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Description

Title of Invention

CELL PREPARATION CONTAINING MESENCHYMAL STEM CELLS, AND METHOD FOR PRODUCING SAME

Technical Field

The present invention relates to a cell preparation containing mesenchymal stem cells and a method for producing the same. The present invention further relates to an additive for a medium for use in the method, a culturing medium, a kit, and a culturing method using any one of them.

Background Art

Mesenchymal stem cells (MSCs) can be isolated not only from tissues of adults, such as bone marrow, fat, a synovial membrane, an alveolar bone, and a peridontal membrane, but also from various cells, such as placenta, cord blood, and umblical cord. In addition, the mesenchymal stem cells can be cultured and proliferated in vitro. Furthermore, the mesenchymal stem cells have multipotency so that the mesenchymal stem cells can be differentiated not only to a plurality of mesenchymal cells (osteoblast, fat cell, cartilage cell), but also to non-mesenchymal cells (neural precursor cell, hepatocyte). Accordingly, the mesenchymal stem cells are expected to be applicable as a cell source for regenerative

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medicine and cell treatment.

A medium containing fetal bovine serum (FBS) has been conventionally used for culturing mesenchymal stem cells. Bovine serum has lot differences, and in addition, causes an immune response in transplantation because the use of bovine serum results in contamination of the mesenchymal stem cells with a serum protein derived from a heterozonic. It is difficult to stably culture the mesenchymal stem cells even by use of human serum due to individual differences. Further, this culturing gives a large physical burden to a donor, and costs a large amount of money.

Therefore, it is known that, in order to supply mesenchymal stem cells which are safer and has a stable quality, a serum-free medium with which no or little contamination with a protein derived from a heterozonic occurs during culturing is suitable. That is, it is preferable that the mesenchymal stem cells are proliferated by a serum-free culturing. Patent Literature 1 and Non-patent Literature 1 disclose serum-free culturing of mesenchymal stem cells. The serum-free culturing mesenchymal stem cells as described in Patent Literature 1 and Non-patent Literature 1 produce a proliferation promoting effect superior to that of culturing of mesenchymal stem cells with a 5 to 15% FBS-containing medium. Such serum-free culturing also allows to produce mesenchymal stem cells whose multipotency is

maintained (improved).

It has been known that mesenchymal stem cells not only have low immunogenicity, but also affect functions of various immune effecter cells (T cell, B cell, NK cell, dendritic cell) (see Non-patent Literatures 2 to 5). Accordingly, the mesenchymal stem cells are expected to be applicable to treatment for various diseases relating to immune responses (Non-patent Literature 6). Non-patent Literature 7 discloses that mesenchymal stem cells down-regulate proliferation of T cells caused by a mouse mixed lymphocyte reaction (MLR).

Citation List

Patent Literature

Citation List

Patent Literature 1

Pamphlet of International Publication WO2007/080919 (Publication date: July 19, 2007)

Non Patent Literature

Non Patent Literature 1

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Non Patent Literature 2

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Non Patent Literature 3

Corcione, A. et. al., Blood 107, 367-372, 2006

Non Patent Literature 4

Ramasamy, R. et. al., Transplantation 83, 71-76, 2007

Non Patent Literature 5

Aggarwal, S. et. al., Blood 105, 1815-1822, 2005

Non Patent Literature 6

Le Blanc K. et. al., J Intern Med., 262, 509-525, 2007

Non Patent Literature 7

Djouad, F. et. al., Blood 102, 3837-3844, 2003

Summary of Invention

Technical Problem

Immunorejection in transplantation is an important problem in regenerative medicine, and an immunosuppressant is used to prevent the immunorejection. The use of immunosuppressant is, however, associated with problems such as a side effect of the immunosuppressant. Mesenchymal stem cells have immunosuppression ability, so that the immunosuppressant is not necessary if the mesenchymal stem cells can be used. Therefore it would be advantageous if mesenchymal stem cells having immunosuppression ability was produced by carrying out serum-free or low-serum culturing having a low risk of heteroprotein and a synthetic medium contamination. Particularly, an FBS-containing medium has a problem not only in an amount of bovine serum albumin but also in containing other soluble allergic

substances. It is therefore necessary to use a culturing medium which does not contain bovine serum albumin and the other soluble substances.

The present invention has been made in view of the aforementioned problem, and an object of the present invention is to provide a method for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained by carrying out the serum-free or low-serum culturing.

Solution to Problem

In order to attain the aforementioned object, the inventors of the present invention diligently studied influence of serum-free culture on immunosuppression ability of mesenchymal stem cells. As a result, the inventors found that the mesenchymal stem cells cultured in a serum-free medium containing a particular additive maintains immunosuppression ability, and an immunosuppressive effect is even improved. Based on this finding, the present invention was accomplished.

Specifically, a method for producing a cell preparation containing mesenchymal stem cells according to the present invention includes the steps of: (A) proliferating mesenchymal stem cells in a serum-free medium "A" containing an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid,

and at least one fatty acid; and (B) screening mesenchymal stem cells whose immunosuppression ability is maintained or improved, from the mesenchymal stem cells thus proliferated in the step (A).

A cell preparation containing mesenchymal stem cells according to the present invention is produced by the aforementioned producing method.

A serum-free additive for a medium for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved, comprising: an FGF; a PDGF; a TGF-β; an HGF; an EGF; at least one phospholipid; and at least one fatty acid.

A serum-free culturing medium according to the present invention is a culturing medium for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved, comprising: the additive for a medium.

A culture method according to the present invention for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved, comprising: (F) culturing mesenchymal stem cells in the culturing medium.

A kit according to the present invention for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved,

comprising at least: the additive "A" for a medium.

Advantageous Effects of Invention

A method for producing a cell preparation containing mesenchymal stem cells according to the present invention, comprising the steps of: (A) proliferating mesenchymal stem cells in a serum-free medium "A" containing an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid; and (B) screening mesenchymal stem cells whose immunosuppression ability is maintained or improved, from the mesenchymal stem cells thus proliferated in the step (A).

For a fuller understanding of the nature and advantages of the invention, reference should be made to the ensuing detailed description taken in conjunction with the accompanying drawings.

Brief Description of Drawings

Fig. 1

Fig. 1 is a graph showing an immunosuppressive effect of hMSCs on anti-CD3 and anti-CD28 stimulation T cell proliferative responses in a case where an activated mouse T cell and the hMSC were co-cultured in an MSCGM medium.

Fig. 2

Fig. 2 is a graph showing an immunosuppressive effect

of hMSCs on anti-CD3 and anti-CD28 stimulation T cell proliferative responses in a case where activated mouse T cells and an hMSC were co-cultured in a serum-free medium "A" according to one embodiment of the present invention.

Fig. 3

Fig. 3 is a graph showing an immunosuppressive effect of hMSCs on a mitogen stimulation T cell proliferative response in a case where activated mouse T cells and the hMSCs were co-cultured in an MSCGM medium.

Fig. 4

Fig. 4 is a graph showing an immunosuppressive effect of hMSCs on a mitogen stimulation T cell proliferative response in a case where activated mouse T cells and the hMSCs were co-cultured in a serum-free medium "A" according to one embodiment of the present invention.

Fig. 5

Fig. 5 is a graph showing an immunosuppressive effect of hMSCs on a mouse mixed lymphocyte reaction stimulation T cell proliferative response (results of the third day in culturing).

Fig. 6

Fig. 6 is a graph showing an immunosuppressive effect of hMSCs on a mouse mixed lymphocyte reaction stimulation T cell proliferative response (results of the fourth day in culturing).

Fig. 7

Fig. 7 is views each illustrating a proliferated state of hMSCs derived from adipose tissues on the eighth day in culturing.

Fig. 8

Fig. 8 is a graph and views showing a proliferation promoting effect of a medium 1 and a medium 2 on hMSCs derived from an initial synovial membrane: (a) of Fig. 8 is a graph showing the number of cells of hMSCs derived from a synovial membrane (on the twelfth day in culturing); and (b) of Fig. 8 is views each illustrating a state of the proliferated hMSCs derived from the synovial membrane (on the twelfth day in the culturing).

Fig. 9

Fig. 9 is a graph showing a chronological change of the number of cells over a period from 0 day (start of culturing) to 68 days.

Fig. 10

Fig. 10 is views each illustrating a proliferated state of hMSCs (cells 1) derived from bone marrow on the fifth day in culturing.

Fig. 11

Fig. 11 is views each illustrating a proliferated state of hMSCs (cells 2) derived from bone marrow on the fifth day in culturing.

Fig. 12

Fig. 12 is views each illustrating a proliferated state of hMSCs (cells 3) derived from bone marrow on the fifth day in culturing.

Fig. 13

Fig. 13 is a graph showing effects of the medium 1 on proliferation of neonatal normal human dermal fibroblasts and human chondrosarcoma cell strains: (a) of Fig. 13 is a graph showing the number of colonies of the neonatal normal human dermal fibroblasts on the fourteenth day in culturing; and (b) of Fig. 13 is a graph showing the number of colonies of the human chondrosarcoma cell strains on the fourteenth day in culturing.

Description of Embodiments

One embodiment of the present invention is described below in detail. Note, however, that the present invention is not limited thereto. The present invention can be modified in many ways within the scope of the description. Note that "A to B" indicative of a numerical range means "A or more and B or less", unless otherwise specified in the Specification.

As described above, Patent Literature 1 and Non-patent Literature 1 disclose a medium (serum-free medium) which does not contain serum and which allows to culture cells without loosing proliferation ability. The medium is made by

adding, to a basal medium, a complex of a particular growth factor group, a phospholipid, and a fatty acid, thereby giving the medium a cell proliferation promoting effect equal to or larger than that of a medium under a 10% serum condition, even if the medium is a serum-free medium. It is possible to carry out serum-free culturing of mesenchymal stem cells in the medium while maintaining or improving differentiation ability of the mesenchymal stem cells. It is also possible to carry out serum-free culturing of MSCs derived from bone marrow, MSCs derived from fat, and MSCs derived from the synovial membrane in the medium.

According to the present invention, a medium having ability of greatly proliferating the mesenchymal stem cells under the serum-free condition further has an effect of realizing culture of the mesenchymal stem cells whose immunosuppression ability is maintained or improved. This makes it possible to produce a cell preparation containing the mesenchymal stem cells which are useful for regenerative medicine in particular.

(1) Method for Producing Cell Preparation Containing Mesenchymal Stem Cells

The present invention provides a method for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved under a serum-free condition. The method for producing the cell

preparation according to the present invention includes the steps of: (A) proliferating mesenchymal stem cells in a serum-free medium "A" containing an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid; and (B) screening mesenchymal stem cells whose immunosuppression ability is maintained or improved from the mesenchymal stem cells thus proliferated which have been subjected to the step (A).

(Proliferation step)

In a proliferation step of this producing method according to the present invention, the mesenchymal stem cells are proliferated by culturing the mesenchymal stem cells in a serum-free medium "A" containing an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid.

In the Specification, the term "serum-free medium" means a medium which does not contain serum, and the term "serum-free culturing" is culturing without serum. Further, in the Specification, the term "low-serum culturing" means culturing by use of a medium whose serum content is lower than that of a general serum-containing medium (e.g., 10% FBS-containing medium), and also means culturing whose culturing period by use of a serum-containing medium is shorter than that of general culturing by use of the serum-containing medium.

In the Specification, the term "cell preparation" is a remedy made by preparing a cell, and is used for regenerative medicine etc. as a material for regenerative medicine. The term "cell preparation" encompasses not only a preparation made by preparing a cell as it is, i.e., a cell whose functions are not changed, but also a preparation made by preparing a cell whose functions (such as a differentiation ability and an immunosuppression ability) are improved by culturing and proliferating the cell under a particular condition.

Further, in the Specification, the term "mesenchymal stem cells" encompasses not only cells isolated from tissues of adults, such as bone marrow, a fat cell, a synovial cell, a alveolar bone, and a peridontal membrane, but also cells isolated from various cells such as fetus, placenta, and cord blood. It is preferable that the term "mesenchymal stem cells" are human mesenchymal stem cells, however, mesenchymal stem cells derived from non-human animals, such as rats and mice, may be used.

A basal medium for constituting the serum-free medium "A" for use in the proliferation step is not particularly limited, provided that the basal medium is a well-known medium for animal cells in this field. A preferable examples of the basal medium encompass a Ham's F12 medium, a DMEM medium, an RPMI-1640 medium, and an MCDB medium. Those basal mediums can be used alone, or two or more types of the basal

mediums can be used in combination. A basal medium for constituting the serum-free medium "A" in one embodiment is preferably a medium in which an MCDB and a DMEM are mixed at a ratio of 1:1.

The serum-free medium "A" whose basal medium is added with an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid may be used in the proliferation step of one embodiment. A content of the FGF in the basal medium is preferably 0.1 to 100 ng/ml at a final concentration, and more preferably 3 ng/ml. A content of the PDGF in the basal medium is preferably 0.5 to 100 ng/ml at a final concentration, and more preferably 10 ng/ml. A content of the TGF-β in the basal medium is preferably 0.5 to 100 ng/ml, and more preferably 10 ng/ml.

A content of the HGF in the basal medium is preferably 0.1 to 50 ng/ml at a final concentration, and more preferably 5 ng/ml. A content of the EGF in the basal medium is preferably 0.5 to 200 ng/ml at a final concentration, and more preferably 20 ng/ml. A total content of phospholipid(s) in the basal medium is preferably 0.1 to 30 μg/ml at a final concentration, and more preferably 10 μg/ml. A total content of fatty acid(s) with respect to the basal medium is preferably 1/1000 to 1/10 of the basal medium, and more preferably 1/100.

The use of such serum-free medium "A" has the

proliferation promoting effect, which is equal to or larger than that of a serum-containing medium, while preventing heteroprotein contamination. This makes it possible to desirably proliferate the mesenchymal stem cells.

In the proliferation step of the producing method according to the present invention, examples of a phospholipid contained in the serum-free medium "A" encompass phosphatidic acid, lysophosphatidic acid, phosphatidylinositol, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidyl glycerol. Those phospholipids can be used alone, or two or more types of the phospholipids can be used in combination. In one embodiment, the serum-free medium "A" may contain an phosphatidic acid and phosphatidyl choline in combination, and those phospholipids may be derived from animals or plants.

Examples of the fatty acids contained in the serum-free medium "A" encompass linoleic acid, oleic acid, linolenic acid, arachidonic acid, myristic acid, palmitoyl acid, palmitic acid, and stearic acid. The additive for a medium according to this embodiment may contain any one of those fatty acids alone or two or more types of the fatty acids in combination. Further, the serum-free medium "A" according to this embodiment may contain not only the fatty acid(s) but also cholesterol.

The term "FGF" used in the Specification means a

growth factor selected from a fibroblast growth factor (FGF) family, and is preferably FGF-2 (bFGF). However, other FGFs of the FGF family, such as an FGF-1 etc., may be selected. The term "PDGF" used in the Specification means a growth factor selected from a platelet derived growth factor (PDGF) family, and is preferably a PDGF-BB or a PDGF-AB. The term "TGF-β" used in the Specification means a growth factor selected from a transforming growth factor-β (TGF-β) family, and is preferably TGF-β3. However, other TGF-βs of the TGF-β family may be selected.

The term "HGF" used in the Specification means a growth factor selected from a hepatocyte growth factor family, and the term "EGF" means a growth factor selected from an epidermal growth factor (EGF) family.

In one embodiment, the serum-free medium "A" may further contain at least two factors selected from the group consisting of a connective tissue growth factor (CTGF), a vascular endothelial growth factor (VEGF), and an ascorbic acid compound.

The term "ascorbic acid compound" used in the Specification means ascorbic acid (vitamin C), ascorbic acid-2-phosphoric acid, or a compound similar to those ascorbic acids.

Note that the growth factors contained in the serum-free medium "A" may be natural or may be produced by

genetical modification.

In one aspect, the serum-free medium "A" preferably contains a lipid antioxidant. The lipid antioxidant contained in the serum-free medium "A" may be DL-α-tocopherol acetate (vitamin E) in one embodiment. The serum-free medium "A" may further contain a surfactant. The surfactant contained in the serum-free medium "A" may be Pluronic F-68 or Tween 80 in one embodiment.

The serum-free medium "A" may further contain insulin, transferrin, and selenate. The term "insulin" used in the Specification may be also an insulin-like growth factor and may be derived from a natural cell or may be genetically modified. The additive for a medium according to the present invention may further contain dexamethasone, or another glucocorticoid.

In the proliferation step, the mesenchymal stem cells isolated from an animal (such as humans) tissue or cell by a conventionally public-known method is inoculated on the serum-free medium "A" described above, and is cultured until the mesenchymal stem cells are proliferated to the desired number of cells. It is preferable that, as a culture condition: the mesenchymal stem cells (1 to 2×10^4) are inoculated in a medium (1 ml) and incubated at a culture temperature of 37 °C ± 1 °C for a culture time period in a range of 48 to 96 hours under 5% CO_2 environment. By culturing the

mesenchymal stem cells as described above, it is possible to efficiently produce a large number of mesenchymal stem cells whose immunosuppression ability is maintained or improved.

In the proliferation step, a culture vessel for use in culturing is not particularly limited, provided that the mesenchymal stem cells can be proliferated. For example, a 75 cm^2 flask (Falcon), a 75 cm^2 flask (manufactured by SUMITOMO BAKELITE CO., LTD.), etc. can be preferably used. Note, however, that proliferation of some cells are affected by kinds of culture vessels to be used. It is therefore preferable that, in order to proliferate more efficiently the mesenchymal stem cells, the mesenchymal stem cells to be proliferated (hereinafter, also referred to as a "proliferation target cell") in the proliferation step is subjected to the proliferation step by use of a culture vessel suitable for proliferation of these mesenchymal stem cells.

Examples of a method for selecting a culture vessel suitable for proliferation of a proliferation target cell encompass a method in which an optimum culture vessel is selected by the proliferation target cell. More specifically, multiple kinds of culture vessels are prepared, and proliferation target cells are proliferated under the same conditions other than the kinds of culture vessels. The number of cells in each vessel is measured by a public-known method on fourteenth day in culturing. It can be determined

that a culture vessel having the largest number of cells is the most suitable for proliferating the proliferation target cell. Further, in a case where proliferation speed of the proliferation target cell is high, it can be determined that a culture vessel having a shortest period in which the proliferation target cells reach the number of cells of 80 to 90% of a confluent state is the most suitable for proliferating the proliferation target cell even if the culturing is carried out for less than two weeks.

In a case where a culture vessel suitable for the proliferation of the proliferation target cell has been already found out, the culture vessel may be used in the proliferation step of the producing method according to the present invention. On the contrary, in a case where a culture vessel suitable for proliferation of the proliferation target cell has not been found out yet, the producing method according to the present invention may further include, before the proliferation step, a "culture vessel selecting step" (described later) for selecting a culture vessel suitable for the proliferation of the proliferation target cell.

Note that adhesion of the mesenchymal stem cells to a culture vessel is a necessary condition for proliferating the mesenchymal stem cells. It is therefore preferable that, in a case where adhesion of the proliferation target cell to the culture vessel is not sufficient, the serum-free medium "A"

further contains cell adhesion molecules in the proliferation step. Examples of the "cell adhesion molecules" encompass fibronectin, collagen, and gelatin. Those cell adhesion molecules can be used alone, or two or more types of the cell adhesion molecules can be used in combination.

Cell adhesion molecule content in the serum-free medium "A" is preferably 1 to 50 μg/ml at a final concentration, and more preferably 5 μg/ml at the final concentration. In one embodiment, where the cell adhesion molecules are fibronectin, fibronectin is added so that a final concentration of fibronectin in the serum-free medium "A" is 5 μg/ml. This can improve the adhesion efficiency of the proliferation target cell with respect to the culture vessel.

In the proliferation step, the mesenchymal stem cells may be subcultured at least once. The mesenchymal stem cells are proliferated anchorage-dependently. For example, in a case where the mesenchymal stem cells are locally unevenly proliferated, the culture condition of the mesenchymal stem cells can be improved by subculturing the mesenchymal stem cells in the middle of the proliferation step.

The subculturing of the mesenchymal stem cells may be carried out in any way, and may be performed by a conventionally public-known method of subculturing mesenchymal stem cells. For the sake of good cell conditions of the subcultured mesenchymal stem cells, it is preferable to

peel off the mesenchymal stem cells by use of a cell remover which does not contain any component derived from mammal and microorganism in a case where the mesenchymal stem cells are to be subcultured in the proliferation step. Examples of the term "cell remover which does not contain any component derived from mammal and microorganism" encompass ACCUTASE (Innovative Cell Technologies, Inc.).

The following description will discuss an example of a subculturing method in a case where ACCUTASE is used as "the cell remover which does not contain any component derived from mammal and microorganism". The mesenchymal stem cells are peeled off by processes (i) to (vi), and is subcultured. Note that, in the following subculturing method, a T-25 flask (Falcon) is assumed to be used as a culture vessel.

(i) A cell layer is washed with PBS(-) (5 mL).

(ii) ACCUTASE (2 mL) is added.

(iii) The resultant liquid is let stand at a room temperature for about two minutes, and whether or not cells are released is checked. After that, a cell suspension fluid is moved to a tube.

(iv) PBS(-) (7 mL) is added into a culture vessel, so as to rinse a bottom surface of the flask with PBS (-).

(v) The resultant solution of the process (iv) is transferred to the tube of the process (iii), and is centrifuged at 1500 rpm

(200×g) for five minutes.

(vi) Supernatant is removed, and the solution is inoculated to the serum-free medium "A" at inoculation density of 5,000 cells/cm^2.

Note that, in the proliferation step, it is preferable to provide mesenchymal stem cells which have been subcultured at least once (P1) after collection of mesenchymal stem cells from animal (such as human) tissues.

(Screening step)

In a screening step (also referred to as a "first screening step") of the producing method according to the present invention, mesenchymal stem cells whose immunosuppression ability is maintained or improved is screened from the mesenchymal stem cells which have been subjected to the proliferation step. The mesenchymal stem cells proliferated in the serum-free medium "A" in the proliferation step at least maintain and even improve their immunosuppression ability. By screening such mesenchymal stem cells by use of the immunosuppression ability as a reference, it is possible to select mesenchymal stem cells whose immunosuppression ability is maintained or improved. The mesenchymal stem cells thus selected are used as a cell preparation. This makes it possible to realize a transplantation treatment of the mesenchymal stem cells in which immunorejection is down-regulated, without using an immunosuppressant.

The serum-free medium "A" does not contain heteroprotein, and can grow the mesenchymal stem cells without depriving the function as stem cells from the mesenchymal stem cells. Further, the mesenchymal stem cells cultured in the serum-free medium "A" have a proliferation ability higher than that of mesenchymal stem cells cultured in the bovine fetusserum-containing medium, and also has a high activity indicative of an immunosuppressive effect. In a case where mesenchymal stem cells cultured in the serum-free medium "A" is used for transplantation treatment, a cell having the function as a stem cell and the immunosuppression ability is increased and individual cells thus proliferated have high activity. It is therefore expected that the function and the immunosuppression ability create a synergistic effect.

Meanwhile, the inventors of the present invention discussed tumorigenicity conceivable from an effect of the proliferation ability of the serum-free medium "A" by testing by a soft agar culture method in vitro and a tumorigenicity test by use of a high-sensitive immunodeficient mouse (NOG mouse) in vivo. Specifically, human mesenchymal stem cells derived from synovial membrane (3 lots) and human mesenchymal stem cells derived from bone marrow (1 lot) were cultured in the serum-free medium "A", and 1,000,000 of cultured cells were transplanted to a medium for a soft agar

culture and ten subcutaneous parts of the NOG mouse. As a result, in a case of the soft agar culture, a tumor colony was observed when 1,000 of Hela cells were transplanted, whereas in the test with the mesenchymal stem cells showed negative results (3 lots). Meanwhile, a tumor nodule was not observed in any transplanted tissues of the NOG mouse under such experimental condition that a tumor nodule was detected even if only one Hep G2 cell was mixed in the NOG mouse (data is not shown). From these reasons, it is clear that a cell preparation produced by the present invention is useful in transplantation treatment.

The term "immunosuppression ability" used in the Specification means ability to down-regulate immunorejection which occurs when a different kind or the same kind of cell etc. is xenotransplanted, and means ability to down-regulate an immune reaction by affecting functions of various immune effecter cells (e.g., by down-regulating proliferation of a T cell). This is a part of anti-inflammatory ability. The wording "the immunosuppression ability is maintained" used in the Specification means that the immunosuppression ability which mesenchymal stem cells naturally have is not lost by the proliferation step, and the wording "the immunosuppression ability is improved" means that the immunosuppression ability which mesenchymal stem cells have is improved after the proliferation step, as compared

with the immunosuppression ability before the proliferation step is carried out.

In the screening step, the mesenchymal stem cells thus proliferated and an immune cell are co-cultured in the serum-free medium "A" which has been used in the proliferation step, and the number of immune cells in the medium is evaluated after the co-culturing is carried out. This makes it possible to screen the mesenchymal stem cells whose immunosuppression ability is maintained or improved, however, the present invention is not limited thereto. For example, as described in Example (described later), an increasing amount of the T cell or a production amount of cytokine is evaluated in a case where the T cell is used as the immune cell, and an increasing amount of a B cell or an activated NK cell is evaluated in a case where the B cell or the activated NK cell is used as the immune cell. This makes it possible to screen the mesenchymal stem cells whose immunosuppression ability is maintained or improved. Further, in a case where a dendritic cell is used as the immune cell, differentiation, maturation, activation, etc. of the dendritic cell are evaluated. This makes it possible to screen the mesenchymal stem cells whose immunosuppression ability is maintained or improved. The immune cell for use in such screening is preferably the T cell, the B cell, or the activated NK cell, and more preferably, the T

cell or the B cell, and the most preferably the T cell.

(Second screening step)

The producing method according to the present invention may further include a second screening step in which mesenchymal stem cells which do not have the tumorigenicity are screened from the mesenchymal stem cells which has been subjected to the proliferation step.

The proliferation in the serum-free medium "A" in the proliferation step selectively grows the mesenchymal stem cells so that the number of the mesenchymal stem cells which do not have the tumorigenicity is increased. Such mesenchymal stem cells are screened by use of the tumorigenicity as a reference. This makes it possible to select a mesenchymal stem cells which do not have the tumorigenicity. Meanwhile, the mesenchymal stem cells thus selected are used as a cell preparation, and this realizes transplantation treatment of the mesenchymal stem cells which do not have the tumorigenicity. Therefore, the present invention also provides a method for producing a cell-preparation-containing mesenchymal stem cells which do not have the tumorigenicity under a serum-free condition.

Whether or not the mesenchymal stem cells have the tumorigenicity can be confirmed by a conventionally public-known method, such as the soft agar culture method in vitro or the tumorigenicity test by use of a high-sensitive

immunodeficient mouse (NOG mouse) in vivo, as described in the "Screening step", however, the present invention is not limited to those methods. That is, the term "mesenchymal stem cells which do not have the tumorigenicity" used in the Specification means a mesenchymal stem cells which are confirmed that the mesenchymal stem cells do not have the tumorigenicity by the methods for confirming whether or not the mesenchymal stem cells have the tumorigenicity.

Note that, in a case where the producing method according to the present invention further includes the second screening step, an order in which the first screening step and the second screening step are carried out is not particularly limited. That is, the first screening step may be carried out before the second screening step, or alternatively, the first screening step may be carried out after the second screening step.

(Serum culture step)

The producing method according to the present invention further includes a serum culture step in which the mesenchymal stem cells which have been subjected to the proliferation step are cultured in a serum-containing medium before the screening step is carried out. In the serum culture step, the mesenchymal stem cells which have been cultured under a serum-free condition in the proliferation step are cultured in a serum-containing medium. In a case where the

serum culture step is carried out, the mesenchymal stem cells are subjected to the screening step after being subjected to the serum culture step.

A conventionally public-known serum-containing medium can be used as the serum-containing medium for use in the serum culture step, such as a medium containing 10% FBS made by adding 10% FBS to the basal medium. In the serum culture step, the mesenchymal stem cells obtained in the proliferation step are inoculated and cultured in the serum-containing medium. It is preferable that, as a culture condition, (A) the number of mesenchymal stem cells are 1 to 2×10^4 with respect to a medium (1 ml), (B) the culture temperature is 37 °C ± 1 °C , the culture time period is 48 to 96 hours, and (C) the mesenchymal stem cells are under 5% CO_2. By culturing the mesenchymal stem cells as described above, it is possible to efficiently and earlier obtain a larger number of the mesenchymal stem cells which have immunosuppression ability.

The mesenchymal stem cells cultured in the serum-containing medium are subjected to the screening step, and the mesenchymal stem cells whose immunosuppression ability is maintained or improved are selected.

As described above, the mesenchymal stem cells are pre-cultured in the serum-free medium "A", and are then cultured in the serum-containing medium. Therefore, the

mesenchymal stem cells can obtain the immunosuppressive effect equal to or larger than that of mesenchymal stem cells which are pre-cultured and cultured in the serum-containing medium. Further, the mesenchymal stem cells exhibit the immunosuppressive effect earlier. Further, since serum is used only when the mesenchymal stem cells are cultured, the content of serum is low. This realizes low-serum culturing.

(Pre-proliferation step)

The producing method according to the present invention may further include, before the step (A), the step of: (D) pre-proliferating the mesenchymal stem cells in a serum-free medium B containing an FGF, a PDGF, an EGF, at least one phospholipid, and at least one fatty acid.

The term "serum-free medium B" is different from the serum-free medium "A" described in the "proliferation step" in that the serum-free medium B does not contain an HGF and TGF-β. Components other than the HGF and TGF-β (an FGF, a PDGF, an EGF, at least one phospholipid, and at least one fatty acid) and a basal medium are the same as those of the serum-free medium "A" described in the "proliferation step", so that the description thereof is herein omitted.

Further, in one aspect, the serum-free medium B preferably contains a lipid antioxidant, which is similar to the serum-free medium "A". Further, the serum-free medium B may further include a surfactant. Further, the serum-free

medium B may further contain insulin, transferrin, and selenate. The serum-free medium B may further contain dexamethasone or another glucocorticoid. Those components also have already described in the serum-free medium "A" of the "proliferation step", so that the description thereof is herein omitted.

Note that contents of those components contained in the serum-free medium B may be the same as those of the serum-free medium "A" or may be different from those of the serum-free medium "A", provided that the contents of the components fall within ranges of the contents of the components of the serum-free medium "A" described in the "proliferation step".

In the pre-proliferation step, the mesenchymal stem cells isolated from an animal (such as human) tissue by a conventionally public-known method are inoculated in the serum-free medium B, and are cultured until the number of mesenchymal stem cells is proliferated to become the desired number of the mesenchymal stem cells. It is preferable that, as a culture condition, (A) tissue slices (containing MSCs) of 1 to 500 mg are separated with respect to a medium 1 ml, (B) the mesenchymal stem cells are inoculated, (C) the culture temperature is 37 °C ± 1 °C, (D) a culture time period is 3 to 14 days, and (E) the mesenchymal stem cells are under the 5% CO_2.

The mesenchymal stem cells which are subjected to pre-proliferation step are not particularly limited. However, the mesenchymal stem cells are preferably initial mesenchymal stem cells, i.e., are preferably cells which have never been subcultured after being collected from an animal (such as human) tissue. As described in Examples (described later), initial mesenchymal stem cells are proliferated in advance in the serum-free medium B before the proliferation step is carried out. This makes it possible to remarkably proliferate the number of the mesenchymal stem cells in the proliferation step.

A method for culturing mesenchymal stem cells in the pre-proliferation step in one embodiment is, for example, as follows: the mesenchymal stem cells are inoculated in the serum-free medium B at inoculation density of 2×10^5 cells/cm^2; after that, the mesenchymal stem cells are incubated for about one week while the serum-free medium B is added therein every two days in an amount equivalent to 10% of the amount of the culture fluid at the inoculation until the number of cells reached 70 to 80% of a confluent state. The mesenchymal stem cells thus cultured in advance in the serum-free medium B are subjected to the proliferation step. This makes it possible to obtain a large number of mesenchymal stem cells whose immunosuppression ability is maintained or improved.

Further, in order to efficiently proliferate the mesenchymal stem cells in the pre-proliferation step, it is preferable that a pre-proliferation step is carried out with a culture vessel suitable for proliferation of the particular kind of the mesenchymal stem cells to be proliferated in the pre-proliferation step (hereinafter, these mesenchymal stem cells also referred to as a "pre-proliferation target cell"). A method for selecting a culture vessel suitable for pre-proliferation of the proliferation target cell has been described in the "proliferation step", so that the description thereof is herein omitted.

In a case where adhesion of the pre-proliferation target cell with respect to the culture vessel is not sufficient, the serum-free medium B may further contain cell adhesion molecules in the pre-proliferation step, which is similar to the proliferation step. The cell adhesion molecules have been described in the "proliferation step", so that the description thereof is herein omitted.

Mesenchymal stem cells may be subcultured at least once in the pre-proliferation step, which is similar to the proliferation step. It is possible to improve a culture condition by subculturing the mesenchymal stem cells in the middle of the pre-proliferation step. Note that it is preferable that the pre-proliferation step is preferably carried out during a period from primary cell culture (P0) to third cell culture (P3). A

method for subculturing mesenchymal stem cells in the middle of the pre-proliferation step and a subculturing method to be carried out after the pre-proliferation step have been already described in the "proliferation step", so that the description thereof is herein omitted.

(Culture vessel selecting step)

The producing method according to the present invention may further include, before the proliferation step (or before the pre-proliferation step), a culture vessel selecting step for selecting a culture vessel suitable for proliferation of the mesenchymal stem cells. The method for selecting a culture vessel suitable for proliferation of the mesenchymal stem cells has been described in the "proliferation step", so that the description thereof is herein omitted.

(2) Serum-free additive for a medium for producing a cell preparation

The present invention provides a serum-free additive for a medium for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved. The additive for a medium according to the present invention contains an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid. By adding the additive for a medium according to the present invention to a conventionally public-known basal

medium, it is possible to use the conventionally public-known basal medium as a serum-free medium (serum-free medium "A") for producing the cell-preparation-containing mesenchymal stem cells whose immunosuppression ability is maintained or improved.

Examples of a phospholipid contained in an additive for a medium according to the present invention encompass phosphatidic acid, lysophosphatidic acid, phosphatidylinositol, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidyl glycerol. Those phospholipids can be used alone, or two or more types of the phospholipids can be used in combination. In one embodiment, the additive for a medium according to the present invention contains an phosphatidic acid and phosphatidyl choline in combination. Further, those phospholipids may be derived from animals or plants.

Examples of the fatty acids contained in the additive for a medium according to this embodiment encompass linoleic acid, oleic acid, linolenic acid, arachidonic acid, myristic acid, palmitoyl acid, palmitic acid, and stearic acid. The additive for a medium according to this embodiment may contain any one of those fatty acids alone or two or more types of the fatty acids in combination. Further, the additive for a medium according to this embodiment may contain not only the fatty acid(s) but also cholesterol.

The term "FGF" used in the Specification means a growth factor selected from a fibroblast growth factor (FGF) family, and is preferably FGF-2 (bFGF). However, other FGFs of the FGF family, such as an FGF-1 etc., may be selected. The term "PDGF" used in the Specification means a growth factor selected from a platelet derived growth factor (PDGF) family, and is preferably a PDGF-BB or a PDGF-AB. The term "TGF-β" used in the Specification means a growth factor selected from a transforming growth factor-β (TGF-β) family, and is preferably TGF-β3. However, other TGF-βs of the TGF-β family may be selected.

The term "HGF" used in the Specification means a growth factor selected from a hepatocyte growth factor family, and the term "EGF" means a growth factor selected from an epidermal growth factor (EGF) family.

In one embodiment, the serum-free medium "A" may further contain at least two factors selected from the group consisting of a connective tissue growth factor (CTGF), a vascular endothelial growth factor (VEGF), and an ascorbic acid compound.

The term "ascorbic acid compound" used in the Specification means ascorbic acid (vitamin C), ascorbic acid-2-phosphoric acid, or a compound similar to those ascorbic acids.

Note that growth factors contained in the additive for a

medium according to the present invention may be natural or may be produced by genetical modification.

In one aspect, the additive for a medium according to the present invention preferably contains a lipid antioxidant. The lipid antioxidant contained in the additive for a medium according to this embodiment may be DL-α-tocopherol acetate (vitamin E) in one embodiment. The additive for a medium according to the present invention may further contain a surfactant. The surfactant contained in the additive for a medium according to this embodiment may be Pluronic F-68 or Tween 80 in one embodiment.

The additive for a medium according to the present invention may further contain insulin, transferrin, and selenate. The term "insulin" used in the Specification may be also an insulin-like growth factor and may be derived from a natural cell or may be genetically modified. The additive for a medium according to the present invention may further contain dexamethasone, or another glucocorticoid.

(3) Kit for carrying out serum-free culturing with respect to animal cell

The present invention provides the serum-free additive for a medium for producing a cell preparation containing a mesenchymal stem cells whose immunosuppression ability is maintained or improved. The additive for a medium (additive "A" for the medium) according to the present invention

contains an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid. The additive for a medium according to the present invention may further include cell adhesion molecules.

A kit for the additive for a medium according to the present invention may contain an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid in one vessel, or separately contain those components. The kit for the additive for a medium according to the present invention may further contain cell adhesion molecules.

The cell adhesion molecules have been described in the "proliferation step" of "(1) Method for Producing Cell Preparation Containing Mesenchymal Stem Cells" in the Specification, so that the description thereof is herein omitted.

By using the kit for the additive for a medium according to the present invention to a conventionally public-known basal medium, it is possible to add the additive to the conventionally public-known basal medium, thereby preparing a serum-free medium (serum-free medium "A") for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved.

In the Specification, the term "composition" means a composition in which various main components are contained in one material, and the term "kit" means a kit in which at

least one of the various main components is contained in a material other than one material in which the rest of the various main components is contained. Therefore, it is easily understood that the growth factors, the at least one phospholipid, and the at least one fatty acid included in the kit for the additive for a medium according to the present invention are the same as those described in the additive for a medium.

The kit according to the present invention is a kit for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved, and includes at least the additive for a medium (additive "A" for a medium) according to the present invention. Further, the kit according to the present invention may further include the additive B for the medium containing an FGF, a PDGF, an EGF, at least one phospholipid, and at least one fatty acid. Note that the description of the "serum-free medium B" can be also read as the description for the "additive B for the medium".

(4) Serum-free culturing medium for producing cell preparation

The present invention provides a serum-free culturing medium for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved. A culturing medium according to the

present invention contains an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid. The culturing medium according to the present invention can be used as a serum-free medium (serum-free medium "A") for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved.

The culturing medium according to the present invention contains an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid, and those components may be simultaneously added to the basal medium, or may be separately added. That is, it can be said that the culturing medium according to the present invention only needs to contain the components contained in the additive for a medium or the components contained in the kit for the additive for a medium.

The basal medium for constituting the culturing medium according to the present invention is not particularly limited, provided that the basal medium is a basal medium for animal cells, which is well-known in this field is medium, and examples of preferable basal media encompass a Ham's F12 medium, a DMEM medium, an RPMI-1640medium, and an MCDB medium. Those basal media can be used alone, or two or more types of the basal media can be used in combination. In one embodiment, a medium in which an MCDB and a

DMEM are mixed at a ratio of 1:1 is preferable as the basal medium for constituting the culturing medium according to the present invention.

(5) Culture method for producing cell preparation

The present invention provides a culture method for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved. The culture method according to the present invention includes a step (culture step "A") in which the mesenchymal stem cells are cultured in the serum-free medium (serum-free medium "A") containing an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid. In the culture method according to the present invention, the serum-free culturing medium can be used in order to culture the mesenchymal stem cells.

The culture method according to the present invention may further include, before the culture step "A", a step for culturing mesenchymal stem cells (culture step B) in the serum-free medium B containing an FGF, a PDGF, an EGF, at least one phospholipid, and at least one fatty acid.

Note that the "culture step A" and the "culture step B" correspond to the "proliferation step" and the "pre-proliferation step", respectively, in the method for producing a cell preparation containing mesenchymal stem cells according to the present invention. Accordingly, the

description of the "proliferation step" and the "pre-proliferation step" in "(1) Method for Producing Cell Preparation Containing Mesenchymal Stem Cells" in the Specification can be also read as the descriptions of the "culture step A" and "culture step B", respectively.

In one embodiment, the culture method according to the present invention may include a step for simultaneously adding an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid to the basal medium. In one embodiment, the culture method according to the present invention may include a step for adding, to the basal medium, an FGF, a PDGF, an EGF, at least one phospholipid, and at least one fatty acid at once. The basal medium is not particularly limited, provided that the basal medium is a medium for animal cells, which is well-known in this field.

(6) Further usage

According to the present invention as described above, it is possible to proliferate, even in the serum-free or a low-serum medium, the mesenchymal stem cells at a speed equal to or higher than that obtained in a case where the mesenchymal stem cells are cultured in the serum-containing medium, and immunosuppression ability of the mesenchymal stem cells thus proliferated are maintained or improved. Further, the mesenchymal stem cell proliferated in the

present invention which do not have the tumorigenicity are selectively increased. Differentiation ability of the mesenchymal stem cells proliferated in the present invention (see Patent Literature 1). Accordingly, when a cell preparation containing such mesenchymal stem cells is administered to a patient, it is possible not only to realize a great transplantation treatment because of the mesenchymal stem cells, but also to effectively suppress immunorejection which is a large problem for the transplantation treatment. This makes it possible to reduce the burden on a patient and realize a stable treatment. It is unnecessary to consider a lot difference of the serum, as compared with a conventional cell preparation produced by use of the serum. This makes it possible to realize a stable cure rate in the transplantation treatment.

It can be said that the mesenchymal stem cells contained in the cell preparation produced by the present invention maintain a function of affecting an immune effecter cell, which function the mesenchymal stem cells originally have. The mesenchymal stem cells have an immunoregulation effect and an immune tolerance effect, so that it can be expected that the cell preparation produced by the present invention is used for treatments to exhibit those effects. Further, it can be also expected that the mesenchymal stem cells according to the cell preparation produced by the

present invention also serves as a function of the anti-inflammatory effect of the mesenchymal stem cells in transplantation treatment. Further, the cell preparation is expected to exhibit an anti-aging effect by exhibiting the anti-inflammatory effect of the mesenchymal stem cells.

The cell preparation produced by the present invention can be used for treatment for a local disease, in which treatment the mesenchymal stem cells are administered (locally administered) to a site which needs transplantation of the mesenchymal stem cells. In addition, by administering the mesenchymal stem cells to a whole body, i.e., by administering the mesenchymal stem cells into a vein etc. and conveying the mesenchymal stem cells to a whole body, it is possible to treat strong immunorejection more effectively because of an acute GVHD etc. caused by bone marrow transplantation etc. It is therefore expected to remarkably improve a survival rate of humans. Further, a cell preparation produced by the present invention is a cell preparation produced by serum-free culture. Accordingly, a useful growth factor or a differentiation factor is not nonspecifically adsorbed together with a serum protein to a transplantation material such as ceramics etc. Accordingly, a tissue-regenerative power caused by the transplantation of the cell preparation produced by the present invention is greatly high. This leads a high therapeutic effect. It is needless to say that

it is possible to perform treatment in which both local administration and whole-body administration are performed in combination.

The immunosuppression ability of the mesenchymal stem cells contained in the cell preparation produced by the present invention is maintained or improved, and the mesenchymal stem cells suppress immunorejection caused when transplantation is performed. It is therefore possible to use the mesenchymal stem cells for treatment in which a non-self tissue or cell is transplanted (xenotransplantation) to another person (a donor and a recipient are different). The mesenchymal stem cells can be also preferably used not only for allotransplantation (allogeneic transplantation) by use of a human tissue or a human cell but also for xenotransplantation (heterotransplantation) by use of an animal (other than human) tissue or an animal (other than human) cell.

The cell preparation produced by the present invention exhibits the immunosuppressive effect earlier than a conventional mesenchymal stem cells produced by use of only a serum-containing medium, so that the cell preparation of the present invention is desired to early exhibit a therapeutic effect in a case where transplantation is performed. It is therefore expected that the cell preparation of the present invention increases the cure rate.

The method for producing the cell preparation containing mesenchymal stem cells according to the present invention, may further include, before the step (B), the step of: (C) culturing, in a medium containing serum, the mesenchymal stem cells which have been subjected to the step (A).

It is preferable that the method for producing the cell preparation containing mesenchymal stem cells further includes, before the step (A), the step of: (D) pre-proliferating the mesenchymal stem cells in a serum-free medium B containing an FGF, a PDGF, an EGF, at least one phospholipid, and at least one fatty acid.

The method may further include the step of: (E) screening the mesenchymal stem cells which do not have tumorigenicity, from the mesenchymal stem cells proliferated in the step (A).

In the method for producing the cell preparation containing mesenchymal stem cells according to the present invention, it is preferable that, in the step (A), the mesenchymal stem cells are proliferated by use of a culture vessel suitable for proliferation of the mesenchymal stem cells

In the method for producing a cell preparation containing mesenchymal stem cells according to the present invention, it is preferable that the serum-free medium "A" further contains cell adhesion molecules in the step (A).

In the method for producing the cell preparation containing mesenchymal stem cells according to the present invention, the mesenchymal stem cells may be subcultured at least once in the step (A).

In the method for producing the cell preparation containing mesenchymal stem cells according to the present invention, it is preferable that, in the step (A), the subculturing is performed in such a way that the mesenchymal stem cells are peeled off by use of a cell removing agent which does not contain a component derived from a mammal or a microorganism.

The method for producing the cell preparation containing mesenchymal stem cells according to the present invention, may further include, before the step (A), the step of: selecting the culture vessel suitable for the proliferation of the mesenchymal stem cells.

In the method for producing the cell preparation containing mesenchymal stem cells according to the present invention, the phospholipid is selected from the group consisting of phosphatidic acid, lysophosphatidic acid, phosphatidylinositol, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidyl glycerol.

In the method for producing the cell preparation containing mesenchymal stem cells according to the present

invention, the fatty acid is selected from the group consisting of linoleic acid, oleic acid, linolenic acid, arachidonic acid, myristic acid, palmitoyl acid, palmitic acid, and stearic acid.

An additive for a medium according to the present invention may further include cell adhesion molecules.

A kit according to the present invention, further include an additive B for a medium containing an FGF, a PDGF, an EGF, at least one phospholipid, and at least one fatty acid.

The invention being thus described, it will be obvious that the same way may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

All the professional literatures and Patent Literature described in the Specification are quoted as reference in the Specification.

Examples

Hereinafter, the present invention will be specifically described with reference to Examples, however, the present invention is not limited by the Examples.

[Example 1]

The following experiment was carried out.

(1. Cell culture)

Mesenchymal stem cells derived from a human bone marrow (bone-marrow derived hMSCs) were bought from Lonza Walkersville, Inc. (hereinafter, Lonza Inc.), and the mesenchymal stem cells were cultured in Mesenchymal Stem Cell Basal Medium (MSCBM) (Lonza Inc.) added with Mesenchymal Cell Growth Supplement (MCGS) (Lonza Inc.), a serum-containing medium (MSCGM medium) or in a serum-free medium 1 shown in Table 1.

Table 1

Table 1 (continued to the next page.)

	Source	Effective concentration	Optimum concentration
Basal medium			
DMEM/MCDB201	**Sigma:D6046/M6770**	**1:1**	
Supplement A (Basic factor)			
(human recombinant) Basic fibroblast growth factor (bFGF)	**Pepro Tech:100-18B**	**0.1~100 ng/ml**	**3ng/ ml**
Dexamethasone (Dex)	**Sigma:D1756**	**10^{-6}~10^{-10} M**	**10^{-8} M**
(human recombinant) Insulin	**Wako:090-03446**	**0.5~50 μg/ml**	**6.25 μg/ml**
Transferrin	**Sigma:T0665**	**0.5~50 μg/ml**	**6.25 μg/ml**
Selenous acid	**Sigma:21,117-6**	**0.1~50 ng/ml**	**6.25 ng/ml**
Bovine serum albumin0020(BSA)	**Sigma:A8806**	**0.1~50 mg/ml**	**1.25 mg/ml**
Supplement B-1 (Basic lipid 1)			
Chemically defined lipid concentrate (CD)	**Gibco:11905-031**	**1/1000~1/10**	**1/100**

(Concentration of undiluted solution: Arachidonic Acid 2.0 μg/ml, Cholesterol 220.00 μg/ml, DL-α-Tocopherol-Acetate 70.00 μg/ml, Linoleic Acid 540.00 μg/ml, Linolenic Acid 10.00 μg/ml, Myristic Acid 10.00 μg/ml, Oleic Acid 10.00 μg/ml, Palmitoleic Acid 10.0 μg/ml, Palmitic Acid 10.0 μg/ml, Stearic Acid 10.00 μg/ml)

Table 1 (continued from the previous page.)

	Source	Effective concentration	Optimum concentration
Supplement B-2 (Basic lipid 2)			
Lecithin from Soybean (LS)	**Waco:120-00832**	**0.5~50 μg/ml**	**10 μg/ml**
cholesterol lipid concentrate (chol)	**Gibco:12531-018**	**0.1~30 μg/ml**	**3 μg/ml**
(+α)-Tocopherol-Acetate (VE)	**Sigma:T1157**	**0.1~50 μg/ml**	**2 μg/ml**
Supplement C			
(human recombinant) Hepatocyte growth factor (HGF)	**Sigma:H1404**	**0.1~50 ng/ml**	**5 ng/ml**
(human recombinant) Transforming growth factor-β_3 (TGF-β_3)	**Pepro Tech:100-36**	**0.5~100 ng/ml**	**10 ng/ml**
(human recombinant) Platelet derived growth factor (PDGF-BB)	**Waco:160-19741**	**0.5~100 ng/ml**	**10 ng/ml**
Others			
(human recombinant) Epidermal growth factor (EGF)	**Waco:050-07141**	**0.5~200 ng/ml**	**20 ng/ml**
Ascorbic acid (VC)	**Sigma:A8960**	**0.5~200 μg/ml**	**50 μg/ml**
Phosphatidylcholine (PC)	**Wako:163-21181**	**0.5~100 μg/ml**	**10 μg/ml**
Phosphatidic acid sodium salt (PA)	**Sigma:P9511**	**0.5~100 μg/ml**	**10 μg/ml**
(human recombinant) Vascular Endothelial Growth Factor (VEGF)	**Sigma:V3388**	**0.5~100 ng/ml**	**10 ng/ml**
(human recombinant) Connective Tissue Growth Factor (CTGF)	**Wako:036-19471**	**0.1~20 μg/ml**	**1 μg/ml**

A spleen BALB/c (H-2d) as a mouse splenocyte bought from CHARLES RIVER LABORATORIES JAPAN, INC. was ground and hemolized, then was suspended in 1%FBS/Advanced PRM1 (GIBCO) was used in an experiment.

(2. Activation of spleen cell)

<2-1: Stimulation by mitogen>

Mouse spleen cells (1×10^5) were inoculated on each well of a 96-well-plate, and stimulated by Phorbol 12-myristate 13-acetate (hereinafter, referred to as "PMA") (2.5 ng/ml) and ionomycin (125 ng/ml).

<2-2: Stimulation by anti-CD3/anti-CD28>

The mouse spleen cells (1×10^5) were inoculated on each well of the 96-well-plate, and were stimulated by anti-CD3 (2.5 μg/ml) and anti-CD28 (0.5 μg/ml).

(3. Co-culture of activated spleen cell and hMSC derived from bone marrow)

As a pre-process of bone-marrow derived hMSCs, the bone-marrow derived hMSCs (1×10^4) is inoculated on each well of the 96-well-plate, and adhesion of the hMSCs to each well was confirmed (in few-hour or one-night culturing), and after that, the hMSCs were irradiated with gamma irradiation by use of a gamma cell 40 exactor. This inhibits cell division of the hMSCs. Then, the mouse spleen cells activated as described above were inoculated on each well to which the hMSCs were adhered, and then co-cultured with the bone-

marrow derived hMSCs.

(4. Cell proliferation measurement)

[^{3}H]-Thymidine was added to each well on the third day and the fourth day in the co-culturing, and the hMSCs were cultured for another 8 hours. The cultured cells were adsorbed to a glass filter, and Thymidine intake was measured by a liquid scintillation counter, thereby measuring proliferation of the cells .

(5. Results)

Results of the co-culture of the hMSC derived from bone marrow and the activated mouse T cell in the MSCGM medium containing 10% FBS or in the medium 1 (STK2 (registered trademark)) which is a serum-free medium "A", are shown in Figs. 1 to 4. In Figs. 1 to 4, each value is shown by average ± standard deviation. Similar results can be obtained from results of independent experiments which were carried out three or more times. #4 and #5 show respective results of hMSCs derived from bone marrow of two different experiments.

Fig. 1 is a graph showing an immunosuppressive effect of bone-marrow derived hMSCs on anti-CD3 and anti-CD28 stimulation T cell proliferative responses in a case where activated mouse T cells and bone-marrow derived hMSCs are co-cultured in the MSCGM medium. Fig. 2 is a graph showing the immunosuppressive effect of bone-marrow derived hMSCs on anti-CD3 and anti-CD28 stimulation T cell proliferative

responses in a case where activated mouse T cells and bone-marrow derived hMSCs are co-cultured in the medium 1.

Fig. 3 is a graph showing an immunosuppressive effect of hMSCs derived from bone marrow on PMA and ionomycin stimulation (mitogen stimulation) T cell proliferation reactions in a case where activated mouse T cells and bone-marrow derived hMSCs were co-cultured in the MSCGM medium. Fig. 4 is a graph showing the immunosuppressive effect of bone-marrow derived hMSCs on the PMA and ionomycin stimulation (mitogen stimulation) T cell proliferation reactions in a case where activated mouse T cells and bone-marrow derived hMSCs were co-cultured in the medium 1.

As shown in Figs. 1 to 4, the bone-marrow derived hMSCs in the medium 1 suppressed proliferation of the T cells activated by the mitogen stimulation and proliferation of the T cells activated by the anti-CD3/anti-CD28 stimulation, which is similar to the bone-marrow derived hMSCs in the MSCGM medium containing serum. That is, Figs. 1 to 4 show that the bone-marrow derived hMSCs, which were cultured by use of the serum-free medium 1, maintained an immunosuppression effect.

[Example 2]

An immunosuppressive effect of bone-marrow derived hMSCs on T cell proliferation caused by a mouse mixed lymphocyte reaction (MLR) was tested. An experiment method

and cells and mediums used in this experiment are the same as those of Example 1. The hMSCs (2×10^4) derived from bone marrow were inoculated on each well of a 96-well-plate as a pre-process of the bone-marrow derived hMSCs, and Example 2 was carried out in the same way as Example 1. Mouse spleen cells were activated by co-culturing mouse spleen cells (2×10^5) and dendritic cells (BMDCs) derived from mouse bone marrow (3.3×10^4) in each well of the 96-well-plate (MLR stimulation).

Note that the BMDCs for use in the experiment were cells which were inhibited from dividing, and those cells were prepared by: collecting and hemolysizing bone marrow cells from C3H (H-2k) bought from CHARLES RIVER LABORATORIES JAPAN, INC.; culturing the bone marrow cells with a medium changed on alternate days, where the medium was 1% FBS/Advanced PRMI (GIBCO) containing a GM-CSF; stimulating the bone marrow cells by use of LPS (100 ng/ml) on the sixth day in this culturing; culturing the bone marrow cells over night; then washing the bone marrow cells; and irradiating the bone marrow cells with gamma rays by use of a gamma cell 40 exactor.

The bone-marrow derived hMSCs were pre-cultured in the MSCGM medium and the medium 1, and then the bone-marrow derived hMSCs subjected to this pre-culturing were co-cultured, in the MSCGM medium, with the mouse spleen

cells activated as described above. Results of the experiment are shown in Figs. 5 and 6. Figs. 5 and 6 are graphs each showing the immunosuppressive effect of bone-marrow derived hMSCs on the T cell proliferation caused by the MLR. Each value is shown by average ± standard deviation. Similar results can be obtained from results of independent experiments which were carried out three or more times. Fig. 5 shows results of the third day in the culturing, and Fig. 6 shows results of the fourth day in the culturing. In the drawings, the letter "M" shows results of a case where the hMSCs were pre-cultured in the MSCGM medium and cultured in the serum-containing medium, and the letter "S" shows results of a case where the hMSCs were pre-cultured in the medium 1 and were cultured in the serum-containing medium.

As shown in Figs. 5 and 6, the bone-marrow derived hMSCs, pre-cultured in the medium 1, maintained an activated T cell proliferation suppression effect like the bone-marrow derived hMSCs pre-cultured in the MSCGM medium. The bone-marrow derived hMSCs pre-cultured in the MSCGM medium showed the immunosuppressive effect on the fourth days of the co-culturing, meanwhile, the bone-marrow derived hMSCs pre-cultured in the medium 1 already showed the immunosuppressive effect on the third day in the co-culturing. That is, the bone-marrow derived hMSCs pre-cultured in the medium 1 showed the immunosuppressive effect earlier.

Further, although data was not shown, the bone-marrow derived hMSCs pre-cultured in the medium 1 showed similar results with respect to the mitogen stimulation T cell proliferation and the anti-CD3 /anti-CD28 stimulation T cell proliferation.

It was confirmed that, as shown in the results of Examples 1 and 2, the bone-marrow derived hMSCs cultured in the medium 1 not only maintained the activated T cell proliferation suppression effect, but also exhibited the activated T cell proliferation suppression effect earlier than the bone-marrow derived hMSCs cultured in the MSCGM medium (serum-containing medium) did. Further, in a case where the bone-marrow derived hMSCs pre-cultured in the MSCGM medium and the activated mouse spleen cells were co-cultured, the T cells before the bone-marrow derived hMSCs showed the immunosuppressive effect are proliferated more actively than those in a control medium (which is a culturing medium of the activated mouse spleen cells, which culturing medium does not contain the bone-marrow derived hMSCs).

The experiment showed that, in a case where the bone-marrow derived hMSCs were cultured in the medium 1 as described above, the hMSCs not only had the advantage of proliferating the necessary number of cells in a short period, but also maintained the immunosuppressive effect. For this

reason, it can be said that the medium 1 is effective at culturing the bone-marrow derived hMSCs especially for the purpose of clinical application.

[Example 3]

The following experiment was carried out.

(1. Cell culture)

Mesenchymal stem cells derived from human adipose tissues (adipose tissue derived hMSCs) were separated and cultured in the following processes (i) to (vii).

(i) Adipose tissues were collected from a human, and were washed twice or three times with a serum-free DMEM medium.

(ii) The adipose tissues thus washed were cut into small pieces by use of scissors (1 mm^3).

(iii) The adipose tissues were treated with the use of a 0.1~0.2% collagenase (GIBCO 17100-017) solution under agitation by a stirrer bar (37°C, 30~60 minutes).

(iv) The adipose tissues subjected to the collagenase process were filtered via a filter (100 mm), and were centrifuged (100×g) for 10 minutes. Thereby, hMSCs derived from the adipose tissues were separated from the adipose tissues.

(v) The centrifuged cells were treated with a Red lysis buffer (sigma R7757) under agitation by a stirrer bar(for 5~10 minutes).

(vi) The cells subjected to the Red lysis buffer process were

washed twice or three times with a serum-free DMEM medium.

(vii) The cells thus washed were inoculated on a culture plate (BECTON, DICKINSON (Falcon) 353047), and were cultured in the serum-free medium 1 shown in Table 1, or in an MEM medium containing 10% FBS (sigma D6046) (referred to as a "10%FBS-MEM").

(2. Cell proliferation measurement)

A proliferated state of the cells was visually observed by use of an optical microscope on the eighth day in the culturing.

(3. Result)

Results were shown in Fig. 7. Fig. 7 is views each illustrating a proliferated state of hMSCs derived from adipose tissues on the eighth day in the culturing. In Fig. 7, the hMSCs derived from the adipose tissues were observed with a magnification of forty times. It was shown that, as shown in Fig. 7, the number of the hMSCs derived from the adipose tissues cultured in the medium 1 was remarkably increased in comparison with the number of the hMSCs derived from the adipose tissues cultured in the 10% FBS-MEM (the hMSCs in the medium 1 were twice to three times as many as those in the 10% FBS-MEM).

[Example 4]

The following experiment was carried out.

(1. Cell culture)

Mesenchymal stem cells derived from a human synovial membrane (synovial membrane derived hMSCs) were separated in the following processes (i) to (iii).

(i) A synovial membrane was collected from a human.

(ii) Cells of the synovial membrane thus collected were washed with PBS, tissues were added into and mixed with a 0.4% collagenase solution (10 ml), and were reacted at 37°C for 1 to 4 hours.

(iii) The reacted mixture was filtered and centrifuged.

Initial synovial membrane derived hMSCs obtained by centrifugation were cultured with the following media. Note that a medium 2 which is a serum-free medium B is a medium obtained by removing an HGF and a TGF-β from the medium 1 shown in Table 1.

· 10% FBS containing DMEM (10%FBS-DMEM) (sigma D6046, FBS; Hyclone, PS(+))

· medium 1

· medium 2

The initial synovial membrane derived hMSCs were cultured in a carbon dioxide incubator (95% air and 5% CO_2) at 37°C.

(2. Cell proliferation measurement)

A proliferated state of those cells was visually observed by use of an optical microscope on the twelfth day in culturing (no subculturing). Further, the number of cells were

counted by a coulter counter.

(3. Result)

Results are shown in Fig. 8. Fig. 8 is a graph and views showing a proliferation promoting effect of a medium 1 and a medium 2 on an initial synovial membrane derived hMSCs: (a) of Fig. 8 is a graph showing the number of synovial membrane derived hMSCs on the twelfth day in the culturing; and (b) of Fig. 8 is views each illustrating a proliferated state of the synovial membrane derived hMSCs on the twelfth day in the culturing. In (b) of Fig. 8, the synovial membrane derived hMSCs were observed with a magnification of ten times.

It was confirmed that, as shown in (a) of Fig. 8, the medium 2 had a remarkably high proliferation promoting effect on the initial synovial membrane derived hMSCs (eight times as many as a case of using the medium 1: forty times or more as many as a case of using the 10% FBS containing DMEM).

[Example 5]

The following experiment was carried out.

(1. Cell culture)

Mesenchymal stem cells derived from a human synovial membrane (synovial membrane derived hMSCs) were separated by the processes (i) to (iii) described in the above item "1. Cell culture" of Example 4 .

The initial synovial membrane derived hMSCs obtained

by centrifugation were cultured by use of the medium 2 for eleven days, and then were cultured in the following medium.

· 10% FBS containing DMEM (10%FBS-DMEM) (sigma D6046, FBS; Hyclone, PS(+))

· medium 1

· medium 2

The initial synovial membrane derived hMSCs were cultured in a carbon dioxide incubator (95% air and 5% CO_2) at 37°C.

(2. Cell proliferation measurement)

The number of cells was measured by a coulter counter.

(3. Result)

A result was shown in Fig. 9. Fig. 9 is a graph showing a chronical change of the number of synovial membrane derived hMSCs during a period from the 0 day to sixty-eighth day.

It was confirmed that, as shown in the graph of Fig. 9, the synovial membrane derived hMSCs were efficiently amplified by culturing the initial synovial membrane derived hMSCs in the medium 2 and then culturing the synovial membrane derived hMSCs in the medium 1 (on the forty-eighth day in culturing, the synovial membrane derived hMSCs were hundred thousand times as many as those cultured in a 10% FBS-DMEM from the eleventh day in the culturing, and were ten thousand times as many as those

cultured in the medium 2 during the period from the 0 day to sixty-eighth day).

[Example 6]

The following experiment was carried out.

(1. Cell culture)

Mesenchymal stem cells derived from a human synovial membrane (synovial membrane derived hMSCs) were separated by the processes (i) to (iii) described in the above item "1. Cell culture" of Example 4.

In order to separate and proliferate the synovial membrane derived hMSCs, the synovial membrane derived hMSCs obtained by centrifugation were moved to a flask, and were cultured in the medium 2 from primary cell culture (P0) to third cell culture (P3), then were cultured in the medium 1 shown in Table 1 from primary cell culture (P0) to fourth cell culture (P4). The synovial membrane derived hMSC was cultured in a carbon dioxide incubator (95% air and 5% CO_2) at 37°C.

The medium was changed twice a week while the synovial membrane derived hMSCs were cultured. Those cells were subcultured every 7 to 21 days. Amplification of the synovial membrane derived hMSCs was performed by inoculating mesenchymal stem cells ($1\sim2\times10^4$) thereof in the medium (1 ml) at 37°C under a 5% CO_2 culture condition.

Claims

Claim 1

A method for producing a cell preparation containing mesenchymal stem cells, comprising the steps of:

(A) proliferating mesenchymal stem cells in a serum-free medium "A" containing an FGF, a PDGF, a TGF-β, an HGF, an EGF, at least one phospholipid, and at least one fatty acid; and

(B) screening mesenchymal stem cells whose immunosuppression ability is maintained or improved, from the mesenchymal stem cells thus proliferated in the step (A).

Claim 2

A method according to claim 1, further comprising, before the step (B), the step of:

(C) culturing, in a medium containing serum, the mesenchymal stem cells which have been subjected to the step (A).

Claim 3

A method according to claim 1 or 2, further comprising, before the step (A), the step of:

(D) pre-proliferating the mesenchymal stem cells in a serum-free medium B containing an FGF, a PDGF, an EGF, at least one phospholipid, and at least one fatty acid.

Claim 4

A method according to any one of claims 1 to 3, further comprising the step of:

(E) screening the mesenchymal stem cells which do not have tumorigenicity, from the mesenchymal stem cells proliferated in the step (A).

Claim 5

The method according to any one of claims 1 to 4, wherein

in the step (A), the mesenchymal stem cells are proliferated by use of a culture vessel suitable for proliferation of the mesenchymal stem cells.

Claim 6

A method according to any one of claims 1 to 5, wherein

the serum-free medium "A" further contains cell adhesion molecules in the step (A).

Claim 7

The method according to any one of claims 1 to 6, wherein:

the mesenchymal stem cells are subcultured at least once in the step (A).

Claim 8

The method according to claim 7, wherein:

in the step (A), the subculturing is performed in such a way that the mesenchymal stem cells are peeled off by use of a cell removing agent which does not contain a component derived from a mammal or a microorganism.

Claim 9

A method according to claim 5, further comprising, before the step (A), the step of:

selecting the culture vessel suitable for the proliferation of the mesenchymal stem cells.

Claim 10

The method according to any one of claims 1 to 9, wherein:

the phospholipid is selected from the group consisting of phosphatidic acid, lysophosphatidic acid, phosphatidylinositol, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidyl glycerol.

Claim 11

The method according to any one of claims 1 to 10,

wherein:

the fatty acid is selected from the group consisting of linoleic acid, oleic acid, linolenic acid, arachidonic acid, myristic acid, palmitoyl acid, palmitic acid, and stearic acid.

Claim 12

A cell preparation comprising mesenchymal stem cells which have been produced by a method according to any one of claims 1 to 11.

Claim 13

A serum-free additive for a medium for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved, comprising:

an FGF;

a PDGF;

a TGF-β;

an HGF;

an EGF;

at least one phospholipid; and

at least one fatty acid.

Claim 14

An additive for a medium according to claim 13, further

comprising

cell adhesion molecules.

Claim 15

A serum-free culturing medium for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved, comprising:

an additive for a medium according to claim 13 or 14.

Claim 16

A culture method for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved, comprising:

(F) culturing mesenchymal stem cells in a culturing medium according to claim 15.

Claim 17

A kit for producing a cell preparation containing mesenchymal stem cells whose immunosuppression ability is maintained or improved, comprising at least:

an additive "A" for a medium according to claim 13 or 14.

Claim 18

A kit according to claim 17, further comprising:

an additive B for a medium containing an FGF, a PDGF, an EGF, at least one phospholipid, and at least one fatty acid.