Fibrin patch-based insulin-like growth factor-1 gene-modified stem cell transplantation repairs ischemic myocardium

Jun Li^{1,2}, Kai Zhu^{1,2}, Shan Yang³, Yulin Wang^{1,2}, Changfa Guo^{1,2}, Kanhua Yin^{1,2}, Chunsheng Wang^{1,2} and Hao Lai^{1,2}

¹Department of Cardiac Surgery, Zhongshan Hospital, Fudan University, Shanghai 200032, P.R. China; ²Shanghai Institute of Cardiovascular Disease, Shanghai 200032, P.R. China; ³Department of Radiology, Zhongshan Hospital, Fudan University, Shanghai 200032, P.R. China

Corresponding authors: Hao Lai. Email: lai.hao@zs-hospital.sh.cn; Chunsheng Wang. Email: cswang@medmail.com.cn

Abstract

Bone marrow mesenchymal stem cells (BMSCs), tissue-engineered cardiac patch, and therapeutic gene have all been proposed as promising therapy strategies for cardiac repair after myocardial infarction. In our study, BMSCs were modified with insulin-like growth factor-1 (IGF-1) gene, loaded into a fibrin patch, and then transplanted into a porcine model of ischemia/reperfusion (I/R) myocardium injury. The results demonstrated that IGF-1 gene overexpression could promote proliferation of endothelial cells and cardiomyocyte-like differentiation of BMSCs *in vitro*. Four weeks after transplantation of fibrin patch loaded with gene-modified BMSCs, IGF-1 overexpression could successfully promote angiogenesis, inhibit remodeling, increase grafted cell survival and reduce apoptosis. In conclusion, the integrated strategy, which combined fibrin patch with IGF-1 gene modified BMSCs, could promote the histological cardiac repair for a clinically relevant porcine model of I/R myocardium injury.

Keywords: Insulin-like growth factor-1, mesenchymal stem cells, fibrin patch, myocardial infarction, cardiac repair

Experimental Biology and Medicine 2015; 240: 585–592. DOI: 10.1177/1535370214556946

Introduction

Myocardial infarction (MI) is projected to remain the leading cause of death globally in the coming decades. Bone marrow mesenchymal stem cells (BMSCs) transplantation has been proposed as a potential treatment to promote cardiac repair after MI. Meanwhile, engineered cardiac patch is considered as an applicable method for BMSCs transplantation.^{1–3}

To improve the curative effect of BMSCs, many strategies have been introduced to improve the paracrine effect of grafted cells, one of which is to modify BMSCs with therapeutic genes before transplantation.^{4,5} Insulin-like growth factor-1 (IGF-1) is a cardiogenic proliferation and differentiation factor.⁶ Evidence suggests that mice deficient in IGF-1 have an increased cardiomyocyte apoptosis after MI, while IGF-1 overexpression can protect cells against apoptosis.^{7,8} These findings provide us a clue that IGF-1 overexpression in ischemic myocardium might be beneficial to cardiac repair.

Here, we transplanted a fibrin patch, which was loaded with IGF-1 gene-modified BMSCs, into a porcine model of myocardial ischemia-reperfusion (I/R) injury, which closely

replicated the clinical relevant results. We hypothesized that IGF-1 overexpression-based tissue engineering method could represent an effective strategy for cardiac repair in the preclinical application.

Materials and methods

Isolation and culture of primary porcine BMSCs

Isolation of BMSCs was performed from the bone marrow of 8- to 12-week-old Shanghai white pigs (~22–28 kg) as previously described.⁹ Briefly, bone marrow was collected from the femur with a 16-gauge needle containing 200 U/mL heparin; then, the aspirates were depleted of mature blood-cell lineages and purified by centrifugation in a 1.073 g/mL Percoll (GIBCO, Grand Island, NY, USA) density gradient. The isolated mononuclear cells were transferred to tissue culture flasks and cultured in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% FBS (Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO, St. Louis, MI, USA). Non-adherent cells were discarded after 48 h, and adherent cells were cultured until confluent.

IGF-1 gene manipulation in BMSCs

The cDNA encoding IGF-1 gene was cloned and inserted into pCDH-CMV-MCS-EF1-coGFP vector at EcoRI and Xba I sites. IGF-1 shRNA was synthesized as following: IGF-1-shRNA sense, CCGGTGTTTCAGGAAACAAGAATACTCGAGTAGTTCTTGTTCCTGAACCT TTTTG, IGF-1-shRNA antisense, AATTCAAAAAAGTTCAGGAAA CAAGAAC TACTCGAGTAGTTCTTGTTCCTGAACA. After annealing, IGF-1-shRNA fragment was cloned into pLKO.1-TRC vector. The recombinant plasmids pCDH-IGF-1-GFP and pLKO.1-shRNA-IGF-1 were identified by DNA sequencing. These plasmids were amplified in *E. coli* DH5 α , extracted with a plasmid purification kit (Qiagen, Düsseldorf, GER) according to manufacturer's instructions, and transfected into 70% confluent 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The lentiviral particles were then harvested from the media 48 h after transfection, purified with ultracentrifugation, and assessed by FACS analysis for GFP on transduced control cells. One day before transplantation, BMSCs of passage 2 were transfected with IGF-1 gene or shRNA-IGF-1.

In vitro cardiomyocyte-like differentiation induction assay

To verify the effect of IGF-1 overexpression on cardiomyocyte-like differentiation, BMSCs of passage 2 were divided into four groups as follow: (1) control group: BMSCs were untreated; (2) MSC group: BMSCs were transfected with control lentivirus; (3) oeIGF-1 group: BMSCs were transfected with lentivirus encoding IGF-1 gene; (4) siIGF-1 group: BMSCs were transfected with lentivirus encoding shRNA-IGF-1. Cardiomyocytes were isolated from the left ventricle of neonatal Shanghai white pig (5 days old) (Shanghai Jiaotong university school of agriculture and biology, Shanghai, CHN) as previously described and plated at 5×10^5 cells per dish.¹⁰ Afterwards, BMSCs were co-cultured with cardiomyocytes for 21 days. The morphological changes in BMSCs from day 0 (before treatment) to day 21 were observed under microscopy. On day 7 and day 21, IGF-1 expression and cardiomyocyte specific markers (GATA-4, Nkx2.5, β -MHC and MEF2c) of treated BMSCs were identified with Western blot.

In vitro endothelial cell proliferation assay

To determine the angiogenic proliferation induced by IGF overexpression *in vitro*, time-course endothelial cell proliferation assays were performed. Human umbilical vein endothelial cells (HUVECs) (Type culture collection of the Chinese academy of sciences, Shanghai, CHN) were seeded in 96-well plates at a density of 5×10^3 cells/well. BMSCs in oeIGF-1 group were seeded in six-well plates. From day 0 to day 5, 100 mL of culture medium from the oeIGF-1 group was collected and transferred to HUVECs everyday. HUVEC proliferation was measured by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide blue-indicator dye]-based (MTT) assay on days 0, 1, 3, and 5. Culture medium from the MSC group was used to treat HUVECs as controls. HUVEC proliferation at day 0 was normalized at 100%.

Porcine model of ischemia/reperfusion injury

All animal experiments were approved by the Institutional Review Board and Institutional Animal Care and Use Committee Protocols of Fudan University.

Myocardial ischemia/reperfusion (I/R) injury was surgically induced in female Shanghai white pigs (~22–28 kg) (Shanghai Jiaotong University School of Agriculture and Biology, Shanghai, CHN) as described previously with some modifications.⁹ Briefly, pigs were anesthetized with pentobarbital (60 mg/kg, administered intravenously), intubated, and ventilated with a respirator; then, a left thoracotomy was performed, and the roots of 2–4 diagonal and/or obtuse marginal branches were dissected free of the underlying myocardium and occluded individually with ligatures. Lidocaine (2 mg/kg bolus before ligation, followed by 0.05 mg/kg per min) and amiodarone (5 mg/kg bolus before ligation, followed by 0.04 mg/kg per min) were administered intravenously at the time of occlusion; if ventricular fibrillation occurred, electrical defibrillation was performed immediately. Ischemia was confirmed by a change in color from red to purple (over a 2.5 to 3 cm² area), significantly weakened contractile activity, and significant ST-T changes in ECGs. The coronary arteries were reopened 60 min later to induce ischemia-reperfusion injury, which typically damaged 10–15% of the left ventricular (LV) mass.

Three out of 31 treated pigs were dead postoperatively. One pig died of ventricular fibrillation on the table 30 min after coronary ligation; the other two deaths occurred 10 min after reperfusion due to malignant cardiac arrhythmia. The rest 28 living pigs were divided into four groups (seven pigs per group).

Fibrin patch-based BMSCs administration

Fibrin patch application was performed as described previously with some modifications.¹¹ Briefly, 1 mL of thrombin solution (400 IU/mL thrombin in PBS with 4 mM CaCl₂ and 2 mM 6-aminocaproic acid [EAC]) was co-injected with 1 mL of fibrinogen solution (100 mg/mL lyophilized human fibrinogen in PBS with 4 mM CaCl₂ and 2 mM EAC) through a synchronous dual syringe into a ring-shaped mold positioned over the epicardial surface of the infarcted region. For animals in the cell-treatment groups, 1×10^7 BMSCs were added to the thrombin solution before injection. The solutions typically solidified within a few seconds to produce a circular patch of 3 cm in diameter and 3 mm in thickness.

Animals of I/R injury were treated randomly with cell-free fibrin patch (control group, $n=7$), 1×10^7 BMSCs transfected with the lentivirus encoding GFP only (MSC group, $n=7$), 1×10^7 BMSCs transfected with the lentivirus encoding GFP and shRNA-IGF genes (siIGF-1 group, $n=7$) and 1×10^7 BMSCs transfected with the lentivirus encoding GFP and IGF-1 genes (oeIGF-1 group, $n=7$). All the patches were positioned on the site of infarction. Animals in all four groups were administered cyclosporine (15 mg/kg per day with food) for immunosuppression as previously described.¹²

Heart function assessment

One week and four weeks after cell transplantation, MRI was performed on a 1.5-Tesla clinical scanner (AERA, Siemens AG, Erlangen, GER) with a phased-array 4-channel surface coil and ECG gating as previously described.¹² Briefly, animals were anesthetized with 2% inhaled isoflurane and positioned in a supine position within the scanner.

LV ejection fraction (LVEF) and systolic thickening fraction was evaluated from short- and long-axis cine images, and scar size was evaluated with delayed-enhancement MRI. Images were evaluated independently by two radiologists, and the data were analyzed with Argus flow tool and Syngo MMWP software (Version VE36A, Siemens, Munich, GER). Hemodynamic parameters were measured via a polyvinyl chloride catheter (3 mm outer diameter) inserted through the apical dimple and into the LV. Wall stress was calculated from the MRI and hemodynamic data according to the law of Laplace.¹³

$$\text{LV Wall stress} = \text{LV systolic pressure} \times \text{LV chamber radius} / (2 \times \text{LV thickness})$$

Histological evaluations

Four weeks post-transplantation, animals were sacrificed and the hearts were collected for subsequent histological analysis. LV weight (LVW) and right ventricular weight (RVW) were measured to assess the hypertrophy of myocardium after MI. Weight ratio was calculated by LVW or RVW versus body weight (BW). The LV was cut in a bread-loaf manner into six transverses, 1 cm thick sections from the apex to base. Tissues were fixed in 10% formalin/saline for 24 h, embedded in paraffin, cut in 4-mm sections, and then stained for histological and immunohistological assessments; evaluations were performed under a bright-field microscope.

Cardiomyocyte size was evaluated in hematoxylin-and-eosin (H&E)-stained sections. Endothelial cells were identified by staining for CD31 (ab24590, Abcam, Cambridge, UK). When counting capillary density, five sections of the peri-infarct area were chosen in each porcine. For each section, five fields were evaluated at high magnification. Total CD31 RNA of myocardium was extracted with TRIzol (Invitrogen) and then converted into cDNA with a Reverse Transcription kit (Takara, Otsu, JPN) as directed by the manufacturer's instructions. qRT-PCR analysis was performed using the SYBR Green kit (Qiagen). BMSCs' retention and survival were assessed via GFP expression, and cardiomyocytes were identified via cTnT expression (ab10214, Abcam). IGF-1, Bcl-2, and BAX expressions were evaluated with IGF-1 (ab80548, Abcam), Bcl-2 (sc492, Santa Cruz, Dallas, TX, USA), and BAX (1063-1, Epitomics, Burlingame, CA, USA) antibodies and via Western blot. Immunofluorescence was used to observe the grafted cell and fibrin patch in the myocardium.

Statistics

The data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA). All values were expressed as the

mean $\pm$ standard error of the mean. One-way analysis of variance with the *post hoc* Bonferroni test was performed to assess the significant difference among multiple groups. The significant difference between two groups was evaluated using the Student's *t*-test.

Results

Cardiomyocyte-like differentiation *in vitro*

The expression of cardiomyocyte specific makers (GATA-4, Nkx2.5, β -MHC and MEF2c) was analyzed by Western blot. Seven days after transfection, there was no significant difference in four groups ($P > 0.05$). However, compared with other three groups, oeIGF-1 group demonstrated the obviously enhanced cardiomyocyte specific makers expression on day 21 (Figure 1a) ($P < 0.05$).

HUVEC proliferation *in vitro*

HUVEC proliferation was stimulated and enhanced by IGF-1 overexpression. As shown in Figure 1(b), with three days of incubation, HUVECs treated with culture medium from oeIGF-1 group demonstrated greater proliferation than cells from control group ($P < 0.01$). With five days of treatment, HUVEC proliferation was significantly accelerated ($P < 0.01$).

Heart function assessment

The temporal changes in heart function as measured by MRI are illustrated in Figure 2. For LVEF, there was no significant difference among the four groups at baseline. The three cell-treated groups showed a significant improvement of LVEF than the control group ($P < 0.05$). The beneficial effect in LV chamber function persisted up to four weeks ($P < 0.05$). However, oeIGF-1 group did not show obvious advantage of LVEF than other cell-treated groups (Figure 2a). The systolic wall stress of the infarct zone (IZ) was significantly reduced in cell-treated groups compared to the control group ($P < 0.05$). However, no significant improvement was observed in the border zone (BZ) and remote zone (RZ) (Figure 2b). One week after MI, the systolic thickening fraction of the infarct zone (IZ) was significantly improved in cell-treated groups compared to the control group ($P < 0.05$), but no significant difference was found between oeIGF-1 and siIGF-1 groups. Four weeks later, oeIGF-1 group exhibited better systolic thickening fraction than siIGF-1 group ($P < 0.05$) (Figure 2c). The infarct size decreased with time elapsing. At four weeks after MI, cell-treated groups exhibited less infarct size than control group. And siIGF-1 group demonstrated more deteriorated infarct size than oeOGF-1 group ($P < 0.05$) (Figure 2d).

Histological improvement *in vitro*

MSC (3.38 ± 0.17 g/kg) and oeIGF-1 (3.10 ± 0.14 g/kg) groups resulted in a significant decrease in LV hypertrophy as reflected by a decrease in LVW/BW ($P < 0.05$) (Figure 3a) while it was compared to the hearts of control group

Figure 1 Cardiomyocyte-like differentiation and angiogenic proliferation induced by IGF-1 gene modification. (a) Western-blot analysis of the cardiomyocyte specific marker expression at 7 days and 21 days after gene transfection. (b) HUVEC proliferation stimulated by IGF-1 overexpression. * $P < 0.05$ as compared to oeIGF-1 group; ** $P < 0.01$ as compared to oeIGF-1 group

(3.80 ± 0.12 g/kg). The beneficial effect was impaired significantly in siIGF-1 group (3.6 ± 0.15 g/kg).

Compared with other groups, oeIGF-1 group exhibited significant decrease of BAX level and enhancement of Bcl-2 level ($P < 0.05$) (Figure 3b). And oeIGF-1 group demonstrated reduced cardiomyocyte size and increased angiogenesis ($P < 0.05$) (Figure 3(c) to (f)). Under fluorescence microscope, we found more grafted cell survival in oeIGF-1 group ($P < 0.05$) (Figure 3g). IGF-1, BAX, and Bcl-2 staining cell counting is similar with the Western-blot analysis results ($P < 0.05$) (Figure 3(h) to (j)).

Under fluorescence microscope, we observed that GFP-labeled BMSCs did not exhibit cTnT expression in the myocardium (Figure 4(a) and (b)). The myocardium contacted closely with the fibrin patch, which allowed cardiomyocyte and capillary ingrowth successfully (Figure 4(c) and (d)).

Discussion

Transplantation of therapeutic gene modified BMSCs have been proved to be more advantageous for cardiac repair than BMSCs alone.^{4,5} Engineered cardiac patch could

Figure 2 Heart function assessment. (a-d) MRI results of LVEF, systolic wall stress, systolic thickening fraction, and infarct size (arrow). * $P < 0.05$ as compared to control group. # $P > 0.05$ as compared to control group

deliver the cells effectively into myocardium.¹⁴ In our study, BMSCs were modified with IGF-1 gene, loaded in a fibrin patch, and then transplanted into a porcine I/R injury model. To our knowledge, this experiment is the first to demonstrate the therapeutic properties of fibrin patch loaded with IGF-1-overexpressing BMSCs in a clinically relevant large animal model.

Engineered cardiac patch is recently receiving growing attention as a new strategy for delivery stem cells. It aims to provide: (a) a mechanical support for myocardium replacement or regeneration; (b) a niche that favors cell reside, migration, integration, and proliferation for the desired therapeutic effect; and (c) a permeable construct that allows nutrient and waste transfer.¹⁴⁻¹⁶ In this study, fibrin was chosen as the patch material due to its

biocompatibility and capacity to be remodeled both *in vitro* and *in vivo*.^{17,18} We seeded IGF-1 gene-modified BMSCs into fibrin patch, and transplanted them into porcine ischemic myocardium. It demonstrated that the myocardium contacted closely with the fibrin patch, which allowed cardiomyocyte and capillary ingrowth successfully (Figure 4). Based on the findings mentioned earlier, we believe that cardiac patch loading with gene-modified BMSCs could be applied as a novel clinical therapy for cardiac repair following heart injuries.

Cytokines secreted from grafted cells have been demonstrated to be an important paracrine phenomenon in an ischemic animal model.¹⁹ Therapeutic gene manipulation in BMSCs enhances their paracrine action *in vivo*. IGF-1 binds and activates its receptor IGF-1 receptor, which

Figure 3 Histological assessment *in vivo*. (a) Comparison of LVW/BW and RVW/BW of the explanted heart. (b) Western-blot analysis of IGF-1, BAX, and Bcl-2 expressions. (c, d) H&E evaluation of cardiomyocyte size. (e) Capillary density based on CD31 immunostaining. (f) Western-blot analysis of CD31 expression. (g) Grafted cell survival counting *in vivo*. (h) IGF-1-positive cell counting *in vivo*. (i) BAX-positive cell counting *in vivo*. (j) Bcl-2-positive cell counting *in vivo*. * $P < 0.05$ as compared to oelGF-1 group; ** $P < 0.01$ as compared to oelGF-1 group. Scale bar = 100 μm . (A color version of this figure is available in the online journal.)

prevents cells from hydrogen peroxide-induced oxidative stress, mitochondrial dysfunction, and apoptosis. Subsequently, the biochemical event is followed by activation of downstream signaling cascades, including the PI3K/Akt pathway, a central regulator of cell growth, proliferation, survival, and metabolism.^{6,20,21} In our study, compared with other groups, oelGF-1 group demonstrated improved HUVECs proliferation *in vitro* (Figure 1c) and CD31 expression *in vivo* (Figure 3(d) and (e)). And oelGF-1 group also exhibited less LVW/BW ratio and cardiomyocyte size during the cardiac remodeling process (Figure 3(a) and (c)). Besides that, increased GFP/IGF-1-expressed BMSCs and decreased apoptosis level were observed in oelGF-1 group (Figure 3(b), (g) to (j)). Therefore, IGF-1 overexpression was proven to promote angiogenesis, inhibit remodeling, increase grafted cell survival, and reduce apoptosis in the present study. We believe that IGF-1 overexpressing could enhance paracrine effect of IGF-1 from BMSCs, which could benefit for cardiac repair eventually.

It is well known that IGF-1 could improve cardiogenic proliferation and differentiation.⁶⁻⁸ Here, it did demonstrate that overexpressing IGF-1 in BMSCs improved cardiomyocyte-like differentiation obviously *in vitro* (Figure 1(a) and (b)). But the IGF-1-overexpressed BMSCs did not exhibit cTnT expression in the myocardium (Figure 4(a) and (b)). It indicated that no obvious cardiomyocyte-like

differentiation occurred in grafted cells. We speculated that the harsh microenvironment in ischemic myocardium blocked the differentiation capacity of grafted BMSCs. In other words, the cardiomyocyte-like differentiation of grafted BMSCs might be not necessary for ultimate cardiac repair *in vivo*. Therefore, our study verified that the grafted BMSCs worked effectively through other mechanisms, such as the paracrine action.²²

MRI has been used to evaluate cardiac function since imaging of the heart with high temporal resolution became possible by cine MRI. It has been also established as golden standard for heart function assessment.²³ In our study, MRI results demonstrated that oelGF-1 group had better heart function than MSC group, but no significant difference was observed between two groups. It suggested that IGF-1 gene-modified BMSCs transplantation might be associated with moderate and weak improvement in global cardiac function despite histological results demonstrated benefit *in vivo*. Nowadays, stem cells therapeutic effect in clinical randomized controlled trials is still heterogeneous and disparate. Many trials demonstrated significant yet modest heart function improvement, while others achieved no significant enhancement.^{24,25} For effective application in clinic, optimization of cell delivery route, cell dose, and gene modification in BMSCs are necessary for improving heart function to a larger extent in future studies.

Figure 4 Immunofluorescence observation of grafted cells and fibrin patch in the myocardium. (a, b) GFP-labeled grafted cells (arrow) in myocardium. (c) Close junction between the transplanted fibrin patch and myocardium (arrow). (d) Angiogenesis (arrow) in the fibrin patch. Scale bar = 50 μ m. (A color version of this figure is available in the online journal.)

Conclusion

Our finding demonstrated that IGF-1 overexpression in BMSCs enhanced the paracrine action both *in vitro* and *in vivo*, which in turn benefited the cardiac repair in a porcine I/R injury model, especially delivered through a fibrin patch. Further studies are ongoing to amplify their therapeutic effect for clinical application.

Authors' contributions: HL and CW designed the study; JL, KZ, SY, and YW conducted the experiments; JL, KZ, CG, and KY analyzed the data; and JL and KZ wrote the manuscript. JL and KZ contributed equally to this work. JL and KZ are co-first authors and contributed equally to this article.

ACKNOWLEDGEMENTS

We would like to thank Prof. Jianyi Zhang from University of Minnesota Medical School for his kindly help and excellent technical support. The paper was supported by the National Science Foundation of China (Grant Nos. 81301312 and 81270326) and Youth Foundation of Zhongshan Hospital (2013ZSQN15).

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(Received May 19, 2014, Accepted September 8, 2014)