

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
22 December 2005 (22.12.2005)

PCT

(10) International Publication Number
WO 2005/121317 A2

(51) International Patent Classification⁷: C12N 5/00

(21) International Application Number:
PCT/IB2005/002414

(22) International Filing Date: 4 April 2005 (04.04.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2004110701 9 April 2004 (09.04.2004) RU

GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant and

(72) Inventor: TEPLYASHIN, Alexander, S. [RU/RU]; Kusnezki most 17, Moskau, 107031 (RU).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR OBTAINING MESENCHYMAL STEM CELLS

(57) Abstract: The invention relates to the field of cell biology. In detail it relates to the obtainment of mesenchymal stem cells from human tissue. This invention might be applicable in healing within the frame of the treatment of several diseases. Due to the invention it will be possible to obtain mesenchymal stem cells from human tissue with high homogeneity of the cell suspension, since the used method for obtaining mesenchymal stem cells from human tissue comprises the crushing and enzymatical treatment of the tissue with collagenase solution in Eagle medium in the Dulbecco modification, removal of erythrocytes by the aid of the lysis solution and subsequent filtration of the prepared suspension; as human tissue fat tissue or decidual or amniotic placenta membrane or chorion placenta stroma is used, whereas the filtration is performed sequentially by the use of filters comprising a pore size of 100 pm and 10 pm. In the enzymatical treatment of the fat tissue, of the decidual or amniotic placenta membrane collagenase of the type I is used, and in the enzymatical treatment of the chorion placenta stroma collagenase of the type IV is used.



WO 2005/121317 A2

METHOD FOR OBTAINING MESENCHYMAL STEM CELLS

The invention relates to the field of cell biology. In detail it relates to the obtainment of mesenchymal stem cells from human tissue. This invention might be applicable in healing within the frame of the treatment of several diseases.

Currently in biomedical sciences a new field is developing: cell therapy. By means of cell transplantation insufficient activities of tissue functions can be redressed and affected organs can be regenerated. The function of renewal and restoration is taken over from stem cells in vivo which form an agglomeration of non-differentiated precursors of different cells types which are kept in reserve. As a result of this the use of stem cells is a promising field of cell therapy. In this connection of particular importance is the obtainment of stem cells from human tissue.

At present one has been succeeded in obtaining different types of stem cells from the adult human organism. In detail they concern hematopoietic (blood cell precursors), neuronal (precursors of nerve tissue cells), mesenchymal cells (cells which are capable to differentiate into cells of mesenchymal origin as well as into other embryonic layer cells) and other.

It is characteristic for mesenchymal stem cells that they can be obtained and cultivated comparatively easily; they are also capable to proliferate in vitro over prolonged periods of time. Furthermore, they excel in a broad spectrum of differentiation. In this connection the attention is especially directed to the obtainment of mesenchymal stem cells from the adult human organism. However, till this day there is no universal method for the obtainment of stem cells.

For the first time stem cells could be obtained from the spinal marrow due to their ability of adhering to the surface of culture dishes (Fridenshtein A.J., Deriglazova U.F., Kulagina N.N. et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp. Hematol. 1974 Vol. 2. P. 83-92).

The disadvantage of this method consists in the lacking homogeneity of the obtained cell population. Nevertheless the adhesion of mesenchymal stem cells to plastics has been established as starting basis for further improved methods for obtaining mesenchymal stem cells.

A method for obtaining mesenchymal stem cells is known that results from the cells' ability of adhering to the surface of culture dishes, whereby specific batches of the embryonic bovine serum are used. Thus it was managed to obtain cells with a high ability of adhesion, high rate of proliferation and long maintaining multipotency (Heynesworth S.E., Goshima J., Goldberg V. M., Calplan A.I. Characterization of cells with osteogenic potential from the human bone marrow // Bone. 1995. Vol. 13. P. 81-85.)

The disadvantage of this method consists in the fact that the analysis of the serum during the search for a batch that is suitable for the cultivation of cells plenty of time and work is required; furthermore the reproduction of results is not possible.

It is known to obtain mesenchymal stem cells by a method by means of which a selection for antibodies against Stro-1 (antigen with unknown function) is performed, Stro-1 is temporarily expressed on the surface of mesenchymal stem cells (Grontos S., Simmons P.J. The growth factors requirements of Stro-1 positive human stromal precursors under serum-deprived conditions in vitro // Blood. 1995. Vol. 85. P. 929-940).

This method concerns an extreme complex process that is very time consuming due to the preliminary antibody preparation.

A method for obtaining mesenchymal stem cells is known by means of which mononuclear cells are obtained by centrifugation in a Ficoll gradient, a selection for antibodies against the surface antigen CD105 which is expressed on the surface of mesenchymal stem cells, as well as by a cultivation of cells which adhere to plastics. The portion of CD105⁺ cells amount to 2 to 3% of the mononuclear cells. As a result it was possible to obtain a cell population which shows the morphology and the expression profile of the surface antigens which are characteristic for mesenchymal stem cells and which also have chondrogenic potential (Majumdar M.K., Banks V., Peluso D.P., Morris E.A. Isolation, characterization, and chondrogenic potential of

human bone marrow-derived multipotential stromal cells // J. Cell. Physiol. 2000. Vol. 185. P. 98-106).

This method enables the obtainment of the CD105⁺ cell fraction which is enriched in mesenchymal stem cells, however this method requires an additional selection stage in which antibodies immobilized to magnetic beads are used. Thereby a portion of cells gets lost so that additional efforts are required.

It is known that with increasing age the number of mesenchymal stem cells in the human organism is diminished conspicuously. This also apply to their ability to proliferate and differentiate (Rao M.S., Mattson M.P. Stem cell and aging: expanding the possibilities // Mech. Ageing Dev. 2001. Vol. 122. P. 713-734). For this reason one is working on the preparation of mesenchymal stem cells with high proliferative activity and potential for differentiation, which are for example originating from fetal tissue or larvae organs, respectively.

A method for obtaining mesenchymal stem cells from fetal progeny blood is known in the art. To obtain them the mononuclear cell fraction is prepared by centrifugation in the Ficoll gradient and they are cultivated under conditions which are beneficial for the growth of mesenchymal stem cells. The so obtained cells show osteogenetic or adipogenetic potential, respectively (Campagnoli C., Roberts I.A., Kumar S. et al. Identification of mesenchymal stem/progenitor cells in human first trimester fetal blood, liver, and bone marrow // Blood. 2001. Vol. 98. P. 2396-2402).

The disadvantage of the obtainment of mesenchymal stem cells from this source consists in the fact that fetal tissue is problematic in view of its accessibility. Moreover one has to face ethic problems which are associated with its use. Furthermore, it has turned out that the obtained cell populations are not homogeneous: 76% of the samples contained osteoclast-like cells; they expressed the characteristic antigens CD45, CD51/CD61, and were negative for CD64 (marker for macrophages), SH2 (marker for mesenchymal stem cells), CD31 (marker for endothelial cells). Only 26% of the samples were composed of cells which were similar to mesenchymal stem cells and expressed SH2, SH3, SH4, MAB1470, CD13, CD29, CD49e, CD54, CD90, ASMA, and were negative for CD31 and vWF (marker for endothelial cells).

Known is the method for obtaining mesenchymal stem cells from the subendothelial layer of the umbilical vein (Romanov Y.A., Svinitskaya V.A., Smirnov V.N. Searching for alternative sources of postnatal human mesenchymal stem cells; candidate MSC-like cells from umbilical cord // Stem Cells. 2003. Vol. 21. P. 105-110). For these purposes the umbilical vein was treated inside with a collagenase IV solution for a short time (for 15 minutes). The so obtained cells were cultivated in DMEM-LG supplemented with 10% FBS. In this manner a cell population with fibroblast-like morphology was obtained; it expressed a range of antigens which was similar to mesenchymal stem cells: ICAM1^{+/-}, VCAM1⁺, CD34⁻; MySM⁻ (smooth muscle myosine); CD31⁻, vWF⁻ (marker for endothelial cells); CD14⁻, CD45⁻, CD68⁻ (marker for monocytes/macrophages), however it did express smooth muscle fibre actin ASMA. The so obtained cell population showed osteogenetic or adipogenetic potential in vitro.

The disadvantage of this method consists in the heterogeneity of the obtained cell population: The primary culture contained endothelial and smooth muscle cells, whereby the endothelial cells have not proliferated under these conditions, whereas the myocytes portion was maintained during the cultivation.

A method for obtaining mesenchymal stem cells from human lipoaspirate is known in the art. In this method fat tissue reduced to small pieces is exposed to the effect of collagenase (type I). After neutralization of the collagenase and rinsing of the cell suspension a purification is performed in order to remove cell residues by the use of filters having a pore size of 100 μm . By doing so a population of mesenchymal stem cells could be obtained that showed a characteristic morphology, immunophenotype and that was able to differentiate into bone, cartilage, fat, muscle or nerve tissues, respectively (Zuk P.A., Zhu M., Ashjian P., De Ugarte D.D., Huang J.I., Mizuno H., Alfonso Z.C., Fraiser J.K. Benhaim P. and Hedrick M.H. Human adipose tissue is source of multipotent stem cells // Molecular Biology of the Cell. 2002. Vol. 13. P. 4279-4295).

This method has the disadvantage that the obtained cell suspension is heterogenous and the yield is low.

With the present invention it is now possible to obtain mesenchymal stem cells from human tissue, whereby the cell suspension has a high homogeneity.

Against this background, an object of the present invention is a method for obtaining and/or isolating mesenchymal

stem cells from human tissue, comprising the following steps: (a) providing human tissue, (b) enzymatical and mechanical treatment of said human tissue for obtaining a cell suspension, (c) removal of erythrocytes from said suspension, and (d) filtration of said suspension for obtaining mesenchymal stem cells, wherein in step (a) fat tissue or/and placenta tissue is used as human tissue.

Within the frame of the method for obtaining mesenchymal stem cells from human tissue fat tissue or placenta is used as human tissue. This method might comprise the crushing and enzymatic treatment of the tissue with a solution of collagenase in Eagle medium in the Dulbecco modification, the removal of erythrocytes by the aid of lysing buffer solution and the subsequent filtration of the obtained suspension. The filtration can be performed sequentially by means of filters comprising a pore size of 100 μm and 10 μm . The filtration step can be performed by the use of a first filter comprising a pore size of 100 μm in diameter, and the subsequent use of a second filter comprising a pore size of 10 μm in diameter.

Collagenase type I can be used for the enzymatic treatment of the fat tissue, the decidual or amniotic membrane of the placenta. For the enzymatic treatment of the stroma of the chorionic placenta collagenase type IV can be used.

The technical results of the invention (increase of the homogeneity of the cell suspension, increase of the yield of the target product and improved viability of the cells) attribute to the conditions for the filtration: Specific size of the filter pores and specific ratio of the pores of the used fil-

ters. The change (increase or decrease) of these parameters will have the result that the stated technical results will not be achieved, since in this case the yield of the target product and the homogeneity of the obtained cell suspension will be conspicuously reduced. In comparison to known similar methods a multiple yield of cells can be obtained. According to the applicant by performing the known methods the yield amounts to 10^4 cells each tissue sample having a mass of 1 gram, whereas by performing the method according to the invention the yield of cells amounts to between 1.5 to 3×10^4 and 10^7 cells each 1 gram of several tissue samples. The increase of the homogeneity of the cell suspension is proved by the data of the morphological analysis and the determination of the immunophenotype. According to the data of the applicant by using the known method by which the population of mesenchymal stem cells is obtained from lipoaspirate, the population contains multiple morphological cell types; not before the 4th passage the culture becomes homogenic in view of their cell shape and granulation or the expression of surface markers, respectively.

The method is realized as follows:

First of all a tissue sample is rinsed with physiological saline solution with phosphate buffer (PBS) at pH 7.2 without Ca^{2+} and Mg^{2+} ions, supplemented with antibiotics (penicillin 100 units/ml, streptomycin 100 microgram/ml) and antimycotics (amphotericin B 0.25 microgram/ml). The tissue to be treated is reduced to small pieces; the eagle medium in the Dulbecco modification with above-mentioned antibiotics and antimycotics is added with a volume ratio between tissue and medium of 1:5 to 1:10 (DMEM, Dulbecco's Modified Eagle Medium). For the enzy-

matic treatment collagenase solution is added to the suspension until a final concentration of 0.075% is reached. The suspension is incubated for 30 minutes at 37°C, whereby the suspension is swung carefully.

The so prepared mixture is agitated until a homogenic suspension is produced; subsequently for neutralization of the collagenase an equivalent volume of the DMEM medium is added to the prepared mixture, which contains 10% by volume of fetal bovine serum (FBS). Afterwards a centrifugation at 1000 g for 10 minutes is performed. The pellet is resuspended in the erythrocyte lysis buffer solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA). The mixture is thoroughly mixed and incubated for 3 to 5 minutes at room temperature.

The suspension is diluted with an equivalent volume of the DMEM medium that contains antibiotics and antimycotics; subsequently the cells are pelleted by centrifugation for 10 minutes at 1000 g. The pellet is rinsed with DMEM medium and again pelleted by centrifugation in the manner as described. The cells are brought into suspension in DMEM-LG medium with a concentration of glucose of 1 g/l, supplemented with 20% FBS, antibiotics and antimycotics. The so prepared cell suspension is filtered by the use of filters comprising a pore size of 100 μm and 10 μm, and 1 million cells each are plated per 1 cm².

The so obtained cell population is characterized by the high homogeneity of mesenchymal stem cells, whereby the alteration of the filtration data (increase and/or decrease of the

pore size) results in a reduction of the homogeneity of the target product or the yield of cells, respectively.

Against this background another object of the present invention is the use of human fat tissue and/or placenta tissue as source for obtaining and/or isolating mesenchymal stem cells.

The invention is illustrated by the following embodiments:

Example 1

The decidual membrane is separated from the placenta by means of small scissors. A tissue sample of 2 g is rinsed with PBS (Gibco) at pH 7.2 without Ca^{2+} and Mg^{2+} ions for three times, whereby the PBS contains a one-fold solution of antibiotics or antimycotics (Gibco), respectively, in which the final concentration of penicillin is 100 units/ml, streptomycin 100 microgram/ml, amphotericin B 0.25 microgram/ml.

The tissue is reduced to small pieces in a Petri dish by means of scissors; then the DMEM medium (Gibco) having a volume of 25 ml is added, which contains antibiotics and antimycotics; the tissue is resuspended and given into a 50 ml test tube (Costar).

To the so prepared suspension for the enzymatic treatment 1 ml of a solution containing 2% collagenase of type I (Gibco) is added until a final concentration of 0.075% is reached. The suspension is incubated for 30 minutes at 37°C in a shaker, whereby slow swinging movements should be performed.

The mixture is thoroughly stirred until a homogenic suspension is produced; afterwards 25 ml DMEM medium is added to the mixture that contains 10% FBS (HyClone, PerBio) in order to neutralize the collagenase. The cells are pelleted by centrifugation for 10 minutes at 1000 g.

The supernatant is removed. In order to lyse the erythrocytes the pellet is resuspended in 20 ml of cold buffer solution which contains 155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA . The mixture is thoroughly stirred and incubated for 3 to 5 minutes at room temperature.

The so prepared suspension is diluted with DMEM medium that contains 25 ml antibiotics and antimycotics (Gibco); subsequently the cells are pelleted by centrifugation for 10 minutes at 1000 g.

The supernatant is removed. The cell supernatant is suspended in DMEM medium for washing. The cells are pelleted by centrifugation for 10 minutes at 1000 g.

The so prepared cell pellet is suspended in 25 ml DMEM-LG medium having a glucose concentration of 1 g/l (Gibco), supplemented with 20% FBS (HyClone, PerBio), one-fold solution of essential amino acids (Gibco) as well as a one-fold solution of antibiotics and antimycotics (100 units/ml penicillin, 100 microgram/ml streptomycin, 0.25 microgram/ml amphotericin B, Gibco).

The cell suspension is sequentially filtered by the use of filters comprising a pore size of 100 μm and 10 μm (Millipore) in order to remove cell residues and debris.

The number of purified cells is calculated in the Gorjajev chamber. The total cellular yield amounts to $10^8/1$ g of tissue. The cells are plated in 75 cm^2 flasks at 1 million/ 1 cm^2 each. The portion of adhering cells amounts to about 1%; the yield of mesenchymal stem cells from the decidual membrane amounts to approximately $10^6/1$ g of tissue.

After 24 hours the medium for the cells is replaced by fresh medium. Once the monolayer is reached the cells are subcultivated and visually evaluated in view of their morphology by the use of a phase contrast microscope; the mitosis index and the cellular generation time are calculated.

On account of the morphological analysis two major cell populations have been determined according to their phenotype. The first cell type presents itself as fusiform cells having a diameter of 15 to 35 μm with homogeneous cytoplasm, low nucleus-cytoplasm ratio, a centric nucleus consisting of 4 to 7 nucleoli. The second type comprises larger fibroblast-like, spread cells having a diameter of 90 μm with cytoplasm of diverse homogeneity; it has a low nucleus-cytoplasm ratio, a centric nucleus with 2 to 4 nucleoli. Thus, the cells to be analyzed have a morphology that is characteristic for human mesenchymal stem cells.

The mitosis index is calculated in the phase of logarithmic growth as the ratio of the number of mitosis to the total

cell number. The mitosis index amounts to 29.5%. The cell generation time amounts to 29 hours.

The so obtained cells were immunophenotyped by staining with antibodies against the surface antigens CD10, CD13, CD31, CD34, CD44, CD45, CD90, CD105, CD117 (Becton Dickinson), whereby indirect fluorescence is used. The evaluation is performed by the use of a flowcytometer (Beckman Coulter). The surface marker expression corresponds to the immunophenotype of the mesenchymal stem cells: The cells are positive for CD13, CD44, CD90, CD105 and negative for CD31, CD34, CD45, CD117. The CD10 expression is moderately positive (table 1).

Table 1. Expression of the surface antigens on the surface of mesenchymal stem cells from the decidual placenta membrane.

Immunophenotype of mesenchymal stem cells from the decidual membrane	
CD	%
CD10	50.30
CD13	87.00
CD31	1.50
CD34	1.30
CD44	95.90
CD45	3.40
CD90	93.70
CD105	95.50
CD117	7.00

Example 2

The chorion stroma is separated from the placenta by means of small scissors. The tissue sample of 5 g is rinsed three times with PBS (Gibco) at pH 7.2, without Ca^{2+} and Mg^{2+} ions. PBS (Gibco) contains a one-fold antibiotics and antimycotics solution (Gibco). The final concentration of penicillin is 100 units/ml, of streptomycin 100 microgram/ml, amphotericin B 0.25 microgram/ml.

The tissue is reduced to small pieces in 10 cm Petri dishes by means of scissors; subsequently 25 ml DMEM (Gibco) medium comprising antibiotics and antimycotics are added; afterwards, the tissue is suspended and given into a 50 ml test tube (Costar).

To the so prepared suspension for the enzymatic treatment 1 ml of a solution of 2% collagenase type IV (Gibco) is added until a final concentration of 0.075% is reached. The suspension is incubated for 30 minutes at 37°C in a shaker with slow swinging movements.

The so prepared mixture is thoroughly stirred until a homogenic suspension is produced; afterwards 25 ml DMEM medium that contains 10% FBS (HyClone, PerBio) is added in order to neutralize the collagenase. The cells are pelleted by centrifugation for 10 minutes at 1000 g.

The supernatant is removed. For lyses of the erythrocytes the pellet is resuspended in 20 ml of cold buffer solution which contains 155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA . The

mixture is thoroughly stirred and incubated for 3 to 5 minutes at room temperature.

The suspension is diluted with DMEM medium that contains antibiotics and antimycotics (Gibco) in a volume of 25 ml; afterwards the cells are pelleted by centrifugation for 10 minutes at 1000 g.

The supernatant is removed. In order to wash the cell residue the latter is suspended in DMEM medium. The cells are pelleted by centrifugation for 10 minutes at 1000 g.

The so prepared cell pellet is suspended in 25 ml DMEM-LG medium having a glucose concentration of 1 mg/ml (Gibco), supplemented with 20% FBS (HyClone, PerBio), a one-fold solution of essential amino acids (Gibco) and a one-fold solution of antibiotics and antimycotics (100 units/ml penicillin, 100 microgram/ml streptomycin, 0.25 microgram/ml amphotericin B, Gibco).

The cell suspension is sequentially filtered by the use of filters comprising a pore size of 100 μm and 10 μm (Millipore) in order to remove cell residues and debris.

The number of purified cells is calculated by evaluation in the Gorjajev chamber. The total yield of cells amounts to 10^9 /1 g of tissue. The cells are plated in 75 cm² flasks at 1 million/1 cm² each. The portion of the adhering cells amounts to about 1%; the yield of mesenchymal stem cells from the chorion stroma amounts to approximately 10^7 /1 g of tissue.

After 24 hours the medium for the cells is replaced by fresh medium. Once the monolayer is reached the cells are sub-cultivated, visually evaluated in view of the morphology by the use of a phase contrast microscope; the mitosis index and the cell generation time are calculated.

As a result of the morphological analysis two major cell populations were identified according to their phenotypes. The first cell type presents itself as fusiform cells having a diameter of 20 to 40 μm with homogenic cytoplasm, a low nucleus-cytoplasm ratio, a centric nucleus consisting of 4 to 7 nucleoli. The second type comprises larger fibroblast-like, spread cells having a diameter of 100 μm with cytoplasm of various homogeneity; it comprises a low nucleus-cytoplasm ratio, a centric nucleus with 2 to 4 nucleoli. Thus, the cells to be analyzed have a morphology that is characteristic for human mesenchymal stem cells.

The mitosis index is calculated in the phase of logarithmic growth as the ratio of the number of mitosis to the total cell number. The mitosis index amounts to 31.8%. The cell generation time amounts to 28 hours.

The so obtained cells are immunophenotyped by staining with antibodies against the surface antigens CD10, CD13, CD31, CD34, CD44, CD45, CD90, CD105, CD117 (Becton Dickinson), whereby indirect fluorescence is used. The evaluation is performed by the use of a cytometer (Beckman Coulter). The surface marker expression corresponds to the immunophenotype of the mesenchymal stem cells. The cells are positive for CD13, CD44,

CD90, CD105 and negative for CD31, CD34, CD45, CD117. The CD10 expression is moderately positive (table 2).

Table 2. Expression of the surface antigens on the surface of mesenchymal stem cells from the chorion placenta stroma.

Immunophenotype of mesenchymal stem cells from the chorion stroma	
CD	%
CD10	84.40
CD13	90.90
CD31	0.20
CD34	0.30
CD44	97.60
CD45	1.50
CD90	95.30
CD105	92.70
CD117	3.90

Example 3

The amniotic membrane is separated from the placenta by means of small scissors. The tissue sample having a mass of 2 grams is rinsed three times in PBS (Gibco) at pH 7.2, without Ca^{2+} and Mg^{2+} ions. PBS (Gibco) contains a one-fold antibiotics or antimycotics solution (Gibco), respectively, the final concentration of penicillin is 100 units/ml, streptomycin 100 microgram/ml, amphotericin B 0.25 microgram/ml.

The tissue is reduced to small pieces in 10 cm Petri dishes by means of scissors; subsequently DMEM (Gibco) medium

that contains antibiotics and antimycotics is added in a volume of 25 ml; the tissue is suspended and transferred in a 50 ml test tube (Costar).

For the enzymatic treatment to the so prepared suspension 1 ml of a solution of 1% collagenase of type I (Gibco) is added until a final concentration of 0.075% is reached. The suspension is incubated for 30 minutes at 37°C in a shaker with slow swinging movements.

The so prepared mixture is thoroughly stirred until a homogeneous suspension is produced; then 25 ml DMEM medium that contains 10% FBS (HyClone, PerBio) is added in order to neutralize the collagenase. The cells are pelleted by centrifugation for 10 minutes by 1000 g.

The supernatant is removed. In order to lyse the erythrocytes the pellet is resuspended in 20 ml of cold buffer solution which contains 155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA . The mixture is thoroughly stirred and incubated for 4 minutes at room temperature.

The so prepared suspension is diluted with DMEM medium that contains antibiotics and antimycotics (Gibco) in a volume of 25 ml. Subsequently the cells are pelleted by centrifugation for 10 minutes at 1000 g.

The supernatant is removed. The cell pellet is suspended in DMEM medium for washing. The cells are pelleted by centrifugation for 10 minutes at 1000 g.

The so prepared cellular pellet is suspended in 25 ml DMEM-LG medium having a glucose content of 1 g/l (Gibco), supplemented with 20 % FBS (HyClone PerBio), one-fold solution of essential amino acids (Gibco) as well as one-fold antibiotics and antimycotics solution (100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Gibco)).

The cell suspension is sequentially filtered by the use of filters comprising a pore size of 100 µm and 10 µm (Millipore). The number of purified cells is evaluated by calculation in the Gorjajev chamber. The total yield of cells amounts to 10^8 /1 g of tissue. The cells are plated in 75 cm² flasks of 1 million/1 cm² each. The portion of the adhering cells amounts to about 1 %; the yield of mesenchymal stem cells from the amniotic membrane amounts to approximately 10^6 /1 g of tissue.

After 24 hours the medium for the cells is replaced by fresh medium. Once the monolayer is reached the cells are subcultivated, visually evaluated in view of their morphology by the use of a phase contrast microscope; the mitosis index and the cell generation time are calculated.

As a result of the morphological analysis two major cell populations were determined. The first cell type presents itself as fusiform cells having a diameter of 10 to 30 µm with homogenous cytoplasm, low nucleus-cytoplasm relation, centric nucleus consisting of 4 to 7 nucleoli. The second type comprises larger fibroblast-like, spread cells having a diameter of 80 µm with cytoplasm of various homogeneity; it comprises a lower nucleus-cytoplasm relationship, a central nucleus consisting of 2 to 4 nucleoli. Thus, the cells to be analyzed show

a morphology that is characteristic for human mesenchymal stem cells.

The mitosis index is calculated in the phase of logarithmic growth as the ratio of the number of mitosis to the total cell number. The mitosis index amounts to 31.6 %. The cell generation time amounts to 25.7 hours.

The so obtained cells are immunophenotyped by staining with antibodies against the surface antigens CD10, CD13, CD31, CD34, CD44, CD45, CD90, CD105, CD117 (Beckton Dickinson), whereby indirect fluorescence is used. The evaluation is performed by use of a cytometer (Beckman Coulter). The surface marker expression corresponds to the immunophenotype of mesenchymal stem cells. The cells are positive for CD13, CD44, CD90, CD105, and negative for CD31, CD34, CD45, CD117. The CD10 expression is moderately positive (table 3).

Table 3. Expression of surface antigens at the surface of mesenchymal stem cells from the amniotic placenta membrane.

Immunophenotype of mesenchymal stem cells from the amniotic membrane	
CD	%
CD10	58.70
CD13	93.20
CD31	1.70
CD34	1.40
CD44	98.30
CD45	0.00
CD90	92.60
CD105	96.90
CD117	1.70

Example 4

A fat tissue sample having a mass of 10 g is rinsed three times in PBS (Gibco) at pH 7.2 without the ions Ca^{2+} and Mg^{2+} . PBS (Gibco) contains a one-fold antibiotics and antimycotics solution (Gibco). The final concentration of penicillin is 100 units/ml, streptomycin 100 $\mu\text{g}/\text{ml}$, amphotericin B 0.25 $\mu\text{g}/\text{ml}$. The portions of compact connective tissue are removed.

Afterwards the tissue is subjected to mechanic fragmentation using medical scissors in 10 cm culture dishes (Costar), until a fine-dispersed mass is produced. It is transferred into two 50 ml test tubes having a cone-shaped bottom (Costar).

Afterwards each sample is suspended in 25 ml DMEM medium that contains antibiotics and antimycotics.

Afterwards the enzymatic treatment is performed: to the so prepared suspension 1 ml of a solution of 2 % collagenase of the type Gibco in the PBS buffer solution without Ca^{2+} and Mg^{2+} is added until a final enzyme concentration of 0.075 % is reached. The suspension is incubated for 30 minutes at 37°C at slow swinging movements.

The so prepared mixture is thoroughly mixed; then an equivalent DMEM volume is added that contains 10 % FBS, antibiotics and antimycotics. The centrifugation lasts 10 minutes at 1000 g. The supernatant and the fat drops are removed. The pellets are pooled and suspended in 10 ml of cold lysine buffer solution (+4°C) for 10 minutes, which contains 155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA . The mixture is thoroughly stirred and incubated for 3 to 5 minutes at room temperature.

Subsequently, 10 ml DMEM medium which contains antibiotics and antimycotics are added to the cell suspension. The cells are pelleted by centrifugation for 10 minutes at 1000 g.

The supernatant is removed and rinsed. The cell pellet is resuspended in 30 ml DMEM medium that contains antibiotics and antimycotics, and is centrifuged for 10 minutes at 1000 g.

The so obtained pellet is resuspended in 25 ml DMEM medium that contains antibiotics and antimycotics.

The so prepared cell suspension is filtered by the use of a filter comprising a pore size of 100 μm and is centrifuged for 10 minutes at 300 g. The pellet is resuspended in DMEM medium that is supplemented with antibiotics and antimycotics, 10% FBS and a one-fold solution of non-essential amino acids (Gibco) in a volume of 25 ml. The suspension is filtered by the use of a filter comprising a pore size of 10 μm .

In doing so, a homogenous cell fraction is produced that is free of cellular debris and blood cells.

The so prepared cell suspension is evaluated by calculation in the Gorjajev chamber and plated in 75 cm^2 flasks (10^6 cells/ cm^2). The portion of adhering cells amounts to about 1 % to 1.5 %; the yield of mesenchymal stem cells which could be obtained from 1 g of fat tissue amounts to about $1.5\text{-}3 \times 10^4$ cells.

After 24 hours the medium is replaced by DMEM that contains antibiotics (100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco), 10 % FBS and a one-fold solution of non-essential amino acids (Gibco).

Once the monolayer is reached, the cells are sub-cultivated, visually evaluated in view of the morphology by the use of a phase contrast microscope; the mitosis index and the cell generation time are calculated.

After the obtainment of cell fractions as a result of the morphological analysis two sub-populations were determined. The first cell type presents itself as a sub-population of fusiform

cells having a diameter of 10 to 15 μm with exactly adjusted nucleus and homogenous cytoplasm. The second type is characterized by round cells with a flat cytoplasmatic outgrowth elongated on one side. The cell size amounts to 40 μm ; a dark nucleus is observable which is displaced to the side, and a heterogeneous cytoplasm. Also an increased granulation in the area of the nucleus is observable.

The mitosis index and the cell generation time is calculated in the phase of logarithmic growth. The ratio of the portion of cells being in mitosis to the total cell number amounts to 34 %. The doubling time is about 54 to 62 hours.

The so obtained cells are immunophenotyped by the use of indirect fluorescence. By the use of a cytometer (Beckman Coulter) a high expression status of the following antigens could be determined: CD10 (CALLA), CD13 (APN), CD44 (hyaluronic acid receptor), CD90 (Thy-1), CD105 (endoglin) (Becton Dickinson). Furthermore, the lacking expression of the markers of hematopoietic cells - CD34, CD45 and CD117 (Becton Dickinson) - (table 4) was determined. The results of the determination of the immunophenotype prove that the population of the obtained cells corresponds to mesenchymal stem cells in view of their surface antigen expression.

Table 4: Surface antigen expression on the surface of mesenchymal stem cells from fat tissue

Immunophenotype of mesenchymal stem cells from fat tissue	
CD	%
CD10	68.08
CD13	96.53
CD34	4.38
CD44	93.08
CD45	3.28
CD90	98.36
CD105	90.18
CD117	2.30

Claims

1. Method for obtaining and/or isolating mesenchymal stem cells from human tissue, comprising the following steps:

- (a) providing human tissue,
- (b) enzymatical and mechanical treatment of said human tissue for obtaining a cell suspension,
- (c) removal of erythrocytes from said suspension, and
- (d) filtration of said suspension for obtaining mesenchymal stem cells,

characterized in, that in step (a) fat tissue or/and placenta tissue is used as human tissue.

2. The method according to claim 1, characterized in that in step (a) the decidual membrane is used as human tissue.

3. The method according to claim 1 or 2, characterized in that in step (a) the amniotic membrane is used as human tissue.

4. The method according to any of claims 1 to 3, characterized in that in step (a) chorion stroma is used as human tissue.

5. The method according to any of claims 1 to 4, characterized in that in step (d) at least one filter is used comprising filter pores which are of a size suitable for removing cell residues and/or debris.

6. The method according to any of claims 1 to 5, characterized in that in step (d) said suspension is sequentially

filtered by the use of filters comprising a pore size of about 50 μm to 150 μm , preferably of 100 μm , and of about 5 μm to 15 μm , preferably of 10 μm .

7. The method according to any of claims 1 to 6, characterized in that in step (d) said suspension is firstly filtered through a first filter comprising a pore size of about 50 μm to 150 μm , preferably of 100 μm , and secondly filtered through a second filter comprising a pore size of about 5 μm to 15 μm , preferably of 10 μm .

8. The method according to any of claims 1 to 7, characterized in that in step (b) for said enzymatic treatment collagenase is used.

9. The method according to any of claims 1 to 8, characterized in that in step (b) collagenase of type I is used in case where decidual membrane, amniotic membrane, and/or fat tissue is used as human tissue, and collagenase of type IV is used in case where chorion stroma is used as human tissue.

10. The method according to any of claims 1 to 9, characterized in that in step (b) for said enzymatic treatment collagenase is provided in Eagle medium, preferably in the Dulbecco modification.

11. The method according to any of claims 1 to 10, characterized in that in step (b) for said enzymatic treatment collagenase is provided in a final concentration of about 0.075%.

12. The method according to any of claims 1 to 11, characterized in that in step (c) said removal of erythrocytes is performed by the aid of buffer capable of lysing said erythrocytes.

13. Use of human fat tissue or/and placenta tissue as source for obtaining and/or isolating mesenchymal stem cells.

14. Use according to claim 13, characterized in that as source for obtaining and/or isolating mesenchymal stem cells human decidual membrane, amniotic membrane, and/or chorion stroma is used.

15. Method for obtaining mesenchymal stem cells from human tissue, comprising the crushing and enzymatical treatment of said tissue with collagenase solution in Eagle medium in the Dulbecco modification, removal of erythrocytes by the aid of the lysis solution and subsequent filtration of the prepared suspension, characterized in that as human tissue fat tissue or decidual membrane or amniotic membrane or chorion placenta stroma is used, whereas the filtration is performed sequentially by the use of filters comprising a pore size of 100 μm and 10 μm .

16. Method according to claim 1, characterized in that in the enzymatical treatment of the fat tissue, the decidual or amniotic placenta membrane collagenase of type I is used, and in the enzymatical treatment of chorion placenta stroma collagenase of type IV is used.