer resulted in significant improvement of left ventricular in AMI rat and prevention of ventricular remodeling.⁴ But the limited time of transgene expression and the low level of transient protein secretion limited the use of plasmid/vector.^{9,10}

Our study aimed to investigate the use of engineered tissues as stable, long-term vehicles for growth factor delivery in cardiovascular disease. Genetically modified skeletal muscle myoblasts can be injected into muscle tissues to deliver proteins systemically or locally, but cells are not possible to predict *in vivo* dosing, especially when they are injected into diseased muscle. In some studies, genetically engineered myoblasts were tissue-engineered *ex vivo* into bioartificial muscles (BAMs). When implanted in nonmuscle or muscle sites, they survived for a long term and deliver predictable levels of gene products, such as GH, insulin-like growth factors, and erythropoietin for months.⁸ Vandenburg et al⁸ reported that the BAM-secreted rhGH was found to be more effective in attenuating acute skeletal muscle disuse atrophy induced by hindlimb unloading than daily rhGH injections.

However, the feasibility of implanting BAMs that are expressing hGH to treat acute myocardial infarction remains unknown. We hypothesized that BAMs genetically modified to secrete recombinant hGH can also improve LV myocardial structure and function in rats with CHF and alleviate the development LV remodeling. To test our hypothesis, we transferred a recombinant gene encoding hGH into myoblasts with a retroviral vector. Genetically modified myoblasts are tissue-engineered *ex vivo* into BAMs. To evaluate the effect of hGH delivered by genetically modified BAM on cardiac function in rats.

METHODS

Generation of replication-deficient retroviral producer cell lines

Retroviral producer cell lines were generated for pLgGHSN and pLgGFPSN (provided by our laboratory)

after a 2-step transfection/transduction protocol optimized for primary rat myoblasts using E86 ecotropic and PT67 amphotropic packaging cells. Virus-containing medium was collected from high-titer PT67 clones and stored at -80°C .⁵

Primary rat myoblast culture and transduction

Primary rat myoblasts were isolated from the hind limbs of 1- to 3-day-old Sprague-Dawley rats (Department of Test Animals Research Center, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) and maintained in culture medium according to standard procedures.¹¹ The isolated cells were transduced with polybrene-supplemented virus-containing medium according to a centrifugation protocol.⁵

Genetically modified myoblasts are tissue-engineered into BAMs

The tissue-engineering techniques for BAMs formed from myoblasts were as described elsewhere.^{8,12} Briefly, transduced rat skeletal myoblasts were tissue engineered into BAMs by suspending the cells in a collagen-matrigel extracellular matrix solution and casting the suspension into silicone rubber molds with artificial end attachment points. BAMs for subcutaneous implants were formed from 2×10^6 transduced myoblasts and were 1×15 mm. Non-rhGH-secreting myoblasts were formed in an identical fashion from normal nontransduced myoblasts. BAM was frozen, and cryo-section was analyzed by fluorescence microscopy to examine the expression of green fluorescent protein.

Growth factor analyses in culture medium from BAMs

rhGH levels in culture medium from BAMs were measured by a radioimmunoassay (RIA) technique that does not cross-react with rat GH (GH RIA Kit, Jiuding Medical & Bioengineering, Tianjin, China).

Western blotting

For Western blotting, aliquots of conditioned culture medium containing 0–50 ng of rhGH were subjected to electrophoresis on 15% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, probed with anti-hGH (sc27091, Santa Cruz Biotechnology), and developed with ECL detection reagent (Amersham).

Surgical procedures: CHD model and implantation of BAMs

The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8523, revised 1996). Myocardial infarction was produced in male Sprague-Dawley rats weighing 220 to 250 g (Department of Test Animals Research Center, Tongji Medical College, Huazhong University of Science and Technology) by left coronary ligation as described previously.⁵ The control rats underwent a sham operation consisting of

thoracotomy and cardiac exposure but without coronary artery ligation. rhGH-secreting BAM (GH-BAMs) and Non-rhGH-secreting BAM (GFP-BAMs) were implanted subcutaneously into syngeneic rats with ligation of the left coronary artery or sham operation was performed. Infarct rats were randomly assigned to 2 groups: CHF treatment group ($n=6$) and CHF control group ($n=6$). The CHF treatment group received GH-BAMs implant, and the CHF control group received GFP-BAMs implant. Similarly, 12 sham-operated rats were randomly divided into 2 groups: sham treatment group ($n=6$) and sham control group ($n=6$). The sham treatment group received GH-BAMs implant, and the sham control group received GFP-BAMs implant. The rate of success in this operation was higher than 90%.

BAMs were treated with cytosine arabinoside (1 $\mu\text{g}/\text{ml}$) for 4 to 6 days before implantation to eliminate proliferating cells. Subcutaneous BAM implants were as described elsewhere¹² with 2 BAMs implanted into the back of each animal. All animals received daily injections of CsA (60 mg/kg) to block rhGH antibody formation.

Echocardiographic studies

Echocardiographic studies were performed after 8 weeks of surgical implantation with GH-BAMs or GFP-BAMs. After an injection of intraperitoneal pentobarbital, the rats underwent transthoracic 2D guided M-mode echocardiography before and after 8-week surgical implantation. M-mode measurements were averaged from 5 cycles. From the cardiac short axis (papillary level), LV end-diastolic dimension (LVEDD) and LV end-systolic dimension (LVESD), anterior end-diastolic wall thickness (AWT) and posterior end-diastolic wall thickness (PWT), fractional shortening (FS), and ejection fraction (EF), EF was calculated as $\text{EF} = (\text{LVEDV} - \text{LVESV}) / \text{LVEDV}$.

Hormone analyses in rat serum

After the echocardiographic measurements, blood was obtained in a heparinized syringe from the LV cavity and centrifuged. The plasma obtained was stored at -70°C for subsequent analysis. Rats' GH and IGF-1 were measured by a sensitive and specific radioimmunoassay (RIA). hGH levels in rat serum were measured by a RIA technique that does not cross-react with rat hGH (hGH RIA Kit, Jiuding Medical & Bioengineering, Tianjin, China). IGF-1 levels in rat serum were measured with IGF-1 RIA kits (Jiuding Medical & Bioengineering). TNF- α was measured with a solid-phase sandwich ELISA using a monoclonal antibody specific for rat TNF- α (Euroclone, UK).

Histological examination

The hearts were removed from the chest and the ventricles. Three transverse sections of the hearts were made from the apex to the base. The heart samples were embedded in paraffin and cut into 3 μm and 5 μm thick sections. The 3 μm thick slices were stained with hematoxylin and eosin for measurements of infarct size

and muscle fiber diameter. Myocardial infarct size was measured as previously described.⁵ Rats with an infarct size of $<25\%$ were excluded from analysis. Muscle fiber diameter was evaluated by direct measurements at $400\times$ magnification only in cross sections that included a nuclear profile. The 5 μm thick slices were stained with collagen-specific sirius red stain. Interstitial fibrosis was assessed according to the previously described methods.⁵ After 3 sections per animal and 20 fields per section were scanned and computerized with a HMIAS-2000 analysis system (Wuhan Championimage technology LTD, China), the area of interstitial fibrosis was calculated as the ratio of the sum of total area of interstitial fibrosis to the sum of total connective tissue area and cardiomyocyte area in all LV fields of the section.

Statistical analysis

Statistical analysis was done by SPSS 12.0. Parametric data were expressed as mean \pm standard deviation (SD). Comparisons of parameters among the 4 groups were made with one-way ANOVA, followed by the Newman-Keuls test. Comparisons of parameters between 2 groups were made by unpaired Student's t test. A P value less than 0.05 was considered statistically significant.

RESULTS

Myoblast immunocytochemistry identification

Myoblasts immunocytochemistry stained by desmin antibody 4 days after the primary culture. The results showed that 98% of myoblasts were desmin positive cells (Figure 1).

Myoblast transduction

The green fluorescence in primary myoblasts infected by pLgGFPSN could be observed under a fluorescence microscope (Figure 2).

Genetically modified myoblasts are tissue-engineered into BAMs

Transduced myoblasts were tissue engineered into BAMs by suspending the cells in a collagen-matrigel extracellular matrix solution and casting the suspension into silicone rubber molds with artificial end attachment points (Figure 3). The internal longitudinal tensions developed within the cell-gel mixture as it dehydrated, causing the formation of a cylindrical structure of 1 mm in diameter and containing parallel arrays of multinucleated postmitotic myofibers. Cryosection of BAM was analyzed by fluorescence microscopy to examine the expression of green fluorescent protein. The expression of green fluorescent protein cryosection could be observed by fluorescence microscopy (Figure 4).

Growth factor analyses in culture medium from BAMs

The BAMs *in vitro* secreted consistent levels of hGH (1.0 to 2.0 $\mu\text{g}\cdot\text{BAM}^{-1}\cdot\text{d}^{-1}$) for several weeks. BAMs formed from nontransduced myoblasts have no detectable hGH.

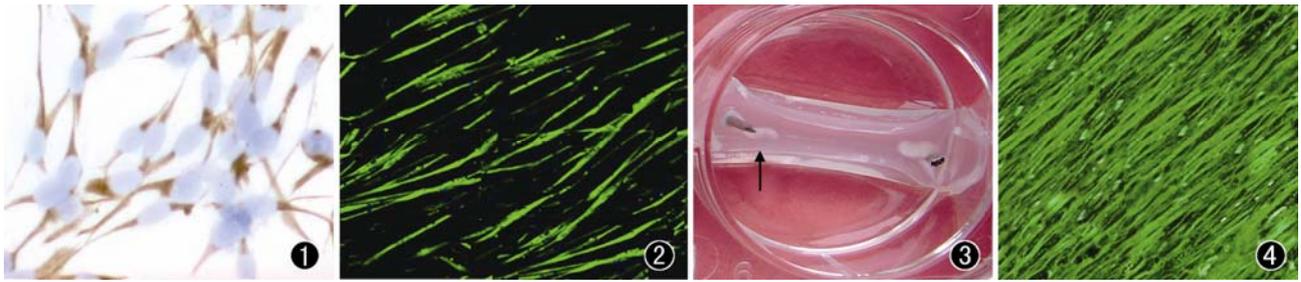


Figure 1. Myoblast immunocytochemistry identification. Myoblasts immunochemistry stained by desmin antibody 4 days after the primary culture. Cytoplasm shows desmin expression positive (original magnification $\times 400$).

Figure 2. Fluorescence microscope detection of GFP expression in myoblast. Positive expression of GFP in myoblast-GFP (original magnification $\times 200$).

Figure 3. The BAM in casting mold. The arrows indicate detached BAM.

Figure 4. The expression of green fluorescent protein in GFP-BAM (original magnification $\times 200$). Cryosection of BAM was analyzed by fluorescence microscopy to examine the expression of green fluorescent protein.

Western blotting

Western blotting of GH-BAM tissue culture medium showed that the major form of rhGH secreted by the GH-BAM myofibers *in vitro* was the 22-kDa monomer, GFP-BAMs have no detectable hGH (Figure 5).

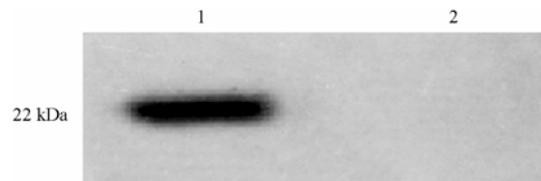


Figure 5. Western blotting of rhGH secreted from GH-BAMs. The arrow points to the 22-kDa monomeric form of rhGH. 1: GH-BAM; 2: GFP-BAMs.

Echocardiographic studies

The levels of EF, FS were significantly higher in CHF rats treated with GH-BAM than in CHF rats treated with GFP-BAM (65.0 \pm 6.5)% vs (48.1 \pm 6.8)%; (41.3 \pm 7.4)% vs (26.5 \pm 7.1)%. $P < 0.05$). The levels of LVEDD were significantly lower in CHF rats treated with GH-BAM than in CHF rats treated with GFP-BAM (7.2 \pm 0.42 vs 8.25 \pm 0.31, $P < 0.05$). There were no significant differences in EF, FS and LVEDD between the sham control group and sham treatment group (Figure 6).

in the animals implanted with GH-BAMs averaged (4.1 \pm 0.1) ng/ml ($n=6$). Two weeks after implantation, the serum levels of rhGH in the animals implanted with GH-BAMs averaged (3.7 \pm 0.2) ng/ml ($n=6$), and in the animals implanted with GFP-BAMs, no hGH was detected. The level of serum IGF-1 was significantly higher in both CHF and sham rats implanted with GH-BAMs than in those implanted with GFP-BAM.

Hormone analysis in rat serum

One weeks after the experiment, the serum level of rhGH

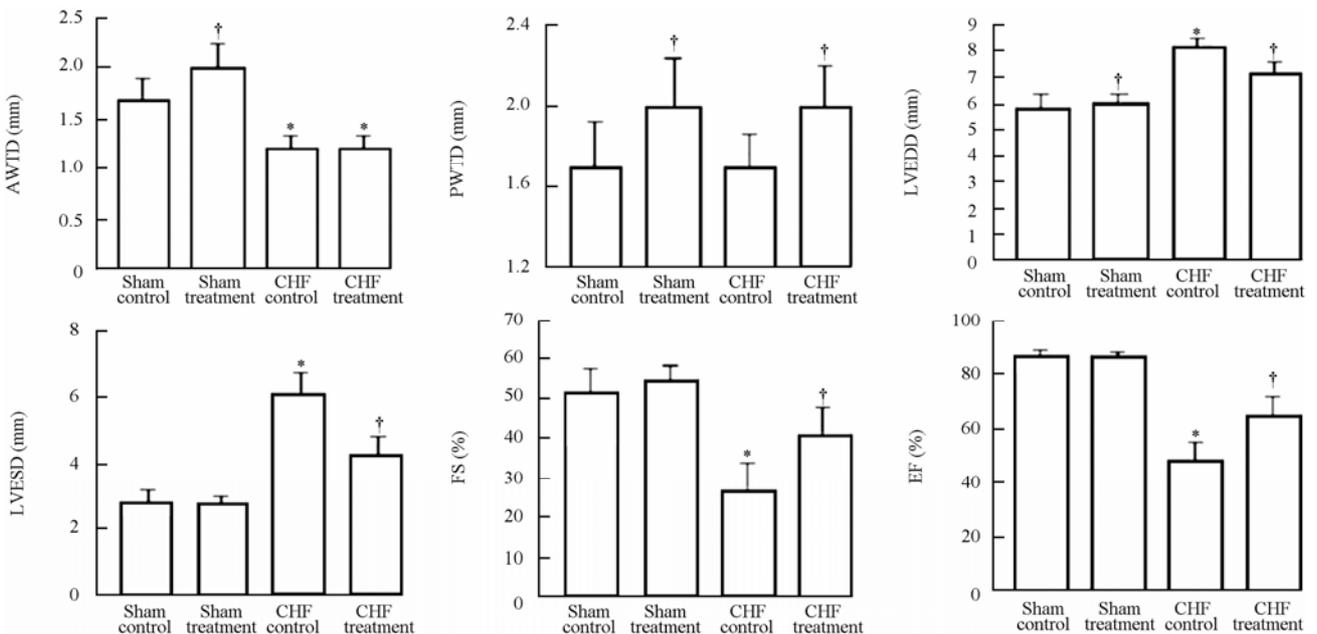


Figure 6. Echocardiographic parameters in rats treated with GH-BAMs and rats treated with GFP-BAMs. LVEDD: LV end-diastolic dimension; EF: ejection fraction; FS: LV fractional shortening. AWT: anterior end-diastolic wall thickness posterior; PWT: posterior end-diastolic wall thickness. * $P < 0.05$ vs respective sham group; † $P < 0.05$ vs respective control group.

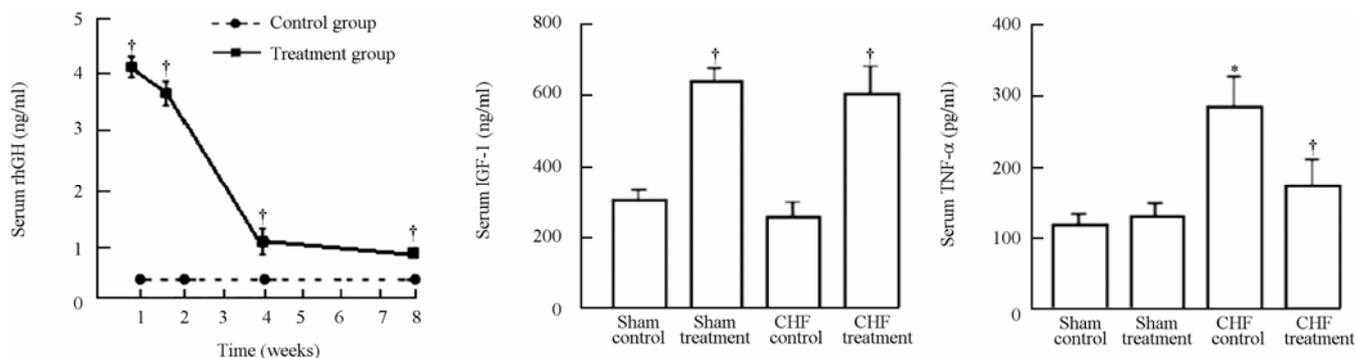


Figure 7. Effects of BAM on serum rhGH, IGF-1 and TNF- α levels in rat. Data are mean \pm SD. * P < 0.05 vs sham group; † P < 0.05 vs control group.

After 8 weeks of implantation, the serum levels of TNF- α in the CHF animals were increased significantly compared with the controls ((282 \pm 100) vs (114 \pm 12) pg/ml; P < 0.04, Figure 7). GH-BAMs treatment produced a slight decrease in TNF- α levels in CHF rats implanted with GH-BAMs compared with CHF rats implanted with GFP-BAMs (P < 0.05).

Histological analysis

The infarct sizes was similar between the CHF groups, there was no significant difference between the treated and control groups ((43.9 \pm 9.4)% vs (45.4 \pm 11.1)%, respectively). Compared with the CHF rats implanted with non-rhGH-secreting BAM, the CHF rats implanted with rhGH-secreting BAM exhibited a significant increase in muscle fiber diameter of the non-infarcted myocardium. There was no significant difference in collagen volume fraction between the CHF rats treated with rhGH-secreting BAM and those treated with non-rhGH-secreting BAM (Figure 8).

DISCUSSION

In this study, we found for the first time the effect of rhGH bioartificial muscles administration on heart failure. Primary rat myoblasts were genetically engineered to secrete rhGH tissue-engineered into bioartificial muscles. GH-BAMs secreted rhGH with molecular weights of 22-kDa. Subcutaneous implantation of GH-BAMs into syngeneic rat improved cardiac performance in CHF rats, as indicated by increases in LVFS, LVEF, diastolic thickness of the non-infarcted posterior wall (PWT) and decreased LVEDD. In addition, GH-BAMs treatment increased serum GH and IGF-1 levels in rats treated with GH-BAM, decreased serum TNF- α levels in the CHF treatment group. These results suggest that tissue-engineered skeletal muscle may be a practical platform to secrete biologically active rhGH in order to improve left ventricular dysfunction.

CHF (a cardiac condition that impairs the ability of the ventricle to fill with or eject blood) is the ultimate consequence of a vast number of cardiovascular diseases and constitutes one of the leading worldwide causes of

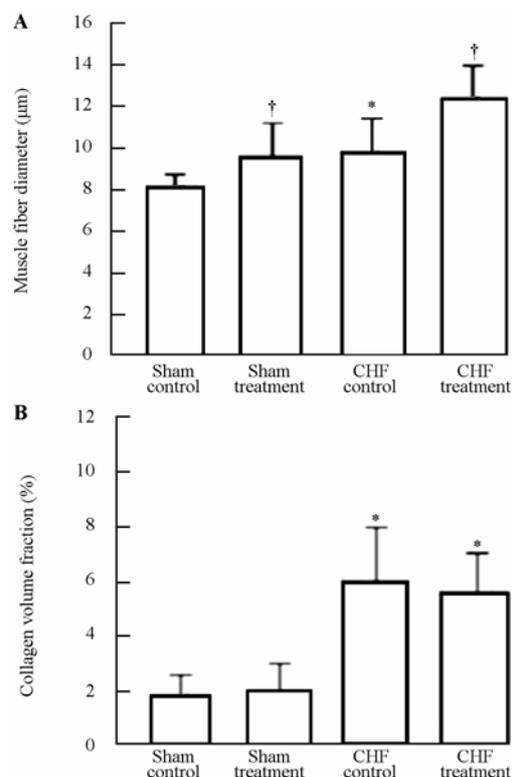


Figure 8. Effects of BAM on myocyte growth and collagen density in CHF and sham rats. Data are mean \pm SD. * P < 0.05 vs sham group; † P < 0.05 vs control group.

morbidity and mortality. Although progress in conventional treatment modalities is making steady and incremental gains to reduce this disease burden, there remains a need to explore new and potentially therapeutic approaches. Recently, many considerations have made heart failure an appealing clinical candidate for gene therapy, with progressive understanding of the molecular mechanism of heart failure.^{13,14}

The main target organs for CHF gene therapy, in addition to arteries and veins, are the myocardium, skeletal muscles of the lower limbs, and the liver. Two approaches for delivering genes coding for a therapeutic protein are used, namely *ex vivo* or *in vivo* gene transfer. The *ex vivo* approach involves the transduction of cells in culture

followed by the injection of these modified cells into the target tissue. The genetically modified cells secrete the desired protein at the site of injection. The second strategy, *in vivo* approach to genetic manipulation involves the transfer of genes to target tissue by either systemic administration or direct injection with plasmid/vector.¹⁵ The significant technical problem with genetically modified cells lies in the majority of injected cells dying within 48 hours *in vivo*, possibly due to ischemia, apoptosis, inflammation, or immunological rejection. The limitations of plasmid/vector include limited time of transgene expression and low transient protein secretion levels.^{8-10,15}

Therefore, in this study, we explored a new method of gene therapy. Our study is focused on the use of engineered tissues as stable, long-term vehicles for growth factor delivery in cardiovascular disease. Tissue engineering is a multidisciplinary approach for the *in vitro* construction of implantable tissues such as pancreatic islets, liver, skin, cartilage, bone, muscle, and blood vessels. While a primary goal of tissue engineering is to repair damaged tissue, engineered tissues may also serve as vehicles for protein delivery.⁸ Previous studies showed^{16,17} that skeletal muscle cells can be tissue engineered *in vitro* into organized functional organ-like muscle structures (BAMs) and, when implanted in non-muscle or muscle sites, they may survive for a long-term and deliver predictable levels of gene products, such as growth hormone, insulin-like growth factors, and VEGF for months. In this study, we found that primary rat myoblasts could be genetically engineered to secrete rhGH tissue-engineered into bioartificial muscles. Subcutaneous implantation of GH-BAMs into syngeneic rats improved cardiac performance in CHF rats.

Another advantage of implanting genetically engineered postmitotic myofibers (BAM) is that secretion levels of growth factors can be monitored *in vitro* before implant surgery. *In vivo* systemic levels of rhGH from implanted BAMs could be predicted from pre-implantation secretion levels. With BAM technology, the desired *in vivo* biological effect can be regulated by engineering BAMs with varying numbers of growth factor-secreting myofibers or by implanting varying numbers of BAMs into each animal. Protein delivery by injected myoblasts or by intramuscular injection of plasmid DNA is limited by the variability in the number of postmitotic muscle fibers that take up and express the foreign gene, making secretion levels difficult to predict.⁸

GH, a 191-amino acid protein produced by the anterior pituitary gland. Evidence suggests that the heart is a target organ of GH and IGF-1. GH and its mediator, IGF-1, are anabolic hormones that are essential to skeletal and myocardial growth and metabolic homeostasis.⁴ GH may increase myocardial contractility, produce peripheral vasodilatation, improve cardiomyocyte metabolic imbalance, stimulate cardiac hypertrophy, and inhibit

inflammatory cytokine generation and apoptosis.¹⁸⁻²⁰ Abnormal serum levels of GH, which acts on cardiac myocytes primarily through IGF-1, are associated with abnormalities of myocardial growth and function that can be ameliorated with restoration of GH/IGF-1 homeostasis, as demonstrated by experience with patients with deficiency or hypersecretion of GH.^{21,22} Clinical trials^{23,24} have demonstrated that GH therapy may increase left ventricular wall thickness, reduce chamber size, and improve hemodynamics and clinical status in adults with heart failure.

But in a number of clinical studies, rhGH did not result in the expected benefits in many patients and this was attributed to the inability to deliver the protein in a consistent manner. In any event, for clinical use, lack of patient compliance, side effects, and high cost make multiple daily injection of rhGH unlikely a widespread use.⁸ It is well known that the method of rhGH delivery determines the serum level obtained. For example, constant infusion of rhGH in rats has been shown to be more effective in stimulating insulin-like binding protein complexes (an important anabolic marker) than twice-daily injections.²⁵ Constant delivery of physiological levels of rhGH by infusion pumps might also be advantageous in optimizing its effects while minimizing side effects, but these devices are not currently available for long-term delivery of rhGH because of the instability of the protein at 37°C. Vandenberg et al⁸ demonstrated that the BAM-secreted rhGH was found to be more effective in attenuating acute skeletal muscle disuse atrophy induced by hind-limb unloading than daily rhGH injections. Thus, a major advantage of the constant synthesis and release of rhGH from a cell-based system (BAM) may be in obtaining more consistency in response to the hormone. In the present study, treatment with rhGH secreted from BAM increased posterior wall thickness, inhibited the progressive LV enlargement in rats with CHF, decreased serum TNF- α levels in the CHF treatment group. Histological analysis also demonstrated that rhGH induced a cardiac hypertrophic response without development of significant fibrosis.

This is the report that rhGH secreted from tissue-engineered bioartificial muscle improves left ventricular function in AMI rat. The use of retroviral vectors in our studies resulted in the stable integration of the rhGH gene into the host cell genome and long-term expression when implanted *in vivo*. Other advantages of this vector include its broad host range and the availability of packaging cell lines for the large-scale production of high-titre vectors.²⁶⁻²⁸

In summary, the cell-based delivery of rGH from a "living protein delivery platform" composed of fused, postmitotic muscle cells results in the improvement of left ventricular function in rats with CHF.

Extending rhGH BAM technology to human skeletal

muscle offers great potential for the treatment of clinical disease. Powell et al²⁹ found that human adult skeletal muscle cells isolated from elderly patients with congestive heart failure and genetically engineered to secrete rhGH can be formed into GH-BAMs. The subsequent implantation of human BAMs for gene therapy would offer the advantage of a predictable delivery platform having a high protein synthesis capacity and long-term survival (decades for skeletal myofibers). Several issues remain to be addressed in both small and large animal models before effective clinical trials are possible, including the regulation of secretion levels, effectiveness of implants at different target sites, and the potential existence of unfused myoblasts in the BAMs that could migrate to distant sites. With the resolution of these issues, rGH-BAM gene therapy could provide a new option for the future clinical treatment of heart failure as well as other cardiovascular disease.

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(Received January 4, 2009)

Edited by WANG Mou-yue and LIU Huan

Correction

In the original article entitled *Critical role of Δ DNMT3B4/2 in regulating RASSF1A promoter-specific DNA methylation in non-small cell lung cancer* published in September 5 issue, 2008 (*Chin Med J* 2008; 121(17): 1712-1721), the author LIU Nin-hong on page 1712 should be changed into LIU Ning-hong.