

## Original Article

# Cell transplantation into ischemic myocardium using mesenchymal stem cells transfected by vascular endothelial growth factor

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**Abstract:** Aims: To investigate the effects of mesenchymal stem cells (MSCs) transplantation combining with vascular endothelial growth factor (VEGF) gene therapy on myocardium rebuilding, angiogenesis, and heart function improvement in rats with myocardial infarction. Methods: SD rat MSCs were isolated, cultured in vitro, labeled with BrdU and transfected by Ad.VEGF gene. Four weeks after left anterior descending artery was ligated to create rat myocardial infarction, cardiac function was examined with echocardiography. Rats were randomly divided into four groups (n = 10 in each group): Group I: MSCs/Ad.VEGF implantation; Group II: MSCs implantation; Group III: Ad.VEGF injection; Group IV: Control. MSCs differentiation was observed 4 weeks after transplantation. Immunohistochemistry and angiogenesis were observed. Echocardiography was performed to detect the effects on heart function. Results: MSCs labeled with BrdU could be identified in host hearts in group I and II, most of them positively stained with cTnT antibody. Echocardiography indicated that the improvement of the LVEF value in group I was more significant than that in the other three groups ( $P < 0.01$ , respectively). Some cells were incorporated into the coronary capillaries in the infarcted region. The capillary density in group I was higher than that in the other three groups ( $P < 0.01$ , respectively). Conclusion: MSCs implantation combining with VEGF gene therapy can obviously repair damaged myocardium and enhance the angiogenesis in ischemic heart tissue.

**Keywords:** Mesenchymal stem cells, vascular endothelial growth factor, cell transplantation, myocardial ischemia

## Introduction

Cell transplantation, as a modern therapy for heart failure posterior to myocardial infarction, has been focused by scientists in recent years. Although researchers previously succeeded in performing the allogeneic myocardial transplantation with cells including embryonic stem cells and fetal cardiomyocytes, the application of this method was confined for immunological and ethic concerns. After that, autologous cells including autologous skeletal muscle satellite cells and smooth muscle cells, which could partially improve the cardiac function after transplantation, were used to replace the infarcted myocardia. However, with the aging of body, the number of satellite cells within the skeletal muscle would decrease and the remains were inadequate and difficult to be isolated for cell transplantation. Skeletal muscle cells differ

from myocardial cells in their excitation contraction coupling and mechanic properties, so it is difficult to establish an excitation contraction coupling between the donor satellite cells and recipient cardiomyocytes. The application of skeletal muscle cells in transplantation was thereby confined. Certainly, transplantation with myocardial cells also shows diverse defects such as insufficient myocardial cells supply and low survival of cultured cells which limit its use.

In recent years, with the development of stem cell engineering, mesenchymal stromal cells (MSCs) became increasingly involved in the treatment of ischemic heart disease. As a type of multipotent stem cell, myeloid MSCs can directionally differentiate to cardiac-like cells under certain conditions, indicating the application of myeloid stem cells in the treatment for

## Mesenchymal stem cell transplantation into ischemic myocardium

heart diseases. Autologous MSCs transplantation is characterized by the following advantages: (1) MSCs can multipotently differentiate to diverse mesoderm cells including contractible myocardial cells and vascular endothelial cells; (2) autologous MSCs can be free from the immunological reactions; (3) as autologous cells, MSCs are easy to be harvested and amplified and easy to differentiate both in vivo and in vitro; (4) MSCs are good cellular vector which can carry target gene during transfection; (5) no carcinogenic evidence for MSCs is found. Therefore, it is currently believed that MSCs are good donor cells in cell transplantation. Some researches showed that MSCs transplantation could promote vascular regeneration and improve cardiac function despite the lower survival of transplanted cells.

As a common specific and potent cytokine in accelerating angiogenesis, vascular endothelial growth factor (VEGF) has currently been used in the treatment of myocardial ischemia. Because the principal pathological change of heart failure induced by ischemic heart disease is left ventricular remodeling and myocardial blood supply deficiency caused by scars formed posterior to the infarction, the transplantation of myeloid MSCs transfected with VEGF gene is expected to improve the myocardial ischemia and heart function via the following two routes: 1. myocardial tissue regenerates from the transplanted cells to improve left ventricular remodeling; 2. the myocardial perfusion is improved and surroundings for the survival of transplanted cell are optimized by VEGF-induced angiogenesis. Both are achieved by combining cell therapy to gene therapy.

In this study, based on the self-made VEGF encoded replication deficient adenovirus (Ad-VEGF), the myeloid MSCs were transfected with VEGF in vitro and transplanted into animal ischemic heart disease model. The efficacy of this method was also evaluated through investigating the myocardial revascularization, survival rate of transplanted cells and improvement of heart function.

### Materials and methods

#### *Reagents and animals*

The following reagents were used in the study: Iscove's modified Dulbecco's medium (IMDM, Gibco, US), fetal bovine serum (FBS, Hyclone, US), Ficoll-paque solution (Pharmacia, US),

trypsin (Shanghai Biochemistry, Shanghai, China), EDTA (Shanghai Biochemistry, Shanghai, China), Bromodeoxyuridine (BrdU, Sigma, US), rabbit anti-mouse anti-factor VIII polyclonal antibody (Maixin, Fuzhou, China), immunohistochemistry kit (Boster, Wuhan, China) and restriction enzymes HindIII and Sal I.

Forty one month SD rats, half males and half females, weighing 90-100 grams, were supplied by Laboratory Animal Center from Sun Yat-Sen University. After the experiment was approved by Ethics Committee, the animals were randomized into four groups.

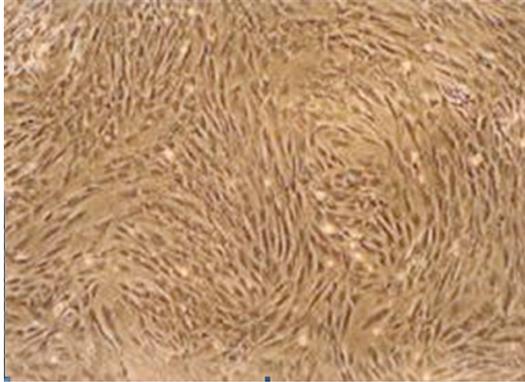
#### *Isolation, culturing, purification and labeling of rat MSCs*

After the animals were sacrificed and immersed in 75% alcohol for 5 minutes, their lower long bones were aseptically sampled. The bone marrow cavity was then exposed and rinsed in IMDM containing 15% FBS before the rinse solution was collected and centrifuged at 2000 rpm for 15 minutes. The precipitation was suspended in IMDM, softly added on 1.077 g/ml lymphocyte separation liquid and then centrifuged at 2000 rpm for 10 minutes. After the monocytes located in the central layer were collected, suspended in IMDM and centrifuged at 1000 rpm for 10 minutes, the product cells were harvested, suspended in IMDM containing 15% FBS and inoculated in culture bottles which were incubated in a CO<sub>2</sub> incubator at 37°C, with the culture medium changed per three days. When 90% of cells fused, primary cells were digested in 0.25% trypsin and 0.1% EDTA and subject to passage as a ratio of 1:3. The well grown second generation cells were labeled with BrdU before they were used in the transplantation. The labeling was performed because the labeled MSCs could be traced in the heart specimen posterior to the transplantation. As an analogue of thymidine, BrdU can be incorporated into nuclei during DNA synthesis before mitosis and can be detected using immunohistochemistry. In this study we used BrdU to label cells for transplantation and found a vast number of BrdU labeled cells in the infarct area, suggesting the survival of transplanted cells.

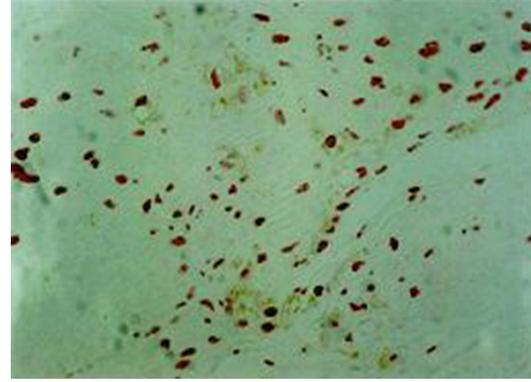
#### *Establishment of experimental myocardial ischemia model in rats*

SD rats, weighing 250~300 grams, aged 6~8 weeks, were subject to tracheal cannula using

## Mesenchymal stem cell transplantation into ischemic myocardium



**Figure 1.** Ten days after the passage, MSCs showed spindle appearance and fused together ( $\times 100$ ).



**Figure 2.** Four weeks after MSCs transplantation, BrdU immunohistochemical staining showed yellow BrdU-labeled MSCs in the infarct area ( $\times 200$ ).

intravenous catheter after they were anesthetized with ketamine hydrochloride intraperitoneally administered (50~70 mg/kg). Their breath was supported by a breathing machine before the continuous inhalation anesthesia was performed. Animals' left anterior descending coronary were exposed via the incision at the left fifth lateral intercostal space and ligated along the lower margin of the left auricle using 6-0 atraumatic suture. After ligation, the local myocardia at left ventricular anterior wall turned pale and synchronous ECG monitoring showed typical ischemic manifestation which indicated a successful establishment of experimental model. 5-10 minutes later, the thorax was closed before the circulation of animals was demonstrated stable.

### *In vitro transfection of VEGF gene to MSCs*

Cloning and sequencing of VEGF cDNA, construction of deficient recombinant adenovirus plasmid and adenovirus mediated production and amplification of VEGF (Ad.VEGF) were performed in Central Laboratory of our hospital. The experimental MSCs were transfected using Ad.VEGF one day before the transplantation and the multiple of infection (MOI) was designated as 50. The controls were transfected with blank adenoviral vector. To determine the transfection rate, some of the MSCs were transfected with Ad.GFP containing green fluorescent protein gene. Twenty-four hours after transfection, nearly all the cells presented green fluorescence indicating a successful transfection. The transfection rate was up to 98%.

### *MSCs transplantation*

Four weeks after the myocardial ischemia model was successful established, the thorax was opened along the intercostal space next to the original incision for cell transplantation. Forty animals were randomized into four groups: Group I (MSCs/Ad.VEGF group, injected with  $4 \times 10^6/50 \mu\text{l}$  MSCs which were previously transfected with Ad.VEGF in vitro); Group II (MSCs group, injected with MSCs  $4 \times 10^6/50 \mu\text{l}$ ); Group III (Ad.VEGF group, injected with Ad.VEGF  $5 \times 10^7$  pfu/ $50 \mu\text{l}$ ); Group IV (control group, injected with blank IMDM  $50 \mu\text{l}$ ).

### *Heart function determination*

Before and four weeks after the MSCs transplantation, the heart function of the animals was determined based on the value of left ventricular ejection fraction (LVEF) and left ventricular end-diastolic diameter (LVDD) determined by ultrasonography (HP 5500).

### *Pathological examination*

Four weeks posterior to the cell transplantation, after the animals were sacrificed by overdosing of pentobarbital sodium, the hearts of animals were harvested, fixed in formaldehyde, dehydrated, embedded in paraffin and made into  $5 \mu\text{m}$  successive sections ensuring the apex, base and infarct area of the heart could be sampled. In order to determine the survival and transformation of the transplanted cells, the labeling substance BrdU and a specific myocardial protein marker cTnT were examined immunohistochemically and routinely as

# Mesenchymal stem cell transplantation into ischemic myocardium

**Table 1.** LVEF values from the groups prior and posterior to the transplantation

Groups	MSCs/Ad.VEGF	MSCs	Ad.VEGF	Controls
Prior to the transplantation	0.427 ± 0.036	0.431 ± 0.022	0.419 ± 0.053	0.432 ± 0.027
Four weeks posterior to the transplantation	0.633 ± 0.051*	0.601 ± 0.017*	0.428 ± 0.056	0.391 ± 0.042

Notes: \*P < 0.01, as compared with the controls.

**Table 2.** LVDd values from the groups prior and posterior to the transplantation (mm)

Groups	MSCs/Ad.VEGF	MSCs	Ad.VEGF	Controls
Prior to the transplantation	6.63 ± 0.75	6.78 ± 0.44	6.71 ± 0.92	6.77 ± 0.91
Four weeks posterior to the transplantation	6.08 ± 0.66*	6.21 ± 0.47*	6.66 ± 0.55	9.68 ± 0.71

Notes: \*P < 0.01, as compared with the controls.

described in the instruction of the kits. Meanwhile, the factor VIII was examined in the specimen from all the groups to determine the number of new blood vessels. After immunohistochemically stained with factor VIII, the sections were observed microscopically to calculate the numbers of new capillaries: numbers of new capillaries from 5 microscopic visual fields were averaged to obtain the final number of new capillaries.

## Statistical analysis

All data presented were mean ± S.D. One way ANOVA was used in the comparison of multiple means. The statistical analysis was performed by using SPSS 10.0 software. *P*-value < 0.05 was considered statistically significant.

## Results

### Properties of MSCs

The primary MSCs began to adhere to the wall at 6 hours after culturing, divided and showed a spindle appearance as fibroblasts had at 48 hours after culturing and proliferated and showed diverse appearances like fibroblasts and large flat cells at 72 hours after culturing. Because of absent of adherence properties, the myeloid hematopoietic cells would be gradually removed during the solution change and these cells would disappear at 5 to 6 days after culturing. With the continuous proliferation of the colony, the cells would arrange in a certain rule called "whirlpool" growth. 7 to 12 days after culturing, the number and diameter of colonies in the culture bottle increased. The tightly arranged central cells began to fuse together and showed a spindle appearance. The transferred MSCs were round, distributed in cluster and totally adhered and extended

within 24 hours when they would change to long spindle or large polygonal cells. The transferred MSCs had short growth latency and good proliferating ability. 7 to 10 days later, the cells fused together (**Figure 1**).

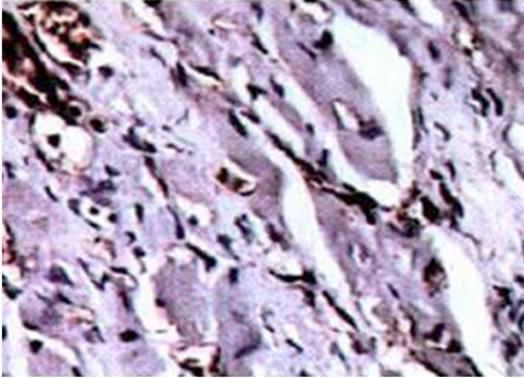
### Survival and differentiation of transplanted cells

Four weeks posterior to MSCs transplantation, in groups I and II, the yellow transplanted cells, labeled with BrdU, were found inside the infarct area, instead of the normal myocardial area. The transplanted cells, resembling fetal cardiac cells, were smaller than the autologous mature myocardial cells, but with a larger nucleus (**Figure 2**). In groups III and IV, the myocardial cells in the infarct area disappeared and were replaced by fibrous tissue.

Meanwhile, a routine HE and cTnT immunohistochemistry assay were used to evaluated the transplanted cells in groups I and II. It was found that the transplanted cells, not the controls, were positively stained with cTnT, showing myoid cell appearance and displaying yellow particles in their cytoplasm.

### Changes of heart function prior and posterior to MSCs transplantation

Four weeks posterior to MSCs transplantation, the heart function in groups I and II (MSCs/Ad.VEGF group and MSCs group) was improved, i.e., LVDd and volume of ventricular chamber decreased and LVEF increased in groups I and II when compared with those in group IV (*P* < 0.01). The LVEF in group I (those transplanted with VEGF transfected MSCs) was more improved than that in group II (those merely transplanted with simple MSCs) (**Tables 1 and 2**).



**Figure 3.** Four weeks after transplantation, factor VIII immunohistochemical staining showed the new capillaries in MSCs/Ad.VEGF group were significantly more than those in MSCs group ( $\times 200$ ).

Four weeks posterior to Ad.VEGF injection, LVDd and LVEF in group III (Ad.VEGF group) did not change significantly. However, 4 weeks posterior to serum free IMDM injection, LVDd and volume of ventricular chamber increased and LVEF decreased when compared those prior to cell transplantation.

### *Angiogenesis*

In this study, it was found that, 4 weeks posterior to MSCs transplantation, a number of BrdU stained cell differentiated to endothelial cells and were involved in the angiogenesis in local infarct area. A few red blood cells were also seen in blood vessels made of transplanted MSCs.

As compared with that in group IV, there was a significant angiogenesis in groups I and III ( $P < 0.01$ ). The new derived capillaries distributed uniformly between transplanted cells and remaining myocardial cells, both in center and in border. Among the groups, the new derived capillaries in group I was more than that in group III ( $P < 0.05$ ); those in group II was fewer than that in groups I and III ( $P < 0.01$ ), but more than that in group IV where no obvious angiogenesis was seen ( $P < 0.01$ ) (Figures 3, 4).

### **Discussion**

As a terminally differentiated cell, myocardial cells can not regenerate and are only substituted by scars when they are necrotized, which will lead to a refractory heart failure or even death. Because the clinical means currently used for myocardial infarction treatment did



**Figure 4.** Immunohistochemical findings in MSCs group 4 weeks after transplantation ( $\times 200$ ).

not contribute to the regeneration of necrotized myocardia, rebuilding of originally ischemic myocardia became a key topic in the research of myocardial infarction treatment. Stem cell transplantation is such a method which provides a new means for the treatment of heart infarction. Due to the presence of multi-potent stem cells, myeloid MSCs can differentiate into diverse tissues such as muscles and vascular endothelia under certain conditions. Additionally, the cells are easy to be obtained and amplified in vitro, so they are considered as ideal donor cells in ischemic myocardium rebuilding [1-3].

Because the principal pathological changes of heart failure induced by ischemic heart disease are left ventricular remodeling and myocardial blood supply deficiency which were caused by the scars formed posterior to the infarction, we combined the VEGF transfection and MSCs transplantation together on the basis of the previous experimental outcomes [4]. As an exclusively specific cytokine to promote the mitosis of vascular endothelial cells, VEGF can accelerate the proliferation of vascular endothelial cells and plays an important role in the angiogenesis in vivo. The previous studies showed that, at the ischemic area during heart infarction, exogenous VEGF gene therapy could promote the regeneration of capillaries, enhance the density of blood vessels, change the endothelium-dependent structure and function and improve the collateral blood circulation and heart function through continuous VEGF expression [5].

As detected by using cardiac ultrasonography, the animals in groups I and II (MSCs/Ad.VEGF

group and MSCs transplantation group) showed an improved LVEF and LVDD, indicating that MSCs could improve the heart function and inhibit the left ventricular remodeling after they were transplanted into the infarct area. In the present study, the more improved heart function occurred in MSCs/Ad.VEGF group than in simple MSCs transplantation group, suggesting that combination of cell transplantation to VEGF gene transfection would produce a synergetic effect. This may be because MSCs transfected with VEGF would cause a significant increase of new capillaries at local area after they were transplanted. This could be demonstrated by our immunohistochemical factor VIII and local vascular density determinations. We also revealed that simple Ad.VEGF transplantation contributed to a particular angiogenesis at the local infarct area rather than an improvement of heart function when compared with the controls. This may be because: although it had been reported that VEGF could arouse the local dormant myocardia surviving during the infarction to improve the heart function, few myocardia would survive at the local area of a chronic ischemic infarction model since the condition the model presented was a transmural myocardial infarction where few myocardial cells would remain alive; without new supplement of exogenous myocardial cells, the simple VEGF administration could not definitely improve the heart function.

Though MSCs were reported differentiating to endothelial cells in the microenvironment in vivo, involved in angiogenesis posterior to the transplantation and secreting VEGF [6], we revealed that the survival of MSCs was closely related to the blood supply. During the early phase of transplantation, the transplanted MSCs were vulnerable because the VEGF the cells secreted was inadequate for the growth of new blood vessels. The relationship between transplanted cells and new blood vessels can be described as "seed" and "soil" [7, 8]. Under this condition, VEGF supplement was of importance. Therefore, we transfected Ad.VEGF into MSCs in vitro to make the cells synchronously express VEGF, which could strengthen the therapeutic effects from the following two aspects: 1. the dormant myocardial cells at the border of infarct area received an enhanced blood supply which could arouse and improve their systolic function via a vast number of new capillaries induced by secreted VEGF [9, 10]; 2. The local

blood vessels were directly dilated to provide an ideal environment for the proliferation of MSCs and improve the survival rate of transplanted cells [11, 12].

In this study, through combining MSCs transplantation and VEGF transfection together, the transplanted cells could secrete VEGF to promote regeneration of myocardia and formation of blood vessels thus improving the heart function. Moreover, as providing a good environment for the growth of transplanted cells and having a synergetic effect, the combination of MSCs transplantation and VEGF transfection has a good prospect for clinical application.

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### Disclosure of conflict of interest

None.

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### References

- [1] Wang JS, Shum-Tim D, Galipeau J, Chedrawy E, Eliopoulos N, Chiu RC. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. *J Thorac Cardiovasc Surg* 2000; 120: 999-1005.
- [2] Ji GR, Lin X, Han JF, et al. Study on osteogenic potential of cultured mesenchymal stem cells transfected with Ad-BMP-2. *Chin J Microsurgery* 2004; 27: 46-47.
- [3] Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, Jia ZQ. Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation* 1999; 100: 247-56.

## Mesenchymal stem cell transplantation into ischemic myocardium

- [4] Hua P, Xiong LH, Zhang H, Chen J, Zhang HZ, et al. Experimental study on the effect of repairing and reconstruction by mesenchymal stem cells. *Chin J Microsurgery* 2004; 27: 122-124.
- [5] Yoon YS, Johnson IA, Park JS, Diaz L, Losordo DW. Therapeutic myocardial angiogenesis with vascular endothelial growth factors. *Mol Cell Biochem* 2004; 264: 63-74.
- [6] Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerated infarcted myocardium. *Nature* 2001; 410: 701-705.
- [7] Kobayashi T, Hamano K, Li TS, Katoh T, Kobayashi S, Matsuzaki M, Esato K. Enhancement of angiogenesis by the implantation of self bone marrow cells in rats ischemic heart model. *J Surg Res* 2000; 89: 189-195.
- [8] Akhaldi A, Eliopoulos N, Martineau D, Lejeune L, Lachapelle K, Galipeau J. Postnatal bone marrow stromal cells elicit a potent VEGF-dependent neoangiogenic response in vivo. *Gene Therapy* 2003; 10: 621-629.
- [9] Choudhery MS, Khan M, Mahmood R, Mehmood A, Khan SN, Riazuddin S. Bone marrow derived mesenchymal stem cells from aged mice have reduced wound healing, angiogenesis, proliferation and anti-apoptosis capabilities. *Cell Biol Int* 2012; 36: 747-53.
- [10] Khan WS, Hardingham TE. Mesenchymal stem cells, sources of cells and differentiation potential. *J Stem Cells* 2012; 7: 75-85.
- [11] Caplan AI. Why are MSCs therapeutic? New data: new insight. *J Pathol* 2009; 217: 318-324.
- [12] Nuri MM, Hafeez S. Autologous bone marrow stem cell transplant in acute myocardial infarction. *J Pak Med Assoc* 2012; 62: 2-6.