ORIGINAL ARTICLE

Bone marrow mesenchymal stromal cells with support of bispecific antibody and ultrasound-mediated microbubbles prevent myocardial fibrosis via the signal transducer and activators of transcription signaling pathway

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Abstract

Background aims. This study was initiated to investigate the efficacy of myocardial fibrosis intervention via signal transducer and activators of transcription (STAT) signaling using bone marrow (BM) mesenchymal stromal cells (MSC) with the aid of bispecific antibody (BiAb) and ultrasound-mediated microbubbles (MB).

Methods. BiAb (anti-CD29/H11003 anti-myosin light chain antibody; AMLCA) was prepared and combined with isolated MSC from male mice and transfused into female mice with isoproterenol-induced myocardial fibrosis via the tail vein, followed by MB (MSC/H11001 BiAb/H11001 MB). This study included seven groups: MSC/H11001 BiAb/H11001 MB; MSC; BiAb; MB; MSC/H11001 BiAb; untreated; and control. Five weeks after treatment, expression levels of the sex-determining region of Y-chromosome (SRY), matrix metalloproteinases (MMP)-9, tissue inhibitor of metalloproteinase (TIMP)-1 and vascular endothelial growth factor (VEGF) in myocardium were detected by fluorescent quantitative real-time polymerase chain reaction (qRT-PCR). Collagen distribution was observed using Sirius Red staining. The protein expression of signal transducer and activators of transcription (STAT)1 and STAT3 was detected by Western blot.

Results. The highest homing number of MSC was in the MSC/H11001 BiAb/H11001 MB group, second highest in the MSC/H11001 BiAb group, and lowest in MSC alone. Compared with the untreated group, MSC/H11001 BiAb/H11001 MB, MSC/H11001 BiAb and MSC groups had decreased levels of MMP-9, TIMP-1, STAT1 and collagen deposition, and increased levels of STAT3. Upregulated STAT3 and downregulated TIMP-1 were significantly different in MSC/H11001 BiAb/H11001 MB compared with MSC alone or MSC + BiAb. Conclusions. The homing rate and repairing efficacy of MSC improved with treatment utilizing a combination of BiAb and MB. MSC can improve MMP–TIMP expression in injured myocardium and interfere with myocardial fibrosis after homing, a mechanism that may be related to the STAT-mediated signaling pathway.

Key Words: bispecific antibody, mesenchymal stromal cell transplantation, microbubbles, myocardial fibrosis, signal transducer and activators of transcription

Introduction

Stem cells have unique advantages in the treatment of myocardial damage. Specifically, bone marrow (BM) mesenchymal stromal cells (MSC) have many desirable characteristics, including low immunogenicity, liability for isolation and amplification, and multiple differentiation (1), making these cells one of the most frequently used stem cells for the repair and reconstruction of injured myocardium. In investigations of cellular cardiomyoplasty, MSC transplantation via the peripheral vein pathway reduced local trauma and had a high feasibility compared with alternative treatments. However, the repairing efficacy is limited by the migratory number and colonization amount of targeted stem cells. In order to promote the homing of MSC and understand its repairing mechanism, we combined bispecific antibody (BiAb) and ultrasound-mediated microbubbles (MB) to guide MSC to improve the homing number of MSC and evaluate the therapeutic efficacy of this treatment on myocardial fibrosis.
Methods

Isolation and culture of MSC from mice

MSC were cultured according to the whole BM adherence method (2,3). Healthy specific pathogen free (SPF)-grade male BALB/c mice, 3–4 weeks old (12–15 g, provided by the Experimental Animal Center of Chongqing Medical University, Chongqing, China), were killed by cervical dislocation, and femoral bones were isolated under sterile conditions. Five milliliters of Dulbecco’s modified Eagle medium (DMEM)/F12 culture medium (Gibco, Grand Island, NY, USA) was used to force the BM out and blow out the cells. Cells were then centrifuged at 2000 r.p.m. for 10 min and the supernatant discarded. Three milliliters of DMEM/F12 culture medium was used to prepare the BM cell suspension, and the suspension was slowly transferred to centrifuge tubes with an equal volume of lymphocyte-separating medium against the tube wall (Second Reagent Factory, Shanghai, China). These tubes were then centrifuged at 2000 r.p.m. for 10 min. The cells were carefully collected and rinsed with DMEM/F12 culture medium twice, then counted using a hemocytometer and inoculated into culture flasks of 25 mL at a density of $4 \times 10^5 / \text{cm}^2$. The conditions for culture were as follows: DMEM/F12 + 10% fetal bovine serum, 37°C incubator with 5% CO$_2$ and saturated humidity, static culture, and the cells were subcultured after they reached 90% confluency. Cells in the exponential phase of growth were collected for further tests.

MSC identification

Morphologic and functional indexes and cellular phenotype were used to identify the cultured MSC (4). The morphology of stem cells was observed under an inverted light microscope ($100 \times$). Induced osteogenic and adipogenic cultures were created. For bone formation induction, MSC were kept in six-well plates and cultured until cells were 80–90% confluent. The original culture solution was removed and replaced with 2 mL complete medium for bone formation induction and differentiation (OriCell™; Cyagen Biosciences, GuangZhou, GuangDong, China). The solution was changed every 3 days and the cells were observed continuously. After induction for 3 weeks, the cells were subjected to Alizarin Red staining, formalin fixation and photographing.

For adipogenic induction, MSC were kept in six-well plates and cultured until cells were 80–90% confluent. The original culture solution was removed and replaced with 2 mL complete medium for bone formation induction and differentiation (OriCell™; Cyagen Biosciences). After 3 days, the medium was replaced with complete medium B for adipogenic induction, and after 24 h replaced with complete medium A for adipogenic induction. This cycle was performed three times. The cells were subjected to Oil Red O staining, formalin fixation and photographing.

The phenotypes of MSC were identified using flow cytometry. MSC were prepared in a unicell suspension, and fluorescent-labeled anti-CD29, anti-CD44 or anti-CD117 added. The cells were incubated at 4°C in the dark for 30 min, washed with cold phosphate-buffered saline (PBS), and then fixed with 1% paraformaldehyde. The positive expression rate of corresponding labeled antigen in the cells was analyzed quantitatively using Cell Quest software for the flow cytometer (FACSCalibur, BD Company); isotypic IgG served as the negative control.

Preparation of BiAb

Rabbit anti-mouse CD29 (Bioss, Beijing, China) was dissolved in Traut’s reagent (Pierce, Rockford, IL, USA) for 1 h according to the chemical crosslinking method (5,6). Anti-myosin light chain antibody (AMLCA) (Abcam, Cambridge, UK) was dissolved in Sulpho-SMCC reagent (Pierce) for 1 h. Both monoclonal antibodies were mixed immediately in equimolar proportions and kept overnight at 4°C. The BiAb was identified using non-reductive sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE).

Management of ultrasound-mediated MB

MB were awarded the National Invention Patent of China in 2005 and have been widely applied in experimental studies (7–10). The ultrasound treatment meter Type UGT 1025 (10–12) was developed by the Institute of Ultrasound Imaging, Chongqing Medical University. The concentration of MB used was approximately $8.5 \times 10^8$ bubbles/mL. Ultrasound irradiation was performed at a frequency of 1 MHz and an intensity of 1.5 W/cm$^2$ for 1 min.

Establishment of the myocardial fibrosis model

Seventy healthy female BALB/c mice, 6 weeks old (18–20 g; provided by the Experimental Animal Center of Chongqing Medical University), were used in the study. The mice were subjected to hypodermic injection with isoproterenol (batch number 080701; Shanghai Hefeng Pharmaceutical Co. Ltd, Shanghai, China) at 50 mg/kg. The injection was carried out twice a day for a continuous 10 days.

The prepared models (13) were divided into the seven groups, each containing 10 animals. The groups were: untreated; pure BiAb treatment (BiAb
MSC with the aid of BiAb and MB prevent myocardial fibrosis

Group; pure MB treatment (MB group); pure MSC transplantation (MSC group); MSC + BiAb group; MSC + BiAb + MB group; and control group (physiologic saline but not isoproterenol hypodermically injected during the model preparation). The handling of animals during these experiments was approved by the Animal Use Ethics Committee of Chongqing Medical University.

Cellular transplantation

One day after the last isoproterenol injection, all mice in each group were subjected to tail vein transfusion. The mice in the untreated group were transfused with 0.1 mL PBS. The mice in the BiAb group were transfused with 0.1 mL MB. The mice in the MB group were transfused with 0.1 mL MB treatment, and ultrasound wave irradiation (frequency 1 MHz, intensity 1.5 W/cm², duration 1 min) on the precordial region was performed. In the MSC group, the mice were transfused with 4 × 10⁶ MSC/kg. The mice in the MSC + BiAb group were transfused with 4 × 10⁶ MSC/kg and 50 ng BiAb mixture. The mice in the MSC + BiAb + MB group were first transfused with 0.1 mL MB and then ultrasound wave irradiation (frequency 1 MHz, intensity 1.5 W/cm², duration 1 min) was performed on the precordial region. Subsequently, 4 × 10⁶ MSC/kg and 50 ng BiAb mixture were transduced. The mice in the control group were transfused with 0.1 mL PBS.

Fluorescent quantitative polymerase chain reaction analysis on expression of the sex-determining region of Y-chromosome, vascular endothelial growth factor, matrix metalloproteinases-9 and tissue inhibitor of metalloproteinase-1 in myocardium

Mice were killed 5 weeks after cell transplantation and their hearts collected. The cardiac apaxes were sampled and subjected to fluorescent quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The trizol one-step method was used to extract the total RNA and its purity was verified using an ultraviolet spectrophotometer. Reverse transcription and cDNA synthesis were carried out using conventional methods.

Specific primers (Table I) were designed according to the sequences of sex-determining region of Y-chromosome (SRY), matrix metalloproteinase (MMP)-9, tissue inhibitor of metalloproteinase (TIMP)-1, vascular endothelial growth factor (VEGF) and β-actin in GenBank. Primers were synthesized by Shinegene Biotechnological Co. (Shanghai, China).

The FTC2000 (Canada) fluorescent qRT-PCR detection system was used for amplification. An SYBR green fluorescent quantitation PCR kit (Shinegene Biotechnological Co.) was used for quantitative detection of the target genes. Each reaction system included 1 μL cDNA, 25 μL 2 × PCR buffer, 0.6 μL of each primer for the target gene (25 pmol/μL), 22.5 μL RNase-free water and 0.3 μL SYBR green I (20×). The signals were detected at 72°C after 35 cycles (94°C for 4 min, 94°C for 20 s, 60°C for 30 s, 72°C for 30 s). The expression level of β-actin was also detected as an internal control. The cycle threshold was read and the relative ration method was used for the calculation. The standard curve, amplification curves and melting curve were plotted.

Assessment of myocardial collagen with Sirius Red staining and polarized light

The transverse plane of the left ventricle with a thickness of 2 mm was collected for the preparation of successive paraffin sections to a thickness of 5 μm. This was followed by carbozatic acid–Sirius Red staining. Myocardial collagen was observed under a polarized light microscope. Image J software (version 1.43; http://rsb.info.nih.gov/ij, 2010-01) was used for the quantitative analysis. Collagen with Sirius Red staining was analyzed using image enhancements, color processing and measuring in Image J software. Significant differences were determined by analysis of variance (ANOVA) with appropriate post-hoc testing.

Western Blot analysis of signal transducer and activators of transcription 1 and 3 expression in myocardium

Fresh cardiac tissue (250–500 mg) was collected and 1 mL total protein extraction reagent containing protease inhibitor added. Total proteins were extracted

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Image 489x15 to 599x29
after homogenization. Coomassie brilliant blue staining was used to determine the protein concentration. Subsequently, SDS–PAGE electrophoresis was used to separate the proteins, and proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated with rabbit anti-mouse signal transducer and activators of transcription (STAT)1 or STAT3 antibodies (Aviva Systems Biology, San Diego, CA, USA.), followed with anti-rabbit IgG (Sigma, Santa Clara, CA, USA.) staining, and then subjected to film development and further analysis.

**Statistical analysis**

SPSS 16.0 statistical software was used for the data analysis. The measurement data were represented by mean ± standard deviation. An ANOVA was used to compare differences among the seven groups. A P-value of 0.05 was used to determine statistical significance.

**Results**

**Identification of MSC**

The in vitro cultured MSC grew in adherence and most of them grew in parallel arrays or a whirlpool-like pattern. The cells had a fusiform or polygonal shape and the karyoplasmic ratio was large (Figure 1A).

The MSC became polygonal 1 week after osteogenic induction, and calcium content deposition between cells were observed 2 weeks after induction (Figure 1B). Significant formation of calcium nodes with Alizarin Red staining was observed 3 weeks after induction (Figure 1C). The morphology of MSC became irregular 1 week after adipogenic induction, and small fatty granules with a relatively strong refraction rate were found in the cytosol 2 weeks after induction (Figure 1D). Lipid droplet accumulation with Oil Red O staining was found 3 weeks after induction (Figure 1E).

Flow cytometry results indicated that the positive expression rates of CD29, CD44 and CD117 on MSC were 99.96%, 99.66% and 2.10%, respectively. These results indicated that the cultured cells expressed CD29 and CD44, but not CD117, which is in accordance with the phenotypic characteristics of MSC (14,15) (Figure 1F). The above-mentioned morphologic data and induced differentiation results in conjunction with the classification of cell phenotypes indicated that these cells were indeed MSC.

**BiAb preparation**

CD29 × AMLCA was prepared by using the chemical conjugation method (5,16,17). The prepared product contained BiAb (268 kDa), conjugated multimer (>300 kDa), unconjugated monoclonal antibody CD29 (88 kDa) and AMLCA (180 kDa), as shown by SDS–PAGE (Figure 2).

**Homing efficiency of MSC and its functions in the treatment of myocardial fibrosis**

The mRNA expression of SRY, MMP-9, TIMP-1 and VEGF in myocardium of the seven groups of...
Almost no expression of SRY gene was found in the control, untreated, BiAb and MB groups.

Expression of MMP-9 mRNA in the control group was relatively low, while its expression in the untreated, MB and BiAb groups was relatively high ($P < 0.05$) (Figure 3B). In comparison with that of the untreated group, expression of MMP-9 in the MSC, MSC + BiAb and MSC + BiAb + MB groups was significantly lower, but it was still higher than that of the control group ($P < 0.05$).

Expression of TIMP-1 in the control group was relatively low, while its expression in the untreated, MB and BiAb groups was relatively high ($P < 0.05$).

Compared with the untreated, BiAb and MB groups, expression of TIMP-1 in the MSC, MSC + BiAb and MSC + BiAb + MB groups was significantly lower; however, it was still higher than that of the control group ($P < 0.05$) (Figure 3C).

Expression of VEGF in mice with myocardial fibrosis (whether treated or not) was relatively low compared with the control group ($P < 0.05$).

Expression of myocardial VEGF in the MSC group was relatively high ($P < 0.05$) (Figure 3D). However, there were no significant differences in myocardial VEGF expression between the MSC + BiAb, MSC + BiAb + MB and untreated groups ($P > 0.05$).
determined by Western blot. The results are shown in Figure 5, with separate bands for STAT1 and STAT3. The analysis of the optical density of each band is shown in Figure 5C,D.

STAT1 expression in mice with myocardial fibrosis significantly increased compared with that of the control group (\(P < 0.05\)). Higher levels of STAT1 in the untreated, BiAb and MB groups were seen than in the MSC, MSC + BiAb, MSC + BiAb + MB and control groups (\(P < 0.05\)) (Figure 5A,C).

STAT3 expression in the MSC, MSC + BiAb and MSC + BiAb + MB groups significantly increased compared with that of the control, untreated, BiAb and MB groups (\(P < 0.05\)). The expression of STAT3 decreased in succession in different groups: the MSC + BiAb + MB group ranked first, MSC + BiAb second, MSC third, and the untreated, BiAb, MB and control groups fourth (Figure 5B,D).

**Discussion**

We investigated the homing and therapeutic efficacy of MSC transplantation in isoproterenol-induced myocardial fibrosis mice with a combination treatment.
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of BiAb and ultrasound-mediated MB. With the combination of BiAb and ultrasound-mediated MB, the homing number of transplanted MSC increased, MMP-9 and TIMP-1 decreased, and pathologic myocardial fibrosis was improved. These results may indicate a potential mechanism relevant to the STAT signaling pathway.

Previous investigation had found that only a few MSC can be recruited to the area of injured myocardium via intravenous injection of MSC (18). A problem facing clinical applications of stem cell transplantation is the need to improve the homing efficiency of MSC. In the past decade, BiAb have become more prominent in the immunologic treatment of tumors. As BiAb has a binding site for two antigens, it is possible that such a molecule can guide the effector cells to the target cells, and thus make it possible to target MSC and cause them to migrate to injured myocardium. The homing rate of hematopoietic stem cells that are assembled with BiAb in cardiomyocyte transplantation is significantly increased (5,16,17) and the structure and functions of ischemic hearts are significantly improved. Whether BiAb that is not assembled on stem cells (free BiAb) is able to promote the homing of stem cells has yet to be elucidated.

Prepared CD29 × AMLCA is the connecting bridge between injured myocardium and MSC. AMLCA can specifically recognize injured myocardium (19), and CD29 is the molecular marker that shows a positive rate of more than 99% on the surface of MSC. Therefore CD29 × AMLCA can bind to both MSC and injured myocardium, and it can act as the bridge between them. In the present study, we transfused MSC and BiAb in a free state into mice that suffered from isoproterenol-induced myocardial fibrosis and found that BiAb can increase the homing number of MSC, and the homing rate was even higher with the aid of ultrasound-mediated MB. These findings indicate that BiAb and ultrasound-mediated MB can guide the targeted migration of MSC. Previous work has revealed that the transfection of BiAb and effector cells via intravenous infusion into lymphoma mice and BiAb can assist the effector cells to bring tumor inhibiting activities into play (20). Additionally, BiAb in a free state can guide effector cells to damaged tissues containing target antigen in animal models (21). This may be attributed to the maintenance of stronger biologic activity by MSC and BiAb, as they were not subjected to the assembly process in vitro.

BiAb in the transplantation receptor has a short half-life, but this factor did not affect its targeting and guiding functions. Based on the homing and synergistic effect, we presumed that the function of BiAb as an ‘engager’ began early after transplantation. Rossi et al. (22) presumed that the function can be attributed to the rapid binding of BiAb to the target antigen. Although the half-life of the transplanted BiAb was shorter than that of the autologous antibody, BiAb exerted its functions before its elimination.

The combination of ultrasound-mediated MB with BiAb improves the microenvironment of target sites. In fact, ultrasound-mediated MB can be thought of as improving the ‘soil’ of the microenvironment, while the BiAb itself can be thought of as improving the ‘seed’ quality and rate of ‘sowing’. The homing rate of MSC with the aid of the ultrasound-mediated MB and BiAb combination significantly increased
compared with the MSC + BiAb and MSC groups, indicating that ultrasound-mediated MB can promote the targeted migration and colonization of MSC. Ultrasound-mediated MB may improve the microenvironment in the target site for homing, which is helpful for the survival of stem cells. Previous investigations have found that cavitation erosion can increase the permeability of vascular endothelium and lead to the formation of extraction points (23), which improves the adhesion and transplantation of MSC (7).

The bioeffects of ultrasound-mediated MB would also help homing MSC secrete cytokines and promote the functions of these cytokines. Ultrasound-mediated MB functioned indirectly on STAT. Some cytokines or factors, such as caspase-1 (24), act as a bridge between ultrasound-mediated MB and the STAT pathway.

The homing amount of MSC increased after improving the 'seeds' and 'soil'. A large number of investigations have shown that, only after homing, stem cells can undergo proliferation and differentiation. This may be attributed to the secretion of multiple kinds of cytokines, growth factors and adhesion molecules by MSC, which slightly change the structure and function of the microenvironment in the target site for homing. On the other hand, this may be explained by the correct targeted differentiation of MSC in the local microenvironment. Stem cells have multidirectional differentiation potency, and they can accept local induction, flexibly differentiate and proliferate under the effects of environment (such as acute damages on tissues), and repair tissue by various kinds of mechanisms (such as paracrine secretion). It has been confirmed that MSC can differentiate into vascular endothelial cells (25), cardiomyocyte-like cells (26) and other cells after recruitment to injured myocardium, and they can promote vascular neogenesis (27), interfere with myocardial fibrosis (28) and improve the function of an injured heart.

MMP-9 and TIMP-1 in untreated myocardial fibrosis mice were significantly upregulated and fibrous degeneration of hearts was expedited. MMP-9 and TIMP-1 levels decreased 5 weeks after MSC transplantation, but they were still higher than those of normal mice. Meanwhile, collagen deposition decreased and interstitial fibrosis was alleviated. Among the treatment groups, the homing number of MSC in the MSC + BiAb + MB group was the highest, and the degree of myocardial fibrosis was moderate, while the expression of TIMP-1 and MMP-9 was relatively low, indicating that MSC can not only be recruited efficiently with the aid of BiAb and ultrasound-mediated MB, but can also prevent myocardial fibrosis at the homing site. These results indicated that the intervention of MSC on myocardial fibrosis is closely related to its homing amount.

The upregulation and imbalance in MMP–TIMP expression after myocardial damage affects the degree of fibrous degeneration of heart (29,30). Therefore, the internal mechanism for the intervention of MSC on MMP–TIMP needs to be elucidated. First, more transplanted MSC (which can ensure a greater homing amount) can regulate a pathologic MMP–TIMP imbalance (31), indicating that the amount of stem cells at the local target site can affect the efficacy of MMP–TIMP. Second, some investigations have found that the culture solution of MSC (not containing MSC) can regulate the expression of MMP–TIMP and inhibit the collagen secretion of cardiac fibroblasts (32). These results indicate that MSC can secrete many kinds of biologically active molecules, such as various growth factors, cytokines and chemical mediators (although these mechanisms remain unclear), that help MSC to function in repair at injured sites (28,33). Some investigations also indicate that MSC have anti-inflammatory functions in the target position of the transplantation, and thus exhibit anti-inflammatory mechanisms and participate in protection of the heart in ischemic heart disease (34).

Myocardial fibrosis is a complex pathologic process that is accompanied by hypoxia and inflammatory responses. The STAT signaling pathway mediates the expression of myocardial MMP (35) and participates in the inflammatory process of myocardial fibrosis, and thus affects the prognosis of myocardial fibrosis (36). In addition, STAT1 and STAT3 are the most representative targets in the STAT signaling pathway (37).

Our investigation shows that myocardial STAT1 expression increases in mice that suffer from myocardial fibrosis compared with normal mice. When tail vein transplantation of MSC was performed, the high expression of STAT1 was inhibited, while the expression of STAT3 was stimulated and upregulated. With the aid of BiAb and ultrasound-mediated MB, STAT3 expression increased and TIMP-1 was downregulated. At the same time, the homing amount of MSC was increased, and the improvement in myocardial fibrosis was significant. These results indicate that STAT1 is involved in myocardial fibrosis and STAT3 is related to anti-fibrosis of MSC.

Previous investigations (37–40) have also shown that STAT1 can promote apoptosis and inflammation while STAT3 can prevent apoptosis and protect the myocardium. The mediating functions of these two factors on injured myocardium are different. Some drugs, such as myricetin, can protect ischemic myocardium by inhibiting the activity of STAT1 (38). Some cytokines, such as granulocyte–colony-stimulating factor (41) and VEGF (42) can improve the functions of mitochondria and protect ischemic myocardium by upregulating STAT3. BiAb and
ultrasound-mediated MB have two possible effects on the STAT pathway. First, they promote the homing number of MSC and subsequently affect the expression of STAT1 and STAT3. With enhanced MSC migration ability, the STAT pathway is activated (43,44). Second, MSC secretes cytokine and chemi-

cal mediator (25). These secretory products activate the STAT pathway (45,46) and have the potency to treat diseases, including myocardial fibrosis. With the aid of BiAb and ultrasound-mediated MB, factors excreted from MSC have a significant effect on the STAT pathway. Our investigation indicates that the intervention functions of MSC on myocardial fibrosis are related to the STAT-mediated signaling pathway. Therefore direct stimulation and/or indirect effects (cytokines and others) of MSC is the intermediate mechanism between the STAT path-

way and the BiAb and ultrasound-mediated MB.

Although many investigations have indicated the superior effects of MSC on the repair of ischemic myocardium, some investigations presume that MSC transplantation is not always optimal. Furlani et al. (47) found that regional injections of MSC, carried out for the treatment of rats with acute myocardial infarction, for 6 weeks did not improve heart function. These conflicting results may occur because of different treatment details and evaluation methods utilized during the early research stages in MSC trans-

plantation therapy. In the present study, MMP-9 and TIMP-1 expression was significantly downregulated.

This indicates that MSC transplanted via the peripheral intravenous route can significantly improve the mesenchyma structure of the fibrotic heart to some extent, but its improvement on vascular neogenesis is not significant. Such results could be attributed to the short duration of the treatment, or intrinsic limitations of the isoproterenol-induced myocardial fibrosis model. Further treatment and investigations in long-term studies are needed.

It is still unclear whether BiAb and ultrasound-

mediated MB lead to the mobilization of autolo-
gous stem cells and how many autologous stem cells are mobilized. The present study was a short-term transplantation investigation of MSC, and BiAb was administered by a single intravenous infusion. Further observations are needed on the therapeutic efficacy of multiple infusions of BiAb.

Acknowledgments

The present study was completed in the Center of Molecular Medicine and Tumor Research and the Institute of Basic Medical Sciences in Chongqing Medical University from September 2009 to May 2010. This work was supported by a grant from the National Natural Science Foundation of China (number 30970826). The authors are thankful to all of the involved laboratories and related research members.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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