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Review

Recent progress in the differentiation of bone marrow derived mesenchymal stem cells (BMMSCs) to cardiomyocyte- like cells and their clinical application

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Bone marrow mesenchymal stem cells (BMMSCs) are one of the cells found in bone marrow stromal. A large number of studies have shown that BMMSCs cannot only differentiate into hematopoietic stromal cells, but can migrate and position themselves in multiple non-hematopoietic organizations and differentiate into the corresponding tissue cells; this characteristic demonstrates their multilineage differentiation potential. In different conditions, BMMSCs can differentiate into bone, cartilage, fat, cardiomyocyte, endothelial cells and nerve cell, etc. Because BMMSCs are easy to acquire, they can proliferate *in vitro*, have multi-differentiation potential after implantation *in vivo*, and therefore have wide application prospects for the treatment of cardiovascular disease as the ideal seed cells. This review focuses on the biological characteristics of BMMSCs, the induction and differentiation of cardiomyocyte-like cells and the application in the cardiovascular field.

Key words: Bone marrow mesenchymal stem cells (BMMSCs), cardiomyocyte-like cells, cardiovascular disease.

INTRODUCTION

Cardiovascular disease has become one of the major diseases causing mortality in human. In spite of continuous improvement to cardiovascular drug therapy and cardiac interventional technology, the mortality rate of cardiovascular disease remains very high. Conventional theory is that cardiac myocytes (CM) are terminally differentiated cells and do not have the capacity to regenerate themselves. Once damage has occured, they cannot be repaired. Some studies have shown that a small amount of CM can divide after myocardial infarction (MI), but the limited proliferation ability of the cells is too insufficient to replace the large number of lost cells after myocardial necrosis. Bergmann et al. (2009) have shown that myocardial cells will slowly proliferate, but the updating rate declines slowly. Therefore, the repairment of damaged myocardium is a serious challenge that researchers and clinicians face after MI. Heart transplant is difficult to apply in clinical practice because of the scarcity of donor hearts, the need for specialised surgical skills, and expensive fees. Autologous heart cell transplantation for cardiac replacement therapy is used for myocardial reconstruction, but due to the difficult culture of autologous heart cells, limited number of CM obtained and low survival rate post-transplant, only limited success has been achieved. The recent stem cell technology could be our source of hope. In accordance with the genetic source, stem cells can be divided into embryonic stem cells and adult stem cells. Adult stem cells are undifferentiated cells but can exist in differentiated tissues. Currently, most studies are focused on bone marrow mesenchymal stem cells (BMMSCs). In 1867, Cohnheim (1875) first proposed that bone marrow contained bone marrow stromal stem cells (BMMSCs). In 1974, Friedenstein et al. (1976) first discovered that the cells present in bone marrow stromal and can differrentiate into bone cells, cartilage cells and fat cells. Bone marrow is an organ consisting of hematopoietic stem cells and non-hematopoietic stem cells. Non-hematopoietic stem cells (BMMSCs) are composed of bone precursor cells, cartilage precursor cells, fat precursor cells, nerve cells and muscle cell precursor cells.

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Therefore, BMMSCs are cells which have a group of complex composition and function and are derived from the bone marrow stem cells or precursor cells and somatic stem cells (Deng et al., 2001). A large number of studies have shown that BMMSCs can not only differentiate into hematopoietic stromal cells, but also migrate and position to multiple non-hematopoietic organs and differentiate into the corresponding tissue cells; this property demonstrates multilineage differentiation potential. In different conditions, BMMSCs can differentiate into bone, cartilage, fat, cardiomyocyte, endothelial cells and nerve cell etc. Because BMMSCs are easy to acquire, can proliferate, be expanded *in vitro*, have multi-differentiation potential, these cells have enormous potential for the treatment of cardiovascular disease as they are the ideal seed cells. This review focuses on the biological characteristics of BMMSCs, the induction and differentiation of cardiomyocyte-like cells and the application of this technology in cardiovascular disease.

THE BIOLOGICAL CHARACTERISTICS OF BMMSCs

Morphological characteristics of BMMSCs

BMMSCs derived from bone marrow are non-hematopoietic cells and early mesoderm cells. Using an optical microscope and phase contrast microscopy, BMMSCs are similar to uniform fibroblasts. BMMSCs cultured in vitro have a low volume, simple structure and a spindle-shaped appearance; they have a high nuclear-cytoplasm ratio and make up 0.001 ~ 0.01% of nucleated cells in the bone marrow (Pittenger et al., 2004a). BMMSCs have three kinds of cell morphology: These include spindle cells; great squamous cells and small spherical cells (Bianco et al., 2001).

Biological characteristics of BMMSCs

BMMSCs have a number of biological characteristics. The first of these is the capacity of self-renewal. They also have the property of adhesion and are able to quickly adhere when cultured at low-density *in vitro*. This allows them to be separated from hematopoietic stem cells by repeated washing. Thirdly they have the potential of multi-lineage differentiation. In the appropriate micro-environment, BMMSCs can differentiate into bone cells, cartilage cells, fat cells, muscle cells, astrocytes, oligodendrocytes and nerve cells. They can also differentiate cross germ layer. BMMSCs or precursor cells of different germ layers can differentiate into an unrelated germ layer organization. BMMSCs also have the ability to proliferate rapidly and form clones. These clones are derived from single cell cloning (Vescovi et al., 2007). Finally they have the ability to be transplanted. These characteristics Including potential capacity of self-renewal and multi-lineage differentiation fulfills the prerequisites for transplantation.

Immunological phenotype of BMMSCs

BMMSCs can not only differentiate into hematopoietic substance and stromal cells, but also differentiate into non hematopoietic cells, especially those derived from the mesoderm and neuroectodermal tissue. A BMMSC-specific marker has not yet been identified and therefore there is no direct method of identifying these cells. We can only speculate whether the cells are BMMSCs through a differentiated phenotype in the culture process. Despite the lack of specific surface markers on BMMSCs, a number of recent screening studies have found that BMMSCs have some markers of mesenchymal cells, endothelial cells and muscle cells rather than hematopoietic cells. Studies have shown that bone marrow-derived BMMSCs expressed SH_2 (CD_{105}), SH_3 (CD_{73}), SH_4, $STRO_{21}$, ASMA, MAB_{1470}, CD_{29}, CD_{44}, CD_{71}, CD_{90}, CD_{106}, CD_{120a}, CD_{124}, CD_{126}, and a variety of other surface proteins, They did not however express surface markers of hematopoietic stem cell lines, such as CD_{14}, CD_{34} , CD_{45}, vWF factor and HLA-DR, etc. BMMSCs have a variety of cytokines and growth factor receptor, such as IL-1R (CD_{121}), IL-3R (CD_{123}), IL-4R, IL-6R (CD_{126}), IL-7R (CD_{127}), LIFR, TNF-Ar (CD_{120a}), FGFR, PDGFR (CD_{140a}), IFNγR (CD_{w119}), SCFR, G-CSFR, TGFR, bFGFR, EGFR, etc. BMMSCs also expressed a number of adhesion molecules such as Integrinβ1 (CD_{29}), VLA-α (CD_{49}), ICAM-1 (CD_{54}), ICAM-2 (CD_{102}), ICAM-3 (CD_{50}), VCAM (CD_{106a}), NCAM (CD_{56}), HCAM (CD_{44}), L-selectin (CD_{62L}), LFA-3 (CD_{58}), etc. Surface markers on BMMSCs are not unique. In general, BMMSCs can be screened by combining positive and negative markers (Pittenger et al., 2004a; Larghero et al., 2008; Pittenger et al., 1999b; Sottile et al., 2002; Majumdar et al., 1998; Gronthos et al., 2001; Yuehua et al., 2002; Alhadlaq and Mao, 2004).

ISOLATION, CULTURE AND PURIFICATION OF BMMSCS

BMMSCs derived from bone marrow reticular stromal can be separated using four different methods:

Adherent screening method, also known as whole bone marrow method

A cell suspension is made from the extracted bone marrow is made to cell suspension by adding culture medium, and then inoculated into a flask at a concentration of 1×10^6/ml cell. During culture, the blood cells which are not adherent are discarded when the medium is changed. The

stromal cells are adherent, they continuously proliferate into a fibroblast-like appearance, and gradually form a monolayer of fibroblast-like colonies. This method is simple, but the final purity of mesenchymal stem cells is not high (Phinney et al., 1999).

Density gradient centrifugation

According to the different density of BMMSCs to other cells, bone marrow can be centrifuged using density gradient. This is performed using a medium with a density of 1.073 g/ml. The isolated cells obtained from the interface are inoculated into the culture flask or dish. The cells grow at the bottom of the flask or dish, and are similar to fibroblasts. The cells in suspension are removed by changing the medium; this method can obtain the mesenchymal stem cells with a purity of 95%. This approach, which is often used in association with adherent screening method, is easy to perform. If conditions are standardized and remain unchanged, the time taken from primary culture with density gradient centrifugation is shorter than that of the whole bone marrow method. More than 95% cells obtained by density gradient centrifugation express CD_{44} and CD_{49} (Pittenger et al., 1999b).

Flow cytometry screening

Using the difference in the surface antigen expression between BMMSCs and hematopoietic cells, a high purity of BMMSCs can be obtained using fluorescent activated cell sorting. The process of cell sorting can however cause cell damage and death to the BMMSCs (Orlic et al., 1994).

Immunomagnetic bead method

Another technique utilized to separate BMMSCs is the use of magnetic beads which are combined to antibodies which are specific to a surface of antigen specifically expressed on the surface of BMMSCs. If the antigen is present the magnetic bead will attach itself to the cell and the cells can be sorted using a magnetic field (Orlic et al., 1994).

INDUCTION AND DIFFERENTIATION OF BMMSCS INTO CARDIOMYOCYTE-LIKE CELLS

Induction and differentiation using chemical substances

5-Aza, a chemical inducing agent, is an anti-cancer drug which induces demethylation. It also is an effective chemical agent to induce the differentiation of BMMSCs into cardiomyocyte-like cells. However, the induction mechanisms are not yet fully understood. A number of animal studies have shown that rat BMMSCs isolated and cultured *in vitro* can differentiate into cardiomyocyte-like cells after induction by 5-aza. Wakitanis et al. (1995) isolated and cultured BMMSCs *in vitro* and induced differentiation using 5-aza. After 7-10 days, the long multinucleus myotubes were seen, proving that rat BMMSCs can be induced to differentiate into cardiomyocyte-like cells in vitro. Makino et al. (1999) reported that if the BMMSCs were immortalized and then induced with 5-aza, 30% of then the cells demonstrated morphological changes. The cells showed fibroblast-like morphology and had characteristics of cardiomyocyte-like cells. Many studies have shown that the cells induced by 5-aza showed not only demonstrated phenotypic characteristics of myocardial cells, but also similar biological and electrophysiological features to myocardium. However, Liu et al. (2003) considered that this stimulation cannot be used for non-immortalized BMMSCs. Bone marrow-derived cells were treated with primary culture, and the adherent BMMSCs become immortalized cells through continuous passage after 4 months. Presently, however, it is not clear how the differentiation of BMMSCs into cardiomyocytes is regulated. The structure of 5-aza is similar to cytidine, and can lead to demethylation of certain cytosine, a component of DNA. Some studies have shown that mouse embryonic cells can transform into mesodermal organization cells after treatment with 5-aza. The mechanism of this process may be related to activation of the myogenic genes MyoD1. In addition, some resear-chers have reported that angiotensin II and dimethyl sulfoxide (DMSO) can induce hBMMSCs to differentiate into cardiomyocyte-like cells in vitro. In addition to some of these chemicals, some certain cytokines, such as insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), can also induce differentiation of BMMSCs into cardiomyocyte-like cells. Furthermore, cell number, doubling time, dose of inducing agent, induction time, concentration of extracellular calcium and culture conditions may also affect the induction and differentiation of BMMSCs into cardiomyocyte-like cells. Currently, 5-aza is still the most affective chemical inducing agents, but its cytotoxicity limits clinical application.

Induction and differentiation in a cardiac micro-environment

Li et al. (2007) co-cultured labeled BMMSCs with neonatal rat ventricular myocytes and demonstrated ultrastructural characteristics of sarcomere formation and inward rectifier potassium current. This indicated differentiation into cardiomyocyte-like cells which could contract together with the rat cardiomyocytes. Some scholars proposed that this phenomenon is due to chemical factors within the myocardial micro-environment, these include cytokines, hormones, ion gradient, and the other

soluble factors produced by the surrounding cells and physical factors, such as the traction force and the electrical-physical environment between cells. Additional influences such as direct contact between cells and the stimulation signal provided by extracellular matrix also contribute to differentiation of BMMSCs into cardiomyocyte-like cells. Some researchers have co-cultured rat BMMSCs and myocardial cells using biomimetic electrical stimulation, and the results showed that this method can also promote the differentiation of BMMSCs differentiation into cardiomyocyte-like cells. Therefore the in vitro micro-environment appeared to stimulate earlier changes into cardiomyocyte-like cells.

Other inducing factors

In recent years, many studies have confirmed that certain monomer of traditional Chinese medicine and Chinese medicine can effectively induce BMMSCs into cardiomyocyte-like cells. Therefore, Chinese medicine has also become another important area of research in the investigation of BMMSCs differentiation into cardiomyocyte-like cells.

Induction and differentiation *in vivo*

Wang et al. (2000) injected BMMSCs cultured and marked with DAPI (4, 6 - 2 Mi-base -2 - phenyl indole hydrochloride) into the same species of rat heart. He removed the heart after 4d-12w, and detected marked karyotypic changes of BMMSCs at the transplant region. After 4 weeks of transplantation, BMMSCs expressed MHC and spectrin, which proved that BMMSCs had differentiated into myocardial cells, and positive staining of connexin-43 suggested the existence of gap junctions, indicating that there were intercalated disk connections between induced myocardial cells and the original myocardial cells. All of this showed that the structure of the transplanted cells had undergone adaptive changes in the myocardial micro-environment, and the formation of intercalated disc connections provided a possibility for signal transmission. It also provided the anatomical basis for functional integration of the transplanted cells and host myocardium.

THE APPLICATION IN CARDIOVASCULAR DISEASE

Animal studies utilizing BMMSCs transplantation for the treatment of myocardial infarction have increased. At the same time, many scholars at home and abroad have carried out this related research in a clinical setting. Currently, the most common methods include intracoronary transplantation, partial transplantation and intravenous infusion of BMMSCs after purification and amplification *in vitro*. Strauer et al. (2002) divided 20 patients of STEMI into two groups; both groups accepted routine standard treatment. The experimental group included intracoronary transplantation of autologous BMMSCs. After three months, the BMMSCs transplantation group had reduced myocardial infarct size and an increased ventricular contraction-rate. The study showed that BMMSCs transplantation was associated with myocardial cells and vascular regeneration, and assisted repair of the heart repair after infarction. Janssens et al. (2006) carried out a double-blind, randomized controlled trials using autologous BMMSCs transplantation for the treatment of STEMI patients. Sixty seven (67) patients of STEMI were randomly divided into a placebo coronary perfusion group (n = 34) and BMMSCs group (n = 33), while being given drugs as treatment. LV ejection fraction of cells in the transplantation group was not significantly increased; however, those with a similar myocardium region at risk, MRI detection found that the infarct volume of the BMMSCs group was significantly reduced compared with the control group after 4 months follow-up. This suggested that BMMSCs have a remarkable biological potential to effect ventricular remodeling after infarction. Meyern et al. (2004) carried out a randomized and controlled study by injecting autologous BMMSCs into intracoronary for STEMI treatment. 60 STEMI patients were selected. The study confirmed autologous BMMSCs transplantation in the early stage of infarction can effectively enhance the left ventricular systolic function and improve LVEF, while not adverse clinical events, restenosis, and cardiac arrhythmia were caused by BMMSCs transplantation. BOOST tests had confirmed the efficacy and safety of BMMSCs transplantation. Cao et al. (2009) successfully treated successfully 86 STEMI patients with percutaneous coronary intervention (PCI), randomly divided into BMMSCs transplantation group (n = 41) and the placebo group (n = 45), while given the drug treatment. 6 months, 1 year and 4-year follow-up results showed that the left ventricular ejection fraction (LVEF) in the BMMSCs transplantation group was significantly improved compared with that of in the placebo group; however, there was no significant difference in improvement of the myocardial infarct size. This study shows that intracoronary transplantation of autologous BMMSCs is safe and feasible, and contributes to long-term improvement of cardiac function. Lago et al. (2006) injected BMMSCs directly into the coronary artery of eight patients with non-ischemic dilated cardiomyopathy. The postoperation ejection fraction increased significantly from 18.3 ± 7 to 26.4 ± 10% ($P < 0.005$), but the left ventricular diastolic diameter was not significantly reduced. The classification of cardiac function (NYHA) decreased from 2.5 ± 0.8 to 1.4 ± 0.5% ($P < 0.001$), and clinical symptoms improved significantly. In addition, the study did not report any deaths or major complications.

Wang et al. (2006) treated idiopathic dilated cardiomyopathy (DCM) by transplanting autologous BMMSCs through the coronary artery. The 24 cases of DCM were randomly divided into a control group (12 cases) and a

cell transplantation group (12 cases). The two groups were treated by routine anti-heart failure medication. The transplantation group received intracoronary autologous BMMSCs transplantation, and the control groups were injected with normal saline. The two cohorts were treated using echocardiography, holter monitoring and the 6 min walking test was performed pre and post-operation. The results showed that 6 months later the 6 min walking distance in the transplantation group had increased significantly. NO serious adverse events such as arrhythmia in the peri-operative period and at 6 months follow-up were reported. This demonstrated that the intracoronary autologous BMMSCs transplantation in the treatment of DCM increased exercise tolerance and had no significant arrhythmia, embolism or immune inflamematory response, however, no significant improvement in LVEF was demonstrated. The above studies have shown that although the improvement of LVEF using intracoronary autologous BMMSCs is still disputed, this is due to small sample sizes and different experimental conditions. It can however, significantly improve clinical symptoms and quality of life in patients with DCM. It has no significant complications, and is safe and effective. This new treatment method has brought new hope for patients with DCM.

PROBLEMS AND PROSPECTS

Human bone marrow-derived mesenchymal stem cells can be obtained from the bone marrow, have no ethical problems, does not cause immune rejection, can be induced into cardiomyocyte-like cells through a variety of methods and have wide and promising clinical applications. Current studies of BMMSCs in clinical myocardial cell transplantation are still confronted with many problems. These include the imperfect isolation and culture of BMMSCs; the low inducing ratio, and this results in BMMSCs being cultured *in vitro* with a variety of different cells. Thus, BMMSCs isolation, purification, identification and cultivation require further studied. Secondly, regulatory mechanisms of BMMSCs differentiation into cardiomyocyte-like cells remain unclear, and therefore methods to accurately control differentiation into myocardial cells need to be investigated. Thirdly, the question of whether induced and differentiated BMMSCs eventually develop into mature and functional myocardial cells and can effectively improve damaged myocardial cells also requires further study. The timing and choice of migration method is still under debate and needs further research and finally the high expansion capacity of BMMSCs *in vitro* could induce the formation of tumours and question of whether this could occur *in vivo* is still under discussion but as yet has not been proven.

CONCLUSIONS

Further research and investigation in the field of BMMSCs transplantation would bring about great changes for the treatment of cardiovascular disease. A large number of animal experiments and clinical studies have already demonstrated that BMMSCs transplantation for the treatment of heart disease has significant effect. With the ongoing research of BMMSCs, we believe that the utilization of BMMSCs in cardiovascular disease will have great significance in the future.

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