**ABSTRACT**

Chondrocytes are prepared from perichondrocytes. The present invention provides a cell derived from a perichondral tissue, the cell being capable of differentiating into a chondrocyte. The present invention also provides a method of preparing the above-described cell and a composition comprising the same. A method of preparing a chondrocyte and a medium for use in the method are also provided.

19 Claims, 20 Drawing Sheets

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(54) METHOD FOR PREPARATION OF CARTILAGE CELL

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See application file for complete search history.

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(57) Chondrocytes are prepared from perichondrocytes. The present invention provides a cell derived from a perichondral tissue, the cell being capable of differentiating into a chondrocyte. The present invention also provides a method of preparing the above-described cell and a composition comprising the same. A method of preparing a chondrocyte and a medium for use in the method are also provided.

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European office action dated Nov. 25, 2011 for corresponding European application 08704109.1 cites the non-patent literature above.


* cited by examiner
Fig. 4

Chondrocytes enchondrocytes

Alcian Blue Staining

Triple-layered

Mono-layered
Fig. 5

RT-PCR

Perichondrocytes

Chondrocytes

Type I Collagen

Type II Collagen

18S

After 1 week (Mono-layer)

After 2 weeks (2nd layer)

After 3 weeks (3rd layer)
Fig. 6

Realtime PCR

<table>
<thead>
<tr>
<th>Type I Collagen</th>
<th>Type II Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono-layer (2nd layer) (3rd layer)</td>
<td>Mono-layer (2nd layer) (3rd layer)</td>
</tr>
</tbody>
</table>
Fig. 13

Cells in Perichondrium-Cartilage Transition Zone

Perichondrocytes

Chondrocytes

Number of Colonies Composed of 50 or More Cells
Fig. 14

- **Perichondrocytes**
- **Cells in Cartilage-Perichondrium Transition Zone**
- **Chondrocytes**

**Graph:**
- **Cell Count**
- **Days of Culture**
- Log scale for Cell Count: 1E+27, 1E+24, 1E+21, 1E+18, 1E+15, 1E+12, 1E+09, 1E+06, 1E-03, 1E-06, 1E-09, 1E-12, 1E-15, 1E-18, 1E-21, 1E-24, 1E-27, 1E-30, 1E-33, 1E-36
- Log scale for Days of Culture: 0, 50, 100, 150, 200
**Fig. 17**

**A. 1 Month after Transplant**

- Perichondrocytes-Derived Regenerated Cartilage
- Chondrocytes-Derived Regenerated Cartilage

**B. 3 Months after Transplant**

- Perichondrocytes-Derived Regenerated Cartilage
- Chondrocytes-Derived Regenerated Cartilage
Fig. 19

Perichondrocytes

Cells in Perichondrium-Cartilage Transition Zone

Chondrocytes
Fig. 20

Perichondrocytes

Cells in Perichondrium-Cartilage Transition Zone

Chondrocytes

10% Human Autoserum Medium

10% Bovine Serum Medium
METHOD FOR PREPARATION OF CARTILAGE CELL

The present invention relates to a method of preparing chondrocytes. More specifically, the present invention relates to a method of preparing chondrocytes from perichondrium.

BACKGROUND ART

When human cartilage is congenitally deficient or becomes damaged or deficient in the course of lifetime, usually the cartilage is not regenerated. For treating diseases of such human cartilage, a method has been used in which a cartilage tissue is taken from a site of a patient and transplanted into the deficient site of the patient. However, this method has problems that the donor site and the amount of the tissue that can be taken are limited. Then, methods in which a part of autologous chondrocytes is taken, cultured ex vivo and returned to the deficient site have been developed (Non-Patent Documents Nos. 1-4) and applied clinically (Non-Patent Documents Nos. 5, 6 and 7).

However, these methods using chondrocytes have two problems: invasion into the donor site and retention of the shape of a regenerated tissue for a long time. The first one (invasion) is a problem that the donor site from which a cartilage tissue has been taken for culture may result in deformities, such as defect or recess, or dysfuction. The second one (retention of the shape for a long time) is a problem whether a tissue regenerated with cultured cartilage can retain its shape for a long time without being absorbed.

In order to solve these problems, other sources of chondrocytes have been sought for. That is, an idea has been contemplated in which cells other than chondrocytes are differentiated ex vivo into chondrocytes and returned into the living body. Examples of these cells include embryonic stem cells, mesenchymal stem cells, cells derived from the synovial membrane of knee joint, and adipocytes (Non-Patent Documents Nos. 8-10). All of these cells have been confirmed to differentiate into chondrocytes. However, clinical application of embryonic stem cells is difficult from an ethical viewpoint; collecting mesenchymal stem cells or cells derived from the synovial membrane of knee joint, and adipocytes (Non-Patent Documents Nos. 1-4) and applied clinically (Non-Patent Documents Nos. 5, 6 and 7).

It is an object of the present invention to provide a method of preparing chondrocytes which is less invasive to a donor site.

Means to Solve the Problem

Toward the solution of the above-described problems, the present inventors have developed a method of using perichondrium covering the outside of auricular cartilage or costicartilage. The present inventors have succeeded in producing proteoglycan and type II collagen (matrixes peculiar to chondrocytes) by isolating and proliferating perichondrocytes from perichondrium and differentiating the resultant perichondrocytes into chondrocytes in vivo or ex vivo. The present invention may be summarized as follows.

1. A cell derived from a human perichondrial tissue, the cell being capable of differentiating into a chondrocyte.
2. A method of preparing the cell according to (1), wherein the human perichondrial tissue consists of its outermost layer and fibroblast layer.
3. The cell according to (1), wherein the human perichondrial tissue consists of its outermost layer, fibroblast layer and innermost layer.
4. A method of preparing the cell according to (1), wherein the method comprises culturing cells isolated from a human perichondrial tissue.
5. A composition comprising the cell according to any one of (1) to (3).
6. The composition according to (5) for use in proliferating a cell derived from a human perichondrial tissue, the cell being capable of differentiating into a chondrocyte.
7. The composition according to (5) for use in preparing human chondrocytes.
8. The composition according to (5) for use in cell transplant.
(9) The composition according to (8), wherein the cell transplant aims at one or a treatment of congenital auricular deformity, treatment of costicartilage defect, treatment of damage to articular cartilage, treatment of tracheal cartilage defect, rhinoplasty, genioplasty, plastic surgery of small facial recesses, corrective surgery of facial left-right asymmetry, corrective surgery around eyelids, or cosmetic surgery of face.

(10) The composition according to any one of (5) to (9), which further comprises a matrix produced by the cell according to (1).

(11) The composition according to any one of (5) to (10), which further comprises a scaffold.

(12) A method of preparing chondrocytes, comprising differentiating the cell according to (1) into chondrocytes.

(13) The method according to (12), wherein cell masses are formed by culturing the cell according to (1) in a centrifuge tube.

(14) The method according to (12), wherein the cell according to (1) is multi-layered by plate culture.

(15) The method according to any one of (12) to (14), wherein the cell according to (1) is proliferated and/or differentiated in a medium containing a serum.

(16) The method according to (15), wherein the serum is bovine serum.

(17) The method according to (15), wherein the serum is an autoserum.

(18) The method according to any one of (12) to (17), wherein the cell according to (1) is differentiated into chondrocytes in a medium containing DEME/F 12, a serum, antibiotics and antimycotics.

(19) The method according to (18), wherein the medium further contains dexamethasone and/or L-ascorbic acid.

(20) The method according to (18) or (19), wherein the medium further contains an insulin-like growth factor and/or a basic fibroblast growth factor.

(21) Chondrocyte prepared by the method according to any one of (12) to (20).

(22) A composition comprising the chondrocytes according to (21) and/or a cartilage tissue formed by the chondrocytes.

(23) The composition according to (22) for use in transplant treatment.

(24) The composition according to (23), wherein the transplant treatment aims at any one of treatment of congenital auricular deformity, treatment of costicartilage defect, treatment of damage to articular cartilage, treatment of tracheal cartilage defect, rhinoplasty, genioplasty, plastic surgery of small facial recesses, corrective surgery of facial left-right asymmetry, corrective surgery around eyelids, or cosmetic surgery of face.

(25) The composition according to any one of (22) to (24), which further comprises a matrix produced by the chondrocytes according to (21).

(26) The composition according to any one of (22) to (25), which further comprises a scaffold.

(27) A method of transplanting the cell according to any one of (1) to (3) into a living body.

(28) A method of transplanting the chondrocytes according to (21) and/or a cartilage tissue formed by the chondrocytes into a living body.

(29) Use of the cell according to any one of (1) to (3) for cell transplant.

(30) Use of the chondrocytes according to (21) and/or a cartilage tissue formed by the chondrocytes in transplant treatment.

(31) A method of preparing a matrix produced by chondrocytes, comprising differentiating the cell according to (1) into chondrocytes and allowing the chondrocytes to produce the matrix.

(32) The method according (31), wherein the matrix is type II collagen and/or proteoglycan.

**EFFECT OF THE INVENTION**

The method of preparing chondrocytes of according to the present invention need not collect a cartilage tissue. Therefore, it is possible to minimize the invasion into donor sites. Further, by using perichondrocytes comprising cartilage stem/progenitor cells, the method of the present invention makes it possible to retain the shape of a regenerated tissue for a long time.

The present specification encompasses the contents described in the specification and/or the drawings of Japanese Patent Application No. 2007-012160 based on which the present patent application claims priority.

**BRIEF DESCRIPTION OF THE DRAWINGS**

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings(s) will be provided by the Office upon request and payment of the necessary fee.

**FIG. 1.** Histological Examination of Human Perichondrium at the Time of Collection.

While cartilage tissue is stained blue with Alcian blue, perichondrium is not stained. Only the outermost layer and the fibroblast layer of perichondrium may be collected. Alternatively, the outermost layer, the fibroblast layer and the innermost layer (zone of transition to the cartilage matrix) may be collected. a: before collection; arrow: perichondrium; b: perichondrium alone is being detached; c: perichondrium has been detached; d: the innermost layer of perichondrium (zone of transition to the cartilage matrix) may also be collected; e: collected perichondrium consisting of the outermost layer and the fibroblast layer alone. Alcian blue staining. Magnification: 200

**FIG. 2.** Centrifuge Tube Culture of Perichondrocytes

Like a cell mass of chondrocytes, a cell mass of perichondrocytes forms a cartilage tissue in a centrifuge tube. a: cartilage tissue regenerated from perichondrocytes; b: cartilage tissue regenerated from chondrocytes. Alcian blue staining. Bar: 200 μm

**FIG. 3.** The matrix (proteoglycan)-producing capacity of perichondrocytes is enhanced by multi-layering in vitro, in the same manner as seen in chondrocytes. a: bright field; b: Alcian blue staining.

**FIG. 4.** The matrix (proteoglycan)-producing capacity of perichondrocytes is enhanced by multi-layering in vitro, in the same manner as seen in chondrocytes. The matrix-producing capacity of perichondrocytes is comparable to that of chondrocytes. a: mono-layered perichondrocytes; b: multi-layered (triple-layered) perichondrocytes; c: mono-layered chondrocytes; d: multi-layered (triple-layered) chondrocytes. Alcian blue staining.

**FIG. 5.** RT-PCR in Multi-Layered Culture of Perichondrocytes and Chondrocytes

In perichondrocytes, the type I collagen decreases and type II collagen increases as a result of multi-layering.

**FIG. 6.** Quantitative Determination by Real Time PCR in Multi-Layered Culture of Perichondrocytes and Chondrocytes
In perichondrocytes, type I collagen tends to decrease and type II collagen tends to increase as a result of multi-layering.

FIG. 5. Proteoglycan is produced in the supernatant as a result of multi-layering in vitro. The proteoglycan-producing capacity of perichondrocytes is almost comparable to that of chondrocytes.

FIG. 6. Cultured human perichondrocytes form a cartilage tissue in vivo.

This Figure shows the state after two months of subcutaneous transplanting of cultured perichondrocytes in the back of NOD/SCID mouse. Alcian blue staining: a: magnification 200; b: magnification 400.

FIG. 7. Cultured human perichondrocytes produce type I and type II collagen and form a cartilage tissue in vivo.

This Figure shows the state after two months of subcutaneous transplanting of cultured perichondrocytes in the back of NOD/SCID mouse. Alcian blue staining. Type I collagen (red) and type II collagen (green): a: magnification 100; b: magnification 400.

FIG. 8 shows the layer structure of perichondrium and cartilage such as elastic cartilage and hyaline cartilage.

FIG. 9 supplements FIG. 1.

A human perichondrial tissue is collected in outermost layer and separate two layers of fibroblast layer and innermost layer. Those other than these layers fall under cartilage tissue: a: outermost layer and fibroblast layer; b: innermost layer; c: cartilage tissue. Alcian blue staining. Magnification: 200.

FIG. 10 shows the layer structure of perichondrium and cartilage such as elastic cartilage and hyaline cartilage.

FIG. 11 supplements FIG. 1.

The extracellular matrix-producing capacity was increased by (A) mono-layered, (B) double-layered and (C) triple-layered culture of perichondrocytes in a differentiation inducing medium. When perichondrocytes were stained with type I collagen (red), type II collagen (green) and DAPI (blue), it was also confirmed that the extracellular matrix-producing capacity was increased by (D) mono-layered, (E) double-layered and (F) triple-layered culture. When a differentiation inducing medium was not used, extracellular matrix was not produced even when (G) mono-layered, (H) double-layered and (I) triple-layered culture was performed.

A-C, G-I: Alcian blue staining; D-F: type I collagen staining (red), type II collagen staining (green) and DAPI staining (blue). Scale bar: 200 μm.

Panel B: Induction of Cartilage Differentiation in Human Chondrocytes

The extracellular matrix-producing capacity was increased by (A) mono-layered, (B) double-layered and (C) triple-layered culture of chondrocytes in a differentiation inducing medium. When chondrocytes were stained with type I collagen (red), type II collagen (green) and DAPI (blue), it was also confirmed that the extracellular matrix-producing capacity was increased by (D) mono-layered, (E) double-layered and (F) triple-layered culture.

When a differentiation inducing medium was not used, extracellular matrix was not produced even when (G) mono-layered, (H) double-layered and (I) triple-layered culture was performed. Scale bar: 200 μm.

Thus, perichondrocytes differentiated into cartilage in vitro in almost the same manner as chondrocytes differentiated.

A-C, G-I: Alcian blue staining; D-F: type I collagen staining (red), type II collagen staining (green) and DAPI staining (blue). Scale bar: 200 μm.

FIG. 17 Histological Examination of Cartilage Tissues Regenerated in vivo from Human Perichondrocytes and Chondrocytes

Panel A: Human Perichondrocytes-Derived and Human Chondrocytes-Derived Regenerated Cartilage Tissues after One Month of Transplant (A-D: perichondrocytes-derived cartilage tissue; E-H: chondrocytes-derived cartilage tissue)

While the human perichondrocytes-derived regenerated cartilage was covered with type I collagen, the chondrocytes-derived regenerated cartilage was not covered with type I collagen.

Panel B: Human Perichondrocytes-Derived and Human Chondrocytes-Derived Regenerated Cartilage Tissues after Three Months of Transplant (A-D: perichondrocytes-derived cartilage tissue; E-H: chondrocytes-derived cartilage tissue)

While the human perichondrocytes-derived tissue was covered with type 1 collagen in the same manner as seen one month after transplant, the chondrocytes-derived regenerated cartilage was not covered with type I collagen.
with perichondrium. This suggests that perichondrocytes-
3) innermost layer (zone of transition to the cartilage
Anat 158:173-187 (1988)). The “outermost layer” is the
the thus isolated chondrocytes are cultured for transplant
three months passed after transplant, a decrease in cell count
while no change in cell count was observed in
perichondrocytes-derived tissue whether one month or
passing through a filter with a mesh size of 500 µm. While
chondrocytes in a long-term shape-retaining ability.
FIG. 18 Cell Count per mm² of in vivo Reconstructed Cartilage
This figure shows the cell count per mm² of the cartilage
portion in tissues removed one month and three months after
perichondrocytes-derived tissue whether one month or
three months passed after transplant, a decrease in cell count
was observed in the chondrocytes-derived tissue three
months after transplant.
These results indicate that perichondrocytes are superior to
chondrocytes in long-term shape-retaining ability.
FIG. 19 Comparison of Colony Forming Activities in
10% Human Autoserum Medium between Human Perichondrocytes, Cells in Perichondrium-Cartilage Transition Zone, and Chondrocytes
At day 9 of culture, human perichondrocytes formed a
larger colony than human chondrocytes. Magnification: 40
FIG. 20 Microscopic Comparison of Human Perichondrocytes, Cells in Perichondrium-Cartilage Transition Zone, and Chondrocytes after culture in 10% Human Autoserum Medium
Each of the three types of cells cultured in 10% human
autoserum medium reached confluency more quickly than in
10% bovine serum medium. Magnification: 40

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinbelow, the present invention will be described in
detail.
Conventionally, regeneration of human cartilage has been
performed as follows: a cartilage tissue is collected as a
mass, which is then treated with enzymes such as collagenase
to isolate chondrocytes from the matrix components;
the thus isolated chondrocytes are cultured for transplant
treatment, especially autotransplantation.
In the present invention, however, not those chondrocytes
present in the matrix but the very thin perichondrium
surrounding such cells need be collected. Therefore, the
collected perichondrium has substantially no chondrocytes
present.
For example, perichondrium and cartilage such as elastic
cartilage and hyaline cartilage are composed of four layers
(Fig. 10). They are 1) outermost layer (including capillary
vessels); 2) fibroblast layer (mainly consisting of perichondrocytes)
expressed as “perichondrocyte layer” in Fig. 10); 3)
inmost layer (zone of transition to the cartilage
matrix), and 4) mature cartilage layer (surrounded by the
cartilage matrix) (see, for example, Bairati A, Comazzi M,
Gioria M. et al., Tissue Cell 28: 455-68. (1996); Tonna et al.,
Labor. et al., Invest 3:609-632 (1974); Ellender et al., J.
Anat 158:173-187 (1988)). The “outermost layer” is the
uppermost of those layers expressing type I collagen but not
expressing type II collagen, and includes capillary vessels.
The “fibroblast layer” is a layer expressing type I collagen
but not expressing type II collagen, and is entirely composed
of perichondrocytes excluding the outermost layer. The
“innermost layer” includes the “fibroblast layer” and the
cartilage matrix that is expressing type II collagen and
proteoglycan. The “mature cartilage layer” is a layer that is
expressing type II collagen and proteoglycan but not
expressing type I collagen.
In conventional cartilage collection methods, all the four
layers 1) to 4), or two layers of 3) and 4), or layer 4) alone
are collected. According to such conventional methods,
the cartilage tissue at the donor site becomes deficient or can not
be recovered. As a result, the donor site may present an ugly
appearance such as recession or deformity.
In the present invention, a tissue section comprising 1) and
2) above or 1) to 3) above may be collected with sharp
tools such as tweezers scissors. Alternatively, dull tools such
as raspatories may be used. The layer 4) in which chondrocytes
occupying the major part of cartilage tissue reside need not
be collected. Therefore, it is possible to collect a tissue
section with minimum invasion. It is a great advantage of the
method of the present invention that no deficiency is caused
in the cartilage tissue and thus no ugly appearance will
occur. The thus obtained tissue section is isolated with
collagenase or the like under specific conditions (e.g., 0.1-0.3% collagenase, 37° C., 1-3 hours). This is an isolation
method peculiar to perichondrocytes. Alternatively, all the
four layers 1) to 4) may be collected. When the resultant
tissue section is isolated with collagenase or the like,
perichondrocytes and chondrocytes may be separated from each
other. For example, when the tissue section is treated with
collagenase, perichondrocytes are isolated within three
hours while chondrocytes need 10 to 16 hours for isolation.
Using this time difference, it is possible to separate
perichondrocytes and chondrocytes.
The thus isolated perichondrocytes are plated in culture
dishes and cultured for about one week in a growth medium.
Subsequently, the cells are centrifuge tube cultured, or
mono-layer or multi-layer cultured in a differentiation
medium. By these procedures, it is possible to differentiate
perichondrocytes into chondrocytes.
In the present invention, it is possible to differentiate
perichondrocytes into chondrocytes by culturing those
human perichondrocytes obtained from cartilage-free
human perichondrium.
The present invention provides a cell derived from a
perichondrial tissue, which is capable of differentiating into
a chondrocyte. The cell of the present invention derived
from a perichondrial tissue is believed to be a cartilage stem
cell and/or a cartilage progenitor cell. The perichondrial
tissue is part of the tissue composing cartilage tissue such as
articular or costochondral, and includes perichondrium. Specif-
ically, the perichondrial tissue is a tissue comprising the
outermost layer and the fibroblast layer (perichondrocyte
layer) or a tissue comprising the outermost layer, the fibro-
blast layer (perichondrocyte layer), and the innermost layer.
The human perichondrial tissue from which the cell of the
present invention is derived may consist of the outermost
layer and the fibroblast layer. Alternatively, the human
perichondrial tissue may consist of the outermost layer, the
fibroblast layer, and the innermost layer. The perichondrial
tissue may be a perichondrial tissue section collected from
a human, especially a patient in need of cartilage transplant.
The cell derived from a perichondrial tissue may be a cell
isolated from a perichondrial tissue or a cell obtained by
subculturing the isolated cell.
In order to obtain the cell of the present invention, a
perichondrial tissue section may be collected and cells may
be isolated from the resultant tissue section. For collecting a perichondrial tissue section, sharp tools such as tweezers and scissors may be used. Alternatively, dull tools such as raspatories may be used. For isolation of cells from the perichondrial tissue section, the cell may be cultured in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F12 (DMEM/F12) or Nutrient Mixture F-12 Ham (F-12 Ham), both supplemented with a serum (e.g., 10% fetal bovine serum (FBS) or a serum derived from the patient who is to receive transplant), at about 37°C. The medium may be exchanged every 2 to 4 days.

It is difficult to culture chondrocytes for a sufficiently long time to proliferate. On the other hand, it is possible to culture perichondrocytes for a relatively long time.

In order that the cells isolated from a perichondrial tissue section are differentiated into chondrocytes, the cells may be cultured in a medium containing a serum (e.g., a medium containing DEEM/F12, a serum (e.g., 10% FBS or a serum derived from the patient who is to receive transplant), antibiotics and antimycotics) at about 37°C. As a drug containing both antibiotics and antimycotics, antibiotic antimycotic solution SIGMAAS9555 or the like may be used. To the medium, 40-60 µg/ml dexamethasone and/or 30-60 µg/ml L-ascorbic acid may be added further. Still further, an insulin-like growth factor (e.g., 5-10 ng/ml insulin-like growth factor-1 (IGF-1) or 5-10 ng/ml basic fibroblast growth factor (bFGF)) may also be added. Still further, 5-10 ng/ml insulin or the like may be added. The medium may be exchanged every 2 to 3 days.

It is possible to allow formation of a cell mass by culturing the perichondrial tissue-derived cell in a centrifuge tube. For example, a cell mass is formed when the cell is cultured in a centrifuge tube in serum-free DMEM/F12 medium containing 5 ng/ml insulin-like growth factor-1 (IGF-1), 5 ng/ml basic fibroblast growth factor (bFGF), 40 ng/ml dexamethasone and L-ascorbic acid, 1% antibiotic antimycotic solution, and insulin/transferrin/serine (ITS) at about 37°C. For 2 to 4 weeks.

The perichondrial tissue-derived cell may be mono-layered or multi-layered by plate culture. For example, when the cell is plate cultured in DMEM/F12 medium containing 10% FBS and 1% antibiotic antimycotic solution or DMEM/F12 medium containing 10% FBS, 1% antibiotic antimycotic solution, 5 ng/ml IGF-1, 5 ng/ml bFGF and 40 ng/ml dexamethasone and layered every one week, the matrix (e.g., proteoglycan) producing capacity of the cell is enhanced. Although the number of times of layering varies depending on the purpose or the size of tissue needed, usually 3 to 5 times is appropriate.

The above-described composition of the medium and the contents of individual components may be changed appropriately, and such changed composition and contents are also within the scope of the present invention.

Therefore, the present invention provides a method of preparing a cell capable of differentiating into a chondrocyte, comprising culturing cells isolated from a perichondrial tissue.
absorbable or non-absorbable material at the bottom of a culture vessel during cell culture, it is possible to allow the perichondrocytes to enter the material. The resultant material may directly be transplanted into an affected area.

Transplant of chondrocytes may be performed by injecting the cells into an affected area with a syringe. Transplant of a cartilage tissue using a scaffold may be performed by a surgical transplant operation. For example, surgical techniques such as surgery practiced in rhinoplasty or otoplasty may be used.

Further, the present invention provides a method of preparing a matrix produced by chondrocytes, comprising differentiating perichondrocytes into chondrocytes and allowing the chondrocytes to produce the matrix. Examples of matrices produced by chondrocytes include type II collagen and proteoglycan. These matrices may be used in cosmetics, foods, health foods, pharmaceuticals, and so on. Hereinbelow, the present invention will be described more specifically with reference to the following Examples. However, the scope of the present invention is not limited by those Examples.

EXAMPLE 1

Hereinbelow, the term "perichondrial tissue" at the time of collection refers histologically to the outermost layer and the fibroblast layer; and the term "tissue of perichondrium-cartilage transition zone" refers to the innermost layer.

Collection of Perichondrocytes

In order to collect a perichondrial tissue from a cartilage tissue such as auricular cartilage or costialcartilage (perichondria obtained from human auricular cartilage and left over after surgical operation were used with an approval obtained from patients or their parents; approval was also obtained from Kanagawa Prefectural Children Medical Center and the Ethics Committee of Yokohama City University Hospital), sharp tools such as tweezers and scissors were used. Alternatively, dull tools such as raspatories may be used. When sharp tools were used, a tissue section was prepared for each individual and examined for confirmation that the perichondrium alone had been collected (FIG. 1).

From the resultant tissue section, every tissue on the perichondrium (such as adipose tissue) was removed. Subsequently, the perichondrium was collected manually with sharp tools such as scissors. The thus obtained perichondrial tissue was stained with Alcian blue (staining peculiar to cartilage matrix), and the staining was examined for each individual. While the perichondrial tissue was not at all stained with Alcian blue, a part of the tissue in perichondrium-cartilage transition zone and the entire part of cartilage tissue were stained (FIG. 11).

Isolation of Perichondrocytes

The thus obtained perichondrial tissue was cut into pieces with scissors or surgical knives and shaken in 0.1-0.3% collagenase at 37° C. for 1-3 hours. Subsequently, the perichondrium was centrifuged (1500 rpm/5 min; twice) and the resultant precipitate was recovered. By these procedures, perichondrocytes could be isolated.

Primary Culture of Perichondrocytes

The perichondrocytes obtained as described above were plate cultured in Dulbecco's Modified Eagles Medium/Nutrient Mixture F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) at 37° C. while exchanging the medium twice a week. The cells were grown densely within 2 weeks. These cells were used in subculture. The cells were grown densely in 7 to 10 days. It was possible to continue subculture for at least 6 months.

Differentiation of Perichondrocytes into Cartilage

a) Perichondrocytes were precipitated by centrifugation (1500 rpm/5 min; twice) to form a cell mass. This cell mass was cultured in serum-free DMEM/F12 medium containing 5 ng/ml insulin-like growth factor-I (IGF-I), 5 ng/ml basic fibroblast growth factor (bFGF), 40 ng/ml dexamethasone, 30-60 μg/ml L-ascorbic acid, 1% antibiotic antifungal solution, and 1% insulin/transferrin/serine (ITS) for 3 to 4 weeks. The resultant white material was stained with Alcian blue. As a result, extracellular matrices were stained blue, suggesting the presence of aggrecan (a cartilage marker). This result was almost comparable to the result on a cell mass formed by chondrocytes obtained by a similar technique (FIG. 2).

b) Over plate-cultured perichondrocytes in a dense state, perichondrocytes were further seeded. As a medium, DMEM/F12 containing 10% FBS and 1% antibiotic antifungal solution, or DMEM/F12 containing 10% FBS, 1% antibiotic antifungal solution, 5 ng/ml IGF-I, 5 ng/ml bFGF and 40 ng/ml dexamethasone was used.

Cells were cultured using the latter medium. Perichondrocytes and chondrocytes at week 1 and week 3 of culture were stained with Alcian blue (FIGS. 3 and 4). Reverse transcription-polymerase chain reaction (RT-PCR) (FIG. 5) and quantitative PCR (FIG. 6) were performed, followed by comparison of the results. Alcian blue staining was performed after formalin fixation in a similar manner as used on the tissue section. As a result of Alcian staining, cells were stained blue on cell culture dishes. For use in RT-PCR and quantitative PCR, RNA was extracted from perichondrocytes and chondrocytes. RNA extraction was performed with RNeasy (QIAGEN) according to kit’s the protocol. From the resultant RNA, cDNA was obtained using RNA PCR kit (Takara). As RT-PCR primers, F: atgctcagctttgtggacatcc (SEQ ID NO: 5) and R: caggtgaagactttgtggacatcc (SEQ ID NO: 6) were used for type I collagen; F: caggtgaagactttgtggacatcc (SEQ ID NO: 5) and R: atgctcagctttgtggacatcc (SEQ ID NO: 5) were used for type II collagen; and F: caggtgaagactttgtggacatcc (SEQ ID NO: 5) and R: atgctcagctttgtggacatcc (SEQ ID NO: 5) were used for aggrecan. As quantitative PCR primers, type I collagen: Hs00266273_ml, type II collagen: Hs00164099_ml and aggrecan: Hs00202971_ml of Taqman Gene Expression Assays (Applied Biosystems) were used.

The results revealed that type I collagen (perichondrium marker) decreased and type II collagen (cartilage marker) increased. Thus, it was suggested that perichondrocytes differentiated into chondrocytes. Further, proteoglycan produced by chondrocytes was quantitatively determined by collecting the supernatant in the cell culture dish and subjecting it to enzyme linked immunosorbent assay (ELISA) using Blyscan (Biocolor). As a result, perichondrocytes exhibited a proteoglycan producing capacity comparable to that of chondrocytes (FIG. 7).

From these results, no big difference was recognized between perichondrocytes and chondrocytes with respect to differentiation into chondrocytes. It was suggested that per-
ichondrocytes possess a cartilage differentiation capacity almost comparable to that of chondrocytes. Transplant of Perichondrocytes

The cells from the above-described culture h) were harvested with a cell lifter and transplanted subcutaneously at the back of severe immunodeficiency mice (Sankyo, Japan). The cells were collected two months after the transplant and examined histologically. In order to examine the collected tissue histologically, the tissue was thinly sliced to prepare tissue samples. The tissue samples were subjected to Alcian blue staining to stain proteoglycan, a matrix peculiar to cartilage tissue. Further, immunostaining was performed for type II collagen that is a matrix of cartilage tissue and for type I collagen that is a cover around the cartilage. As a result, the extracellular matrix of cartilage tissue was stained blue in Alcian blue staining; this suggested the presence of proteoglycan, a cartilage marker (FIG. 8). Further, the extracellular matrix of cartilage tissue was stained with type II collagen, and the tissue around the cartilage was stained with type I collagen (FIG. 9). Thus, it was confirmed that cultured perichondrocytes form a cartilage tissue when transplanted.

Comparison of Perichondrocyte and Chondrocyte Colonies

In Vitro Colony assay was performed on the resultant perichondrocytes and chondrocytes. Each type of cells was seeded in 35 mm easy grip cell culture dishes to give a density of 1 cell/cm². Cells were cultured using Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 10% fetal bovine serum and 1% antibiotic antimyotic solution in an incubator set at 37°C under 5% CO₂. Starting 24 hours after the seeding, the medium was exchanged once in every 3 days. After one-month culture, formed colonies were observed under a microscope and photographed. As a result, perichondrocyte colonies were larger than chondrocyte colonies (FIG. 12).

Comparison of Colony Forming Activities between Human Perichondrocytes, Cells in Perichondrium-Cartilage Transition Zone, and Chondrocytes

Colony assay was performed on the resultant perichondrocytes, cells in perichondrium-cartilage transition zone, and chondrocytes. Each type of cells was seeded in 35 mm easy grip cell culture dishes to give a density of 52 cells/cm². Cells were cultured using Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 10% fetal bovine serum and 1% antibiotic antimyotic solution in an incubator set at 37°C under 5% CO₂. Starting 24 hours after the seeding, the medium was exchanged once in every 3 days. Colony numbers were counted after 14-day culture. A cell population consisting of 50 or more cells was counted as one colony. Comparison of colony numbers revealed the following order of colony forming activities: perichondrocytes>cells in perichondrium-cartilage transition zone>chondrocytes (FIG. 13).

*: p<0.001 (Mann Whitney U-Test with Bonferroni correction) n=27 (patient No.:3) Comparison of the Long-Term Growth Capacities of Human Perichondrocytes, Cells in the Cartilage-Perichondrium Transition Zone, and Chondrocytes

Long-term growth capacity was examined on the resultant perichondrocytes, cells in perichondrium-cartilage transition zone, and chondrocytes. Each type of cells was seeded in 35 mm easy grip cell culture dishes to give a density of 1200 cells/cm². Cells were cultured using Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 10% fetal bovine serum and 1% antibiotic antimyotic solution in an incubator set at 37°C under 5% CO₂. Starting 24 hours after the seeding, the medium was exchanged once in every 3 days. Cells reached confluency after culture for about 14 days. Then, cells were detached using 0.2% type II collagenase-containing Hank’s balanced solution, followed by cell counting using a hemocytometer. Subsequently, the cells were seeded in 35 mm easy grip cell culture dishes to give a density of 1200 cells/cm². These operations were performed repeatedly. As a result of 14 passages over 182 days, it was revealed that perichondrocytes possess a growth capacity remarkably higher than that of cells in perichondrium-cartilage transition zone or chondrocytes (FIG. 14). In addition, it was revealed that cells in perichondrium-cartilage transition zone possess a remarkably higher than that of chondrocytes (FIG. 14).
transplant, the resultant tissue was removed from each mouse and examined histologically. The removed tissue was stained with hematoxylin-eosin, Alcian blue, Elastica van Gieson, and type I and II collagen. As a result, both tissues derived from perichondrocytes and chondrocytes were stained with Alcian blue and Elastica van Gieson (see panels B, C, F and G in both FIGS. 17A and 17B). Further, the perichondrocytes-derived tissue was stained with type I and type II collagen (see panel D in both FIGS. 17A and 17B). On the other hand, the chondrocytes-derived tissue was stained with type II collagen but not with type I collagen (see panel H in both FIGS. 17A and 17B).

Cell Count Per mm² of In Vivo Reconstructed Cartilage

Cell counts were determined per mm² of the cartilage portion in the tissues removed one month and three months after transplant in the above-described “Histological Examination of Cartilage Tissues Regenerated in vivo from Human Perichondrocytes and Chondrocytes”. While no change in cell count was observed in the perichondrocytes-derived tissue whether one month or three months passed after transplant, a decrease in cell count was observed in the chondrocytes-derived tissue three months after transplant (FIG. 18).

*p<0.05, **: p<0.01 (Mann Whitney U-Test)

Method of Preparing Human Autoserum

Human autologous blood obtained from the same patient from whom cartilage had been collected was left to stand at room temperature for 20 min and then centrifuged at 3000 rpm for 10 min. The resultant supernatant was recovered as human autoserum. For the purpose of culture, a human autoserum medium reached confluency more quickly than in 10% bovine serum medium.

Preparation of Human Autoserum Medium

Human perichondrocytes in centrifuge tubes, culturing such cells in a layered form. The resultant perichondrocytes-derived tissue was removed from each mouse, examined histologically, and stained with type II collagen but not with type I collagen (see panel H in both FIGS. 17A and 17B). Further, the cell mass may be transplanted either as it is or after being embedded in a cartilage treatment material such as collagen gel. Thus, such a cell mass is applicable to cartilage-related diseases (e.g., congenital auricular deformity, costicartilage defect, damage to articular cartilage such as knee osteoarthritis, tracheal cartilage defect, etc.). Further, the cell mass is also useful in treatment for aesthetic improvement in the field of cosmetic surgery, e.g., such as treatment for cartilage transplant in rhinoplasty, genioplasty, plastic surgery of small facial recesses, corrective surgery of facial left-right asymmetry, corrective surgery around eyelids, or cosmetic surgery of face.

INDUSTRIAL APPLICABILITY

According to the present invention, it has become possible to culture human chondrocytes by proliferating and differentiating human perichondrocytes. Further, it is possible to obtain a mass of human chondrocytes by culturing human perichondrocytes in centrifuge tubes, culturing such cells three dimensionally with collagen gel or the like, or culturing such cells in a layered form. The resultant cell mass may be transplanted either as it is or after being embedded in a cartilage treatment material such as collagen gel.
The invention claimed is:

1. A method of preparing chondrocytes, comprising:
   separating an outermost layer and a fibroblast layer of human perichondrium away from a perichondrium-cartilage transition zone of the human perichondrium to produce a tissue section from the outermost layer and the fibroblast layer without the perichondrium-cartilage transition zone,
   then treating the tissue section from the outermost layer and the fibroblast layer with collagenase to produce an isolated perichondrocyte population, and
   then differentiating the perichondrocytes from the isolated perichondrocyte population from the tissue section from the outermost layer and the fibroblast layer into chondrocytes in a medium containing a serum and further containing an insulin-like growth factor and/or a basic fibroblast growth factor.

2. The method according to claim 1, wherein the serum is bovine serum.

3. The method according to claim 1, wherein the serum is an autoserum.

4. The method according to claim 1, wherein the isolated perichondrocyte population is differentiated into chondrocytes in the medium further containing DMEM/F12, antibiotics and antimycotics.

5. The method according to claim 4, wherein the medium further contains dexamethasone and/or L-ascorbic acid.
6. A method of transplanting chondrocytes: comprising:
preparing the chondrocytes according to the method of
claim 1; and
transplanting the chondrocytes and/or a cartilage tissue
formed by said chondrocytes into a living body.

7. A method of preparing a matrix produced by chondro-
cytes, comprising
preparing the chondrocytes according to the method of
claim 1; and
allowing said chondrocytes to produce the matrix.

8. The method according to claim 7, wherein the matrix
is type II collagen and/or proteoglycan.

9. The method according to claim 1, wherein the medium
contains the insulin-like growth factor.

10. The method according to claim 1, wherein the method
further comprises proliferating the differentiated cell popu-
lation.

11. A method of transplanting a cell derived from a human
perichondrial tissue, comprising:
separating an outermost layer and a fibroblast layer of
human perichondrium away from a perichondrium-
cartilage transition zone of the human perichondrium to
produce a tissue section from the outermost layer and
the fibroblast layer without the perichondrium-cartilage
transition zone,
then treating the tissue section from the outermost layer
and the fibroblast layer with collagenase to produce an
isolated perichondrocyte population;
then culturing the isolated perichondrocyte population in
a centrifuge tube;
then proliferating and differentiating the perichondrocytes
from the isolated perichondrocyte population into
chondrocytes in a medium containing a serum and
further containing an insulin-like growth factor and/or
a basic fibroblast growth factor; and
then transplanting the chondrocytes or a cartilage tissue
formed by the chondrocytes into a subject.

12. The method of transplanting a cell according to claim
11, wherein differentiating the cell into chondrocytes in the
presence of the insulin-like growth factor and/or the basic
fibroblast growth factor is accompanied by allowing said
chondrocytes to produce a matrix.

13. The method according to claim 11, wherein the serum
is bovine serum.

14. The method according to claim 11, wherein the serum
is an autoserum.

15. The method according to claim 11, wherein the medium
further contains DMEM/F 12, a serum, antibiotics,
and antimycotics.

16. The method according to claim 11, wherein the medium
further contains dexamethasone and/or L-ascorbic
acid.

17. The method according to claim 11, wherein the medium
contains the insulin-like growth factor.

18. The method according to claim 11, wherein the method
comprises maintaining chondrogenic phenotype of
the chondrocytes or the cartilage tissue formed by the
chondrocytes for a month after the transplanting.

19. The method according to claim 11, wherein the method
comprises maintaining cell count of the chondro-
cytes or the cartilage tissue formed by the chondrocytes for
a month after the transplanting.

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