Culture Medium Composition for Culturing Amnion-Derived Mesenchymal Stem Cell, and Method for Culturing Amnion-Derived Mesenchymal Stem Cell by Using Same

TECHNICAL FIELD

The present invention relates to a medium for culturing mesenchymal stem cells, and more particularly to a medium composition for culturing mesenchymal stem cells, which contains basal medium, L-ascorbic acid 2-phosphate, fetal bovine serum, basic fibroblast growth factor (b-FGF), non-essential amino acids (NEAAs), insulin, N-acetyl-L-cysteine, calcium chloride, and hydrocortisone, and a method of culturing mesenchymal stem cells using the same.

BACKGROUND ART

Stem cells refer to cells having not only self-replicating ability but also the ability to differentiate into at least two types of cells, and can be divided into totipotent stem cells, pluripotent stem cells, and multipotent stem cells.

Adult stem cells are obtained by taking cells from various human organs and developing the cells into stem cells and are characterized in that they differentiate into only specific tissues. However, recently, experiments for

differentiating adult stem cells into various tissues, including liver cells, were dramatically successful, which comes into spotlight.

Multipotent stem cells were first isolated from adult bone marrow (Y. Jiang et al., *Nature*, 418:41, 2002), and then also found in other several adult tissues (C.M. Verfaillie, *Trends Cell Biol.*, 12:502, 2002). In other words, bone marrow is the most widely known source of stem cells, multipotent stem cells were also found in the skin, blood vessels, muscles, and brain (J.G. Toma *et al.*, *Nat. Cell Biol.*, 3:778, 2001; M. Sampaolesi *et al.*, *Science*, 301:487, 2003; Y. Jiang *et al.*, *Exp. Hematol.*, 30:896, 2002). However, stem cells are very rarely present in adult tissue such as bone marrow, and such cells are difficult to culture without inducing differentiation, and thus difficult to culture in the absence of specifically screened media. Namely, it is very difficult to maintain the isolated stem cells *in vitro*. Meanwhile, the results of studies on the isolation of mesenchymal stem cells from fetal tissue revealed that there are abundant mesenchymal stem cells in fetal tissue. However, the use of fetal tissue as the source of cell therapeutic agents has been limited due to ethical concerns. Mesenchymal stem cells were also isolated from umbilical cord blood (UCB) as the source of fetal mesenchymal stem cells (MSCs), but

their numbers were very small, and they showed poor proliferation.

In recent years, it was known that mesenchymal stem cells are present in the amnion (amniotic membrane or amniotic lining membrane), i.e., the thin innermost membrane sag surrounding the placenta and a developing mammalian embryo. Thus, technologies for isolating and culturing such mesenchymal stem cells have been developed (PCT International Patent Publication No. WO 2006/019357, Korean Patent Registration Nos. 0795708 and 0818214).

However, conventional methods for culturing amnion-derived stem cells have a shortcoming in that the time taken for stem cells to proliferate is long.

Accordingly, the present inventors have made extensive efforts to develop a method capable of increasing the proliferation rate of amnion-derived stem cells while maintaining the ability to differentiate, and as a result, have found that, when amnion-derived stem cells are cultured in a medium containing DMEM-P and KSFM-P, the ability of the cells to proliferate can be increased while maintaining the ability to differentiate, thereby completing the present invention.

DISCLOSURE OF INVENTION

TECHNICAL PROBLEM

It is an object of the present invention to provide a medium for culturing mesenchymal stem cells, which shows a high cell proliferation rate while maintaining the ability of the cells to differentiate.

Another object of the present invention is to provide a method of culturing mesenchymal stem cells using the above medium.

TECHNICAL SOLUTION

To achieve the above objects, the present disclosure provides a medium composition for culturing mesenchymal stem cells, which contains basal medium, L-ascorbic acid 2-phosphate, fetal bovine serum, basic fibroblast growth factor (b-FGF), non-essential amino acids (NEAAs), insulin, N-acetyl-L-cysteine, calcium chloride, and hydrocortisone.

In one aspect, there is provided a medium composition for culturing mesenchymal stem cells, the medium comprises:
(i) DMEM-P containing DMEM-HG, 0.2mM of L-ascorbic acid 2-phosphate, 10% fetal bovine serum, 10ng/ml of basic fibroblast growth factor (b-FGF), 10㎕/ml of non-essential amino acid (NEAA); and

(ii) KSFM-P containing defined Keratinocyte-SFM, 0.2mM of L-ascorbic acid 2-phosphate, 5μg/ml of insulin, 2mM of N-acetyl-L-cysteine, 0.09mM of calcium chloride, 74ng/ml of hydrocortisone, 5% fetal bovine serum,

wherein the non-essential amino acid (NEAA) consists of 750mg/L of glycine, 890mg/L of L-alanine, 1,320mg/L of L-asparagine, 1,330mg/L of L-aspartic acid, 1,470mg/L of L-glutaminic acid, 1,150mg/L of L-proline, and 1,050mg/L of L-serine, and the volume ratio of DMEM-P : KSFM-P is 2:1, 1: 1 or 1:3.

The present disclosure also provides a method for culturing mesenchymal stem cells, the method comprising culturing the mesenchymal stem cells in the above medium composition.

In one aspect, there is provided a method for culturing mesenchymal stem cells, the method comprising culturing the mesenchymal stem cells in the medium composition as defined herein.

Other features and embodiments of the present disclosure will be more apparent from the following detailed descriptions and the appended claims

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows photographs of amnion-derived mesenchymal stem cells cultured in DMEM-P, KSFM-P or a mixed medium of DMEM-P and KSFM-P for 3 days.

FIG. 2 photographs of amnion-derived mesenchymal stem cells cultured in DMEM-P, KSFM-P or a mixed medium of DMEM-P and KSFM-P for 4 days.

FIG. 3 shows the results of flow cytometry analysis of each group for CD31.

FIG. 4 shows the results of flow cytometry analysis of each group for CD34.

FIG. 5 shows the results of flow cytometry analysis of each group for CD45.

FIG. 6 shows the results of flow cytometry analysis of each group for CD29.

FIG. 7 shows the results of flow cytometry analysis of each group for CD44.

FIG. 8 shows the results of flow cytometry in each group for CD73.

FIG. 9 is a set of photographs showing amnion mesenchymal stem cells cultured in a mixed medium of DMEM-P and KSFM-P (Group 2) in passage 1 (P1).

FIG. 10 is a set of photographs showing amnion mesenchymal stem cells cultured in a mixed medium of DMEM-P and KSFM-P (Group 2) in passage 2 (P2).

FIG. 11 shows the results of analyzing the osteogenic differentiation of amnion mesenchymal stem cells, cultured in a mixed medium of DMEM-P and KSFM-P (Group 2), using Alizarin red S staining under various conditions.

FIG. 12 is a graph showing a comparison between the quantified concentration of Alizarin red S in amnion-derived mesenchymal stem cells, cultured in DMEM-P or a mixed medium (Group 2) and differentiated into bone cells under various conditions, and that in a control group (whose osteogenic differentiation was not induced).

BEST MODE FOR CARRYING OUT THE INVENTION

In one aspect, the present invention is directed to a medium composition for culturing mesenchymal stem cells, which contains basal medium, L-ascorbic acid 2-phosphate, fetal bovine serum, basic fibroblast growth factor (b-FGF), non-essential amino acids (NEAAs), insulin, N-acetyl-L-cysteine, calcium chloride, and hydrocortisone.

In the present invention, the basal medium may be selected from the group consisting of DMEM-HG, defined keratinocyte-SFM, and a mixture thereof.

In the present invention, the medium composition may contain 0.05-1 mM of ascorbic acid 2-phosphate, 2-20% fetal bovine serum, 1-100 ng/ml of basic fibroblast growth factor

(b-FGF), 1-100 μL/ml of non-essential amino acid (NEAA), 0.1-100 μg/ml of insulin, 0.2-20 mM of N-acetyl-L-cysteine, 0.01-1 mM of calcium chloride, and 5 ng/ml-1 μg/ml of hydrocortisone.

In the present invention, the non-essential amino acids (NEAAs) may consist of glycine, alanine, asparagines, aspartic acid, glutaminic acid, proline, and serine. In addition, the non-essential amino acids (NEAAs) may consist of 750 mg/L of glycine, 890 mg/L of alanine, 1,320 mg/L of asparagine, 1,330 mg/L of aspartic acid, 1,470 mg/L of glutaminic acid, 1,150 mg/L of proline, and 1,050 mg/L of serine.

In the present invention, the mesenchymal stem cells may be derived from amnion.

In another aspect, the present invention is directed to a method for culturing mesenchymal stem cells, the method comprising culturing the mesenchymal stem cells in the above medium composition.

In the present invention, the mesenchymal stem cells may be derived from amnion.

Amnion-derived mesenchymal stem cells used in one example of the present invention are not various kinds of cells extracted from the whole amnion isolated from the placenta, but are only amnion-derived mesenchymal stem cells.

In one example of the present invention, with respect to a method for isolating amnion-derived mesenchymal stem cells, amnion tissue was isolated from placental tissue collected during delivery, and the isolated amnion tissue was washed with sterile saline and cut as finely as possible with surgical scissors. A collagenase solution was added to and mixed well with the finely cut tissue. The mixture was placed in a flask which was then incubated in a 5% CO_2 incubator at 37 ℃ for 90 minutes to lyse the tissue. The lysed tissue was filtered through a cell strainer and centrifuged. After centrifugation, the supernatant was removed, and the cell precipitate was suspended in DMEM-P and inoculated into a T-flask, which was then incubated in a 5% CO_2 incubator at 37 ℃ for 3-4 days. Then, only mesenchymal stem cells attached to the culture flask were collected.

In one example of the present invention, amnion-derived mesenchymal stem cells showed a higher cell proliferation rate in a mixed medium of DMEM-P and KSFM-P than in DMEM-P or KSFM-P alone. Particularly, amnion-derived mesenchymal stem cells showed the highest proliferation rate in a medium consisting of DMEM-P and KSFM-P mixed at a ratio of 66:33.

EXAMPLES

Hereinafter, the present invention will be described in further detail with reference to examples. It will be obvious to a person having ordinary skill in the art that these examples are illustrative purposes only and are not to be construed to limit the scope of the present invention.

Example 1: Isolation of mesenchymal stem cells from amnion tissue

Amnion tissue used in the example of the present invention was isolated from the placenta, and the isolated tissue was placed in an antibiotic-containing saline or DMEM (Dulbecco modified Eagle medium) medium and then transferred to the laboratory.

In a clean bench, 5 g of amnion tissue was washed with sterile saline, and in a Petri dish, the washed amnion tissue was cut as finely as possible with surgical scissors. A collagenase solution was added to and mixed well with the finely cut tissue. The mixture was placed in a flask which was then incubated in a 5% CO_2 incubator at 37 ℃ for 90 minutes to lyse the tissue. The lysed tissue was filtered through a cell strainer in a 50 mL tube and centrifuged. After centrifugation, the supernatant was removed, and the cell precipitate was suspended in DMEM-P and inoculated into a T-flask, which was then incubated in a 5% CO_2 incubator at

37 ℃ for 3-4 days. Then, only mesenchymal stem cells attached to the culture flask were collected.

Example 2: Culture of isolated amnion mesenchymal stem cells in mixed medium of DMEM-P and KSFM-P

The amnion-derived mesenchymal stem cells obtained in Example 1 were dispensed in a T175 flask at a density of $1.0x10^6$ cells and cultured in mixed media consisting of DMEM-P medium (Table 1) and KSFM-P medium (Table 3) mixed at the ratios shown in Table 4, while the media were replaced at 2-day intervals. Cell photographs taken at 3 days and 4 days of culture are shown in FIGS. 1 and 2.

Table 1: Components of DMEM-P medium

Components	Purchase Source	Concentration
DMEM-HG	WelGENE, Inc.	
L-ascorbic acid 2-phosphate	Sigma-aldrich	0.2 mM
Fetal Bovine Serum	Invitrogen	10%
b-FGF	Invitrogen	10 ng/㎖
NEAA	Invitrogen	10 ㎕/㎖

Table 2: Composition of NEAA

NEAA Components	Concentration (mg/L)
Glycine	750

L-Alanine	890
L-Asparagine	1,320
L-Aspartic acid	1,330
L-Glutamic Acid	1,470
L-Proline	1,150
L-Serine	1,050

Table 3: Components of KSFM-P medium

Components	Purchase Source	Concentration
Defined Keratinocyte-SFM	Invitrogen	
L-ascorbic acid 2-phosphate	Sigma-aldrich	0.2 mM
Insulin	Millipore	5 μg/mℓ
N-acetyl-L-cysteine	Sigma-aldrich	2 mM
Calcium chloride	Sigma-aldrich	0.09 mM
Hydrocortisone	Sigma-aldrich	74 ng/mℓ
Fetal Bovine Serum	Invitrogen	5%

Table 4: Mixing ratio of DMEM-P and KSFM-P

Groups	DMEM-P	KSFM-P
1	100	0
2	66	33
3	50	50
4	25	75
5	0	100

At 4 days of culture, the amnion-derived mesenchymal stem cells were washed with HBSS buffer, and then incubated in TrypLE-Express (Gibco) or 0.25% Trypsin-EDTA at 37 ℃ for 10 minutes. FBS-containing medium was added thereto to inactivate the trypsin, and then the number of the resulting amnion-derived mesenchymal stem cells was counted.

As a result, as can be seen in Table 4 and FIGS. 1 and 2, the amnion-derived stem cells grew much faster in a mixed medium of DMEM-P medium and KSFM-P medium than in DMEM-P medium or KSFM-P medium alone, and the number of the cells at 4 days of culture was also significantly larger when the cells were cultured in the mixed medium of DMEM-P medium and KSFM-P medium.

As shown in Table 5 below, a medium of Group 2 consisting of DMEM-P medium and KSFM-P medium mixed at a ratio of 66:33 showed the highest cell proliferation rate. Thus, the mixed medium of Group 2 was used in subculture.

Table 5: Change in cell number as a function of ratio of mixing of media

Groups	DMEM-P : KSFM-P	The number of cells
1	100 : 0	1.2×10^7
2	66 : 33	3.7×10^7
3	50 : 50	2.4×10^7
4	25 : 75	2.0×10^7

5	0 : 100	1.4×10^7

Example 3: Immunological characteristics of amnion-derived mesenchymal stem cells cultured in mixed medium

Flow cytometry analysis of surface antigen expression

The amnion-derived mesenchymal stem cells cultured in the mixed culture in Example 2 were characterized with CD series antigen markers. FACS analysis were performed using CD29 (mononuclear cell marker), CD31 (endothelial cell and stem cell marker), CD34 (hematopoietic stem cell marker), CD44, CD45 (PTPR, ASV, leukocyte marker) and CD73.

The amnion-derived mesenchymal stem cells obtained in Example 1 were washed with PBS and treated with trypsin, after which the cells were collected and centrifuged at 1500 rpm for 5 minutes. The supernatant was removed, and the cells were incubated in a blocking buffer solution (5% serum (normal goat serum + normal horse serum)) at 4 ℃ for 60 minutes, followed by centrifugation at 1500 rpm for 5 minutes. After the supernatant has been removed, the cells were suspended in PBS and dispensed in each well at a density of 1 x 10^5 cells corresponding to the number of negative control and CD antigen markers. R-phycoerythrin (PE)/FITC (fluorescein isothiocyanate)-conjugated mouse anti-human monoclonal antibody was added to each well and incubated at

4 ℃ for 40 minutes, followed by centrifugation at 1500 rpm for 3 minutes. The supernatant was removed, and the cells were washed with PBS and centrifuged at 1500 rpm for 3 minutes. Then, the process of removing the supernatant, washing the cells with PBS and centrifuging the cells at 1500 rpm for 3 minutes was repeated once more. After the supernatant has been removed, the cells were analyzed using a flow cytometer.

As a result, the amnion-derived mesenchymal stem cells of each group were all immunologically positive for CD29, CD44 and CD73 and immunologically negative for CD31, CD34 and CD45 (see FIGS. 3 to 8).

Example 4: Comparison of proliferation ability between amnion-derived mesenchymal stem cells subcultured in mixed medium

The cells cultured in Example 1 were subcultured at a concentration of 1.0×10^6 cells/T175 flask, and the abilities of passage 1 (P1) and passage 2 (P2) cells to proliferate were examined.

The subculture was performed under the same conditions as those of Example 1.

As a result, the P1 cells of the group cultured in DMEM-P medium alone (Group 1) were measured to be 5.4×10^6 cells

after 5 days of culture, whereas the P1 cells of the group cultured in the mixed medium of DMEM-P and KSFM-P (66:33) (Group 2) were measured to be $1.25x10^7$ cells after 4 days of culture, suggesting that the cells showed a significantly high ability to proliferate in the mixed medium (see FIG. 9).

The P2 cells of the group cultured in DMEM-P medium alone (Group 1) were measured to be $3.4x\ 10^6$ cells after 4 days of culture, and thus the number of the proliferated cells decreased compared to the number of the P1 cells. However, the P2 cells of the group cultured in the mixed medium of DMEM-P and KSFM-P (66:33) (Group 2) were measured to be $1.38x10^7$ cells after 4 days of culture, and thus the number of the P2 cells increased compared to the P1 cells (see FIG. 10).

Example 5: Comparison of immunological characteristics between amnion-derived mesenchymal stem cells subcultured in mixed medium

In the same manner as Example 2, P1 and P2 cells were obtained by subculture using each of DMEM-P medium alone and a mixed medium of DMEM-P and KSFM-P (66:33). The immunological characteristics of the P1 and P2 cells were examined, and as a result, the amnion-derived mesenchymal stem cells of each group were all immunologically positive for CD29, CD44, CD73, CD90, CD105 and HLA-ABC and

immunologically negative for CD31, CD34, CD45 and HLA-DR.

Example 6: Quantification of osteogenic differentiation of amnion-derived mesenchymal stem cells cultured in mixed medium

The amnion-derived mesenchymal stem cells cultured in each of DMEM-P medium alone and the mixed medium of DMEM-P/KSFM-P in Example 1 were cultured in osteogenesis induction medium (nonhematopoietic OsteoDiff medium, Miltenyi Biotec) for 18 days (37 ℃; 5% CO_2; medium replacement cycle: 3-4 days) to induce differentiation into bone cells, and the differentiation medium was replaced with fresh medium at 2-day intervals. The differentiation of the amnion-derived mesenchymal stem cells into bone cells was performed in each of a normoxic state and a hypoxic state (1-5%) under the conditions shown in Table 6 below.

At 18 days after the start of culture, it was confirmed by Alizarin red S staining that the amnion-derived mesenchymal stem cells differentiated into osteogenic cells. In addition, the cells cultured in the mixed medium (Group 2) differentiated at a rate higher than the cells cultured in DMEM-P medium alone, and the cells (M-H-N) cultured in the hypoxic state showed a higher ability to differentiate (see FIGS. 11 and 12).

Table 6

P2 Culture Conditions		P3 Differentiation Conditions	OD_{405}	Alizarin Red Concentration	Symbols
Culture Media	Incubation	Incubation			
DMEM-P	Normoxia	Normoxia	0.125	4.20712	P-N-N
	Normoxia	Hypoxia	0.045333	1.6289	P-N-H
	Hypoxia	Normoxia	0.134333	4.509159	P-H-N
DMEM-P+ KSFM-P-Mix	Normoxia	Normoxia	0.164	5.469256	M-N-N
	Hypoxia	Normoxia	0.208	6.893204	M-H-N

INDUSTRIAL APPLICABILITY

According to the present invention, a number of mesenchymal stem cells required for stem cell therapy can be obtained in a short time, and the ability of mesenchymal stem cells to differentiate is improved so that they are useful for stem cell therapy.

Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope

of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

【CLAIMS】

【Claim 1】

A medium composition for culturing mesenchymal stem cells, the medium comprises:

(i) DMEM-P containing DMEM-HG, 0.2mM of L-ascorbic acid 2-phosphate, 10% fetal bovine serum, 10ng/ml of basic fibroblast growth factor (b-FGF), 10 $\mu\ell$/ml of non-essential amino acid (NEAA); and

(ii) KSFM-P containing defined Keratinocyte-SFM, 0.2mM of L-ascorbic acid 2-phosphate, 5μg/ml of insulin, 2mM of N-acetyl-L-cysteine, 0.09mM of calcium chloride, 74ng/ml of hydrocortisone, 5% fetal bovine serum,

wherein the non-essential amino acid (NEAA) consists of 750mg/L of glycine, 890mg/L of L-alanine, 1,320mg/L of L-asparagine, 1,330mg/L of L-aspartic acid, 1,470mg/L of L-glutaminic acid, 1,150mg/L of L-proline, and 1,050mg/L of L-serine, and the volume ratio of DMEM-P : KSFM-P is 2:1, 1: 1 or 1:3.

【Claim 2】

The medium composition of claim 1, wherein the mesenchymal stem cells is derived from amnion.

【Claim 3】

A method for culturing mesenchymal stem cells, the method comprising culturing the mesenchymal stem cells in the medium composition of claim 1.

【Claim 4】

The method of claim 3, wherein the mesenchymal stem cells is derived from amnion.