METHOD OF PREPARING CELL FOR TRANSPLANTATION

Technical Field

The present invention relates to a method of producing cells for transplantation into myocardial tissue of a mammal. More specifically, it relates to a method of treating bone marrow stem cells of a mammal with biological growth factors to differentiate into cardiac lineage, treating the differentiated bone marrow stem cells *in vitro* for a certain period to have high rates of cell incorporation and cell survival, transplanting the induced bone marrow stem cells into a mammal having myocardial infarction and assessing the altered condition.

Background Art

The ability of mesenchymal stem cells obtained from bone marrow of adult to differentiate into various cells including adipocytes, osteocytes, chondrocytes, hepatocytes and cardiomyocytes has been shown. Based on the ability, bone marrow stem cells have been proposed to be a source of cell transplantation.

Myocardial infarction has been one of the most important causes of death in the world. Since cardiomyogenic cells cannot amplify in adult heart, the death of cardiomyocytes due to myocardial infarction makes scar in the site where healthy cells exist.

Recent studies have found that bone marrow stem cells have a remarkable flexibility after transplanted into heart. Bone marrow stem cells, which are transplanted into myocardium, have the ability to transdifferentiate into cardiomyogenic cells, which enables bone marrow stem cells to be used in the regeneration of myocardium. Despite their potential therapeutic value, bone marrow stem cells do not differentiate into cardiomyogenic cells enough to show significant improvement in function. Accordingly, there proceeds a study for differentiating bone marrow stem cells into cardiomyocytes.

It has been reported that the treatment of demethylating agent such as 5-azacytidine enables bone marrow stem cells to differentiate into cardiomyocytes. 5-Azacytidine demethylates genome nucleoside sequence randomly to result in

expression of normal silent genes. Bone marrow stem cells treated with 5-azacytidine result in induction of various cells, such as cardiomyocytes (MyoD positive), osteocytes (osteocalcin positive), adipocytes (PPAR-positive), and cardiomyogenic cells (cardiac troponin I positive). When bone marrow stem cells are exposed to 5-azacytidine, the transcription of c-abl and interleukin-6 increases quickly, and the expression of collagen I, main substrate protein, decreases. However, demethylating agent such as 5-azacytidine, which proves to be safe to human body, cannot induce significantly the differentiation into one cell type, cardiomyogenic cells.

Considering the above, the present inventors have supposed that bone marrow stem cells exposed to a certain biological environment before transplantation are facilitated to transdifferentiate into a cardiomyocyte lineage and tried to find adequate biological growth factors and conditions which induce the cells toward one cell type (e.g., cardiomyogenic cells). Consequently, a specific method to induce bone marrow stem cells directly toward a cardiomyocyte lineage by treating biological growth factors has been developed. Further, a method of treating the induced bone marrow stem cells *in vitro* for a certain period before injection to have high rates of cell incorporation and cell survival has also been developed. By means of the methods, it was confirmed that the induced bone marrow stem cells were transplanted into infarcted dog myocardium to improve the myocardial infarction.

Korean Patent Application No. 10-2003-0004565, also owned by the present applicant, ANTEROGEN CO., LTD., describes a method of producing cardiomyogenic cells from bone marrow stem cells with high yield. The present invention provides more specific and improved method compared to that of the prior application.

Disclosure of Invention

Technical Problem

It is an object of the present invention to provide a method of inducing bone marrow stem cells of a mammal into cardiomyocytes using biological growth factors, and treating the induced bone marrow stem cells for a certain period before transplantation in order to have high rates of cell incorporation and

cell survival, and bone marrow stem cells obtained therefrom for treating a mammal (e.g., a human) diagnosed as having a disorder characterized by insufficient cardiac function.

Technical Solution

In accordance with one aspect of the present invention, it is provided a method of producing cells for transplantation into myocardial tissue of a mammal comprising the steps:

(a) providing bone marrow stem cells that have not been immortalized;

(b) passage culturing two times said bone marrow stem cells under conditions that maintain the characteristics of bone marrow stem cells;

(c) culturing said bone marrow stem cells in cardiac specific media to differentiate into cardiomyogenic cells; and

(d) collecting the cells of step (c) when about 80 to 90% of said cells are cardiomyogenic cells.

In the method of the present invention, said bone marrow stem cells can be derived from the mammal to be transplanted. In step (c), said bone marrow stem cells are preferably cultured for 1 hour to 21 days, more preferably, for 6 days.

The cardiac specific media in step (c) contain bFGF (basic fibroblast growth factor), BMP-2 (bone morphogenic protein-2) and IGF-1 (insulin-like growth factor-1). Preferably, the media contain bFGF, BMP-2 and IGF-1 in each concentration of 1 to 200 ng/ml, and further contain 2 to 20% fetal bovine serum, 1 to 1000 μM L-ascorbic acid-2-PO_4, 5 to 15 ng/ml LIF, and 1 to 200 nM dexamethasone.

According to the method of the present invention, the cultured bone marrow stem cells express a cardiomyogenic cell specific marker suitable for transplantation. It is preferred to collect the cells in such step that they express MEF2 protein and do not express MF-20.

The production of cells for transplantation into myocardial tissue of a mammal according to the present invention is carried out by the following processes: a method of inducing bone marrow stem cells into cardiomyocyte lineage before transplantation; a method of culturing the induced cells for a certain period to have high rates of cell incorporation and cell survival; and a

method of transplanting the cells into a mammal model of myocardial infarction and assessing the effect.

The method of inducing bone marrow stem cells into cardiomyocyte lineage before transplantation comprises the steps of:

(1) providing bone marrow stem cells that have not been immortalized;

(2) passage culturing said bone marrow stem cells to obtain sufficient number of cells;

(3) culturing said bone marrow stem cells in cardiac specific media containing a biological growth factor to differentiate into cardiomyogenic cells;

(4) monitoring the differentiation state of the cells of step (3); and

(5) collecting the cells of step (3) when about 60 to 90% of said cells are cardiomyogenic cells.

Further, the method of culturing the induced cells for a certain period to have high rates of cell incorporation and cell survival comprises the steps of:

(1) providing bone marrow stem cells that have not been immortalized;

(2) passage culturing said bone marrow stem cells to obtain sufficient number of cells;

(3) culturing said bone marrow stem cells in cardiac specific media containing a biological growth factor for 1 hour to 21 days to differentiate into cardiomyogenic cells;

(4) monitoring the differentiation state of the cells of step (3) at a certain period; and

(5) collecting the cells of step (3) when about 80 to 90% of said cells are cardiomyogenic cells.

Furthermore, the method of transplanting the cells into a mammal model of myocardial infarction and assessing the effect comprises the steps of:

(1) preparing a mammal model of myocardial infarction;

(2) separating bone marrow stem cells from the mammal

(3) passage culturing said bone marrow stem cells to obtain sufficient number of cells;

(4) culturing said bone marrow stem cells in cardiac specific media containing a biological growth factor for 6 days to differentiate into cardiomyogenic cells;

(5) collecting the cells of step (4) when about 80 to 90% of said cells are cardiomyogenic cells; and

(6) transplanting the cardiomyogenic cells into the mammal.

The present inventors have treated bone marrow stem cells of a mammal with a biological growth factor to differentiate into cardiac lineage, and cultured the cells for a certain period before injection to have high rates of cell incorporation and cell survival in a damaged myocardium. Further, the cultured cells were transplanted into an infarcted myocardium of a mammal, and then the state of differentiation was assessed. The cardiomyogenic cells induced according to the method of the present invention are more suitable than those obtained by using demethylating agent such as 5-azacytidine.

Hereinafter, the present invention will be described in detail.

In the step of producing cells to be transplanted into myocardial tissue of a mammal *in vitro*, growth factors can be used in all stages to induce bone marrow stem cells into cardiomyocyte lineage. Especially, all biological growth factors can be used for inducing into cardiac cells during embryonic development. The growth factors include, but are not limited to, bFGF (basic fibroblast growth factor), BMP-2 (bone morphogenic protein-2), IGF-1 (insulin-like growth factor-1), TGF-β, Wnt-inhibitor substance, activin, retinoic acid, Erb-2, all FGF growth factor members, all TGF growth factor members and all IGF growth factor members.

The transplanted cells can amplify actively or differentiate sufficiently. Bone marrow stem cells can be induced specifically into cardiomyocyte lineage, which can be identified using cardiac specific markers, and used. These markers include, but are not limited to, Nkx2.5, GATA4, MEF2 (myosin enhancing factor) and SRF (serum response factor). Differentiated cardiomyogenic cells, which can be identified by the expression of cardiac differentiation marker, can be also used. These markers include, but are not limited to, MHC (myosin heavy chain), cTnI (cardiac troponin I), cTnT (cardiac troponin T), α-cardiac actin, α-actinin and MLC2 (myosin light chain). Bone marrow stem cells can be used as cardiomyocytes which have the characteristic of communicating each other. The phenotype of such cells can be assessed by rhythmical contraction and expression of cardiac-related markers. The identification of cardiac-related markers include, but are not limited to, any detectable method, for example, reporter gene expression (LacZ induced by cardiac specific promoter), RNA expression (RT-PCR, Northern blot, RNase protection method), or protein expression (immunofluorescence assay, Western

blot, flow cytometry).

The cells transplanted according to the above method can be any cells during the differentiation step of cardiomyogenic cells.

As used herein, by "bone marrow mesenchymal stem cell or bone marrow stem cell (BMSC)" is meant a bone marrow mesenchyme-derived stem cell that is $CD45^-$. Bone marrow mesenchymal stem cells are also referred to as bone marrow stem cells and bone marrow multipotent progenitor cells.

By "cardiac cell" is meant a differentiated cardiac cell (e.g., a cardiomyocyte) or a cell committed to producing or differentiating as a cardiac cell (e.g., a cardiomyoblast or a cardiomyogenic cell).

By "cardiomyocyte" is meant a muscle cell in heart that expresses detectable amounts of cardiac markers (e.g., alpha-myosin heavy chain, cTnI, MLC2v, alpha-cardiac actin, and, *in vivo*, Cx43), contracts, and does not proliferate.

By "cardiomyoblast" is meant a cell that expresses detectable amounts of cardiac markers, contracts, and proliferates.

By "cardiomyogenic cell" is meant a cell expressing detectable amounts of Csx/Nkx2.5 RNA or protein, and does not show organized sarcomeric structures or contractions, and preferably does not express detectable amounts of myosin heavy chain protein.

By "cardic specific" is meant an initial stage of cardiac differentiation in which initial cardiac transcription factors express prior to the expression of finally differentiated markers.

By "cardic specific media" is meant a medium including bFGF, BMP-2 and IGF-1 which induce bone marrow stem cells into cardiomyocytes.

By "transdifferentiation" is meant a differentiation into other cell type which differs its origin of blastoderm.

Other features and advantages of the present invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Advantageous Effects

The cardiomyogenic cells induced according to the present invention can be clinically applied more suitably than those induced using demethylating agent

such as 5-azacytidine. According to the present invention, there are provided induced bone marrow stem cells of a mammal into cardiomyocytes having high rates of cell incorporation and cell survival, and the induced bone marrow stem cells are used for treating a mammal (e.g., a human) diagnosed as having a disorder characterized by insufficient cardiac function.

Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Figure 1 is a phase contrast micrograph (X100) of isolated, cultured bone marrow stem cells from dog.

Figure 2 is a series of micrographs (X100) showing the morphology and the expression of MEF2 (A) and Desmin (B) after culturing bone marrow stem cells of a dog in cardiac specific media, while (C) is that of negative control of MF-20.

Figure 3 is a series of micrographs (X200 in A to F, X100 in G to N) showing the expression of MEF2 after treating BMSCs with cardiac specific media and staining. A and D show untreated cells; B and E show the cells 1 day after the treatment with cardiac specific media; C and F, 2 days after the treatment; G and K, 3 days after the treatment; H and L, 6 days after the treatment; and I and M are negative controls which are treated for 8 days and then stained with rabbit-IgG.

Figure 4 is a micrograph (X40) showing the localization of implanted BMSCs in infarcted dog myocardium 15 days after implantation. Red fluorescence indicates DiI-marked BMSCs implanted into myocardium of a host, green fluorescence indicates cardiomyogenic cells stained with MF-20 antigen, and blue fluorescence indicates a nucleus marked with DAPI.

Figure 5 is a micrograph (X40) showing the localization of implanted BMSCs in infarcted dog myocardium 70 days after implantation. Red fluorescence indicates DiI-marked BMSCs implanted into myocardium, green fluorescence indicates cardiomyogenic cells reacted with MHC antigen, and blue fluorescence indicates a nucleus marked with DAPI.

Figure 6 is a micrograph (X40) showing the localization of implanted

BMSCs in infarcted dog myocardium 70 days after implantation. Red fluorescence indicates DiI-marked BMSCs implanted into myocardium, and green fluorescence indicates cardiomyogenic cells reacted with cTNI antigen.

Figure 7 is a micrograph (X40) showing the localization of implanted BMSCs in infarcted dog myocardium 96 days after implantation. Red fluorescence indicates DiI-marked BMSCs implanted into myocardium, and green fluorescence indicates cardiomyogenic cells reacted with MF-20 antigen.

Figure 8 is a graph showing the changes of heart rate before (BL) and after (4, 8 and 12 weeks) the transplantation of the induced BMSCs into infarcted dog myocardium.

Figure 9 is a series of graphs showing the changes of fractional shortening and area shortening before (BL) and after (4, 8 and 12 weeks) the transplantation of the induced BMSCs into infarcted dog myocardium in echocardiogram.

Figure 10 is a graph showing the changes of thickness of posterior wall before (BL) and after (4, 8 and 12 weeks) the transplantation of the induced BMSCs into infarcted dog myocardium in echocardiogram.

Best Mode

Hereinafter, the present invention will be described in detail, in conjunction with various examples. These examples are provided only for illustrative purposes, and the present invention is not to be construed as being limited to these examples. Further, even though bone marrow stem cells isolated from a dog are illustrated in the Examples, the present invention cannot be limited thereto but be applicable to those isolated from all kinds of mammal including human, rat, mouse, monkey, etc.

Example 1: Method of inducing cardiomyogenic cells from bone marrow stem cells by treating with biological growth factors

15~20 ml of bone marrow stem cells were obtained from iliac bone of a dog by using injector soaked with heparin more than 4 weeks after creating myocardial infarction. The bone marrow stem cells were cultured in medium containing 2~20% fetal bovine serum, 1~1,000 μM L-ascorbic acid-2-PO_4, 5~15 ng/ml LIF (leukemia inhibitory factor), and 1~200 nM dexamethasone. All

culture devices and slides were coated with 5 ng/ml collagen for 15 minutes at a room temperature before receiving bone marrow stem cells. This *in vitro* condition allows the bone marrow stem cells to maintain their self-renewing character and to expand by passaging without losing responsiveness to the differentiation agents such as growth factors. Further, this condition also allows the bone marrow stem cells to maintain a mesenchymal morphology and karyotype by passage culture. Figure 1 is a phase contrast micrograph (X100) of isolated, cultured bone marrow stem cells from dog.

After two times of passage culture, the bone marrow stem cells were inoculated into 4-well chamber slide and treated with myocardium differentiation medium. The differentiation medium includes 2~20% fetal bovine serum, 1~1,000 μM L-ascorbic acid-2-PO_4, 5~15 ng/ml LIF, 1~200 nM dexamethasone, 1~200 ng/ml bFGF, 1~200 ng/ml BMP2, and 1~200 ng/ml IFG-1 in DMEM. The medium contains the growth factors which are known to play an important role in cardiac development during embryonic development.

After 14 days of culture in the presence of growth factors, about 50~90% of the bone marrow stem cells expressed MEF2 protein. Figure 2 is a series of micrographs (X100) showing the morphology and the expression of MEF2 (A) and Desmin (B) after culturing bone marrow stem cells of a dog in cardiac specific media, while (C) is that of negative control of MF-20.

In these cells, MEF2 protein was discovered, while the expression of cardiac differentiation markers, which can be identified by immune response with MF-20, was not seen. Therefore, it appears that the cells express cardiac specific markers, but do not express terminal differentiation markers.

The expression of MEF2 is used as a cardiac lineage specific marker, and the expressed protein is positioned in nucleus. MEF2 transcription factors are expressed in cardiac progenitor during embryonic development stage before the induction of cardiac differentiation. These genes are expressed in early precardiac mesoderm during embryonic development. Further, MEF2 proteins are important transcription factors in the expression of many structure genes required for complete cardiac differentiation.

MEF2 or myosin heavy chain was labeled to be detectable in cells by the following processes: Cultured cells were refrigerated and fixed in 4% formaldehyde for 20 minutes, and then treated with PBS containing 0.2% Triton X-100 for 15 minutes. After three times of washing with PBS, the resultant

was treated with blotting solution (PBS containing 1% fetal bovine serum albumin and 0.2% Tween 20) for 15 minutes. The treated sample was reacted with anti-MEF2 (1:200, sc-10794, Santa Cruz Biotechnology, Santa Cruz), MF-20 (1:100, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa), anti-Desmin (1:100~200, Sigma-Aldrich) and, if wanted, one of isotype antibodies (rabbit IgG2b for MEF2, mouse IgG2b for MF-20, mouse IgG1 for Desmin) in moist chamber at 4 ℃ overnight. Sample slides were washed three times with washing solution (0.5% Tween 20 in PBS), and then reacted with second antigen (donkey anti-rabbit IgG for MEF2, donkey anti-mouse IgG for MF20 and Desmin; Molecular Probe) according to the manufacturer's guide. After three times of washing, the sample slides were observed by a fluorescence microscope (Nikon TS 100).

Example 2: Method of inducing bone marrow stem cells into cardiomyogenic cells having high rates of cell incorporation and cell survival for a certain period of time

Bone marrow stem cells can be treated with biological growth factors for various time period from 1 hour to 21 days in cardiac specific media, and in this period, about 40 to 90% of the cells express nucleus protein MEF2. The induction toward cardiomyogenic cells can increase up to 60 to 90% depending on the period of treatment with cardiac specific media. Treatment for optimum period enables 80 to 90% of the cells to express MEF2 protein. The transplanted cells are those of which 80 to 90% express MEF2 protein, and therefore regenerate infarcted heart most suitably.

Bone marrow stem cells isolated from adult dog were cultured in cardiac specific media for 6 days to show an optimum quantity of MEF2 protein existed in nucleus under fluorescence microscope. Transplantation of the cells into infarcted dog showed that the cells were incorporated into myocardium and the dog survived 96 days after implantation. Figure 7 is a micrograph (X40) showing the localization of implanted BMSCs in infarcted dog myocardium 96 days after implantation. Red fluorescence indicates DiI-marked BMSCs implanted into myocardium, and green fluorescence indicates cardiomyogenic cells reacted with MF-20 antigen. This *in vivo* test was carried out as follows:

In order to determine the optimum time period for the induction, bone marrow stem cells of dog were passage cultured two times and the cultured cells

were inoculated on collagen-coated 4-well chamber slides in a concentration of 10,000 cells/chamber. After 24 hours, the cells were washed two times with PBS and added with 1 ml of cardiac specific media. One of the slides became a negative control without using cardiac specific media. 1, 2, 3, 4, 5, 6, 8 and 10 days after the treatment of cardiac specific media, the cells were fixed, stained with MEF2 antibody, and then observed by fluorescence microscope. Figure 3 is a series of micrographs (X200 in A to F, X100 in G to N) showing the expression of MEF2 after treating BMSCs with cardiac specific media and staining. A and D show untreated cells; B and E show the cells 1 day after the treatment with cardiac specific media; C and F, 2 days after the treatment; G and K, 3 days after the treatment; H and L, 6 days after the treatment; and I and M are negative controls which are treated for 8 days and then stained with rabbit-IgG. As the result of the test for relative intensity of MEF2 staining, 6 days of treatment with cardiac specific media shows maximum expression.

Based on the above result, the cells collected after the treatment with cardiac specific media for 6 days were used in canine model of myocardium infarction as *in vivo* test.

Example 3: Transplantation of bone marrow stem cells and assessing the rates of cell incorporation and survival

Bone marrow stem cells, which were directed toward a cardiomyogenic cell lineage *in vitro*, were transplanted into infarcted dog myocardial tissue. The dog myocardial infarction was created by permanent occlusion of the left coronary artery. The infarction was allowed to stabilize for at least two months prior to transplantation of bone marrow stem cells. In order to prevent immunorejection of the transplants, bone marrow was collected and bone marrow stem cells were prepared from the individual transplant recipient dogs as follows:

About 4 weeks after the ligation, after the myocardial infarction had been confirmed using echocardiogram, iliac bone was punctured to aspirate bone marrow. Bone marrow was immediately mixed with heparin, frozen and transported in dry ice to the tissue culture facility, where the bone marrow was thawed at 37 ℃, perturbated, washed once with regular DMEM, and placed in tissue culture flasks containing culture medium (2~20% fetal bovine serum, 1~1,000 μM L-ascorbic acid-2-PO_4, 5~15 ng/ml LIF, and 1~200 nM

dexamethasone). After two times of passage culturing, bone marrow stem cells were cultured in media containing 1~200 ng/ml bFGF, 1~200 ng/ml BMP2, and 1~200 ng/ml IFG-1 for 6 days. At the time of harvest, bone marrow stem cells were labeled with DiI, a red fluorescent marker, to track the survival and progression of the cells following transplantation. About 2 x 10^8 cells were harvested and injected into the infarcted region of the heart.

Survival of bone marrow stem cells following transplantation was assessed by post-mortem visualization of DiI fluorescence. Figure 4 is a micrograph (X40) showing the localization of implanted BMSCs in infarcted dog myocardium 15 days after implantation. Red fluorescence indicates DiI-marked BMSCs implanted into myocardium of a host, green fluorescence indicates cardiomyogenic cells stained with MF-20 antigen, and blue fluorescence indicates a nucleus marked with DAPI. As shown in Figure 4, large clusters of DiI-positive cells were observed in the myocardium 15 days after transplantation, suggesting long-term viability of the bone marrow stem cells. Specifically, the DiI-labeled stem cells were observed within regions of the myocardium containing MF-20-positive cardiomyocytes and in the infarcted regions which were devoid of MF-20-positive cardiomyocytes.

In order to investigate the long-term viability of implanted cells, the dog was killed and observed. Figure 5 is a micrograph (X40) showing the localization of implanted BMSCs in infarcted dog myocardium 70 days after implantation. Red fluorescence indicates DiI-marked BMSCs implanted into myocardium, green fluorescence indicates cardiomyogenic cells reacted with MHC antigen, and blue fluorescence indicates a nucleus marked with DAPI. Figure 6 is a micrograph (X40) showing the localization of implanted BMSCs in infarcted dog myocardium 70 days after implantation. Red fluorescence indicates DiI-marked BMSCs implanted into myocardium, and green fluorescence indicates cardiomyogenic cells reacted with cTNI antigen.

Further, Figure 7 is a micrograph (X40) showing the localization of implanted BMSCs in infarcted dog myocardium 96 days after implantation. Red fluorescence indicates DiI-marked BMSCs implanted into myocardium, and green fluorescence indicates cardiomyogenic cells reacted with MF-20 antigen. As shown in Figures 5 to 7, in all cases DiI-marked cells were observed in the infarcted area. In some cases, DiI fluorescence was observed in the same place as the cardiac specific marker containing MF-20. Further, the border region of

the infarcted area contained DiI positive stem cells which also express the cardiac muscle specific marker, MHCα/β and TNI.

These data demonstrate that transplanted BMSCs, which have been conditioned *in vitro* according to the above-described methods, survive and incorporate into the host myocardium and express markers characteristic of cardiac differentiation.

Example 4: Improvement of myocardial infarction

The canine myocardial infarction model described above was used for assessment, by echocardiogram (ECG), of the restorative effects of BMSC transplantation. ECGs were performed 3.5, 4.5, and 5 weeks after BMSC transplantation and compared to pre-implantation ECGs.

Figure 8 is a graph showing the changes of heart rate before (BL) and after (4, 8 and 12 weeks) the transplantation of the induced BMSCs into infarcted dog myocardium. Figure 9 is a series of graphs showing the changes of fractional shortening and area shortening before (BL) and after (4, 8 and 12 weeks) the transplantation of the induced BMSCs into infarcted dog myocardium in echocardiogram. Further, Figure 10 is a graph showing the changes of thickness of posterior wall before (BL) and after (4, 8 and 12 weeks) the transplantation of the induced BMSCs into infarcted dog myocardium in echocardiogram.

As shown in Figures 8 to 10, in each animal, contraction of the infarcted area became more synchronized with neighboring area of the myocardium. The ECG results confirm the histological findings of Example 3 and demonstrate that transplantation of cultured BMSCs results in a partial restoration of cardiac tissue following infarction.

CLAIMS

1. A method for producing cells for transplantation into myocardial tissue of a mammal comprising the steps:

(a) providing bone marrow stem cells that have not been immortalized;

(b) passage culturing two times said bone marrow stem cells under conditions that maintain the characteristics of bone marrow stem cells;

(c) culturing said bone marrow stem cells in cardiac specific media to differentiate into cardiomyogenic cells; and

(d) collecting the cells of step (c) when about 80 to 90% of said cells are cardiomyogenic cells.

2. The method of claim 1, wherein said bone marrow stem cells are derived from the mammal to be transplanted.

3. The method of claim 1, wherein said bone marrow stem cells are cultured for 1 hour to 21 days in step (c).

4. The method of claim 3, wherein said bone marrow stem cells are cultured for 6 days in step (c).

5. The method of claim 1, wherein the cardiac specific media contain biological growth factors.

6. The method of claim 5, wherein the biological growth factors are bFGF (basic fibroblast growth factor), BMP-2 (bone morphogenic protein-2) and IGF-1 (insulin-like growth factor-1).

7. The method of claim 6, wherein the media contain bFGF, BMP-2 and IGF-1 in each concentration of 1 to 200 ng/ml, and further contain 2 to 20% fetal bovine serum, 1 to 1000 μM L-ascorbic acid-2-PO_4, 5 to 15 ng/ml LIF, and 1 to 200 nM dexamethasone.

8. The method of claim 1, wherein the cells are cultured to express a cardiomyogenic cell specific marker suitable for transplantation.

9. The method of claim 8, wherein the cells are collected in such step that they express MEF2 protein and do not express MF-20.