

[DESCRIPTION]

[Title of Invention] TRANSPLANT MATERIAL FOR TREATMENT OF
HEART DISEASE

[Technical Field]

5 The present invention relates to a transplant
material for treatment of heart disease including a cell
cluster obtained by causing isolated cells to adhere to
each other with an adhesive agent and a heart disease
treatment method using the transplant material.

10 [Background Art]

 Myocardial infarction is an irreversible damage of
myocardial cells (NPL 1). Ischemic heart disease
accounts for 50% of all cardiovascular mortality causes
and is a leading cause of congestive heart failure. If
15 patients diagnosed with congestive heart failure are not
ameliorated by treatment, their prognoses are very bad and
the 1-year mortality rate is close to 20% (NPL 2).
Currently, many of the treatments available to physician
s can eliminate the causes of these acute myocardial
20 infarctions, but a time period from infarction onset to
reperfusion determines the extent of irreversible
myocardial damage (NPL 3). Cardiomyoplasty has been
proposed as a surgical method for improving left
ventricular (LV) function in patients suffering from
25 congestive heart failure, but the effect on cardiac
function and patients' prognoses are not satisfactory

(NPLs 4 to 7). Any clinically available drugs and treatments are ineffective in replacing a myocardial scar with functionally contractile tissue, and there is a demand for novel treatment for regenerating normal myocardial cells.

In transplantation of an organ (for example, the heart, a blood vessel, or the like) as a replacement treatment, use of graft tissue such as an allogeneic graft (or an allograft) or a xenograft has been reported to cause immunological rejection (NPLs 8 to 11). For example, transplantation of a cardiac graft biologically manipulated using biodegradable scaffolds has demonstrated minimal benefits in improving the cardiac function, because the cardiac graft hardly engraft to the myocardial layer (NPLs 12 and 13). Meanwhile, transplantation of a whole heart is not the first choice for a heart failure due to donor shortage.

Recently, cell transplantations have attracted attention as treatment methods utilizing biological substances such as iPS cells and mesenchymal stem cells (MSC). These cell transplantations have also been reported that they have the possibility of replacing damaged myocardial tissue and improving the cardiac function. For example, there has been studies on methods for preparing and transplanting adipose-derived cells, in which they are directly administered by intramyocardial

injection or intracoronary injection. However, it has been reported that these administration methods cannot obtain sufficient therapeutic effects or even cause serious adverse effects (such as arrhythmia) after the transplantation (PTL 1 and NPLs 14 and 15).

To address this, studies on administration methods other than injections have resulted in development of a method in which a stem cell is cultured on a temperature-responsive culture dish and processed into a sheet, and then the sheet is transplanted onto the cardiac surface. This method can transplant cells without causing serious adverse effects, and has drawn attention as producing the therapeutic effects by the paracrine of the cytokines which are secreted from the transplanted cells and have myocardial protection and angiogenesis effect(PTL 2).

In addition, it has also been reported that adiponectin known as cytokines secreted from adipocytes has a possibility of protecting the cardiomyocyte after injury and suppressing the deterioration of the cardiac function through the effects of anti-apoptosis, anti-inflammation, angiogenesis, anti-fibrosis, and anti-cardiac hypertrophy (NPLs 16 and 17). Furthermore, studies on adipocyte transplantations as a drug delivery system (DDS) have also been done as means for adiponectin administration to target cardiac tissue over a long period

of time. As a result, it has been found that the adiponectin secreted from a cell sheet prepared from matured adipocytes differentiated by culture and transplanted onto the cardiac surface can improve the cardiac function over a long period (PTL 3).

However, preparations of these cell sheets for transplantation require much time and efforts and therefore have a problem in using in clinical situation.

[Citation List]

[Patent Literature]

[PTL 1] Japanese Patent No. 5210155

[PTL 2] Japanese Patent No. 5103626

[PTL 3] Japanese Patent No. 5661048

[Non Patent Literature]

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[NPL 2] American Heart Association., Dallas, Tex: American Heart Association; 2001

[NPL 3] Ryan TJ et al., J Am Coll Cardiol 1999; 34: 890-911

[NPL 4] Corin WJ et al., J Thorac Cardiovasc Surg 1992; 104: 1662-71

[NPL 5] Kratz JM et al., J Thorac Cardiovasc Surg 1994; 107: 868-78

[NPL 6] Carpentier A, Chachques JC., Lancet.1985; 8840:1267

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[NPL 8] Carrel A, J Exp Med 1907; 9: 226-8

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[NPL 10] Calne RY, Transplant Proc 1970; 2: 550

[NPL 11] Auchincloss, Transplantation 1988; 46:1

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Res 2008; 1(1): 55-63

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[Summary of Invention]

[Technical Problem]

20 The present invention has been made in view of the
aforementioned circumstances and has an object to provide
a transplant material which has an excellent therapeutic
effect on heart disease and which can be prepared easily
within a short period of time. A further object of the
present invention is to provide a heart disease treatment
25 method using this transplant material.

[Solution to Problem]

As a result of intensive studies to solve the above-mentioned problems, the inventor of the present invention found that a cell cluster, obtained in such a way that regenerative cells just isolated from living
5 adipose tissue are caused to adhere to each other without culturing, secretes adiponectin after transplantation to the heart and thereby exerts an excellent therapeutic effect on heart disease. In production of this cell cluster, a cell cluster was able to be easily produced by
10 using fibrinogen as an adhesive material of the cells. In addition, the therapeutic effect was further enhanced by using adipose-derived mesenchymal progenitor cells treated with a PPAR γ agonist in the production of the cell cluster.

15 Conventionally, in the case of a treatment of heart disease with a cell sheet produced from adipocytes or stem cells derived from adipose tissue, culturing of cells over a long period of time has been required before the production of the cell sheet. For example, according to
20 PTL 3, in order to differentiate cells into mature adipocytes, the cells are generally cultured in a prescribed medium for 5 to 10 days before induction of differentiation after seeding, for 36 to 60 hours during the induction of differentiation, and for 5 to 14 days
25 after the induction of differentiation. The cell culturing for periods shorter than the above is said to

be unfavorable in practice due to reasons such as one that the obtained cells secrete only a small amount of adiponectin (paragraph 0025).

Meanwhile, a method in PTL 2 produces the therapeutic effect on heart disease by using a cell sheet not containing mature adipocytes but containing mesenchymal stem cells derived from adipose tissue. Also according to this method, collected stem cells are cultured in a prescribed medium generally for several days, and then the cells are sub-cultured for several generations to produce a cell sheet (paragraphs 0013 and 0014). However, PTL 2 only states that the mesenchymal stem cells in this cell sheet differentiate into cardiomyocyte, vascular endothelial cells, and vascular smooth muscle cells after transplantation to the heart, and does not mention anything about the differentiation into mature adipocytes and the accompanying secretion of adiponectin (paragraphs 0017 and 0018).

Under such circumstances, it is extremely surprising that a cell cluster obtained by causing cells just isolated from living adipose tissue to adhere to each other secreted adiponectin after transplantation into the heart, and demonstrated an excellent therapeutic effect on heart disease.

As described above, the present invention is intended to provide a transplant material which can be

prepared easily within a short period of time and has an excellent therapeutic effect on heart disease, and a heart disease treatment method using the transplant material, and more specifically to provide the invention described
5 below.

[1] A transplant material for treating heart disease, the transplant material comprising a cell cluster obtained by causing isolated cells to adhere to each other with an adhesive agent.

10 [2] The transplant material according to [1], wherein the isolated cells are selected from the group consisting of adipose-derived regenerative cells, bone marrow-derived regenerative cells, umbilical cord-derived regenerative cells, smooth muscle-derived
15 regenerative cells, multipotent stem cells, regenerative cells derived from the multipotent stem cells, vascular endothelial cells, and monocytes.

[3] The transplant material according to [1], wherein the isolated cells are adipose-derived
20 regenerative cells treated with a PPAR γ agonist.

[4] The transplant material according to any one of [1] to [3], wherein the isolated cells are cells not cultured.

[5] The transplant material according to any one of
25 [1] to [4], wherein the adhesive agent contains fibrinogen.

[6] A heart disease treatment method comprising covering a cardiac surface of a subject suffering from heart disease with the transplant material according to any one of [1] to [5].

5 [7] The treatment method according to [6], wherein the subject suffering from the heart disease is a subject from whom cells for the transplant material were collected.

[Advantageous Effects of Invention]

10 According to the present invention, the cells just isolated can be processed into a cell cluster, which can be then transplanted. Moreover, the cells can be processed into a cell cluster easily using fibrinogen or the like. Therefore, the time, effort, and cost can be
15 drastically reduced compared with conventional transplantation treatment using a cell sheet.

In addition, the method of the present invention is much safer than the conventional transplantation treatment in which adipose-derived mesenchymal progenitor
20 cells are injected into the coronary artery or the cardiomyocyte, and also achieves superior cardiac function recovery in combination with the secretion of adiponectin.

[Brief Description of Drawings]

25 [Fig. 1A] Fig. 1 includes drawings demonstrating a result of histological examination on a cell cluster

composed of adipose-derived regenerative cells (ADRCs) (hereinafter also simply referred to "ADRCs cell cluster"). Fig. 1A demonstrates photomicrographs (in vitro evaluation) of an ADRCs cell cluster after culture.

5 [Fig. 1B] Fig. 1B includes graphs demonstrating a result of measurement of adiponectin (APN), VEGF, HGF, and IL-6 in the culture supernatant of the ADRCs cell cluster by enzyme-linked immunosorbent assay (ELISA method) (in vitro evaluation).

10 [Fig. 1C] Fig. 1C is a microphotograph (in vitro evaluation) of an ADRCs cell cluster after culture. Spherical ones in the picture are oil droplets.

[Fig. 2A] Fig. 2A is a photomicrograph (in vivo evaluation) of a cardiac tissue of a myocardial infarction model rat taken on day 5 after transplantation of an ADRCs cell cluster into the rat.

20 [Fig. 2B] Fig. 2B is a photomicrograph (in vivo evaluation) demonstrating the expression of neovascularization on the epicardial side and between the cell cluster and the area of myocardial infarction.

[Fig. 3A] Fig. 3 includes diagrams demonstrating results of echocardiographic examinations on how transplantation therapy of ADRCs cell clusters to myocardial infarction model rats affects the cardiac function. Fig. 3A is a graph of left ventricular ejection fraction (EF).

[Fig. 3B] Fig. 3B is a graph of left ventricular end-systolic diameter (LVESd).

[Fig. 3C] Fig. 3C is a graph of left ventricular end-diastolic diameter (LVEDd).

5 [Fig. 3D] Fig. 3D is a graph of survival rate.

[Fig. 4A] Fig. 4 includes diagrams each demonstrating a result of echocardiographic examinations on how transplantation of ADRCs cell clusters, ADRCs intramyocardial injection, and ADRCs intracoronary
10 administration to chronic myocardial infarction model rats affect the cardiac function. Fig. 4A is a graph of left ventricular ejection fraction (EF).

[Fig. 4B] Fig. 4B is a graph of left ventricular end-systolic diameter (LVESd).

15 [Fig. 4C] Fig. 4C is a graph of left ventricular end-diastolic diameter (LVEDd).

[Fig. 5A] Fig. 5 includes diagrams demonstrating results of examinations on how transplantation therapy of ADRCs cell clusters to myocardial infarction model rats affects the myocardial tissue. Fig. 5A is a
20 photomicrograph demonstrating a Picro-Sirius red stained low magnification image of an S group.

[Fig. 5B] Fig. 5B is a photomicrograph demonstrating a Picro-Sirius red stained low
25 magnification image of an A group.

[Fig. 5C] Fig. 5C is a photomicrograph demonstrating a Picro-Sirius red stained low magnification image of an AP group.

[Fig. 5D] Fig. 5D is a graph demonstrating a result of measurements of the left-ventricular end-diastolic wall thickness (LVAWD) of the S group, the A group, and the AP group.

[Fig. 5E] Fig. 5E is a graph demonstrating a result of measurements of the fibrotic area in the left ventricular cross-sectional area of the S group, the A group, the AP group, an ic group, and an im group.

[Fig. 5F] Fig. 5F is a photomicrograph demonstrating a von Willebrand factor-stained image of the infarct-normal border area of the S group.

[Fig. 5G] Fig. 5G is a photomicrograph demonstrating a von Willebrand factor-stained image of the infarct-normal border area of the A group.

[Fig. 5H] Fig. 5H is a photomicrograph demonstrating a von Willebrand factor-stained image of the infarct-normal border area of the AP group.

[Fig. 5I] Fig. 5I is a graph demonstrating a result of measurements of the capillary density in the infarct-normal border area of the S group, the A group, the AP group, the ic group, and the im group.

[Fig. 6A] Fig. 6 includes pictures demonstrating a result of examination on influences on myocardial tissue

of chronic myocardial infarction model rats at week 8 after transplantation therapy of ADRCs cell clusters to the rats. Fig. 6A is a photomicrograph demonstrating a hematoxylin-eosin stained low magnification image of the S group.

[Fig. 6B] Fig. 6B is a photomicrograph demonstrating a hematoxylin-eosin stained low magnification image of the A group.

[Fig. 6C] Fig. 6C is a photomicrograph demonstrating a hematoxylin-eosin stained low magnification image of the AP group.

[Fig. 6D] Fig. 6D is a representative tissue photograph demonstrating a result of Periodic acid-Shiff (PAS) staining of the infarct-normal border area of the S group.

[Fig. 6E] Fig. 6E is a representative tissue photograph demonstrating a result of Periodic acid-Shiff (PAS) staining of the infarct-normal border area of the A group.

[Fig. 6F] Fig. 6F is a representative tissue photograph demonstrating a result of Periodic acid-Shiff (PAS) staining of the infarct-normal border area of the AP group.

[Fig. 6G] Fig. 6G is a graph demonstrating a result of quantification of myocardial cell diameters in the

myocardial infarct-normal border area of the experimental groups.

[Fig. 7A] Fig. 7 includes diagrams demonstrating results of examining anti-inflammatory effects by ADRCs cell cluster transplantation. Fig. 7A is a graph demonstrating a result of quantification of the transcription level of Adiponectin (APN) in the infarct area and the infarct-normal border area.

[Fig. 7B] Fig. 7B is a graph demonstrating a result of quantification of the transcription level of Adiponectin receptor 1 (Adipo-R1) in the infarct area and the infarct-normal border area.

[Fig. 7C] Fig. 7C is a graph demonstrating a result of quantification of the transcription level of Adiponectin receptor 2 (Adipo-R2) in the infarct area and the infarct-normal border area.

[Fig. 7D] Fig. 7D is a graph demonstrating a result of quantification of the transcription level of T-cadherin in the infarct area and the infarct-normal border area.

[Fig. 7E] Fig. 7E is a graph demonstrating a result of quantification of the transcription level of vascular endothelial growth factor (VEGF) in the infarct area and the infarct-normal border area.

[Fig. 7F] Fig. 7F is a graph demonstrating a result of quantification of the transcription level of Tumor

necrosis factor alpha (TNF- α) in the infarct area and the infarct-normal border area.

[Fig. 8A] Fig. 8 includes pictures demonstrating an ability to persistently produce adiponectin and successful intramyocardial engraftment of ADRCs. Fig. 8A is a photograph of tissue taken one week after transplantation of an ADRCs cell cluster derived from a GFP transgenic LEW/Sea rat to the left ventricle of a LEW/Sea rat, the upper part in the picture is the ADRCs cell cluster, and the lower part in the picture is the heart.

[Fig. 8B] Fig. 8B is a photomicrograph demonstrating a result of immunostaining adiponectin in the tissue taken three weeks after the transplantation of an ADRCs cell cluster derived from a GFP transgenic LEW/Sea rat to the left ventricle of a LEW/Sea rat. Regions with light color indicate adiponectin.

[Fig. 8C] Fig. 8C includes photomicrographs demonstrating a result of left ventricular intramyocardial injection of ADRCs derived from a GFP transgenic LEW/Sea rat.

[Fig. 8D] Fig. 8D includes photomicrographs demonstrating a result of intracoronary administration of ADRCs derived from a GFP transgenic LEW/Sea rat.

[Description of Embodiments]

The present invention provides a transplant material for treatment of heart disease, the material comprising a cell cluster obtained by causing isolated cells to adhere to each other with an adhesive agent.

5 The isolated cells may be any cells not particularly limited as long as the cells have a therapeutic effect on heart disease. Examples thereof include adipose tissue (for example, subcutaneous adipose tissue, greater omentum, and epicardial fat)-derived regenerative cells,
10 bone marrow-derived regenerative cells, umbilical cord-derived regenerative cells, smooth muscle (for example, perivascular smooth muscle)-derived regenerative cells, pluripotent stem cells (for example, iPS cells, ES cells, and Muse cells), and regenerative
15 cells derived therefrom. In addition, it is also possible to use differentiated cells such as vascular endothelial cells and monocytes.

 The "regenerative cell" in the present invention means a cell that has an ability to restore the structure
20 and function in an organ or tissue to which the cell is applied. In a preferable embodiment, regenerative cells are of a cell group including mesenchymal stem cells and mesenchymal progenitor cells. Here, the "mesenchymal stem cell" means a stem cell having an ability to
25 differentiate into a mesenchymal cell such as osteoblast, adipocyte, myocyte, or chondrocyte, and the "mesenchymal

progenitor cell" means a cell in the process of differentiation from a mesenchymal stem cell to a mesenchymal cell. Then, the "stem cell" means a cell having a self-renewal ability and pluripotency. Whether or not isolated regenerated cells are mesenchymal stem cells or mesenchymal precursor cells can be evaluated by using a predetermined cell surface marker (Lin K et al., Cytotherapy 2008; 10(4): 417-426).

In the present invention, "adipose-derived regenerative cells" are preferably used in particular. The "adipose tissue" is connective tissue composed mainly of adipocytes. The adipose tissue from which regenerative cells are isolated is not particularly limited, but may be subcutaneous adipose tissue, greater omentum, epicardial fat, or the like. The adipose tissue can be collected from a living body, for example, by aspiration from a small incision or by surgical excision.

In a preferred embodiment, a cell cluster obtained by adhesion of cells contains mesenchymal progenitor cells and mesenchymal stem cells in a total amount of at least 1% or more, and preferably 5% or more (for example, 10% or more, 20% or more, 30% or more, 40% or more, or 50% or more). In addition to these cells, the cell cluster may contain, for example, cells mixed in the isolation process, and so on.

The cells can be isolated from the tissue in such a way that the tissue is first fragmented, and the obtained fragments are treated with collagenase, followed by filtration through a filter and then centrifugation. The centrifugation for isolating regenerative cells can be performed under conditions, for example, at 500 to 800 G for 5 to 10 minutes.

In the present invention, isolated cells are caused to adhere to each other to form a cell cluster. The method for causing cells to adhere to each other is not particularly limited, but fibrinogen can be preferably used. The fibrinogen becomes a pasty fibrin polymer when mixed with thrombin, and thus comes to have an ability to bond cells to each other. Therefore, as presented in Examples, cells can be caused to adhere to each other easily by mixing a suspension of cells to which fibrinogen is added with a solution containing thrombin. If the above mixed solution is kept warm, the adhesion can be made faster.

In this way, cells for forming a cell cluster need not be cultured. Thus, it is possible to immediately process the isolated regenerative cells into a cell cluster and to use the cell cluster in transplantation to the heart. Therefore, a process from collection to preparation of a cell cluster can be accomplished within a very short period of time. For example, unlike the

present invention, the conventional method using a cell sheet causes the cells to adhere to each other by culture (an adhesive agent is used for the purposes such as reinforcement of the cell sheet) and generally requires
5 a period in the order of several days to several weeks as a period from collection of biological tissue to preparation of a cell sheet. In contrast, the present invention requires only several hours from collection of biological tissue to preparation of a cell sheet, which
10 means achievement of significant time reduction.

In the formation of a cell cluster, it is preferable to use adipose-derived regenerative cells treated with a PPAR γ agonist. This allows further enhancement of secretion of adiponectin from the cell cluster after
15 transplantation to the heart. Examples of the PPAR γ agonist include, but are not particularly limited to, thiazolidine-based drugs such as pioglitazone. As substances with which adipose-derived cells are treated, there are, besides the above substances, substances that
20 promote induction of differentiation into adipocytes such as insulin and steroid, angiotensin receptor antagonists (ARB), angiotensin converting enzyme inhibitors (ACEi), cytokines such as VEGF and HGF, and so on.

In the case of use for transplantation treatment of
25 heart disease, the cell cluster can be adjusted in size and thickness as appropriate according to a site to which

the cell cluster is to be transplanted or the like. Generally preferable cell clusters have a disc shape having a diameter of 5 mm to 5 cm or a square shape having a side of 5 mm to 5 cm, and have a thickness of 0.5 to 2 mm so as to have sufficient strength. In order to obtain such a cell cluster, a suspension can be used which has a cell concentration of 2×10^5 to 2×10^7 cells/ml, for example.

The transplant material of the present invention may further contain other elements useful for treating heart disease, as long as it contains the above-mentioned cell cluster. Examples of the other elements include, but are not limited to, a cell cluster or a cell sheet composed of heterologous cells, extracellular matrices, and adhesion factor proteins.

An example of the "heart disease" to be treated by the transplant material of the present invention is, but not limited to, a disease or a disease accompanied by a disorder, the disease selected from the group consisting of heart failure, ischemic heart disease, myocardial infarction, cardiomyopathy, myocarditis, hypertrophic cardiomyopathy, diastolic hypertrophic cardiomyopathy, and dilated cardiomyopathy.

The present invention also provides a heart disease treatment method, the method comprising covering a cardiac

surface of a subject suffering from heart disease with the
aforementioned transplant material.

The treatment according to the present invention can
be applied to human and non-human subjects. Organisms to
5 be treated are not particularly limited as long as they
each have a heart as a treatment target, and examples
thereof include human, mammals other than human, birds,
reptiles, amphibians, and fishes. The mammals other than
humans include, but are not limited to, primates such as
10 chimpanzees and monkeys, as well as livestock and
companion animals such as cattle, swine, horses, chickens,
cats, and dogs.

As the cells from which the transplant material of
the present invention is derived, cells compatible with
15 an organism to be treated may be used. However, from the
viewpoint of suppression of immune rejection reaction or
the like, used are cells derived from the same species,
and particularly preferably autologous cells. Therefore,
particularly preferably, the transplant material of the
20 present invention is transplanted to a subject from whom
the cells were collected.

It should be noted that, even when a rejection
reaction is expected in principle, the rejection reaction
may be inhibited by using publicly known means such as an
25 immunosuppressive agent. In this way, a range of cells
applicable to a particular organism can be expanded.

A site to which the transplant material of the present invention is transplanted is preferably a cardiac surface. In this case, a heart disease can be treated by covering the cardiac surface with the transplant material of the present invention. In the transplantation of the transplant material to the cardiac surface, for example, the transplant material may be attached to a lesion (for example, a myocardial infarction area), and then surgically fixed to the epicardium with a proline needle or fixed to the cardiac surface by fibrin spray. Preferably, the transplant material is placed on the cardiac surface so as to sufficiently cover the boundary between the lesion site (for example, the myocardial infarction site) and the surrounding normal site.

For transplantation, the heart targeted for the transplantation is treated as needed in advance. For example, in the case of an initial surgery, the transplantation is preferably conducted after the blood and moisture on the cardiac surface are wiped off sufficiently. Meanwhile, in the case of a second or following surgery, the transplantation is preferably conducted after the scar tissue on the cardiac surface is removed as quickly as possible.

In the treatment method of the present invention, the treatment with the transplant material of the present invention may be combined with another treatment. For

example, for a chronic myocardial infarction, the treatment of the present invention may be combined with coronary artery bypass surgery, percutaneous coronary angioplasty, or left ventricular reconstruction. For an acute myocardial infarction, the treatment of the present invention may be combined with coronary artery bypass surgery or percutaneous coronary angioplasty. In addition, the treatment of the present invention may be combined with valve replacement surgery or pediatric heart surgery.

[Examples]

Hereinafter, the present invention is described in more details based on Examples, but the present invention should not be limited to Examples described below.

[Example 1]

[Materials and Methods]

(1) Preparation for Adipose-Derived Regenerative Cells (ADRCs = Adipose derived regenerative cells)

Adipose tissue was collected from both inguinal regions of a 9 week-old female LEW/Sea rat. The adipose tissue was cut into fine pieces by scissors, and the obtained tissue pieces were suspended in a 0.1% type II collagenase solution and shaken in a 37°C warm bath for 1 hour. The suspension was filtered through 100 µm and 70 µm mesh filters, followed by centrifugation for 10 minutes at 1800 revolutions per minute. The resultant

sediment suspended in a culture medium (10% fetal bovine serum and antibiotic-containing D-MEM) was used as adipose-derived regenerative (mesenchymal progenitor) cells (ADRCs). These include adipose tissue-derived mesenchymal stem cells and progenitor cells.

(2) Production of ADRCs cell cluster

Subsequent to the extraction of the ADRCs from the adipose tissue, cell clusters each containing 5×10^6 ADRCs were produced immediately. A solution A was prepared by adding 60 μ l of fibrinogen to 140 μ l of the ADRCs cell suspension, whereas a solution B was prepared by adding 30 ml of thrombin to 170 μ l of a culture medium (10% fetal bovine serum and antibiotic-containing D-MEM), followed by thorough mixing. Then, the solution A was first dropped on a culture dish, the solution B was added to the dropped solution A, and the obtained mixture was formed into a shape suitable for transplantation, and then was kept warm in a humid environment at 37°C in a 5% carbon dioxide atmosphere for 5 to 10 minutes to cause the cells to adhere to each other to produce an ADRCs cell cluster. After the production of the ADRCs cell cluster, the cluster was promptly transplanted to an infarct site.

(3) Measurement of Secretion Levels of Cytokines such as Adiponectin by ADRCs Cell Cluster

The supernatant after the ADRCs cell cluster was cultured for 24, 48, 72, 96, 120, and 144 hours was

collected, and the secretion levels of adiponectin, VEGF, HGF, and IL-6 in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA).

(4) Histological Examination of ADRCs Cell Cluster

5 Frozen sections of the ADRCs cell cluster were prepared, fixed with 4% paraformaldehyde, and then subjected to Oil Red O staining and adiponectin immunostaining.

10 5) Production of Cardiac Insufficiency Model and Transplantation of ADRCs Cell Cluster

A myocardial infarction model was prepared using a 7-week old female LEW/Sea rat by a ligation at the anterior descending branch of the left coronary artery. With a general anesthesia given by inhalation anesthesia of isoflurane under artificial respiration (1.5% isoflurane, 15 ventilation volume 4 ml, 110 cycles/minute), left thoracotomy was performed to expose the heart. A site of the left coronary artery at a distance of 2 to 3 mm from the origin of the left coronary artery was ligated with 7-0 proline suture. Two weeks after the coronary artery 20 ligation, an ADRCs cell cluster was placed on tip-curved forceps, and slid to and set on the infarct site of the left ventricular anterior wall. For a sham group, stabilized fibrin not containing ADRCs was transplanted in the same process. 25

(6) Animal Experiment Protocol

Two weeks after the coronary artery ligation, rats were divided into 5 groups, and then treated as follows. Specifically, prepared were: a group in which an ADRCs cell cluster was transplanted to the left ventricular anterior wall (an A group; n = 26); a group in which an ADRCs cell cluster containing pioglitazone (PGZ, 50 mM) was transplanted (an AP group; n = 26); a sham group in which no cell cluster transplantation was conducted (an S group; n = 26); and a group with direct intramyocardial transplantation (an im group; n = 6) and a group with intracoronary transplantation (an ic group; n=6) of ADRCs to peri-infarct regions of the left ventricular anterior walls. Then, histological evaluation and molecular evaluation were conducted on days 12 and 56 after the surgery. In addition, the cardiac function was evaluated by echocardiography every week after the surgery.

(7) Echocardiographic Examination

After a sedation state was obtained by inhalation anesthesia of isoflurane, short axis views of the left ventricle at the papillary muscle level were obtained using a echocardiographic system equipped with a 14 MHz transducer. The left ventricular end-diastolic diameter (LVEDd) and end-systolic diameter (LVESd) were measured. The measurements were repeated three times or more, and the average values were obtained. The left ventricular ejection fraction (EF%) was calculated from the following

equations.

Left ventricular ejection fraction (EF) =
 $\{(\text{LVEDV}-\text{LVESV})/\text{LVEDV}\}\times 100 = (\text{SV}/\text{LVEDV})\times 100$

Left ventricular end-diastolic volume (LVEDV) =
5 $\{7.0/(2.4+\text{LVEDd})\}\times \text{LVEDd}^3$

Left ventricular end-systolic volume (LVESV) =
 $\{7.0/(2.4+\text{LVESd})\}\times \text{LVESd}^3$

(8) Histological evaluation

On days 14 and 56 after the surgery, a sedation state
10 was obtained by inhalation deep anesthesia of isoflurane,
and thereafter the heart continuously beating was removed.
Each tissue section was prepared by slicing the left
ventricle in the short axis direction, compound-embedding
the slice, and freezing the slice in liquid nitrogen.
15 Hematoxylin-eosin staining, Picro-Sirius red staining,
Masson trichrome staining, Oil Red O staining, adiponectin
immunostaining, SMA immunostaining, and von Willebrand
factor immunostaining were performed. The fibrosis rate
was determined by analysis of a Picro-Sirius red stained
20 image. For the living cell size at the peri-infarct border
site, the cell short diameter in one field of view (at a
magnification of 400) of a hematoxylin-eosin stained image
was measured and the average value of 10 fields of view
per sample was calculated. In addition, the number of
25 capillaries per field of view (at a magnification of 400)
of a von Willebrand factor immunostained image was

measured, and the average value of 5 fields per sample was calculated.

(9) Molecular Biological Study

On days 14 and 56 after the transplantation, the removed cardiac sample was divided into an infarct part (scar), a border part between the infarct and normal parts (border), and a non-infarct part (remote), and mRNA extraction in each of the parts was carried out. Then, cDNA was synthesized from mRNA by reverse transcription reaction. The transcription level of each of vascular endothelial cell growth factor (VEGF), adiponectin (APN), adiponectin receptor-1 (Adipo-R1), adiponectin receptor-2 (Adipo-R2), T-cadherin (CDH-13), and tumor necrosis factor (TNF- α) was quantified by the quantitative PCR method, and was divided by the transcription level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is an endogenous control. Then, a ratio of the obtained value to the normal value of each sample was expressed.

(10) Statistical Analysis

Data was expressed as the average value $\pm$ standard error. Comparison between groups was conducted by t test, and a P value less than 0.05 was considered to indicate a significant difference. For survival rate analysis, the survival rate was calculated by the Kaplan-Meier method, and a significant difference for each experimental group was determined by a log rank test.

[Results]

(1) ADRCs Cell Cluster's Abilities to Secrete Cytokines and Differentiate into Adipocytes

ADRCs cell clusters (Fig. 1A) and pioglitazone (PGZ)-containing ADRCs cell clusters were prepared and cultured for 144 hours in a humid environment at 37°C in a 5% carbon dioxide atmosphere, and then their cytokine secretion abilities were examined. The amounts of adiponectin (APN), VEGF, HGF, and IL-6 secreted from the ADRCs cell clusters and the PGZ-containing cell clusters increased over time in the culture media. The adiponectin concentrations after culture for 144 hours in particular were 21.7 ± 0.9 ng/ 5×10^6 cells/day and 51 ± 3.9 ng/ 5×10^6 cells/day, respectively, and the culture medium of the PGZ-containing ADRCs cell cluster has a significantly higher concentration than that of the cell cluster containing only ADRCs (Fig. 1B).

Also, the ADRCs cell cluster was treated with 10 to 15% autoserum or fetal bovine serum (FBS), 0.5 to 2.0 μ M insulin, 0.1 to 1.0 μ M dexamethasone, and 0.2 to 2 mM isobutyl-methylxanthine, and thereby were induced to differentiate into adipocytes. As a result, adipocytes positive for Oil Red O appeared 7 days after the induction of differentiation (Fig. 1C). The number of cells constituting one cell cluster was in the order of 5×10^6 and the cell cluster contained Oil Red O positive cells

with a thickness of about 1000 μm . Thus, it was found that the cell cluster maintained the differentiation ability.

(2) Engraftment of ADRCs Cell Cluster on Myocardial Surface

5 In order to examine the engraftment of an ADRCs cell cluster on the cardiomyocyte, the cell clusters were transplanted to the cardiomyocyte of rats promptly after the preparation. As a result, the cell cluster after several days to one week adhered to the cardiac surface
10 (Fig. 2A). Since the cell cluster was observed communicating with the cardiomyocyte via capillary vessels (Fig. 2B), it was confirmed that the transplanted cell cluster was successfully engrafted on the cardiomyocyte.

15 (3) Influence of Transplantation of ADRCs Cell Cluster on Cardiac Function

Fig. 3 demonstrates evaluation results of the cardiac function on day 28 after the ADRCs cell cluster transplantation on chronic myocardial infarction model
20 rats. Here, the left ventricular ejection fraction (EF), left ventricular end-systolic diameter (LVESd), left ventricular end-diastolic diameter (LVEDd), and left-ventricular end-diastolic wall thickness (LVAWD) in heart echocardiographic examinations were used as
25 evaluation indexes of the cardiac function. Two weeks after coronary artery ligation, model rats were divided

into 5 groups and tested after the following treatments. Specifically, tested were the group in which an ADRCs cell cluster was transplanted to the left ventricular anterior wall (the A group); the group in which an ADRCs cell cluster containing pioglitazone (PGZ, 50 mM) was transplanted to the left ventricular anterior wall (the AP group); the sham group in which no cell cluster transplantation was conducted (the S group); and the group with direct intramyocardial transplantation (the im group) and the group with left ventricular intracoronary transplantation (the ic group) of ADRCs to the peri-infarct regions of the left ventricular anterior walls.

From the echocardiographic examinations of the hearts, the A group and the AP group had significantly larger EF values than the S group, and were found to have an improvement of left ventricular contractility. Furthermore, at week 8 after the transplantation, the AP group demonstrated greater improvement in EF than the A group (Fig. 3A). Meanwhile, as compared with the S group, the A group and the AP group had the LVEDd values with no significant difference (Fig. 3C), and had small LVESd values (Fig. 3B). Therefore, the A group and the AP group were observed having an improvement of left ventricular contractility by the transplantation. In addition, the survival rates of the groups were evaluated. As a result, the survival rates of the A group and the AP group were

significantly higher ($p = 0.0424$) than that of the S group (Fig. 3D). From the above results, transplantation of ADRCs cell clusters was found to have an excellent therapeutic effect on myocardial infarction.

Furthermore, as a result of comparative tests with the administration methods different from the transplantation of a ADRCs cell cluster, that is, with the group with direct intramyocardial transplantation (the im group) and the group with left ventricular intracoronary transplantation (the ic group) of ADRCs to the peri-infarct regions of the left ventricular anterior walls, the A and AP groups had the EF values significantly larger than those of the S, im, and ic groups (Fig. 4A), had the LVESd values smaller than those of the S, im, and ic groups (Fig. 4A), and had no significant difference in LVEDd (Fig. 4C). The results described above demonstrate that the transplantation of an ADRCs cell cluster has a superior therapeutic effect in the treatment of myocardial infarction to that of the method of directly injecting ADRCs cells into the cardiomyocyte or the coronary artery.

(4) Influence on Myocardial Tissue by Transplantation of ADRCs Cell Cluster

Histological analysis was performed on days 14 and 56 after the ADRCs cell cluster transplantation to the myocardial infarction model rats. The untreated S group demonstrated strong thinning of the left ventricular

anterior wall (Fig. 5A), whereas the A group (Fig. 5B) and the AP group (Fig. 5 C) were found to have the left ventricular anterior walls thicker than those of the S group, the im group, and the ic group, and maintain the left ventricular anterior wall tissue (Fig. 5D). As a result of the quantification of the infarct size, the A group and the AP group tend to be smaller in infarct size than the S group, the im group, and the ic group, although there was no statistically significant difference (Fig. 5E). Furthermore, as a result of the measurement of the capillary density at the peri infarct area, the A group (Fig. 5G) and the AP group (Fig. 5H) demonstrated significantly higher capillary densities than those of the S group (Fig. 5F), the im group, and the ic group (Fig. 5I).

(5) Cardiac Protection Effect by ADRCs Cell Cluster

Histological analysis was performed on days 14 and 56 after the ADRCs cell cluster transplantation to the myocardial infarction model rats. A cell diameter at the peri infarct area of each of the groups was detected by hematoxylin-eosin staining (Figs. 6A to 6 C) and PAS staining (Figs. 6 D to 6 F), and the cell short diameters in the A group and the AP group had significantly lower values than those of the S group, the im group, and the ic group (Fig. 6G). The above result suggested the possibility that ADRCs cell cluster transplantation may

have protective effect such as anti-apoptosis on residual myocardial cells.

(6) Anti-Apoptosis and Anti-Inflammatory Effects by ADRCs Cell Cluster

Fig. 7 shows the results of investigations on myocardial protection and anti-inflammatory effects on myocardial tissue on day 56 after the ADRCs cell cluster transplantation. The degrees of transcription of the neovascularization and myocardial protection cytokines at the peri infarct area of each group were evaluated. As a result, the transcription level of adiponectin (APN) at the periphery of the infarct area was significantly higher in the AP group than in the other groups (Fig. 7A). Also, the group A had a significantly higher APN transcription level than the S group. There was no significant difference in the transcription levels of the other Adipo-R1, Adipo-R2, CDH-13, VEGF, and TNF- α (Figs. 7B to 7F). The above finding suggested the possibility that the addition of PGZ to the ADRCs cell cluster may further enhance the improvement effect on the cardiac function after myocardial infarction.

(7) Engraftment and APN Production of ADRCs Cell Cluster on Myocardial Infarction Area

The ADRCs cell cluster transplanted on the left ventricular anterior wall of a myocardial infarction model rat was successfully engrafted on the scar area in the left

ventricular anterior wall on day 28 after the transplantation (Fig. 8A), and demonstrated adiponectin positive at the transplanted site (Fig. 8B). This suggested that the successfully engrafted cell cluster secreted adiponectin persistently. In addition, in the groups, as the comparison targets, treated with the intramyocardial (Fig. 8C) and intracoronary (Fig. 8D) transplantation of ADRCs, engraftment success in the cardiomyocyte was also observed on day 3 after the transplantation.

[Example 2]

In order to analyze constituent cells of ADRCs, cell surface antigens were analyzed using flow cytometry (FACS). Fresh ADRCs extracted from adipose tissue by the method described in Example 1 were suspended in a FACS staining solution (a phosphate buffered saline supplemented with 5% fetal bovine serum). The analysis used mouse antibodies against CD11b, CD31, CD45, CD73, and CD90 as surface antigen markers, and the corresponding mouse IgG1 isotypes as negative markers. The cells were stained for 30 minutes at room temperature, washed and then analyzed using flow cytometry (BD FACS cant II instrument (BD Biosciences, San Jose, CA)).

As a result, the constituent cells of ADRC were mainly mesenchymal stem cells/mesenchymal progenitor cells (CD90+, CD31-, CD45-, and CD73+), endothelial cells

(CD90+, CD31+, and CD45-), vascular smooth muscle (CD90-, CD31-, and CD45-), and hematopoietic cells (CD90+/-, CD31-, and CD45+). The approximate constituent ratio is as shown in Table 1. In particular, 6.5% of adipose tissue-derived mesenchymal stem cells were contained.

[Table 1]

Cell Type	Constituent Ratio
Mesenchymal Stem Cells/Mesenchymal Progenitor Cells (CD90+, CD31-, CD45-, CD73+)	6.5%
Vascular Smooth Muscle Cells (CD90-, CD31-, CD45-)	13.2%
Vascular Endothelial Cells (CD90+, CD31-, CD45-)	5.6%
Hematopoietic Cell (CD90+/-, CD31-, CD45+)	26.5%

[Industrial Applicability]

As mentioned above, the transplant material of the present invention comprises a cell cluster in which isolated cells are caused to adhere to each other, and can be produced easily within a short period of time. Moreover, the transplant material secretes adiponectin, and has an excellent therapeutic effect of heart disease. Therefore, the present invention can greatly contribute to the development of transplant medical care mainly for heart disease.

[CLAIMS]

[Claim 1]

A transplant material for treatment of heart disease comprising a cell cluster obtained by causing isolated
5 cells to adhere to each other with an adhesive agent.

[Claim 2]

The transplant material according to claim 1,
wherein the isolated cells are selected from the group
consisting of adipose-derived regenerative cells, bone
10 marrow-derived regenerative cells, umbilical
cord-derived regenerative cells, smooth muscle-derived
regenerative cells, multipotent stem cells, regenerative
cells derived from the multipotent stem cells, vascular
endothelial cells, and monocytes.

15 [Claim 3]

The transplant material according to claim 1,
wherein the isolated cells are adipose-derived
regenerative cells treated with a PPAR γ agonist.

[Claim 4]

20 The transplant material according to any one of
claims 1 to 3, wherein the isolated cells are cells not
cultured.

[Claim 5]

25 The transplant material according to any one of
claims 1 to 4, wherein the adhesive agent contains
fibrinogen.