Science in China Series C: Life Sciences

www.scichina.com
life.scichina.com
www.springerlink.com

Treatment of chronically myocardial ischemia by adenovirus-mediated hepatocyte growth factor gene transfer in minipigs

YUAN Biao[1*], ZHANG YouRong[2*], ZHAO Zhong[2], WU DanLi[2], YUAN LiZhen[2], WU Bin[2], WANG LiSheng[2] & HUANG Jun[1†]

[1] The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China;
[2] Beijing Institute of Radiation Medicine, Beijing 100850, China

Growth factor gene transfer-induced therapeutic angiogenesis has become a novel approach for the treatment of myocardial ischemia. In order to provide a basis for the clinical application of an adenovirus with hepatocyte growth factor gene (Ad-HGF) in the treatment of myocardial ischemia, we established a minipig model of chronically ischemic myocardium in which an Ameroid constrictor was placed around the left circumflex branch of the coronary artery (LCX). A total of 18 minipigs were randomly divided into 3 groups: a surgery control group, a model group and an Ad-HGF treatment group implanted with Ameroid constrictor. Ad-HGF or the control agent was injected directly into the ischemic myocardium, and an improvement in heart function and blood supply were evaluated. The results showed that myocardial perfusion remarkably improved in the Ad-HGF group compared with that in both the control and model groups. Four weeks after the treatment, the density of newly formed blood vessels was higher and the number of collateral blood vessels was greater in the Ad-HGF group than in the model group. The area of myocardial ischemia reduced evidently and the left ventricular ejection fraction improved significantly in the Ad-HGF group. These results suggest that HGF gene therapy may become a novel approach in the treatment of chronically ischemic myocardium.

gene therapy, hepatocyte growth factor, adenovirus, chronically ischemic myocardium, therapeutic angiogenesis

The main pathological characteristic of coronary heart disease is coronary vessel blockage resulting in myocardial ischemia. A number of therapeutic measures, including percutaneous transluminal coronary angioplasty, coronary bypass surgery, and transmyocardial laser revascularization, have been effectively used in the treatment of coronary heart disease[1,2]. In addition, the application of gene transfer strategies involving angiogenic factor genes, including those encoding vascular endothelial growth factor (VEGF)[3], fibroblast growth factor (FGF)[4], and hepatocyte growth factor (HGF)[5], has become a potential therapeutic approach for coronary heart disease.

HGF, also known as scatter factor, is a multifunctional factor with strong angiogenic activity[6]. It induces angiogenesis via a number of signal pathways, including mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3′-kinase signaling (PI3K), and sphingosine kinase[7,8]. HGF also reduces the necrosis of myocardiocytes during acute myocardial infarction[9,10]. During acute myocardial ischemia, the HGF concentration in the circulating blood and the c-met receptor mRNA levels in heart are elevated significantly[11,12]. HGF exerts a protective effect on acute ischemic cardiomyocytes during reperfusion, and direct injection of

Received December 18, 2007; accepted April 12, 2008
doi: 10.1007/s11427-008-0073-1
†Corresponding author (email: yuan.biao@live.cn)
* Contributed equally to this work
Supported by National “863” Programme (Grant Nos. 2003AA216080 and 2007AA021007)

the HGF protein reduces the infarct area and improves heart function. On the contrary, blocking endogenous HGF leads to enlargement of the infarct area and mortality[13]. These results indicate that the therapeutic effect of HGF on ischemic myocardiocytes is mediated through protection of the myocardium and promotion of blood vessel formation.

In animal models of acutely ischemic myocardium, transduction of the HGF gene into the rat heart was observed to reduce acute injury from reperfusion of the ischemic myocardium[14]. Moreover, bone marrow-derived mesenchymal stem cells highly expressing HGF have a therapeutic effect on acutely ischemic myocardium[15]. The therapeutic effects of HGF gene on ischemic myocardium are attributed to its angiogenic, antifibrotic and antiapoptosis actions[16,17]. Recent studies showed that HGF gene transfer is also beneficial in the treatment of chronic myocardial ischemia in mice and canine models[18,19]. In the present study, we evaluated the curative effect of adenovirus-mediated HGF gene transfer in a minipig model of chronically ischemic myocardium. Because the anatomicophysiological features of the minipig heart are very similar to those of the human heart, the results of this study can provide a more direct basis for conducting clinical trials of HGF.

1 Materials and methods

1.1 Ad-HGF

The replication-deficient adenovirus carrying human HGF cDNA (Ad-HGF) is constructed and prepared by Chinese Academy of Military Medical Sciences. Ad-HGF was measured according to the criteria set up by the National Institute for the Control of Pharmaceutical and Biological Products, China. The viral concentration is higher than 1.0×10^{10} particles/mL.

1.2 Experimental animals

Eighteen minipigs of both sexes of the Huanjiang strain were purchased from the Experimental Animal Center of Chinese Agriculture University. Their body weights ranged from 20 to 30 kg. These minipigs were randomly divided into 3 groups: (1) a control group (n = 5), in which each pig was subjected to 2 thoracotomies conducted at an interval of 3 weeks; (2) a model group (n=5), in which an Ameroid constrictor (Research Instruments @ MFG, USA) was placed around the root of the circumflex branch of the left coronary artery (LCX) during the first thoracotomy, and 1-ml sterile normal saline was evenly injected into the heart muscle at 10 locations chosen randomly at the transitional zone between the ischemic zone and the normal heart muscle during the second thoracotomy[20]; and (3) an Ad-HGF group (n=8), in which the Ameroid constrictor was placed in the same manner as in the model group but in which 1×10^9 pfu Ad-HGF was injected instead of saline. The minipigs were closely observed during the entire experimental period. The general appearance, water and fodder intake, body weight, mental state, respiration rate, mobility, gait, and body temperature of the pigs were monitored and recorded.

1.3 Establishment of the chronic myocardial ischemia model

First, ketamine (10—15 mg/kg) was injected intramuscularly (i.m.) to induce anesthesia. This was followed by the insertion of endotracheal tubing, the mechanical management of respiration, continuous anesthesia using pentobarbital sodium, lateral thoracotomy through the left fourth intercostal space, longitudinal dissection of the pericardium, exposure of the LCX, placing the Ameroid constrictor (inner diameter, 2.0—2.5 mm) around the root of the LCX for the pigs in the model and Ad-HGF groups, closure of the pericardium and the thoracic cavity, and ECG monitoring. After complete recovery of spontaneous respiration, the endotracheal tube was removed and penicillin (8000 U) was injected i.m. Subsequently, the pigs were sent to the animal house, fed with standard forage, and closely monitored. The second thoracotomy was performed 3 weeks after the first one. All these experiments were conducted in the Animal Center of Najing Medical University.

1.4 SPECT imaging

Single-photon emission computed tomography (SPECT) imaging was performed twice for every pig: the first immediately before the second thoracotomy, and the second at the end of the observation period. The radioactivity distribution in each myocardial segment was evaluated using a 5-point scoring method in which 0 signifies no radioactivity; 1, significant reduction in radioactivity; 2, moderate reduction in radioactivity; 3, slight reduction in radioactivity; and 4, normal radioactivity. Because only the LCX branch was targeted in this study, the radioactivity distribution was evaluated only in the lateral wall of the left ventricle.

1.5 Echocardiography

Echocardiography was performed twice simultaneously with SPECT imaging in each pig to observe the systolic and diastolic function of the heart. The systolic parameters included parameters such as fractional shortening of the short axis (FS), ejection fraction (EF), proportion of segmental wall thickening (RSWT), and speed and amplitude of the contraction of the local wall. The diastolic parameters included the slope of the EF, E-point to septal separation (EPSS), left ventricular end-diastolic pressure (LVEP), and the flow-rate ratio between the P wave and A wave (Ev/Av).

1.6 Angiography of the coronary artery

Angiography was performed twice simultaneously with SPECT imaging in each pig. After being narcotized intravenously, each pig was placed in the supine position. A cannula was inserted into the carotid artery; angiography was then performed to determine the degree of closure of the LCX. The Rentrop method was adopted for grading collateral circulation.

1.7 Pathological examination

At the end of the clinical observation period, i.e. 4 weeks after the second thoracotomy, all the pigs were sacrificed by exsanguination and their hearts were removed. Transverse sections were obtained at intervals of 2 mm in the upward direction from the heart apex, and 3 myocardial samples were obtained from each section to observe the ischemic zone (short axis, 30°—120°), the transitional zone (short axis, 120°—135°), and the normal zone (short axis, 135°—180°). These samples were fixed in 40% neutral formaldehyde and embedded in paraffin. Histopathological sections of 5-μm thickness were prepared from each myocardial sample and stained with hematoxylin-eosin and hematoxylin-basic fuchsin-picric acid separately.

1.8 Immunohistochemical staining for blood vessel factor VIII

After the deparaffinization and hydration steps, the histopathological sections were placed in distilled water and then in citrate buffer solution under high temperature and high pressure conditions for antigen recovery. The Elivision^{TM} plus kit was then used for identifying factor VIII-positive endothelial cells.

1.9 Statistical analysis

All data are expressed as mean±standard deviation (x±SD). For intergroup comparison, the t test was used.

2 Results

2.1 The validity of the model

Three weeks after the implantation of the constrictor ring, the LCX was completely closed but the left anterior descending (LAD) branch of the coronary artery was open. The entire Ameroid constrictor was still positioned at the bifurcating site of the LCX and had completely constricted the LCX in all pigs of the model and Ad-HGF groups when they were sacrificed at the end of the observation period (Figure 1).

In the model group, 1 pig died of left heart insufficiency at week 3 after the second operation, while all the pigs in the control group and the Ad-HGF group survived till the end of the observation period.

2.2 Improvement of chronic myocardial ischemia by Ad-HGF

Blood vessel density was evaluated in the coronary angiogram by adopting the Rentrop method. The density in the control group was evidently higher than that in the other 2 groups before the second surgery. However, the density in the Ad-HGF-treated group was remarkably higher than that in the model group, in which not even a slight increase was noted (Table 1).

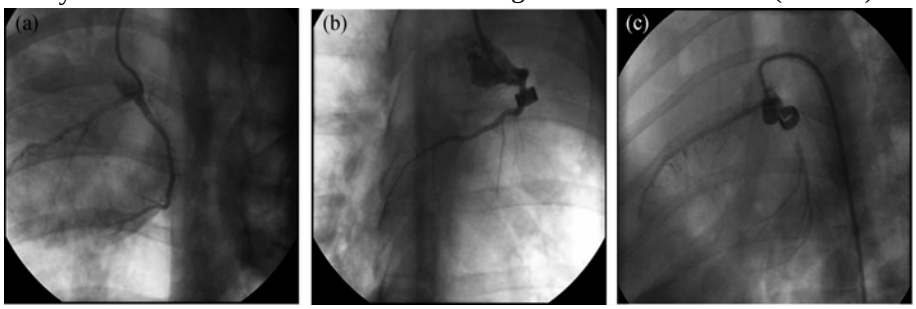

Figure 1 Coronary angiograms taken 4 weeks after the second thoracotomy. (a) Normal image of the LCX and the left anterior descending (LAD) branches of the coronary artery of the control group; (b) the position of the constrictor, absence of LCX, and normal LAD artery of the model group; (c) the position of the constrictor, absence of the proximal segment of the LCX but normal distal segment, indicating the formation of collateral circulation, and normal LAD artery.

Table 1 Semiquantitative evaluation of collateral blood vessel formation[a)]

Time after first surgery (week)	Control group (n=5)	Model group (n=4)	Ad-HGF group (n=8)
3	3.0±0.0	0.25±0.5**	0.5±0.58**
7	3.0±0.0#	0.5±0.58**	2.12±0.23*Δ

a)*, $P < 0.05$ and **, $P < 0.01$ as compared with the control group; Δ, $P < 0.05$ as compared with the model group at the same time point; #, $n = 4$.

The number of myocardial microvessels in the control group was (12.5±1.92)/view field, whereas it was (4.36±2.52)/view field in the ischemic zone of the model group. These 2 values were significantly different (P<0.01). Moreover, in the transitional zone of the model group, the blood vessel density was (7.74±3.99)/view field; this value was also significantly different from those of both the control group and the ischemic zone of the model group (P<0.05). The values of blood vessel density for the ischemic and transitional zones of the Ad-HGF group were (22.81±4.80) and (31.93±3.53)/view field, respectively; All these values were significantly higher than those of the control and model groups (P<0.01 for all values). In addition, in the Ad-HGF group, the blood vessel density in the transition zone was significantly higher than that in the ischemic zone (P<0.05) (Figure 2).

In specially stained histopathological sections, the normal myocardiocytes showed yellow or yellow-brown, whereas ischemic myocardiocytes showed distributed red foci. The total red area in the control group was 2.7%±1.5%. In the ischemic area of the model group, large red patches were noted that had a total area of 77%±4.6%, whereas in the Ad-HGF group, lower red foci with a total area of 41.7%±2% (P<0.01) were observed (Figure 3).

2.3 SPECT imaging of myocardial perfusion

The results showed that myocardial perfusion was normal in the control group (Figure 4(a)), whereas abnormal distribution of myocardial radioactivity was observed in the model group 3 weeks after the first surgery, indicating the presence of a definite defect in the lateral wall of the left ventricle (Figure 4(b)). In the Ad-HGF group, a definite defect was also detected in the lateral

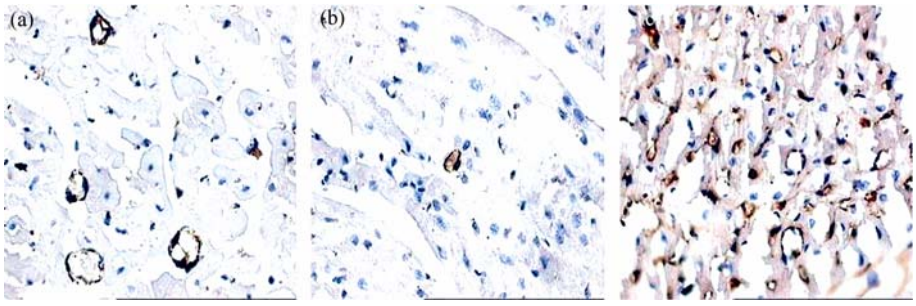

Figure 2 Immunocytochemical staining for factor VIII (single or a cluster of endothelial cells stained brown were counted as 1 vessel). (a) Normal blood vessel density in the myocardium of the control group (×200); (b) density in the model group is much lower than normal (×200); (c) the blood vessel density in the Ad-HGF group (×200).

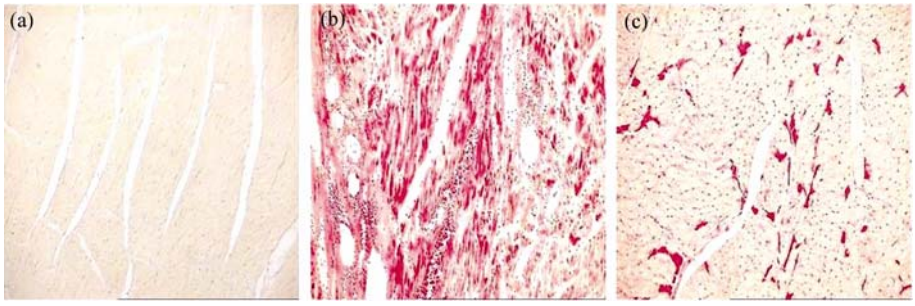

Figure 3 Special staining for ischemic myocardiocytes in the transitional zone (×200). (a) No red colored ischemic myocardiocytes are present in the control group; (b) A large number of ischemic myocardiocytes are present in the model group; (c) lower amount of ischemic myocardiocytes are observed in the Ad-HGF group.

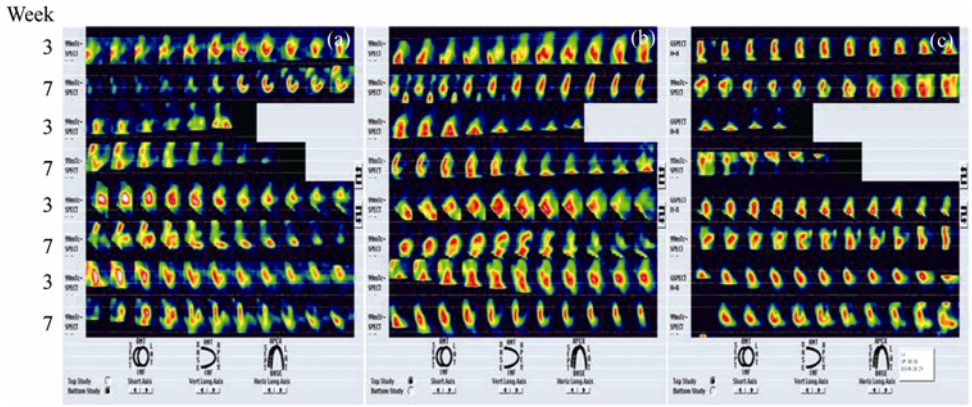

Figure 4 SPECT imaging performed 3 and 7 weeks after the first surgery. (a) Normal perfusion of the myocardium in the control group; (b) Severe reduction of radioactivity distribution in the lateral wall of the left ventricle at week 3 in the model group; this distribution continued to remain low at week 7; (c) Severe reduction of radioactivity distribution in the lateral wall of the left ventricle at week 3 but normal distribution at week 7 in the Ad-HGF group.

Table 2 SPECT imaging results

Time after the first surgery	Control group (n=5)	Model group (n=4)	Ad-HGF group (n=8)
3 weeks	19.60±0.55	10.50±0.58**	9.63±0.82**
7 weeks	19.20±0.45#	10.00±0.82**	15.25±0.27**Δ

a)*, $P < 0.05$ and **, $P < 0.01$ as compared with the control group; Δ, $P < 0.05$ as compared with the model group at the same time point; #, $n = 4$.

wall of the left ventricle 3 weeks; however, 4 weeks later, the abnormal radioactivity distributions had remarkably improved (Figure 4(c) and Table 2).

2.4 Cardiac ultrasonography

The results of body surface echocardiography revealed no evident changes in the cardiac function in the control group at both weeks 3 and 7 after the first surgery. In the model group, both systolic and diastolic cardiac functions were markedly reduced at week 3 after the first surgery and remained low at week 7 after the first surgery. Whereas in the Ad-HGF group, the evident thickening of the left ventricle wall and reduced echo of the endocardium were noted at week 7. The heart function was evidently improved compared to model group (Table 3).

3 Discussion

Therapeutic angiogenesis has become a novel approach for the treatment of myocardial ischemia. Previous studies on the gene transfer of angiogenic factors have shown enhanced angiogenesis in infarcted hearts and subsequently improved heart function. HGF, a multifunctional factor that promotes angiogenesis and anti-fibrosis in the heart, has been shown to have a therapeutic effect on myocardial ischemia[16]. Like several researchers, we have observed its therapeutic effect on both acutely and chronically ischemic myocardial models of mouse, rats, and dogs[21]. In a mouse model of chronic myocardial ischemia, Ad-HGF-gene transduction improved heart remodeling and functional recovery by promoting the hypertrophy of myocardiocytes, thickening of the ventricular wall, preservation of blood vessels, anti-fibrosis, and other pathophysiological processes[18]. In a canine model of chronically ischemic myocardium, the transduced HGF gene improved the ischemic status of the myocardium via the induction of blood vessel formation and the inhibition of myocardial

Table 3 Echocardiography results at week 7 after the first surgery

Index	Control group (n=5)	Model group (n=4)	Ad-HGF group (n=8)
Ejection fraction (EF) (%)	66.0±6.4	47.1±4.0**	66.4±1.84ΔΔ
Shortening of axis (%)	36.7±4.6	24.6±2.1**	34.4±1.45 ΔΔ
Slope of EF (cm/s)	12.5±2.5	5.9±1.3**	11.3±0.78 ΔΔ
Change of EPSS	0.76±0.12	1.29±0.26*	0.79±0.06 Δ
Ev/Av	1.38±0.19	0.89±0.14**	1.24±0.09 ΔΔ

a)*, $P < 0.05$ and **, $P < 0.01$ as compared with the control group; Δ, $P < 0.05$ and ΔΔ, $P < 0.01$ as compared with the model group at the same time point.

apoptosis[19]. These results indicate that HGF has a protective effect on both acutely and chronically ischemic myocardium and supports the application of Ad-HGF gene transfer in the clinical treatment of coronary heart disease.

This research is a preclinical therapeutic evaluation of the use of Ad-HGF in the treatment of chronic myocardial ischemia. The anatomy and physiology of the minipig heart are similar to those of the human heart; moreover, like the human heart, its natural ability for forming collateral blood vessels is low. Thus, the minipig is the most ideal model for evaluating the therapeutic effect of Ad-HGF gene transfer on chronically ischemic myocardium. We established a minipig model in which chronic ischemia was induced by an Ameroid constrictor. The implantation of the constrictor ring resulted in the complete closure of LCX 2 weeks later; however, the LAD branch of the LCA was clear. The HGF gene carried by the recombinant adenoviral vector was injected directly into the local myocardium to observe its effects on blood vessel formation and heart function. Therefore, this model is suitable for studies on blood vessel formation induced by growth factors.

This study showed that adenovirus-mediated transfer of the exogenous gene HGF could reinforce blood perfusion, remarkably increase blood vessel density in the local myocardium and significantly improve both systolic and diastolic functions of the heart. This indicates that HGF expressed by transfected myocardial cells promotes the formation of collateral blood vessels and reinforces blood perfusion, resulting in resuscitation of a certain number of dormant myocardial cells in chronically ischemic myocardium. Thus, using this method, we provide an efficient approach for the treatment of ischemic myocardial diseases. Our result indicates that HGF gene transfection is a potent therapeutic approach for the treatment of ischemic heart diseases such as myocardial infarction.

The basic requirements of gene therapy are safety, high efficiency, and specificity for avoiding toxic side effects; reducing the gene dosage; and enhancing the curative effect. Therefore, it is important to carefully select the *in vivo* gene transduction route to the heart. In this study, we also explored the safety of gene therapy using Ad-HGF in minipig models. After a single intramyocardial injection of Ad-HGF at 5×10^{10} pfu (5-fold the therapeutic dose) and 14 times of i.m. injections in the hind limb of healthy minipigs, the minipigs were sacrificed; subsequently, the survival status, ECG, heart function, peripheral blood cell counts, serum biochemical indices, and pathologic changes in the main organs and tissues were observed. The results revealed no abnormalities for any of the above-mentioned parameters, thus indicating that gene therapy using Ad-HGF for the treatment of ischemic heart diseases is safe.

In conclusion, we observed the therapeutic effect of Ad-HGF gene transfer on chronic myocardial ischemia in a minipig model and evaluated the safety of direct myocardial administration of an Ad-HGF gene. Our results indicate that Ad-HGF gene transfer is a safe and effective approach for the treatment of chronic myocardial ischemia and provide support the future clinical studies of HGF.

The authors thank Prof. WANG JiaXi for modifying this manuscript.

1 Houser F, Culler S D, Becker E R, et al. A retrospective study of 6,671 patients comparing coronary stenting and balloon angioplasty. J Invasive Cardiol, 2000, 12: 354－362
2 Horvath K A. Transmyocardial laser revascularization in the treatment of myocardial ischemia. J Card Surg, 2000, 15: 271－277
3 Su H, Lu R, Kan Y W. Adeno-associated viral vector-mediated vascular endothelial growth factor gene transfer induces neovascular formation in ischemic heart. Proc Natl Acad Sci USA, 2000, 97: 13801－13806
4 Udelson J E, Dilsizian V, Laham R J, et al. Therapeutic angiogenesis with recombinant fibroblast growth factor-2 improves stress and rest myocardial perfusion abnormalities in patients with severe symptomatic chronic coronary artery disease. Circulation, 2000, 102: 1605－1610
5 Funatsu T, Sawa Y, Ohtake S, et al. Therapeutic angiogenesis in the ischemic canine heart induced by myocardial injection of naked complementary DNA plasmid encoding hepatocyte growth factor. J Thorac Cardiovasc Surg, 2002, 124: 1099－1105
6 Furlong R A. The biology of hepatocyte growth factor/scatter factor. Bioessays, 1992, 14: 613－617
7 Sengupta S, Sellers L A, Li R C, et al. Targeting of mitogen-activated protein kinases and phosphatidylinositol 3 kinase inhibits hepatocyte growth factor/scatter factor-induced angiogenesis. Circulation, 2003, 107: 2955－2961
8 Duan H F, Wu C T, Lu Y, et al. Sphingosine kinase activation regulates hepatocyte growth factor induced migration of endothelial cells. Exp Cell Res, 2004, 298(2): 593－601
9 Chen X H, Minatoguchi S, Kosai K, et al. *In vivo* hepatocyte growth factor gene transfer reduces myocardial ischemia-reperfusion injury through its multiple actions. J Card Fail, 2007, 13: 874－883

10 Ryugo M, Sawa Y, Ono M, et al. Myocardial protective effect of human recombinant hepatocyte growth factor for prolonged heart graft preservation in rats. Transplantation, 2004, 78: 1153－1158
11 Yasuda S, Goto Y, Baba T, et al. Enhanced secretion of cardiac hepatocyte growth factor from an infarct region is associated with less severe ventricular enlargement and improved cardiac function. J Am Coll Cardiol, 2000, 36: 115－121
12 Suzuki H, Murakami M, Shoji M, et al. Hepatocyte growth factor and vascular endothelial growth factor in ischaemic heart disease. Coron Artery Dis, 2003, 14: 301－307
13 Nakamura T, Mizuno S, Matsumoto K, et al. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. J Clin Invest, 2000, 106: 1511－1519
14 Jin H, Wyss J M, Yang R, et al. The therapeutic potential of hepatocyte growth factor for myocardial infarction and heart failure. Curr Pharm Des, 2004, 10: 2525－2533
15 Duan H F, Wu C T, Wu D L, et al. Treatment of myocardial ischemia with bone marrow-derived mesenchymal stem cells overexpressing hepatocyte growth factor. Mol Ther, 2003, 8: 467－474
16 Azuma J, Taniyama Y, Takeya Y, et al. Angiogenic and antifibrotic actions of hepatocyte growth factor improve cardiac dysfunction in porcine ischemic cardiomyopathy. Gene Ther, 2006, 13: 1206－1213
17 Jayasankar V, Woo Y J, Pirolli T J, et al. Induction of angiogenesis and inhibition of apoptosis by hepatocyte growth factor effectively treats postischemic heart failure. J Card Surg, 2005, 20: 93－101
18 Li Y, Takemura G, Kosai K, et al. Postinfarction treatment with an adenoviral vector expressing hepatocyte growth factor relieves chronic left ventricular remodeling and dysfunction in mice. Circulation, 2003, 107: 2499－2506
19 Ahmet I, Sawa Y, Yamaguchi T, et al. Gene transfer of hepatocyte growth factor improves angiogenesis and function of chronic ischemic myocardium in canine heart. Ann Thorac Surg, 2003, 75: 1283－1287
20 Roth D M, Maruoka Y, Rogers J, et al. Development of coronary collateral circulation in left circumflex Ameroid-occluded swine myocardium. Am J Physiol, 1987, 253(5Pt2): H1279－288
21 Wu D L, Zhang Y R, Lao M F, et al. Therapeutic induction of angiogenesis by direct myocardial administration of an adenovirus vector encoding human hepatocyte growth factor gene and its safety. Chin Sci Bull, 2004, 49(14): 1464－1469